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Stress effects on ovine behaviour, physiology and the gastrointestinal microbiota

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Declaration

I declare that the present thesis has been composed by me and that the work presented is my own and any assistance has been acknowledged. This thesis has not been submitted for any other degree or professional qualification.

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Abstract

Studies across different species have demonstrated the presence of an interplay between the brain, the gut and the microbiome, most commonly referred to as the gut-brain axis. Furthermore, it has been well documented that stress can affect neuroendocrinological and immunological systems, resulting in altered behaviours, as well as physiological dysregulations. In the past few decades, the effects of stress on the microbiome and the implications of the microbial community structure for the host have been the focus of many studies, aiming to shed light on this intricate and multi-pathway relationship.

Despite this, most studies have been conducted on humans and rodents, with very few on farm animals, particularly ruminants. In ruminants, as in monogastrics, the gut accommodates high microbial concentrations and facilitates host-microbial interactions. What differentiates ruminants is the presence of the rumen, which hosts an equally important microbial community. This organ acts as a primary location for fermentation of feed and plays a pivotal role in animal metabolism, immunity and overall homeostasis. The effects of stress susceptibility, and more specifically psychological or behavioural stress, have been poorly explored in ruminants, despite their well-recognised and important effects on other aspects of animal health and welfare.

This project therefore aimed to explore: 1) the effect of genetic predisposition to stress; 2) the long-term effect of prenatal and early life stressful events; and 3) effects of repeated and unpredictable management stress on the ovine gastrointestinal microbiome in conjunction with various physiology and behavioural aspects.

The first experiment investigated genetic differences in gut and rumen microbial community structure and blood cortisol concentrations in 58 adult Romane ewes, previously selected on the basis of divergent reactivity to stress (30 ewes with high reactivity; 28 ewes with low reactivity). The two groups differed in their behavioural reactivity towards a temporary separation from congeners based on bouts of high bleats. Despite extensive analyses of the microbiota at the phylum, order and genus level, there were only small significant differences in the rumen and faecal microbiota, even when including cortisol levels in the analyses. For example, higher levels of cortisol were positively correlated with *Ruminococcus* abundance in faecal samples and *Lactobacillus* in the rumen, while *Rikenellaceae* abundance was positively correlated with reactive EBV scores in faecal samples.

The second experiment investigated long-term effects of three prenatal stress treatments (Control, Negative and Alternative) and the effect of two early-life treatments (Isolation or

Ewe Recognition tests) on the rumen microbial community structure of 35 8-month-old Scottish Mule lambs, at a stage when the rumen microbiome had assumed a relative stable and mature form. Sex and diet effects were confounded, while Prenatal Treatment did not appear to have an effect. Neonatal Treatment had an effect on relative abundances at the phylum level. The abundance of several bacterial species was correlated with higher or lower cortisol levels, such as *Lactobacillus* in Isolated females and *Proteobacteria* in Isolated males; these significant negative correlations suggested long-lasting effects of early life events.

Finally, in the third experiment, we explored the effect of a 6-week mild unpredictable Chronic Stress paradigm on various behavioural (i.e., time budgets, reactions to a suddenness test) and physiology aspects (i.e. hormonal levels, heart rate and VFAs), as well as the rumen and gut microbiota structure. Forty-eight female Romane lambs were separated into two treatments: Non-Treated (NT, n = 24) and Mild Chronically Stressed Animals (MCS, n = 24). Amongst the most interesting results, indicating a treatment effect, were differences in synchronisation of animals resting and sleeping, duration of resting time, and reactions to novelty, as expressed by latency to approach the ball and contact time with the ball in the suddenness test. Microbiota diversity indices, particularly for the non-treated group, indicated a different development of the microbial community. In MCS animals, cortisol and serotonin levels indicated that several bacteria proliferate in the presence/absence of these hormones, but correlation scores were generally non-significant.

In conclusion, it appears that the microbiota community structure in the rumen is not significantly affected by management stress or stress susceptibility, although the communication pathways between rumen bacteria and host behaviour warrant further exploration.

Lay Summary

Studies across different species have demonstrated that an organism's genetic tolerance to stress and exposure to stress can affect behaviour and physiology (heart rate, stress hormone levels etc.), as well as the microbial species present in various organs and most importantly in the gut. This has mainly been explored in mice, rats and humans, but very few studies have investigated the effects of psychological stress on farm animals and even less so in ruminants such as cattle and sheep. Ruminants have large numbers of bacteria not only in the gut, but also in the rumen, as this is an organ where bacteria and other microorganisms break down indigestible feedstuff high in fibre, so that the animal may use feed effectively. The rumen also plays an important role in animal metabolism and overall health.

In this project, three complementary experiments were conducted to investigate how different levels of stress affect sheep behaviour, physiology and the bacteria present in the gut and rumen of sheep.

In the first experiment, two genetic lines Romane ewes that differed in their responses to stress (30 ewes with high reactivity; 28 ewes with low reactivity) were sampled to explore potential differences in rumen and faecal bacteria present. Cortisol levels, as an indicator of stress response, were also explored. Cortisol levels and genetic breeding values were correlated with a higher or lower presence of several bacterial taxa in the rumen and faecal samples.

In the second project, we explored differences in male and female Scottish Mule lambs at the age of 32-33 weeks. Three groups of animals were used: one Negative group which had been prenatally stressed by exposing the mother to various stressors, one Control group, and an Alternative group whose mothers had received a different diet and were allocated in bigger pens with bigger feed-face surface. The 35 lambs chosen had also been exposed to two different early life tests (isolation or a test to assess if they recognised their mother). Blood samples for plasma cortisol and rumen samples were analysed. Differences seen between males and females in the rumen bacteria could be due to sex differences, or potentially due to a diet effect, as in the last week before sampling males and females were fed differently. Early life treatment had an effect on abundance and presence of several bacterial phyla. Relative abundance correlations of important bacterial species with cortisol, in both females and males, indicated a probable long-lasting effect of early life events.

In the third experiment, a group of female Romane lambs (6 to 7 months of age) were exposed to various mild stressors throughout the day and night (for example the presence of a dog, lights coming on, loud noises, rough handling, social mixing) for a period of 6 weeks. We

observed animal behaviour and investigated differences in physiology (hormone levels, heart rate and more), growth and rumen and faecal bacteria between stressed animals (n = 24) and a non-treated group (n = 24). Differences were observed in resting behaviours, and the reaction the animals had to a suddenness test where a ball fell into the pen. The microbiota in the control animals appeared to be more diverse compared to its pre-trial state, whereas in the stressed animals, diversity remained steady, indicating a potential suppression due to stress. In this study, we also correlated plasma cortisol and serotonin levels with bacterial levels, and several bacteria were correlated with higher or lower concentrations of these hormones.

The results from this project suggest that the bacterial community in sheep, particularly in the rumen is resistant to behavioural and management stressors, but communication between the rumen and gut bacteria and host behaviour should be explored further in farm animals.

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Abbreviation List

ACE: Abundance based Coverage Estimators

ACTH: Adrenocorticotropic Hormone

ADG: Average Daily Gain

ANS: Autonomous Nervous System

ASD: Autism Spectrum Disorder

AVP: Arginine Vasopressin

BDNF: Brain-Derived Neurotropic Factor

BHB: Beta-hydroxy-butyrate

BLUP: Best Linear Unbiased Prediction

CAP: Canonical Analysis of Principal coordinates

CFS: Chronic Fatigue Syndrome

CGRP: Calcitonin Gene-Related Peptide

CLD: Compact letter display

CNS: Central Nervous System

CRH: Corticotrophin-Releasing Hormone

CRHF: Cardiac Rhythm and Heart Failure

CV: Coefficient of Variation

DEFRA: Department for Environment, Food and Rural Affairs

DF: Degrees of Freedom

DM: Dry Matter

DNA: Deoxyribonucleic Acid

e.g.: exempli gratia

EBV: Estimated Breeding Value

EC: Enterochromaffin Cells

ECG: Electrocardiogram

ELISA: Enzyme-Linked Immunosorbent Assay

ELS: Early Life Stress

ENS: Enteric Nervous System

et al. et alia

g: grammar(s)

GABA: Gamma-Aminobutyric Acid

GC: Gas Chromatograph

GCs: glucocorticoids

GF: Germ Free

GH: Growth Hormone

GI: Gastro-Intestinal

GIT: Gastro-Intestinal Tract

GLM: Generalised Linear Model

GLMM: Generalised Linear Mixed Model

GR: Glucocorticoid Receptor

H: hour

HF: High Frequency

HPA: Hypothalamic-Pituitary-Adrenocortical

HPAA: Hypothalamic-Pituitary-Adrenocortical Axis

HR: Heart Rate

HRFI: High Residual Feed Intake

HRV: Heart Rate Variability

i.e. id est

IBS: Irritable Bowel Syndrome

INRAE: Institut national de recherche pour l'agriculture, l'alimentation et l'environnement, French National Research Institute for Agriculture, Food and the Environment

IQR: Interquartile range

Kg: kilogram(s)

LF: Low Frequency

LPS: Lipopolysaccharide(s)

LRFI: Low Residual Feed Intake

MAMP: Microbe-Associated Molecular Patterns

MCS: Mild Chronic Stress

ME: Myalgic Encephalomyelitis

MFS: Methyl-Formaldehyde Solution

mg: milligram(s)

min: minute(s)

ml: millilitre(s)

mm: millimetre(s)

NAA: N-Acetylaspartate

NEFA: Non-Esterified Fatty Acids

NFE: Net Feed Efficiency

NMDS: Nonmetric Multidimensional Scaling

NPY: Neuropeptide Y

NS: Non-Stress

NT: Non-Treated

OUT: Operational Taxonomic Unit

PACAP: Pituitary Adenylate Cyclase-Activating Polypeptide

PCoA: Principal Coordinate Analysis

PCR: Polymerase Chain Reaction

PLS: Partial Least Squares

PNS: Parasympathetic Nervous System

PNS: Pre-Natal Stress

PSD: Partial Sleep Deprivation

PTSD: Post-Traumatic Stress Disorder

QBA: Qualitative Behaviour Assessment

QTL: Quantitative Trait Locus/Loci

RA: Relative Abundance

RFI: Residual Feed Intake

RMSSD: Root Mean Square of the Successive Differences

RNA: Ribonucleic Acid

rRNA: Ribosomal Ribonucleic Acid

s: second(s)

SAM: Sympathetic-Adrenal-Medullary

SARA: Sub-Acute Rumen Acidosis

SCFA: Short-Chain Fatty Acid(s)

SD: Standard Deviation

SDNN: Standard Deviation of RR intervals

RMSSD: Root of the Mean Square of Successive Differences

SNPs: Single Nucleotide Polymorphisms

SNS: Sympathetic Nervous System

SRT: Square Root Transformed/ Transformation

TLR: Toll-Like Receptor(s)

TPH1: Tryptophan Hydrolase1

TPH2: Tryptophan Hydrolase2

UD: Undernutrition Diet

UFV: Feed Unit for maintenance and meat production -Unites Fourrageres Viande (kcal NEmg/kg)

VFA: Volatile Fatty Acid(s)

VIP: Variable Importance in Projection

VNS: Voluntary Nervous System

vs. Versus

µl: microliter(s)

Figure Index

Appendix Figures

Table Index

Appendix Tables

Contents

Chapter 1

General Introduction

1 General Introduction

1.1 Stress and stress response

Stressors are biological, environmental or external stimuli that lead to a cascade of responses (physiological, morphological or behavioural) defined as the stress response. Since Selye's original definition of stress in 1936 as "the non-specific neuroendocrine response of the body to any demand for change" (Selye, 1936, 1955), the way that stress and the stress response has been perceived has evolved in order to incorporate more fields of study (ranging from psychology to endocrinology) and accurately reflect the complex mechanisms that take part in this process. The stress response is a mechanism which acts against any real or perceived threat in order to assist the organism to return to a "normal", non-pathological homeostatic state (Carstens and Moberg, 2000). Adjusting physiology, morphology and behaviour is crucial for survival in all organisms. These adjustments (whether they occur daily or on a seasonal basis) are always necessary, but often unpredictable.

Eustress is considered to be the result of responses that have a neutral or positive effect in order for the animal to respond to its environment and seek out the fulfilment of biological needs, much like an allostatic mechanism (Selye, 1975). This definition has been deemed as insufficient (Kupriianov and Zhdanov, 2014) and now incorporates the psychological aspect of perception to stress and the ways this may affect the outcome of the response (Nelson and Simmons, 2003).

Distress on the other hand, may lead the animal to respond in ways that could negatively affect the fulfilment of short term needs (disruption of coping mechanisms and effective fight-or-flight response) or long term requirements, affecting well-being and resulting in pathological states (Wolff, 1953; Selye, 1955; Romero *et al.*, 2015). Distress, according to Moberg (1985), also leads the animal to exhibit recognisable and "accepted" behavioural signs of suffering and therefore, distress incorporates physiological and psychological internal states as well as observable behavioural cues. In animal welfare literature, the distinction between stress and distress is not frequently addressed.

Events such as disease, injury, and change in social status, or simply ageing, can greatly influence and interrupt life-cycle routines. Therefore, a "normal", non-pathological state of being can only be described as an ever-changing shift from one short-term homeostatic plateau to another, using information acquired from prior stressful events. These changes, defined as allostasis "achieving stability through change" (McEwen and Wingfield, 2010, 2003; Romero

et al., 2009), incorporate perturbations and anticipatory adjustments, as well as altered levels of stress sensitivity in order to activate flight-or-fight responses, with an overall aim of successful survival.

Stress for the purposes of this research project is accepted as "any damaging form of stress that is outwardly expressed by recognisable behaviours" (Moberg, 1985). Acute stress is more likely to be assessed via fluctuations of stress biomarkers (such as glucose, cortisol and heart rate variability), whereas chronic stress is less likely to be defined by similar shifts. However, biomarkers that indicate changes in metabolism and behavioural patterns may be more useful in this context (i.e. weight gain/loss, beta-hydroxy-butyrate, serotonin, frequency and synchronisation of activities) (Merrow, Spoelstra and Roenneberg, 2005; Yamanashi *et al.*, 2017; Wyse *et al.*, 2018; Rojas-Morales, Pedraza-Chaverri and Tapia, 2020).

1.1.1 Types of stress and adaptation processes

Stress for animals can be categorised into: 1) physical stress, due to injury, fatigue or infliction of pain; 2) physiological, due to inadequate fulfilment of biological needs resulting in hunger, thirst or temperature dysregulation and 3) behavioural due to abstract or tangible environmental influences such as light, noise, group density exposure to novelty, social stressors or isolation and in the case of farmed animals, human intervention. Depending on the duration of the stimuli, stress can be acute or chronic (Moberg and Mench, 2000), sequential, episodic, chronically intermittent, sustained and anticipated (Sapolsky, 2000). The two main types of stress discussed further are acute and chronic.

Acute stress occurs after exposure to physical, emotional or psychological negative situations. Upon activation of the sympathetic nervous system (SNS) and hypothalamic pituitary adrenal axis (HPA), a rapid fluctuation of hormones (catecholamines, ACT, opiates, vasopressin, prolactin, glucagon, GH and GCs, serotonin and gonadotrophins), increased heart rate and energy mobilisation (higher levels of glucose and NEFA) follow, mobilising the immune system, resulting in recovery, and usually a complete adaptation.

The HPA axis is responsible for the neuroendocrine adaptation component of the stress response. This response is characterized by hypothalamic release of corticotropin-releasing hormone (CRH). ACTH is produced by the anterior pituitary gland after being stimulated by the neurohormones CRH and arginine vasopressin (AVP), released by neurons in the paraventricular nucleus (PVN). ACTH is released therefore in the circulation and induces glycocorticoid synthesis and release from the adrenal glands, located on the top part of the

kidneys. The main glucocorticoid in humans and in the interest of this study sheep, is cortisol, whereas the main glucocorticoid in rodents (frequently used as model systems), is corticosterone. Therefore, as ACTH binds to receptors on the adrenal cortex it stimulates adrenal release of cortisol. In response to stressors, cortisol is released for several hours after encountering the stressor.

Hypothalamic activation of the HPA axis is managed through a variety of brain signalling (neurotransmitter) systems. Some of these systems have inhibitory effects (e.g., γ-aminobutyric acid [GABA]), while others have excitatory effects (e.g., norepinephrine and serotonin) on the PVN. It is evident therefore that the central nervous system (CNS) and the hormone (i.e., endocrine) system are interconnected, coordinating glucocorticoid activity.

At a certain blood concentration of cortisol, the body and relevant mechanisms assume that protection from the stressor has been achieved and negative feedback is commenced, via cortisol. Negative feedback is exerted to the anterior pituitary gland the PVN and the hippocampus. Two types of cortisol receptors can be found: mineralocorticoid (type-I) (MRs) and glucocorticoid (type-II) (GRs), and both participate in the negative feedback mechanisms. Cortisol has higher affinity with the MRs receptors compared to the GRs (de Kloet and C. Meijer, 2019) . This is particularly important in the regulation of circulating cortisol levels during the normal daily activities (regulation of circadian rhythm) which occurs via the MRs. Circadian and diurnal patterning of cortisol, which in turn is associated with glucose production and free fatty acid availability is particularly important in relation to feed intake (Kakihana and Moore, 1976; Atger *et al.*, 2017).).

When the cortisol concentration is high (e.g., after exposure to a stressor), it is more available and binds more to the GRs with lower affinity. This results in the termination of the stress response. This negative feedback mechanism plays an important homeostatic role because too much or too little exposure to cortisol can have adverse consequences. Repeated exposure to stressors can lead to habituation due to repeated and sustained HPA axis mobilisation.

The means by which an animal responds to stressors can be stressor and species-specific, depending on the complexity of the organism and on particular metabolic and behavioural aspects (Anisman and Merali, 1999; Schneiderman, Ironson and Siegel, 2005). When an animal is exposed to stimuli that are not excessively aversive, it has the means to respond to them to a progressively lesser extent and become habituated, as homeostasis can be restored (Broom *et al.*, 1993b; Lloyd *et al.*, 2014).

If the stressor or stimulus becomes predictable for the animal in terms of frequency, duration and intensity, once again depending on the perceived severity, the animal can anticipate the

action and become habituated (Broom *et al.*, 1993a). Therefore, a response to stimuli would be more likely and more effective when anticipation mechanisms have been set in place. In the case of no previous exposure to a stimulus, the novelty accompanying the event acts as an added stressor. Despite this, on a psychological level, anticipation of an event does not always lead to adaption, but may exacerbate anxiety (Grupe and Nitschke, 2013).

If the repeated stressor persists and occurs in a manner that is uncontrollable from the animal's perspective, perturbations in the response to stress mechanisms may result in long-term effects, defined as chronic stress resulting in psychological and physiological pathologies. For farm animals, these pathologies manifest as altered behaviour, stereotypies, attenuation of immune activities, altered metabolism impacting growth, productivity and reproduction or finally, a combination of these responses (Moberg and Mench, 2000; Garner, 2005; Romero, Dickens and Cyr, 2009; Boonstra, 2013; Romero *et al.*, 2015; Gaskill and Garner, 2017). Based on the duration of the stressor the animal can go through three stages: alarm, resistance and exhaustion. Exhaustion is identified as the end point of being chronically stressed when the body may no longer cope. This is not usually observed, as animals generally tend to develop coping mechanisms to reach a new homeostatic level (Romero *et al.*, 2015; Colditz and Hine, 2016). However, this is possible when combined physical, physiological and environmental stressors persist. In general, a stressor should be defined as acute or chronic, not based on duration of the stressor, but by the duration of its consequences on the physiology of the animal (Animals, 2008).

Furthermore, these maladaptations to stress are more likely to occur in situations where the responsiveness of physiological responses to stress and the ability to adapt has already been altered due to genetic, prenatal or early life events, resulting in a biased susceptibility to the negative effects of stressors throughout life (Maniam, Antoniadis and Morris, 2014).

Finally, when an animal is repeatedly subjected to an aversive stimulus that it cannot escape, the animal may stop responding, attempt to avoid the stimulus and may exhibit an apathetic behaviour described as learned helplessness (Multani *et al.*, 2014; Hoffman, 2016). Regardless of tolerance development, chronic intermittent stress and chronic stress models affect all neuroendocrine systems related to the stressors, as well as their intensity and duration. Exposure to stressors of high intensity can increase SAM and HPA axis activity to respond to ongoing of further stressors, but also inhibit neuroendocrine systems, such as those involved in growth and reproduction (Tsigos *et al.*, 2000; von Borell, Dobson and Prunier, 2007; Godoy *et al.*, 2018; Tsyglakova, McDaniel and Hodes, 2019). Additionally, after consistent exposure to stressors animals may also exhibit an increased sensitivity of the HPA axis and a decrease

in responses to pleasant stimuli, which is described as anhedonia (Schweizer, Henniger and Sillaber, 2009; Hoffman, 2016).

1.1.2 Genetics of stress and temperament

Any physiological response is determined by gene expression. However, the genome too is influenced by phenotype selection, which presents a "cause and effect" cycle, while epigenetic influence also plays a role in shaping these mechanisms. The stress response mechanism is no different, as many genetic loci where functional gene variation is present, determine the structure and functionality of this system (Bouchard, 1994; Uhart *et al.*, 2004; Ising and Holsboer, 2006). Many of these genetic loci are conserved across species, such as the SLC6A4 serotonin transporter gene and the CRH gene (Duman and Canli, 2015; Grone and Maruska, 2015). Despite this, the presence of small genetic differences, such as single nucleotide polymorphisms (SNPs) contribute to the development of individual differences in stress responsiveness mechanisms (Rao *et al.*, 2008; Hough *et al.*, 2013; Pagliaccio *et al.*, 2014; Schneider *et al.*, 2014).

In order to assess the influence these genes exert on the ways individuals respond to stress; the degree of heritability is usually examined. Most studies investigating heritability in humans compare phenotypical similarity between monozygotic and dizygotic twins where "traits" are attributed to facilitate the observations. In terms of behavioural traits in humans, the "Big Five", namely: extraversion, agreeableness, conscientiousness, neuroticism and openness for experience are the most commonly used (Ebner and Singewald, 2017). For example, in humans, a study conducted by Uhart *et al.* (2004) indicated that for people who were homozygous or heterozygous for two SNP variations (T and C) in the gamma-aminobutyric acid ($GABA_A^{a6}$) receptor subunit gene, cortisol response was significantly affected. Specifically, groups of the CT and TT genotype had lower cortisol values compared to the CC group, which also scored lower on the Neuroticism-Extraversion-Openness (NEO) personality scoring system for the factor of extraversion.

Several SNPs have been correlated with the anxiety trait (Savage *et al.*, 2017). One of the most significant and more studied ones is a SNP present in the promoter of the serotonin transporter (5-HTT). 5-HTT is encoded by the SLC6A4 gene and its transcription is modulated by a repetitive sequence, the SLC6A4-linked polymorphic region (5-HTTLPR) (Nakamura *et al.*, 2000).

Further studies have been conducted in farm animal species, to identify genes relevant to the stress response. In pigs, SNP variations in the NR3C1 and AVPR1B genes related to the function of HPA axis, were found to correlate with various stress and aggression parameters (such as front lesion scores) and higher plasma cortisol levels (Muráni *et al.*, 2010). Furthermore, in this study, animals, which were more closely related to the "wild" types and had the relevant gene variants, were affected on a number of physiological stress parameters, and cortisol and glucose levels were significantly affected.

In Charolais cattle, significant associations between SNPs for the genes proopiomelanocortin (POMC) and Neuropeptide Y (NPY), which are dopamine and serotonin mediators, were identified as novel indicators of temperament (Garza-Brenner *et al.*, 2017). In sheep differing in temperament (calm and nervous) and associated cortisol levels, Qiu, Martin and Liu Xiaoyan Qiu (2015) confirmed the influence of a polymorphism in the CYP17 gene involved in cortisol production and the presence of two polymorphisms in dopamine receptors 2 and 4, known to be associated with behaviour and stress.

Individual differences in animal behaviour may be examined 1) by observing the frequencies, durations and/or patterns of particular measures relevant to behaviours a specific species exhibits; or 2) in terms of temperament, i.e., the way animals react to environmental change and stimuli. As such, behavioural traits in animals are often described as temperament and reflect shyness–boldness, emotional reactivity/fearfulness, exploration–avoidance, activity, sociability, and aggressiveness (Boissy, 1995; Réale *et al.*, 2007).

The genetics and heritability of animal temperament traits have been investigated in most farm animals such as pigs (Canario *et al.*, 2014; D'Eath - *et al.*, 2009; König von Borstel *et al.*, 2018), chickens (Rozempolska-Rucińska *et al.*, 2017; Johnsson *et al.*, 2018), cattle (Le Nei*ndre et al*, 1995; Gauly*et al*, 2001; A. Boissy *et al*, 2005; Benhajali *et al*, 2010; Haskell, Simm and Turner, 2014) and sheep (Hazard *et al.*, 2014a, 2016a, 2020; Zambra *et al.*, 2015; Brown *et al.*, 2016). The purpose of this is to assist in selection of animals that will be able to adapt better in variable management systems, exhibit traits relevant to higher productivity and be easier to handle (Colditz and Hine, 2016; Llonch *et al.*, 2016).

Individual differences in response to capture, handling and restraint may lead to different levels of stress response (Grandin and Shivley, 2015). For example, less reactive individuals may be able to respond better than stress-prone individuals in situations where the stress response leads to diverting resources away from immunity, metabolism and reproduction (McNamara and Buchanan, 2005). Stress has also been shown to lead to impaired cognitive abilities (e.g., attention deficit, impaired decision making and memory) which can lead to

learning and adaption difficulties in novel or changeable environments (Mendl 1999). For these reasons, it is important to investigate the genetics of these traits in order to select for animals that are more resilient.

1.2 Physiology of stress

Once a stressor has been perceived, the magnitude of the response is directly linked to the behavioural activity it induces (and therefore the relevant energy requirements), even if the stimulus is not always considered a direct challenge to homeostasis (Koolhaas *et al.*, 2011). Behavioural and subsequent physiological responses are then triggered in anticipation of homeostatic needs (Koolhaas *et al.*, 2011).

Hormone signalling is the first step in the activation of any form of stress response and the regulation of homeostasis, as all endocrine systems respond to specific stressors. Immediate endocrine responses are mediated by the SNS and the adrenal medulla (SAM) (Moberg and Mench, 2000; Al'Absi and Flaten, 2016), whereas longer term effects are the result of hypothalamic signalling and pituitary function, known for activating glucocorticoid production by the adrenal cortex (HPA axis) (Moberg and Mench, 2000; Al'Absi and Flaten, 2016). In co-ordination, these systems activate energy mobilisation and redistribution, synchronisation of peripheral physiology at the cell, tissue and organ level, responding to environmental shifts and changes in circumstance (Smith, 2006).

For the effective functioning of the SNS system, efferent motor neurons carry information from the Central Nervous System (CNS). These neurons are subsequently divided into two systems: the voluntary system (VNS) which controls voluntary movements in skeletal muscles, and the autonomic nervous system (ANS) which controls smooth and cardiac muscles and gland functioning.

The ANS is a control system, regulated by the hypothalamus, which manages functions such as the heart rate, digestion, respiratory rate, pupillary response, urination, and sexual arousal. Neurons of the ANS belong to the sympathetic or parasympathetic pathway and have competing roles, allowing regulation of body function via a finely tuned equilibrium. The sympathetic pathway predominates when the animal is threatened, activating a "fight-or-flight" situation, which involves a coordinated change in organ and tissue function throughout the body, allowing increased amounts of oxygen to reach the skeletal muscles (Al'Absi and Flaten, 2016).

A first step to this effect is achieved by increasing heart rate and myocardial contractility. Furthermore, stimulation of vascular smooth muscle causes vasoconstriction, particularly in the organs of the gastrointestinal system and in the kidneys, which allows redistribution of blood from these tissues to the muscles (McCorry, 2007). In the lungs, bronchodilation facilitates oxygen uptake and carbon dioxide excretion (McCorry, 2007). Stimulation of adrenaline release from the adrenal medulla and noradrenaline from the nerve fibres in the locus coeruleus in the brainstem inhibits protein synthesis and storage of glucose and fatty acids (Moberg, 1987; Moberg and Mench, 2000).

Adrenaline and noradrenaline also stimulate an increase of glucogenolysis and gluconeogenesis in the liver, increasing circulating glucose concentration. Haematic levels of fatty acid molecules are also increased, due to higher lipolysis rates in adipose tissue. These fatty acids are then used by skeletal muscles to supply metabolic energy for contraction (Exton *et al.*, 1972). Other immediate effects include pupil dilation, allowing more light in and adapting the lens for long distance vision in order to prepare the animal to flee and a general alertness and vigilance posture may be assumed (Carstens and Moberg, 2000; Moberg and Mench, 2000; Grandin and Shivley, 2015; Chu and Ayers, 2019). In order to reverse the effects of the processes above, the parasympathetic nervous system (PNS) takes over. The PNS predominates in relaxed states lowering heart rate conditions ("rest and digest"). The vagus nerve is the main contributor of the PNS. The most important function of the vagus nerve is afferent conduction of signals from the gut, liver, heart and lungs to the brain. The parasympathetic innervation causes a dilatation of blood vessels and bronchioles and a stimulation of salivary glands (Breit *et al.*, 2018). In the gastrointestinal tract, the activation of the parasympathetic nervous system increases bowel motility and glandular secretion from the pancreas or gall bladder (Breit *et al.*, 2018). Finally, reproduction and sexual activity rely on the PNS which sends signals to the vas deferens, seminal vesicles, and prostate in males, or vaginal glands in females (Purves *et al.*, 2001).

Another significant player is the enteric nervous system (ENS), which with the SNS and the PNS represent the three branches of the ANS (**Figure 1.1**). The ENS is primarily of vagal origin (Mayer, 2011) and consists of a nerve plexus built in the intestinal wall, extending across the whole gastrointestinal tract from the oesophagus to the anus (Forsythe, Bienenstock and Kunze, 2014; Hao *et al.*, 2016). It consists of ganglionated sub-plexuses – the submucosal plexus, which regulates blood flow and epithelial cell function; the myenteric plexus, which regulates intestinal wall motility (Hao *et al.*, 2016), and the mucosal plexus which contains nerve endings allowing contact between antigens and presenting cells, thus controlling gut immune responses (Campos-Rodríguez *et al.*, 2013). Due to the similarities present between

the ENS and the brain regarding structure, function and chemical coding, it has been described as “the second brain” or “the brain within the gut” (Gershon, 1999).

In the event of stress, the ENS is also activated via the mobilisation of afferent and efferent signalling from the intestinal nerves, as well as from sympathetic (splanchnic) or parasympathetic (vagal) signalling. Neurotransmitters, endocrine, and peptide hormones, particularly glucocorticoids (GCs) and catecholamines, are activated in order to initiate an immune response. These affect cytokine mobilisation and proliferation and influence production of type 1 and 2 cytokines, T-helper cells and most components of cellular immunity (Mayer, 2000).

The above have been linked with reduced gastric emptying, increased colonic motility and intestinal transit time (Mayer, 2000). In the case of persistent or chronic stress, several gut pathologies such as irritable bowel syndrome (IBS), obesity or even cancer have been linked to the disruption of the microbiome due to malfunction of the ENS or miscommunication between the CNS and ENS (Maier and Watkins, 2003; Fichna and Storr, 2012; Coss-Adame and Rao, 2014; Davis, 2016; Pascal *et al.*, 2017; Chong *et al.*, 2019; Pittayanon *et al.*, 2019; Yang *et al.*, 2019).

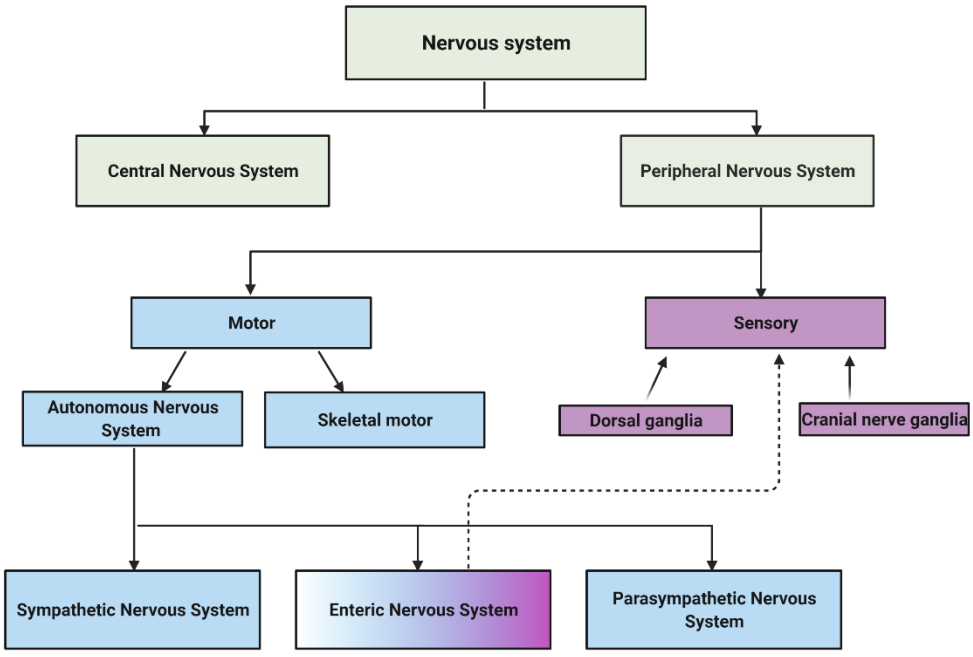


Figure 1.1 Visual representation of the connection and communication pathways between the brances of the Peripheral nervous system. Created with BioRender.com.

As mentioned above, the SNS dominates in the event of immediate perceived or actual threats. When a challenge to homeostasis persists, the HPA axis is mobilised at a slower pace and has a generalised effect on the animal compared to the influence of the SNS (Squires, 2010). The activation and regulation of the HPA axis happens via vagal afferent pathways (Herman *et al.*, 2016b). In the event of external stress or internal stress (in the form of elevated pro-inflammatory cytokines), rostral (limbic and prefrontal cortical regions) and caudal (brainstem) neural pathways, such as the noradrenergic nucleus *tractus solitarius* and serotonergic raphe neurons, coordinate the information about the stimulus and transmit it to the parvocellular paraventricular nucleus of the hypothalamus (Mayer, 2011; Koelsch *et al.*, 2015). These pathways process emotional or physical stressors, and this process initiates a neuroendocrine response via the secretion of CRH and arginine vasopressin (AVP) from the paraventricular nucleus of the hypothalamus. CRH and AVP release stimulates adrenocorticotropic hormone (ACTH) secretion from the anterior pituitary gland (Fink, 2012; Herman *et al.*, 2016a).

This stimulation in turn leads to glucocorticoid (GC) release from the zona fasciculate of the adrenal cortex, the main glucocorticoid in mammals being cortisol (corticosterone in rodents, amphibians, reptiles, and birds) (Smith and Vale, 2006; Herman *et al.*, 2016b). GCs exert their effects through activation of the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (Reul and De Kloet, 1985; Van Lier, Carriquiry and Meikle, 2014; Timmermans, Souffriau and Libert, 2019).

GCs stimulate gluconeogenesis in the liver by increasing enzyme activity, which converts amino acids into glucose, but reduces glucose uptake and use in skeletal muscle and white adipose tissue. Furthermore, GCs increase glycogen storage in the liver, whereas in skeletal muscle they play a permissive role for catecholamine-induced glycogenolysis or inhibit insulin-stimulated glycogen synthesis (Watts *et al.*, 2005; Kuo *et al.*, 2015). Finally, GCs modulate insulin and glucagon secretion from the pancreas (Kuo *et al.*, 2015). The overall role of GCs on glucose is to preserve plasma levels for the brain during stress. This mechanism, in coordination with adrenaline and noradrenaline (mentioned above) act in preparation for the "flight or fight" response. However, chronic GC exposure can lead to pathophysiological conditions due to impaired negative feedback (McEwen 2007). This can result in depletion of energy stores due to energy mobilisation and gluconeogenesis, immunomodulation, growth and reproductive suppression, and suppression of digestion. Hyperglycemia and insulin resistance have also been linked to a number of pathologies such as nephropathy, neurological symptoms, pancreatic dysfunction and more (Gallagher and Oberfield, 2007; Moher, 2018; Van Der Kooij *et al.*, 2018). Negative feedback between the adrenals and the brain fails in

clinical manifestations of chronic stress. Dexamethasone, an artificial glucocorticoid, was used to cause glucocorticoids blood level to fall rapidly, but, in resistant, chronically stressed animals, it fails to do so (Romero, Dickens & Cyr 2009).

Glycocorticoids act via negative feedback at the limbic centres (hippocampus), the hypothalamus, and the pituitary gland (Squires, 2010; Lightman, 2008), thus allowing the HPA axis to self-regulate and terminate the stress response. Two glycocorticoid receptors mediate the process: high affinity mineralocorticoid receptor (or type 1 receptor, MR) and low affinity glucocorticoid receptor (or type 2 receptor, GR) expressed in specific brain structures (Eberwine, 1999). These act on the cell nuclei of the brain as activators of gene transcription factors. MR is expressed in the limbic system, mainly in the hippocampus, as well as in the medial prefrontal cortex and other areas of the limbic system in co-existence with GR (Herman, Prewitt and Cullinan, 1996; Gjerstad, Lightman and Spiga, 2018; R. de Kloet and C. Meijer, 2019). Hippocampal MR are involved in the maintenance of the basal HPA activity, as at the nadir of the circadian rhythm, they are significantly occupied (De Kloet *et al.*, 1998). Cortisol also modifies fat and protein metabolism to support the nutrient requirements of the CNS during stress. However, cortisol also has many other wide-ranging effects when it binds to GRs. For example, it influences cardiovascular function, immunologic status (i.e., inflammatory reactions), arousal, and learning and memory; all of these systems therefore are affected when the HPA axis is activated in response to stress (Dantzer and Mormède, 1983; Ising and Holsboer, 2006; Oomen *et al.*, 2010; Colditz and Hine, 2016; Shaffer and Ginsberg, 2017; Davidson *et al.*, 2018; Tsyglakova, McDaniel and Hodes, 2019).

Thus, cortisol helps maintain or can increase blood pressure by increasing the sensitivity of the blood vessels to signalling molecules, catecholamines. In the absence of cortisol, widening of the blood vessels (i.e., vasodilation) and hypotension occurs. The anti-inflammatory effects of cortisol are a result of proinflammatory cytokine and histamine secretion reduction (Stephens and Wand, 2012).

Cortisol is catabolic and part of the function is to increase glucose and free fatty acids. Cortisol has bidirectional relationships with fatty acid metabolism, it inhibits desaturases and elongases, influences mobilization of fatty acids, and increases oxidation (Mocking *et al.*, 2018). Studies have demonstrated that the hyperactivation of the HPA axis can lead to increased circulating VFA levels (Herman *et al.*, 2016b; Hua *et al.*, 2018a). This has been hypothesized to induce a suppression of HPA activity via a feedback mechanism, as tested in animals by Widmaier, Rosen and Abbott (1992), who found a dose-related increase in ACTH and corticosterone levels after the infusion of Intralipid emulsion in rats. I*n vitro* studies also

showed a direct stimulatory effect of long-chain unsaturated fatty acids, on the adrenal gland (references), although very high FA concentrations have been shown to increase ACTH release from corticotroph cells (Lanfranco *et al.*, 2004). For example lower concentrations of omega-3 fatty acids and higher concentrations of omega-6 fatty acids may lead to HPA-axis hyperactivation (Mocking *et al.*, 2018). However, it has also been observed that oleic acid and linoleic acid stimulate glucocorticoid production in the absence of adrenocorticotropic hormone despite high FA concentrations, inhibiting ACTH action (Widmaier, Rosen and Abbott, 1992).

In terms of fat diposits, individuals under chronic, uncontrollable stress are more likely to have elevated levels of visceral fat (Moyer *et al.*, 1994; Epel, 1999; Epel *et al.*, 2000; Mocking *et al.*, 2018). Despite an increase in overall cortisol production and rates of turnover, circulating cortisol levels have been shown to be normal or low in obesity, which implies other mechanisms may be at play. For example, repeated measurements of salivary cortisol in free-living subjects revealed an abnormal diurnal variation of cortisol, which was positively associated with upper body fat distribution (Lee *et al.*, 2014). This abnormal pattern was characterized by low variability, absent circadian rhythm, low morning cortisol, and lack of meal-induced cortisol response (Lee *et al.*, 2014).

Additionaly, the HPA axis not only responds to stress but is also dominated by an internal circadian mechanism. Cortisol and other HPA-influenced hormones demonstrate a diurnal rhythm over a 24h time period which forms an internal homeostatic basis (Kalsbeek *et al.*, 1996). This fluctuation is variable for each individual in frequency and amplitude depending on feedback latency (Walker, Terry and Lightman, 2010) and plays a significant role in stress responsiveness (Kolbe, Dumbell and Oster, 2015; Koch *et al.*, 2017). In mammals circadian rhythmicity is regulated by a central clock in the suprachiasmatic nucleus (SCN) of the hypothalamus (Honma, 2018) anddisturbances can result in arrhythmicity in behavior as well as loss of other circadian rhythms, such as endocrine and body temperature rhythms (Malyszko *et al.*, 1994; Atger *et al.*, 2017), even reduced appetite. Pecoraro (Pecoraro *et al.*, 2004) suggests that animals also seek "comfort eating" to make up for energy loss. This may be a result of GCs increasing the salience of pleasurable or compulsive activities (ingesting sucrose, fat, and drugs, or wheel-running, depending on the species), which motivates ingestion of "comfort food" (Pecoraro *et al.*, 2004; Dallman, Pecoraro and La Fleur, 2005). Finally, the bidirectional communication between cortisol and fatty acids, lipid mobilisation etc., plus disruptions of cortisol diurnal patterns ultimately may mean disruption in feed intake and feeding patterns (Pickel and Sung, 2020).

1.3 The gut-brain axis, the importance of the rumen and bidirectional communication with microbiome

The digestive system is elaborately innervated through multiple connection pathways with the CNS and the ENS. The ENS serves as intestinal barrier and regulates the major enteric processes such as the immune response, detecting nutrients, motility, microvascular circulation, and epithelial secretion of fluids, ions, and bioactive peptides (Nezami and Srinivasan, 2010). The ENS is capable of operating independently of the brain and spinal cord, but does not normally do so, as the CNS influences enteric behaviour while the gut also sends information to the brain (Rao and Gershon, 2016).

Ten to twenty per cent of all vagal fibres are efferent, which suggests that in this particular mechanism the brain is more of a receiver than a transmitter (Bonaz, Bazin, & Pellissier, 2018; Breit, Kupferberg, Rogler, & Hasler, 2018). The ENS has the ability to produce more than 30 neurotransmitters and has more neurons than the spine (Breit *et al.*, 2018). However, information sent from the bowel to the CNS is regulatory and may not reach consciousness. Despite this, studies attempting to stimulate the vagus in a way that also simulates afferent signalling from the bowel, have shown positive effects on depression and have demonstrated improved learning and memory in animals and humans (Bottomley *et al.*, 2020; George *et al.*, 2000; Rush *et al.*, 2000, 2005), meaning that gut signals can affect mood and behaviour. In addition, the vagus receives neuroactive signals from the microbiota which have also been found to affect mood, behaviour and health aspects (Bonaz, Bazin and Pellissier, 2018; Fülling, Dinan and Cryan, 2019).

According to Furness (2006), 100 million neurons are present in the human small intestine alone, thus making the ENS the largest division of the PNS and the largest collection of neurons and glia outside the brain, while virtually every CNS neurotransmitter is also found in the ENS (Furness, 2006). The neurons of the enteric nervous system control the motor functions of the system, in addition to the secretion of gastrointestinal enzymes. These neurons communicate through many neurotransmitters similar to the CNS, including acetylcholine, dopamine and serotonin. For all of the above, the ENS has frequently been called the “brain within the body” or “the second brain” (Gershon, 1999; Mayer, 2011).

The CNS, furthermore, has a significant role in monitoring the stomach condition and function by controlling its contractile activity and acid secretion through vago-vagal reflexes, whilst also controlling defecation. As such, the importance of coordination between ENS and CNS becomes evident.

This bidirectional communication network includes not only the CNS (both brain and spinal cord) and the ENS, but also the autonomic nervous system (ANS) and the HPA axis and is defined as the gut-brain axis (**Figure 1.2**). The gut-brain axis is responsible for monitoring physiological homeostasis and connecting the emotional and cognitive areas of the brain with the peripheral intestinal functions mentioned previously. To this effect, afferent signals arising from the lumen and transmitted though enteric, spinal and vagal pathways reach the CNS, and efferent signals from the CNS to the intestinal wall are transmitted via the autonomic system via the sympathetic and parasympathetic limbs (Carabotti *et al.*, 2015).

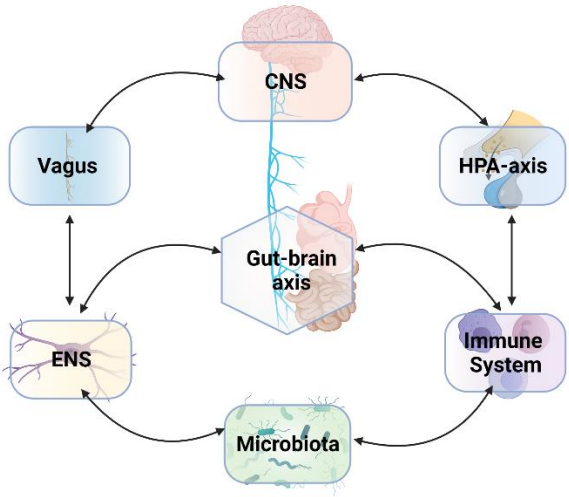


Figure 1.2 Representation of different factors modulating the gut-brain axis. The microbiota and central nervous system (CNS) interact in a bidirectional relationship bridged by the gut-brain axis. This axis communicates and is affected by the immune system, enteric nervous system (ENS), hypothalamic-pituitary axis (HPA), and vagus nerve. Image created with BioRender.com

The HPA axis is considered the core stress efferent axis that coordinates adaptive responses to any kind of stressor. It is a part of the limbic system, which is a crucial zone of the brain, predominantly involved in memory and emotional responses by receiving associational information from subcortical and cortical areas (Godoy *et al.*, 2018). Activation of the HPA and hormone release means that both neural and hormonal pathways are combined to allow the brain to influence the activities of intestinal functional effector cells, such as immune cells, epithelial cells, enteric neurons, smooth muscle cells, the interstitial cells of Cajal and enterochromaffin cells (Carabotti *et al.*, 2015; Breit *et al.*, 2018). These cells have a multitude of neuroreceptors and may produce organoids potentially influencing motility and health

aspects (Zhou, O'Connor and Ho, 2017), and are also under the influence of the gut microbiota (Carabotti *et al.*, 2015).

Overall, there are four major information carriers mediating communication between the gut and the brain as described by Holzer and Farzi (2014): 1) neural messages carried by vagal and spinal afferent neurons, 2) immune messages carried by cytokines, 3) endocrine messages transported by gut hormones and 4) microbial factors that may reach the brain via the bloodstream or interact with the other three transmission pathways. Each communication pathway involves a number of neuropeptides and signalling molecules, which operate as transmitters in the enteric, peripheral and central nervous system, sharing transduction mechanisms with other biologically active peptides such as hormones (Holzer and Farzi, 2014).

A few of the most important molecules produced within the gut which have been studied in depth due to their significant effect, whether localised on the ENS or distal, are: neuropeptide Y (NPY), Substance P (SP), gamma-aminobutyric acid (GABA: regulating fearfulness and anxiety), calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase-activating polypeptide (PACAP) and corticotropin-releasing hormone (CRH). Tryptophan (an essential amino acid and precursor to serotonin), is a key neurotransmitter linking the enteric and central nervous systems and has been thoroughly studied (Holzer, Reichmann and Farzi, 2012; Clarke *et al.*, 2014; Holzer and Farzi, 2014; O'Mahony *et al.*, 2015; Bliss and Whiteside, 2018; Fukui, Xu and Miwa, 2018; Aresti Sanz and El Aidy, 2019; Wei, Keller and Li, 2020). Other hormones that are under the influence of gut "messages" are glucagon-like peptide-1, peptide YY, ghrelin, and leptin (Smitka *et al.*, 2013; Clarke *et al.*, 2014). All of the above are produced or co-produced by the gut microbiome, which can be considered in a sense an endocrine organ (Clarke *et al.*, 2014).

1.3.1 Rumen role and importance

As this study focuses on sheep, a ruminant species, it is important to consider the function and significance of the rumen. Ruminants have three pre-stomachs: the rumen, the reticulum and the omasum, as well as the abomasum, which is functionally comparable to a monogastric stomach. The rumen, located in the centre-left abdominal cavity, is the largest of these compartments and consists of several sacs. Depending on size and breed, in cows the rumen can hold over 100 litres, while in sheep and goats it can hold up to 15 litres of material. The rumen acts as a storage and fermentation site. It serves a complex anaerobic microbial ecosystem, where a large number of microorganisms, mainly bacteria, archaea, protozoa, but

also fungi and viruses interact, and influencing digestion of feed, the metabolism and overall health of the ruminant.

The ruminant host's ability to digest fibrous feeds such as hay and grasses, which are particularly abundant in cellulose, depends on its co-evolved microbiome. This alliance has been investigated in order to define the relationship between ruminant genetics and physiology parameters on the rumen microbiome structure, composition, expression and function.

1.3.1.1 Rumen microbiome

The rumen is often described as a dynamic ecosystem composed of mainly anaerobic bacteria, protozoa, anaerobic fungi, methanogenic archaea and phages. All these microorganisms interact with each other either in antagonistic or symbiotic ways and have a symbiotic relationship with the host (Huws *et al.*, 2018). They have the significant role of providing energy by degrading plant cell wall carbohydrates, whislt many specialise in terms of nutrient utilisation. These microbes manage the rumen ecosystem by regulating subsequent microbial colonisation and nutrient utilisation (Atima, Pereira and Berry, 2017; Huws *et al.*, 2018; Shaani *et al.*, 2018). Bacteria are the predominant microorganisms in the rumen. Bioinformatic analysis of the sequencing results from rumen digesta DNA samples revealed that the bacterial sequences mainly correspond to four phyla: *Bacteroidetes*, *Firmicutes*, *Fibrobacteres*, and *Proteobacteria* (Lee *et al.*, 2012). All these microorganisms comprise the rumen microbiome, which is defined as the microorganisms and genetic material within this environment.

Very briefly, archaea in the rumen mainly consist of methanogenic Euryarchaeota (Yáñez-Ruiz *et al.*, 2010). These methanogens are responsible for methane production in the rumen, serving in elimination of fermentative hydrogen from the rumen to the environment (Hook, Wright and McBride, 2010). The phageome has recently been sequenced (Ross *et al.*, 2013; Huws *et al.*, 2018; Gilbert *et al.*, 2020), but its definitive role remains unknown. Protozoa due to large cell volume represent the largest proportion of the microbial biomass in the rumen and with the fungi consist the eykaryotic part of the microbiome. Their role is controversial and processes such as defaunation have attempted to explore their role in methanogenesis (Ogimoto and Imai, 1981; Newbold *et al.*, 2015; Tapio *et al.*, 2017). Finally, anaerobic fungi are extremely potent fiber-degrading organisms due to their efficient and extensive set of enzymes for the degradation of plant structural polymers (Solomon *et al.*, 2016). Rumen fungi also possess amylolytic and proteolytic activity (Gruninger *et al.*, no date; Yanke *et al.*, 1993; Belanche *et al.*, 2019; Hess *et al.*, 2020) and have been shown to improve feed intake, feed

digestibility, feed efficiency, daily weight gain and milk production (Saxena *et al.*, 2010; Dias *et al.*, 2017; Huws *et al.*, 2018; Elghandour *et al.*, 2020; Zhang *et al.*, 2020).

The rumen microbiome has the unique ability to enzymatically deconstruct and ferment plant biomass, converting it into end products such as VFAs and protein, which the host can then use for maintenance, growth, and lactation purposes. However, changes influencing the rumen microbiome can also result in several negative effects, including production of methane, biohydrogenation of fatty acids, and degradation of dietary protein, which can alter performance of farm animals and product quality. The rumen microbiome may also play a role in animal health and welfare, not only via its digestive purpose but also through beneficial host-microbiome interactions via peptides such as microcin. Genes relative to the production of these peptides were identified in a study by Auffret *et al.* (2017), investigating the role of diet in modulating rumen microbiome, which may play a role in antimicrobial resistance. Furthermore, in weaned animals, digestive disorders such as acute acidosis and sub-acute rumen acidosis (SARA) lead to changes in the rumen microbiota composition and in the animals' behaviour (Commun *et al.*, 2012).

As in the case of the intestinal microbiome, there is evidence that the rumen microbial community is not only controlled by the rumen environment and dietary factors, but that it is also genetically regulated. Proof of this emerged when Weimer *et al.* (2010) exchanged the rumen contents of two cows and the microbial communities reverted close to pre-exchange values within a few weeks, demonstrating a high level of host control over the species composition of the rumen microbiome. This shift was also associated with rumen pH and VFA concentrations, which reverted to their original values within 24h. Again according to Weimer (2015), the stability and host specificity of the rumen community are a hindrance in the manipulation of the bacterial structure in order to improve performance and reduce methanogenesis.

Feed conversion genes from a metagenomic experiment, which focused on two breed types and two diets (72 steers), were found to be associated with host-microbiome cross talk genes (e.g. TSTA3 and FucI), indicating further links between host genetics and the rumen microbiome (Roehe *et al.*, 2016a). Furthermore, a study on 709 beef cattle found that 34% of the microbial taxa and the total copy number of bacteria had moderate heritability $h^2 \geq 0.15$. Fan *et al.* (2020) demonstrated that genetics not only have a direct effect on the rumen microbiome but can also influence it in an indirect manner via microbe-microbe interactions, which were investigated using co-occurrence network analysis. Finally in sheep, Morgavi *et al.* (2015*)* highlighted the influence of gut microbes on animal phenotypes by correlating

microbial community parameters in lambs with fermentation parameters, digestibility and growth rate. A different study linked wool phenotypes with differences in rumen bacterial diversity, as diversity indices and presence/abundance of certain bacteria belonging to the *Firmicutes* and *Bacteroidetes* phyla were significantly different (De Barbieri *et al.*, 2015).

However, an extensive study conducted in rumen and camelid foregut microbial samples using 742 samples from 32 animal species and 35 countries investigated whether microbial composition was influenced by diet, host species, or geography. According to Henderson *et al.* (2015), differences in microbial community composition were diet driven, with the host being less influential. They also identified a few strong co-occurrence patterns between microbes, suggesting that major metabolic interactions are non-selective rather than specific. Similar bacteria and archaea dominated in nearly all samples (*Prevotella*, *Butyrivibrio* and *Ruminococcus*, as well as unclassified *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidales* and *Clostridiales*) (Henderson *et al.*, 2015). The authors suggest that these might be considered a "core bacterial microbiome".

Numerous studies have explored the effect of diet on the rumen microbiome as a means to enhance productivity, immunity and potentially methane mitigation. In lactating Holstein cows, a grain or alfalfa pellet diet aiming to induce SARA indicated that the most significant shift during SARA was a decline in gram-negative *Bacteroidetes* organisms. Real-time PCR data also indicated that *Prevotella* members of the *Bacteroidetes* groups were also less abundant, while severe cases of grain-induced SARA were characterised by *Streptococcus bovis* and *Escherichia coli* (Khafipour *et al.*, 2009).

Bach *et al.* (2019), after supplementing dairy cows' diet with live yeast, explored the relationship between specific bacteria and production traits. Among their findings, live yeast enrichment increased the relative abundance of *Bacteroidetes* and *Lachnospiraceae* before calving, while *Streptococcus* genera abundance also increased 21 d after calving. In terms of correlations with production traits, *Gastranaerophilales* was the only order positively associated with milk yield, while several genera were positively correlated with feed efficiency. *Clostridiales* was the only genus negatively associated with feed efficiency. In the pre-calving period, a *Prevotella* genus and a *Ruminobacter* genus were also negatively correlated with dry matter intake (Bach *et al.*, 2019).

In terms of reducing methane emissions, a number of diets with variable fat, fibre, tannin and lipid concentration (Aboagye *et al*, 2019; Jeyanathan *et al*, 2019a; Richardson *et al*, 2019) have been explored, as well as the inclusion of seaweed (Roque *et al.*, 2019) and various other compounds (Saro *et al.*, 2019).

Kittelmann *et al.* (2014) demonstrated that differences in rumen microbial community structure are linked to high and low CH_4 emissions in sheep. The team analysed the bacterial community structures in 236 rumen samples from high- and low-CH_4 emitting sheep and identified three ruminotypes. Two of these were linked to significantly lower CH_4 yields. One of the low-CH_4 ruminotypes was associated with a significantly lower ruminal acetate to propionate ratio, while relative abundance of the propionate-producing *Quinella ovalis was high in these samples*. The high-CH_4 ruminotype had higher relative abundances of species known to form high quantities of hydrogen, such as *Ruminococcus, Lachnospiraceae, Catabacteriaceae, Coprococcus*, *Prevotella,* and *Alphaproteobacteria.* (Kittelmann *et al.*, 2014).

Early life intervention in lambs using garlic essential oil and linseed oil lead to persistent changes in the bacterial community structure of the rumen but had no effect on methanogenesis (Saro *et al*, 2018b). Earlier studies carried out by Abecia *et al.* (2014, 2013) in goats and Yáñez-Ruiz *et al*. (2010) in lambs had suggested it was possible to promote changes in the rumen microbiota at an early age by manipulating the diet (i.e. forage versus concentrate at an early life led to persistent changes of up to 4 months).

As in the case of the gut microbiota described above, sex, age and environment have also been investigated and found to contribute in rumen microbial diversity and taxonomic aspects (Cunningham, Austin and Cammack, 2018; Pitta *et al*, 2018; Wang *et al*, 2018; Li, Hitch, *et al*, 2019; O'Hara *et al*, 2020). In this study, we have focused on 3 aspects: 1) heritable traits and whether they are associated with gut bacteria in sheep, indicating that the animal's genetics may influence the bacterial profile; 2) maternal effects in the sense of PNS and environmental factors in the form of ELS; and 3) repeated stressors during early development.

1.3.2 Microbiome and pathways of communication with the gut-brain axis and immune system

For the aforementioned system of communication to work between the gut and the brain, the ENS and the CNS have to receive and transmit signals. This signalling is performed by neurotransmitters which may also be classified as hormones (depending on whether they have a distal effect or not, and their production location), or even smaller molecules (granines, chemokines and growth factors) and have a pleiotropic effect (Burbach, 2011).

These neurotransmitters are produced in the cell body of the neurons and are dependent on internal and external signalling. In recent years, the expression and synthesis of these neurotransmitters has been found to be under significant influence of the gut microbiota

(Clarke *et al.*, 2014; Holzer and Farzi, 2014; Strandwitz, 2018), which also manipulates these neurotransmitters and produce hundreds of neuroactive substances (Holzer and Farzi, 2014; Fur*ness et al,* 2014; O'Mahony *et al* , 2015; O'Callaghan *et al* , 2016; Strandwitz, 2018).

1.3.3 Microbiome and microbiota definitions

The gut microbiome is defined as the microorganisms (bacteria, viruses, protozoa, and fungi) and their collective genetic material, present in the gastrointestinal tract and surrounding environment (Lederberg and McCray, 2001). The microbiota has come to be more accepted as the collection of microorganisms in a specific location, although frequently these definitions are used in an interchangeable manner (Marchesi and Ravel, 2015). The gut microbiota is comprised in the greatest part by commensal, opportunistic and pathogenic bacteria that reside in the gastrointestinal tract and differ in abundance depending on the location.

The "gold standard" definition of a "healthy" microbiome has been pursued for almost a decade in humans with the Human Microbiome Project (Lloyd-Price *et al.*, 2017; Proctor *et al.*, 2019), with research in other species, including ruminants, also progressing in the same direction. The notion of a "core" set of taxa that is present in healthy individuals and whose absence could indicate dysbiosis is now challenged, as numerous studies conducted on healthy individuals with no underlying clinical conditions revealed high levels of variability in terms of taxonomic composition, rendering this theory unlikely.

According to Lloyd-Price, Abu-Ali and Huttenhower (2016), the presence of a healthy "functional core" is a more likely hypothesis, where metabolic and molecular functions allow the microbiome to communicate efficiently with the host, resist stress and various perturbations, while being able to adapt and recover to a healthy, functional profile. Dysbiosis, on the other hand, can be characterised by the dominance of harmful microbes or an abrupt shift in the taxonomic abundances present which may lead to different pathologies (Messer and Chang, 2018). In ruminants and camelids, Henderson *et al*. (2015), identified a core microbiome as bacterial species were more consistently observed in varying abundances. They hypothesised that evolutionary pressures led most ruminant species to evolve with similar microbial communities in order to be more efficient.

1.3.4 Factors that influence the microbial community structure

The microbiome is influenced by many factors. Most important are genetics, diet, host health, medications and external stressors – both psychological and physical.

1.3.4.1 Genetics

With regards to the influence genetics play in the microbial structure of the gut, Zoetendal *et al.*, 2001, studying the faecal 16S rRNA profiles of monozygotic human twins, found that they were much more similar than the profiles of unrelated individuals or marital partners. Further research, extensively discussed by Goodrich *et al.* (2016), confirmed the effect genetics plays in shaping the microbiome, and identified correlations between heritable taxa and genes related to diet, metabolism and olfaction.

Similar conclusions were drawn for rodents, where environmental factors were found to exert temporary and reversible effects on the intestinal microbiome. This study was conducted by analysing the microbiota profiles of two mouse strains, C57BL/6J and BALB/c, which were then crossed. Korach-Rechtman *et al.* (2019) then compared the hybrids to one another and the parent lines. Twelve bacterial taxa which were tracked across generations for dominant or recessive inheritance, were found to be under direct genetic influence (Korach-Rechtman *et al.*, 2019). Furthermore, the microbiome of rats genetically predisposed to obesity was significantly different in terms of composition compared to lean animals, so could have specific metabolic phenotypes linked to certain microbial profiles (Waldram *et al.*, 2009).

In farm animals, significant findings on the influence genetics plays on the microbiome were presented in multiple studies for pigs. In a study conducted with 207 pigs that were housed and slaughtered under standardized conditions, the animals were phenotyped for daily liveweight gain, feed intake, and feed conversion rate, and then categorised into High Residual Feed Intake animals (HRFI) and Low Residual Feed Intake animals (LRFI). After sequencing faecal samples, 26 bacterial genera including *Bacteroides*, *Clostridium*, *Oscillibacter*, *Paludibacter*, *Elusimicrobium*, *Bilophila*, *Pyramidobacter*, *TM7* genera, and *Clostridium* clusters were more abundant in LRFI pigs compared to HRFI pigs. Adaptation of the microbiota to a new diet after weaning was also slower in LRFI compared to HRFI pigs, highlighting that RFI profiles which consist of a highly heritable trait, is linked with particular bacterial profiles, influencing animal metabolism (Kubasova *et al.*, 2018). Furthermore, a study conducted by Crespo-Piazuelo *et al.* (2019) identified 39 candidate genes which may be affecting microbial composition and found associations between host genome and gut microbiota in pigs.

Furthermore, Kraimi *et al.* (2019) performed a microbiome transfer of bacteria present in quail lines selected for a high emotional reactivity (E+) and low emotional reactivity (E−) to Germ Free (GF) quails. The bacterial composition of both groups revealed a shift in terms of microbial diversity and richness earlier on, which as time progressed became less pronounced.

Moreover, quails colonised with E− bacteria expressed a lower emotional reactivity at the second week of age, although E+ animals had lower emotional reactivity when behavioural tests were carried out at 4 weeks of age (Kraimi *et al.*, 2019), proving that temperament (a heritable trait) affects the microbiome and HPA axis in bird species.

In cows, host genetics appear to significantly contribute towards shaping the gut microbiota during early life stages (Fan *et al.*, 2020), while Chen *et al.* (2018) also identified 81 and 67 microbial taxa with heritability (h^2) > 0.15 in faecal and cecum luminal samples respectively, as well as 31 taxa with h^2 >0.15 in both types of samples. The researchers also identified significant associations between host genomic loci and the abundance, as well as presence or absence, of certain bacterial taxa in the matrices explored (C. Chen *et al.*, 2018). There have been no publications to date highlighting the influence of genetics on the gut microbiota of sheep, as most studies in ruminants explore the effect of genetics on the rumen microbiome.

1.3.4.2 Diet

Diet irrefutably influences and shapes the gastrointestinal microbiome, and a multitude of studies provide evidence of this in humans, rodents, farm animals including ruminants and even non-mammalian species. In humans, the infant gut microbiota is unstable and reaches a state similar to that of adults at around 3 years of age, which coincides with the establishment of a reasonably variable solid food diet (Voreades, Kozil and Weir, 2014). In adults, according to Voreades *et al.* (2014), several studies have shown that dietary changes induce fluctuations in as little as 24h, but that the community can quickly return to a previous "stable" state.

Diet in humans can affect how the microbiome is shaped during early life and have significant long term effects which may result in food sensitivity, allergic reactions, diabetes and autoimmune disorders (Kelly, King and Aminov, 2007). During adulthood, the dietary choices we make can affect regulation of weight and appetite control, whereas particular diet-influenced bacterial profiles have been linked to IBS, obesity and cancer (Riaz Rajoka *et al.*, 2017; Zmora, Suez and Elinav, 2019). Similar findings have been reported in rodents (Hildebrandt *et al.*, 2009; Jakobsdottir *et al.*, 2013; Siddharth, Holway and Parkinson, 2013; Everard *et al.*, 2014).

In poultry, wheat, barley or rye-based diets which consist of high proportions of indigestible polysaccharides or diets with high animal protein content (fishmeal) can work in favour of the proliferation of *C. perfringens*, the presence of which has been shown to predispose young chicks to necrotic enteritis (Pan and Yu, 2013). On the other hand, additives frequently used in poultry diets such as xylanase have been shown to reduce the abundance of pathogens such

as *E. coli* (Engberg *et al.*, 2004), as do corn-based diets compared to wheat-based ones (Rodríguez *et al.*, 2012). In pigs, early life diet appears to have a significant effect as Frese *et al.* (2015) sampled piglets from the day of birth to the age of 7 weeks and observed clearly distinguishable profiles based on diet treatments.

These findings have been verified for fish as diets containing guar gum, a non-starch polysaccharide, fed to mullet (*Mugil liza) (Ramos et al., 2015)* or soy proteins to rainbow trout (Bruce, Neiger and Brown, 2018) lead to shifts in bacterial abundance and composition in the GIT. Parata *et al.* (2020) described the presence of a core microbiome across the GI tract and diet samples of the convict surgeonfish (*Acanthurus triostegus*), a species with an important role in coral reef ecology. This suggests that these bacteria can be acquired at an early age via the diet, and are retained through adulthood (Parata *et al.*, 2020).

In ruminants, the effects of diet on the microbiome has been investigated in order to improve productivity or reduce methane production via modulation of the microbial populations, but unsurprisingly most studies focus on the effect of diet on the rumen.

Numerous studies have been carried out to investigate the effects of various intervantions on the microbial composition, such as the use of high quality forages or concentrates, modification of forage: concentrate, the addition of essential oils or red clover, yeast, antibiotics, tannins, saponins or bicarbonate (Detailed table: Henderson *et al.*, 2015).

However, there have been a few studies that have explored dietary effects on the microbial composition of other GI locations. The progression of bacterial colonisation of the different segments of the GIT in young goats including the rumen, duodenum, jejunum, ileum, caecum, and colon were investigated by Li, Zhang, *et al.*, (2019), showing that the greatest increase in microbial diversity occurred between 14 and 28 days of age.Additionally, the intestinal microbiota was less sensitive to the introduction of solid feeds compared to the rumen (B. Li *et al.*, 2019).Cui *et al.*, (2020), showed that milk replacer supplemented with alfalfa hay and starter feeding in comparison to alfalfa supplementation, plain starter, or un-supplemented, during the pre-weaning period, enhances rumen microbiological and functional development (i.e., higher bacterial diversity, VFA concentration), intestinal activity and immune function (i.e., higher α-amylase, trypsin, IL-1β, TNF-α, and IFN-β) in yak calves. According to the authors, the availability of different carbon and nitrogen sources from fibrous and non-fibrous carbohydrates benefits GI microbial colonisation (papillae development and fermentation) and intestine anatomical development (villus and crypt) leading overall, to an enhanced animal growth (Cui *et al.*, 2020).

A study conducted on Mongolian sheep where two feeding regimens were followed [free grazing (FG) and barn confinement (BC)], demonstrated that the FG group showed higher levels of *Bacteroides, RC9_gut_group, Alistipes, Phocaeicola, Barnesiella*, and *Oscillibacter*, and lower levels of *Succinivibrio, Treponema*, and *Prevotella*, compared to the BC group (Wang *et al.*, 2020). Additionally, significant associations were observed between several gut microbiota genera and alterations in faecel and plasma metabolites especially those involved in the metabolism of butyric acid, linolenic acid, and L-tyrosine, allowing the authors to hypothesise that feeding regimens influence the composition of gut microbiota and may alter metabolic homeostasis in sheep (Wang *et al.*, 2020).

Diet influences were also observed on intestinal bacteria in young beef cattle after weaning (Liu *et al.*, 2020). A significantly higher level of microbial diversity was documented in feces of grass-fed cattle comparing to grain-fed cattle. Twenty top genera identified with random forest analysis on fecal bacterial community were also assessed as good candidates for microbial biomarkers. The authors also observed that the jejunal bacteria of adult Angus beef cattle exhibited significant differences in microbial composition and metabolic potential under different diets (Liu *et al.*, 2020).

Due to the significant modulatory effect, diet has on the microbiome, it was important in this study to consider it either as factor when diet was not consistent across all animals included in the study, or select animals managed in a way where the effect of diet would be homogenous.

1.3.4.3 Age, sex and health status

The microbial communities present in most locations and organs are ever changing, adapting to hormonal cues and signals sent and received from the ENS, CNS, the immune system and bacteria. The microbial profile of young individuals is defined by low diversity, and as diet is enriched and the individual is exposed to more diverse environmental influences, the microbiota evolves (Langille *et al.*, 2014; Nagpal *et al.*, 2018; de la Cuesta-Zuluaga *et al.*, 2019). Interestingly, the microbiota present can be taxonomically divergent between individuals, which may highlight the early influence of genetics, but then tend to become more homogenous, albeit more abundant (Langille *et al.*, 2014; Nagpal *et al.*, 2018).

Individuals of certain age groups tend to have more similar microbial composition, particularly within certain conditions, whether that is the effect of genetics, a household or diet effect and country effect in humans, and breed and management in animals (Kostic, Howitt and Garrett, 2013; Gupta, Paul and Dutta, 2017; Turner, 2018). There is a theoretical peak in diversity, which often coincides with the peak of physiological ability and health. However, as the

individual ages, microbial diversity declines, something linked with several pathologies more frequently observed in older ages (Karl *et al.*, 2018; Nagpal *et al.*, 2018). This may be related to physiology or the more frequent use of medications and particularly antibiotics.

Antibiotics cause significant disturbances in gut microbiota whether they are beneficial or pathogenic species, permitting proliferation of antibiotic-resistant strains. When the antibiotic ciprofloxacin was given to young healthy volunteers, a significant community structure alteration of the gut microbiome was observed. The microbial structure then shifted to an alternative stable state, the consequences of which were not determined followed by the return to an alternative stable state of undetermined consequences (Dethlefsen and Relman, 2011). In addition, the use of broad-spectrum antibiotics (ampicillin, tetracycline or clarithromycin) has been linked to higher abundance of the opportunistic bacterium *Clostridium difficile*, which can result in severe diarrhoea and colitis (Macfarlane, 2014).

In a study conducted on the association between "inflammaging" and the microbiome in dairy cattle across six farms, old cows suffered from long-term and low-level chronic inflammation, and the gut microbiota genera belonging to *Prevotellaceae* and *Lachnospiraceae* were present in reduced abundances compared to younger cows. Furthermore, beneficial bacteria like *Bacteroidaceae*, *Eubacterium*, and *Bifidobacterium* were less abundant in faeces from the older group, while function related to carbohydrate and lipid metabolism was also affected (G. Zhang *et al.*, 2019). Conversely, age differences were not observed between kids and adult goats in terms of the GI microbiome, though health status (diarrhoeic kids versus healthy kids) did affect the composition of bacteria (Wang *et al.*, 2018).

Males have been shown to be more susceptible to infections (Klein, 2000; Fish, 2008; Ingersoll, 2017), while females have higher occurrence of autoimmune disorders (Fairweather, Frisancho-Kiss and Rose, 2008). This could be linked to different levels of sex steroids (testosterone, progesterone, oestradiol, etc.), which in turn affects the immune system by binding to specific cell receptors, or due to inherent genetic differences, since the X chromosome contains most genes relating to the immune system (Libert, Dejager and Pinheiro, 2010; vom Steeg and Klein, 2016). As the communication between the immune system and the microbiome depends on these mechanisms, microbial differences according to sex have been proven (Kim *et al.*, 2020). Studies in mice showed that female-biased autoimmune disorders may be significantly influenced by sex-dependent differences in the gut microbiome (Yurkovetskiy *et al.*, 2013) and that faecal transfer of bacteria from male mice to females could delay the onset and reduce the severity of the disorder (Markle *et al.*, 2013).

In addition, sex-specific changes in the composition of the gut microbiome can be induced via environmental factors, behavioural differences and stress responsiveness (Cryan and Dinan, 2012; Jašarević, Morrison and Bale, 2016; Kim *et al.*, 2020). These shifts can in turn influence physiological responses, such as metabolism and immunity as well as behaviour (Markle *et al.*, 2013; Jašarević, Morrison and Bale, 2016; Moloney *et al.*, 2016; Fields *et al.*, 2018).

1.3.4.4 The environment

Studies mainly conducted in humans have demonstrated that individuals who live in close proximity have less variation compared to random individuals (Schloss *et al.*, 2014). Cage effects have also been reported in lab animals (McCafferty *et al.*, 2013). Particularly, Ericsson et al. (2018) showed that benign management practices can interact, introducing significant changes in the microbial composition of the GI tract and particularly the cecum of rats. This can have a significant effect when considering reproducibility for experiments.

The effect of bedding and environment is particularly important for bird species, as chickens are coprophagic and cage effect can affect the composition of the GI microbiota due to ingestion of feathers and debris (Meyer *et al.*, 2012). The effect of environment actually appears to be more influential than genetic profiles in birds, as further suggested in a penguin study (Barbosa *et al.*, 2016).

Similarly, animal density and temperature can affect the GI microbiome. Many studies with poultry have demonstrated significant changes in performance and the microbiota composition were observed under the influence of heat stress (Sohail *et al.*, 2015; Jun He *et al.*, 2019; Shi *et al.*, 2019). Specifically, Shi *et al*. (2019) found that heat stress reduced average daily liveweight gain and feed intake and increased the feed conversion ratio. Serum cortisol levels were significantly higher compared to non-stressed animals. Taxonomic differences were observed as *Firmicutes, Tenericutes* and *Proteobacteria* were higher in the heat stress group, while *Bacteroidetes* and *Cyanobacteria* were significantly higher in their control group. Further differences were observed at a genus level (Shi *et al.*, 2019).

Heat stress reduces intestinal barrier functions and increases intestinal permeability in pigs (particularly favouring glucose transport) (Pearce *et al.*, 2013), as well as affecting the gut microbial community (Jianwen He *et al.*, 2019; Le Sciellour *et al.*, 2019). Pigs submitted to heat stress had significantly higher relative abundance of *Clostridiales* and *Halomonas* and lower abundance of the genera *Bacteroidales* and *Streptococcus*. As heat stress has been shown to impair gut permeability and affect immune cell profiles in cows (Koch *et al.*, 2019), it is not unlikely that the microbiota composition would differ too.

Ruminants' response to heat stress involves reduced uptake of dry matter in feed to reduce the metabolic production of heat and maintain a constant temperature. Concentrated feed is then favoured over roughages (Khafipour *et al.*, 2016). These changes can lead to acidosis, in which case a decrease in the rumen pH (about: 6.8–6.5) may be observed and abundances of different bacteria will shift (Khafipour *et al.*, 2016). Such examples include *Fibrobacter* and *Oscillospira* , while *Clostridium coccoides* and *Streptococcus*/*Lactococcus* genera increase (Mizrahi and Jami, 2018; Cholewińska, Górniak and Wojnarowski, 2021). This has a subsequent result the decrease of production of short chain fatty acids and acetate, while propionate and lactate increase (Lettat *et al.*, 2010).

Humidity is an additional factor that affects pH, as higher levels lead to more significant pH drops. Water consumption also usually increases due to heat stress, which may lead to slowdown of flow of food content increasing acidity (Baumgard *et al.*, 2016; Contreras-Jodar *et al.*, 2019; Cholewińska, Górniak and Wojnarowski, 2021). Contreras-Jodar *et al*. (2019), demonstrated that heat stress affcts microbiome homeostasis and milk yields of goats (Contreras-Jodar *et al.*, 2019), while microbiome diversity of the digestive system of cows and production rates were also found significantly affected by Tajima *et al.* (2007). Specifically related to the microbiome, an increase in the level of the *Bacteroidetes, Spirochetes* phylum and a decrease in the level of *Firmicutes* were observed and more prominently in younger animals (Tajima *et al.*, 2007)

In terms of other environmental influences, Fonty *et al.* observed an increase in cellulolytic bacteria in lambs kept in a group compared to animals kept in single pens despite diet and overall environment being the same (Fonty *et al.*, 1987).

1.3.5 Functions of the microbiome

1.3.5.1 Digestive role of the microbiome

For decades, the microbiome has been known to play a significant role in nutrient and mineral absorption, synthesis of enzymes, vitamins and amino acids, and production of VFAs. The main VFAs produced as a result of carbohydrate and protein fermentation are acetate, propionate, and butyrate which play a crucial role in gut health in humans (Jandhyala *et al.*, 2015; Rowland *et al.*, 2018; Valdes *et al.*, 2018), monogastrics (Murphy *et al.*, 2010; Semova *et al.*, 2012; Hanning and Diaz-Sanchez, 2015; Jandhyala *et al.*, 2015; Shang *et al.*, 2018; Jha *et al.*, 2019) and ruminants (Bergman, 1990; Mao, Huo and Zhu, 2016; Shen *et al.*, 2017). With these processes, the microbiome provides energy for epithelial cells, enhances epithelial barrier integrity, and provides immunomodulation and protection against pathogens (Cresci

and Izzo, 2019). More specifically, related to butyrate, Schroeder *et al.* (2007) suggested that sodium butyrate has an effect on brain-derived neurotropic factor (BDNF) and can induce antidepressant-like effects in mice. Finally in bovine cells, butyrate induces the expression of genes associated with cell growth, signal transmission and the immune response (Li, Elsasser and Li, 2008). In ruminants VFAs are produced in large amounts mainly in the rumen via ruminal fermentation, providing greater than 70% of the ruminant's energy, influencing production and product composition in ruminants. The relative proportions in which VFAs are produced, are influenced by a number of factors, including diet (substrate) composition and availability, rate of depolymerization, and microbial community structure (van Houtert, 1993; Dijkstra, 1994). Acetate is the end product of fibre fermentation, meaning that fibrous diets, low in energy such as pasture and forages may lead to bacterial composition that favours an increased ration of acetate: propionate. Acetate is a crucial component for the production of ATP (Bergman, 1990; van Houtert, 1993; Dijkstra, 1994; Bhatia and Yang, 2017). Another important use of acetate is as the major source of acetyl CoA for synthesis of lipids and is therefore crucial for the production of milk fat (Bergman, 1990; van Houtert, 1993; Dijkstra, 1994; Bhatia and Yang, 2017).

Propionate is the end result of carbohydrate fermentation (starch, sugars) and essentially acts as a “building block” for all energy requirements for live weight gain and mammary gland needs, in order to produce lactose. Diets rich in fermentable carbohydrates favour a microbial community that produces more propionate and butyrate (Bergman, 1990; van Houtert, 1993; Dijkstra, 1994; Bhatia and Yang, 2017).

Acetate, proprionate and butyric acids are diffused through the ruminal epithelium, into ruminal veins, to the portal vein and finally the liver (Masson and Phillipson, 1951). Butyrate is metabolised in the epithelium to beta-hydroxybutyric acid, a type of ketone body, before further metabolism in the liver. Ketones which act as a source of energy for further fatty acid synthesis and participates in mobilisation of fat. In the liver, proprionate acts as a substrate for gluconeogenesis (Bergman, 1990; van Houtert, 1993; Dijkstra, 1994; Bhatia and Yang, 2017).

VFA overproduction can lead to pH drop of rumen fluid, therefore circulation of VFAs is crucial (Uribe *et al.*, 1994). This depends on papillae formation, animal age, genetics and health status of the animal (Lane and Jesse, 1997; Diao, Zhang and Fu, 2019).

1.3.5.2 Microbiota influences on the immune system

Novel functions of the microbiota have come to light and have been explored in the past decade. These involve the influence the microbiota has on regulation of the immune system and how it can communicate with the brain utilising the gut-brain pathways available.

The gut microbiota can directly interact with the gastrointestinal immune system via the gastrointestinal mucosa. It has been reported that bacteria can "train" the immune system whilst allowing the host to distinguish commensal and pathogenic bacteria (Arrieta and Finlay, 2012). They can also regulate the immune response in the eukaryotic host cells including the inflammatory process via the nuclear factor-kappaB (NF-κB) pathway (Neish *et al.*, 2000). NF-κB is a transcriptional regulator that migrates to the nucleus to induce inflammatory cytokines and recruit immune cells, a process that only occurs when NF-κB is unbound from IκB. This process can be blocked by commensal bacteria, so NF-κB cannot enter the nucleus to begin the inflammatory response (Hanning and Diaz-Sanchez, 2015). Identifying these bacteria and their products could be useful for treating inflammatory-based diseases (Neish *et al.*, 2000).

As mentioned previously, the microbiota does not only communicate indirectly with the immune system and its immediate gastrointestinal epithelium but can signal the CNS and distant organs via the production of a vast array of molecules. The study of these molecules and the ability of microorganisms to produce and recognise neurochemicals that are produced by them, or that originate from the host they inhabit, involves a combined examination of microbiology, neurobiology and immunology and is defined as microbial endocrinology.

The innate immune system can be influenced via microbe-associated molecular patterns (MAMPs), lipopolysaccharide (LPS) and peptidoglycan components such as meso-diaminopimelic acid, which are produced by bacteria. LPS can activate particular classes of Toll-like receptors (Unsal and Balkay, 2012; Pandey, Kawai and Akira, 2015) while the peptidoglycan structures, present in most bacterial cell walls, stimulate nucleotide-binding oligomerization domain–containing protein-1 (Nod1) and/or Nod2 proteins, a process which initiates intracellular signalling that causes the cell to produce TNF, IL-1, IL-18 and/or IFNα/β (Tak, Saunders and Jett, 2014). NOD proteins are the first free cytoplasmic PRRs identified as detecting PAMPs of intracellular pathogens once the host cell's interior has been compromised. In addition, translocation of peptidoglycans from the gut to the blood impacts on neutrophils in the bone marrow and enhances their capacity to defend the body against bacterial infection via stimulating Nod1 (Clarke *et al.*, 2010).

Furthermore, LPS translocated from the gut carries microbial messages to distant organs and the brain. The behavioural responses to systemic exposure to excess LPS in animals and humans include acute sickness (Dantzer *et al.*, 2008) and delayed depression-like behaviour (McCusker and Kelley, 2013). LPS originating from the gut microbiota may give rise to alterations in brain function via different pathways. Following translocation across the intestinal mucosa, it may on the one hand stimulate the intestinal immune system to produce cytokines which in turn can signal directly to the brain or sensitise/stimulate vagal and spinal afferent neurons (Holzer, 2008; Tarr *et al.*, 2012). On the other hand, the circulation may carry LPS itself to the central nervous system where it may modify brain function (Holzer and Farzi, 2014).

The latter is facilitated by the fact that TLRs are widely expressed at several levels of the gut-brain axis. TLRs, mainly expressed by macrophages in different tissues, are also importantly present on gastrointestinal epithelial cells (Abreu, 2010; Marques and Boneca, 2011), rumen epithelia (Arthanari *et al.*, 2010; Chen, Oba and Guan, 2012), neurons of the enteric nervous system (Barajon *et al.*, 2009; Anitha *et al.*, 2012), primary afferent neurons (Barajon *et al.*, 2009) and various cell types (neurons, microglial cells and astrocytes) in the brain (van Noort and Bsibsi, 2009; Arroyo *et al.*, 2011; Mallard, 2012). By stimulating the TLRs in the brain, LPS and other bacterial factors can stimulate the generation and release of proinflammatory cytokines and in this way give rise to neuro-inflammatory processes (Holzer and Farzi, 2014).

Specifically, increased levels of IgA and IgM against LPS of commensal gut bacteria are found in the circulation of patients with depression or chronic fatigue syndrome, and the hypothesis has been put forward that increased translocation of LPS across a leaky gut may be a factor that contributes to these pathologies (Maes *et al.*, 2012). Taking all of the above results into account, we could hypothesise that the physiological roles of the symbiotic gut microbiota relate not only to the regulation of digestion at the gastrointestinal level, but also extend to systemic immunity and brain function.

The presence of these mechanisms and the influence the microbiome may have on the rumen epithelial response via the production of peptides, via mucus production and reverse signalling to the brain by means of the nervous system are all important issues to be investigated. It is only logical that the same would apply for the rumen, taking into account how it is not only a digestive organ, but also in a sense an endocrine and immune organ due to its complexity (Xiang *et al*., 2016).

1.3.5.3 Microbial neurotransmitters, hormones and metabolites

In recent years, there has been extensive research on the communication pathways between the host and its microbiome in a number of tissues [stomach (Mattarelli *et al.*, 2014), colon (Daniel *et al.*, 2017; Mudd *et al.*, 2017a; Jang *et al.*, 2018), vagina (Aagaard *et al.*, 2012; Jašarević *et al.*, 2015), oral cavity (Takahashi and Yamada, 2000; Duran-Pinedo, Solbiati and Frias-Lopez, 2018), ileum (Kirimlioglu *et al.*, 2006; Cussotto *et al.*, 2019), rumen (Schären *et al.*, 2018; Lima *et al.*, 2019), gut (Heijtz *et al.*, 2011; Kelly *et al.*, 2015; Neuman, Justine W Debelius, *et al.*, 2015)] and in many species(Lettat *et al.*, 2010; Nicholson *et al.*, 2012; Morgavi *et al.*, 2015b; Wong *et al.*, 2016; Mao, Huo and Zhu, 2016; Schirmer *et al.*, 2016; Daniel *et al.*, 2017; Mudd *et al.*, 2017a; Rowland *et al.*, 2018; Lima *et al.*, 2019; Webster, Consuegra and Leaniz, 2020). The reason for this is that the microbiome appears to regulate and co-regulate many functions due to the production and co-production of peptides and hormones (Robinson, Bohannan and Young, 2010).

The first class of molecules of interest are those which are also produced and released by neurons acting as signalling molecules in the brain, participating in a great range of physiological functions. When looking at the link between gut microbiota and the brain, serotonin is of particular interest due to its role in regulation of sleep, learning, anxiety, mood and stress-related disorders (Evans, Morris and Marchesi, 2013). Tryptophan, an essential amino acid and precursor to many metabolites including serotonin, also presents a target of great interest. The gut microbiota appear to have a regulatory effect on serotonin availability in the circulation, as well as on the tryptophan-to-serotonin metabolic pathways in the ENS (Clarke *et al*, 2013; Desbonnet *et al* , 2014; Reigstad *et al*, 2015; Yano *et al*, 2015a; Agus, Planchais and Sokol, 2018; Kaur, Bose and Mande, 2019). Some bacteria such as *Candida spp*., *Strptococcus spp.*, *Escherichia spp*. and *Enterococcus spp*. also have the ability to produce serotonin, therefore potentially influencing the CNS and behaviour (Lyte, 2011, Yano *et al.*, 2015). One result of serotonin regulation from the microbiome is the management of glucose homeostasis (Martin *et al.*, 2019).

Other hormones produced by members of the genera *Lactobacillus*, *Escherichia* and *Saccharomyces* are dopamine and noradrenaline, while other members of *Lactobacillus* produce acetylocholine, and *Bacteroides*, *Lactobacilli* and *Bifidobactrerium* genera manufacture GABA (Dinan and Cryan, 2012; Holzer, Reichmann and Farzi, 2012; Nicholson *et al.*, 2012; Forsythe and Kunze, 2013; Strandwitz *et al.*, 2019).

Neuropeptides such as SP, calcitonin gene-related peptide, somatostatin and corticotropin-releasing factor may also play a role in the bidirectional gut-brain communication (Holzer and

Farzi, 2014). NPY is a neurotransmitter that may play an important role in the microbiota-gut axis as it has multiple implications in brain functions and is also involved in controlling inflammatory processes, pain, emotion, mood, cognition, stress resilience, ingestion and energy homeostasis (Holzer, Reichmann and Farzi, 2012).

In terms of signalling molecules, *Lactobacillus* strains have been shown to produce p-cresol (4-methylphenol) and skatole (Yokoyama and Carlson, 1981). A number of bacteria were also found to produce indolic compounds in the rumen of grazing ruminants (Attwood *et al.*, 2006). Skatole inhibits catalase activity which may lead to endogenous oxidative stress and increases in oxidative metabolites which might lead in turn to damaged cell surfaces (Choi *et al.*, 2014), whereas others have found potential links between skatole with schizophrenia and mental disorders (Nakao, 1960; Bested, Logan and Selhub, 2013). The presence or absence of intestinal microbiota can also significantly affect metabolite profiles present in the brain (Matsumoto *et al.*, 2013; Kaur, Bose and Mande, 2019)

Studies with rodents and rabbits have identified cross-talk between intestinal microbes and the host intestinal epithelium involving metabolism of fucose, a component of salivary and epithelial mucins (Pacheco *et al.*, 2012). Fucose is a component of innate immunity glycoproteins (mucins) produced by the intestinal mucosa and in saliva to help maintain the integrity of the mucosal barrier and is also highly abundant in the intestine. The degradation of mucins often requires enzymes from a range of bacteria, but some *Bacteroides* and *Ruminococcus spp*. are able to degrade mucins completely. For example *Bacteroides thetaiotaomicron* produces multiple fucosidases that cleave fucose from host glycans, resulting in high fucose availability in the mammalian gut lumen (Pacheco *et al.*, 2012).

In ruminants, Roehe *et al.* (2016) noted the abundance of GDP-L-fucose synthetase (TSTA3) and L-fucose isomerase (FucI) in the gut and rumen of cows, which may reveal the importance of host-microbe crosstalk in ruminants. These two genes related to feed conversion efficiency are involved in fucose metabolism. Metabolites identified in the bovine rumen are mainly degraded feed components such as amino acids, sugars, and organic acids (Donze *et al.*, 2004). Traditional methods used in animal nutrition studies can identify rumen metabolites including common amino acids, short-chain fatty acids, organic acids, purine and pyrimidine (Folman *et al.*, 1981).

1.4 Impact of stress

1.4.1 Effects of prenatal and early life stress

1.4.1.1 Effects on physiology, metabolism, immune system and behaviour

A significant number of studies have demonstrated that apart from genetic factors, environmental events acting prenatally on the developing foetus can determine offspring development and health (Archer and Blackman, 1971; Braastad, 1998; Fowden, Giussani and Forhead, 2006). These events, which include all factors capable of eliciting a stress response in pregnant mothers, are defined as prenatal stress (PNS) (Braastad 1998).

Prenatal stress effects have been studied extensively in humans, lab animals, farm animals and of interest to this study, in sheep. Maternal stress can lead to cardiovascular changes in the mother as well as endocrinological modifications which include serotonin, β-endorphin, glucocorticoid and catecholamine concentrations (Kapoor *et al.*, 2006). These are partially screened by the placenta although small concentrations do reach the foetus and can affect hormone production by the placenta or activate the foetal HPA axis.

One of the most significant effects of PNS is hyperactivity of the maternal HPA axis leading to prolonged or dysregulated stress responses in offspring (Clarke *et al.*, 1994; McCormick *et al.*, 1995; Braastad, 1998; Kapoor *et al.*, 2006). PNS alters HPA axis function and consequently the circulating concentration of glucocorticoids. Glucocorticoids have immunosuppressive properties (Munck and Guyre, 1991; Cain and Cidlowski, 2017), therefore the presence of links between PNS and alterations in immune function is not surprising (Coussons-Read, 2012; Christian, 2015). PNS has an inhibitory effect on the immunity of offspring (Merlot, Couret and Otten, 2008; Couret *et al.*, 2009; Veru *et al.*, 2014) affecting both passive and innate immunity.

Effects of PNS on glucocorticoid levels of offspring have mainly been studied in rodents, but several studies on farm animal species have identified changes in cortisol levels. For example, when sows were restrained and injected with ACTH weekly during mid gestation, the offspring showed an increase in cortisol levels and prolonged responses when social mixing was used as a stressor at 8 weeks of age (Haussmann *et al.*, 2000). Similarly, the use of maternal social stress during pregnancy resulted in female offspring exhibiting elevated salivary cortisol concentrations when social mixing was applied at 67 days of age (Jarvis *et al.*, 2006). In contrast, prenatal stress did not appear to affect cortisol levels in pregnant sows subjected to a daily restraint stress for five minutes during the last five weeks of gestation. Their offspring were tested for their endocrine reactions (days 3, 7, 21 and 35) using an

immobilization test and an ACTH challenge test, but despite cortisol levels appearing lower on day 3 of compared to the control group, the stress did not have effect on cortisol levels on any of the test days (Otten *et al.*, 2001).

A study conducted in cattle indicated that calves born to mothers submitted to transport stress early and mid-gestation had a slower decrease in cortisol levels over time compared to control calves when restraint was used as a stressor (Lay *et al.*, 1997). Furthermore, kids born to does exposed to repeated stressor of transport in isolation in the last weeks of gestation differed in terms of cortisol levels the first hour after birth, with the stressed animals exhibiting higher levels of cortisol compared to the control group. The opposite was observed 48h later, whereas no difference was observed 1 month after birth, although the HPA and SAM systems of the stressed animals were affected as indicated by medulla weight and higher phenylethanolamine N-methyl transferase activity (Duvaux-Ponter *et al.*, 2003). In contrast, when goats were stressed via isolated transport and ACTH injections during the last third of pregnancy, their offspring did not differ in basal cortisol concentrations. However, females exhibited higher arousal behaviour, while males were less active (Roussel *et al.*, 2005).

In sheep studies conducted using isolation stress twice a week during the last 5 weeks of gestation, the offspring showed elevated basal cortisol at 25 days of age, but did not differ from controls in the cortisol response to social isolation in the presence of a dog (Roussel *et al.*, 2004). A later study following the same stress protocol did not identify any significant differences in cortisol levels (Roussel-Huchette *et al.*, 2008).

Other than HPA effects on offspring hormone levels due to PNS, retardation of motor and cognitive development (Buitelaar *et al.*, 2003) has also been observed. This is due to the fact that brain plasticity is high (Bock *et al.*, 2015) and the HPA axis is particularly sensitive to glucocorticoids during intrauterine development (Howland, Sandman and Glynn, 2017). In precocious farm animal species, where the offspring is born mature and mobile early on, the major part of neuroendocrine development, particularly of the HPA axis, occurs whilst inside the uterus (Dobbing and Sands, 1979). As a consequence, stress applied during pregnancy may influence precocious animals in more significant ways in later life (Rooke *et al.*, 2015).

In rodent PNS studies, impaired cognitive performance has been associated with specific alterations in brain morphology (Mychasiuk, Gibb and Kolb, 2012; Semple *et al.*, 2013; McGowan and Matthews, 2018; Fatima *et al.*, 2019), while the same has been observed in sheep (Sadowska and Stonestreet, 2014; Petit *et al.*, 2015). These changes can result in learning deficits, an increase in anxiety related behaviours, reduced attention, altered immune function, as well as altered cardiovascular responses to stress in many species (Vallée *et al.*,

1997; Koehl *et al.*, 2001; Buitelaar *et al.*, 2003; Igosheva *et al.*, 2004; Bergman *et al.*, 2007; Weinstock, 2008; Sandman and Davis, 2010; Soares-Cunha *et al.*, 2018), including sheep (Roussel-Huchette *et al.*, 2008; Coulon *et al.*, 2015; Rooke *et al.*, 2015, 2017).

The type of stressor, the period in gestation when stress is applied, the intensity, animal species and susceptibility and finally individual responses affect PNS and its effects on progeny. Prenatal stress can not only affect offspring in direct ways but also indirectly as how mothers are affected by stress can influence the expression of maternal behaviours such as interest in offspring and willingness to nurse or aggressiveness, as the lack of warmth and colostrum could be detrimental during the first hours of life (Dwyer, 2014; Rooke *et al.*, 2015). Finally, colostrum quality has been shown to be affected by maternal stress in many species (Merlot, Quesnel and Prunier, 2013). As such, the effect of PNS in farm animals is not negligible and can have serious implications in terms of financial loss and animal welfare.

1.4.1.2 Prenatal stress effects on the microbiome

There are several ways by which maternal stress can affect the microbiome of the foetus and future microbial colonisation. One of the first mechanisms explored is via the placental microbiome (Aagaard *et al.*, 2014). Initial colonisation may occur by means of translocation of bacterial species from the mother's gut via the bloodstream and placenta (Borre *et al.*, 2014a). Any shifts in the maternal microbiome due to stress effects may therefore have the potential to influence early colonisation of the foetus. Furthermore, several bacterial species were isolated from umbilical cord blood (Jiménez *et al.*, 2008), indicating that translocation could be facilitated via this route.

The vaginal and gastrointestinal microbiome undergo significant changes during pregnancy (Aagaard *et al.*, 2012; Koren *et al.*, 2012; Mueller *et al.*, 2015; Deng *et al.*, 2019), including a decrease in pro-inflammatory proteobacteria from the first to the third trimester and a significant increase in the anti-inflammatory *Faecalibacterium prausnitzii* (Koren *et al.*, 2012; Mueller *et al.*, 2015). In cows, *Bacteroides*, *Enterobacteriaceae*, and *Histophilus* were identified as be the top 3 dominant OTUs in unhealthy (pathological states, including reproductive disorders) pregnant animals (Deng *et al.*, 2019). It is known that stress can alter the gastrointestinal microbiome (Karl *et al.*, 2018) and in rodents stress induced changes in the vaginal microbial community (Jašarevic *et al*, 2015; Jašarević *et al*, 2017, 2018; Amabebe and Anumba, 2018a).

In humans, mothers submitted to high levels of cumulative stress during pregnancy, as assessed via cortisol levels, gave birth to infants that had significantly higher relative

abundances of *Proteobacteria* groups, containing potential pathogens and lower relative abundances of lactic acid bacteria and *Bifidobacteria*, indicating potential inflammation (Zijlmans *et al.*, 2015).

In rodents, maternal stress altered proteins related to vaginal immunity and abundance of *Lactobacillus*, which resulted in decreased transmission of this bacterium to offspring. The microbiota composition in the neonate gut also corresponded with altered metabolite profiles involved in energy balance, and disruptions of amino acid profiles in the neonatal brain (Jašarevic *et al.*, 2015).

In a different study, early prenatal stress influenced offspring bacterial community structure a sex-specific manner. Furthermore, new-born microbiota profiles overlapped with maternal vaginal microbial profiles, whereas microbiota profiles post weaning were more similar to maternal gut microbial communities (Jašarević *et al.*, 2017a). Finally, prenatal stress was associated with alterations in the foetal intestinal transcriptome of male mice and with changes in the adult gut, influenced by additional stress exposure in adulthood (Jašarević *et al.*, 2018). Maternal vaginal transfer also appeared to mediate the effects of prenatal stress on hypothalamic gene expression after exposure to chronic stress in adulthood (Jašarević *et al.*, 2018). Long-lasting effects on the intestinal microbiota composition of PNS rats included decreased *Lactobacillus* counts and a higher abundance of the *Oscillibacter*, *Anaerotruncus* and *Peptococcus* genera. In addition, relative abundance of *Cyanobacteria* was significantly correlated with the HPA axis response to stress.

A final pathway by which maternal stress may influence the foetal microbiome is via increased concentration of glucocorticoids. Prenatal development of the gastrointestinal tract appears to be under the influence of glucocorticoids (Majumdar and Nielsen, 1985) and as mentioned previously, these hormones can significantly influence the microbiota structure. All of the above suggests that prenatal stress can have a lasting effect on the gut microbiome, brain development and stress responsiveness.

As this is an emerging research topic, information on how prenatal stress may affect the microbial structure in ruminant species and particularly sheep does not exist.

1.4.2 Early life stress

1.4.2.1 Effects on physiology, metabolism, immune system and behaviour

Early life stress (ELS), particularly in the perinatal period, can affect long-term basal and stress-induced activity of the HPA axis. ELS, similarly to PNS, has been thought to have a

programming effect on the highly plastic and sensitive neuronal brain networks related to the stress response during critical periods of development which can result in enduring hyper- or hypo-activation of the stress system and altered glucocorticoid signalling. Models most commonly used in order to induce early life stress have been maternal separation, social deprivation, handling and the use of impoverished environments.

In humans, a study based on questionnaires sent to thousands of people, revealed that adults that had been exposed to child abuse and/or neglect present a higher risk of developing effective disorders, addiction and disease vulnerability (Felitti *et al.*, 1998; Maniam, Antoniadis and Morris, 2014). Furthermore, ACTH and cortisol are significantly increased in humans that had experienced childhood sexual or physical abuse. This is potentially due to a persistent hyperactivity of CRH-containing neurons, the effects of which were not only observed on CRH hypothalamic circuits but also in the brain in the limbic area and the amygdala, a centre of depression recognition (Plotsky *et al.*, 2005). In addition, ELS can alter the expression of genes in peripheral tissues, such as the glucocorticoid receptor and 11-beta-hydroxysteroid-dehydrogenase (11β-HSD1) (Maniam, Antoniadis and Morris, 2014).

Increased activity of the amygdala may in turn lead to increased fear, anxiety, vigilance, as well as sympathetic and HPA activation (Clauss, 2019). Furthermore, prior to the development of depression and anxiety disorders, behavioural cues such as high reactivity to novelty can be observed (Clauss, 2019), as well as an alteration in reward, motivation and mood regulatory circuits (Russo and Nestler, 2013).

The means by which early life stress can affect behaviour and lead to an onset of depressive behaviours could be via the immune system, by influencing pro-inflammatory cytokine responses and reducing immune cell sensitivity to anti-inflammatory signals, both in an acute way, but also in a persistent manner in adulthood (Nettis and Mondelli, 2018).

This has been verified in a number of animal species such as rodents, where a single 24h maternal separation test of pups on postnatal day 9 was sufficient in creating a depression-like phenotype in adult 129S1/SvImJ mice (Binder *et al.*, 2011). Stressors, such as handling and maternal separation, resulted in alterations in CRHF receptor type1 mRNA density in rats, resulting in a potential long-term influence of ELS on behaviour and endocrine responses to stress (Plotsky *et al.*, 2005).

A different study using maternal separation on pups from birth to 3 weeks of life resulted in improved memory and learning ability of the pups (Suri *et al.*, 2013). Conversely in the same study, a significant decline in neurogenesis, as well as the onset of cognitive and affective disorders were reported when the early life-stressed rats reached middle age (Suri *et al.*, 2013).

Another study used the model of maternal separation during the postnatal period (days 2-14) to investigate hypothermic effects of a mild chronic stress test in adult rats. Exposure to mild chronic stress led to stronger and longer duration hypothermia in the group which had been separated from the mother for the longest periods early in life (Mrdalj *et al.*, 2014). Similarly, maternal separation induced depression-like behaviour and higher ACTH hormone responses to an acute stressor (forced swimming) in male rats (Veenema *et al.*, 2006). In addition, inter-male aggression was significantly higher in maternally separated rats compared to control rats, whereas serotonin immuno-reactivity in the anterior hypothalamus was significantly lower.

Apart from functional and structural changes in the brain, ELS can lead to changes in circadian rhythm, emotional reactivity, and disruption of proper ANS function. In humans, Dong et al.(2004) were the first to describe the relationship between ELS and ischemic heart disease. Bönke et al. (2019) did not identify a significant effect of ELS on heart rate, although it was lowest in the severest ELS cases. In 2015, a comprehensive study consisting of a 23-year follow up period, revealed that individuals who were exposed to multiple stressors early on life displayed a greater increase in blood pressure levels during early adulthood compared to control individuals (Su *et al.*, 2015). Similarly in rats, maternal separation and similar models of chronic behavioural stress appear to have a long-term effect by priming the physiological systems to overreact in response to a secondary stressor in adulthood (Loria, Pollock and Pollock, 2010; Ho *et al.*, 2016).

ELS maladaptation of the HPA axis and its subsequent effects on energy utilization and expenditure can lead to metabolic dysregulation, influencing production traits in farm animals and reproduction ability (Orihuela and Galina, 2019). In calves, the stress induced by early separation from the mother, or social restriction in rearing systems where calves are individually reared, can lead to short-term negative effects such as reactivity toward novel social companions, feed, hunger disassociated with offered feed and poor growth during the pre-weaning period (Duve *et al.*, 2012; Cantor, Neave and Costa, 2019). Long-term effects of ELS as in rodent models can lead in increased reactivity and aggressiveness and lower productivity (Wagner *et al.*, 2015).

In pigs during the weaning period, maternal separation, the stress of transport, social mixing, fighting and social hierarchy establishment add up to an immense challenge which, in combination with the decline of passive immunity from sow milk can lead to significant disruptions of GI barrier development (Moeser, Pohl and Rajput, 2017). Additionally, ELS induces female-specific effects on the ENS, by affecting cholinergic receptor function, which may represent a mechanistic link between ELS and susceptibility to GI disorders (Medland *et*

al., 2016). In other farm animals particularly dairy cows and sheep, maternal separation is common and can affect social behaviour, immune development and behaviour (Hopster, O'Connell and Blokhuis, 1995; Lidfors, 1996; Sevi *et al.*, 2003).

Overall, an explanation as to why ELS influences long-term responses in terms of behaviour, immune and neuroendocrine response may be given by the match/mismatch theory. This theory describes how encountering ELS prepares an organism for similar ("matching") adversities later in life, while a mismatching environment can potentially lead in an increased susceptibility to psychological and physical disorders (Santarelli *et al.*, 2014).

1.4.2.2 Early life stress effects on the microbiome

The neonatal gut microbiome development is complex and influenced by many factors including mode of delivery, maternal diet and nutrition, diet offered, environmental factors and use of antibiotics (Cong *et al.*, 2015; Cotten, 2016; Tapiainen *et al.*, 2019). The structure it assumes early on in life and its developmental trajectory according to external influences have important implications in future life (Arrieta *et al.*, 2014).

As mentioned above, ELS influences the immune system of neonates for the duration of the perinatal period. The immune system in this time of life is already charged with distinguishing between what is beneficial or dangerous as to avoid over-activation of immune response, but also protect the organism. This process of immune maturation is finely balanced and the impact of stress can dysregulate this, potentially leading to chronic inflammatory and metabolic diseases, from asthma and obesity to diabetes (Raposa *et al.*, 2014; Gollwitzer and Marsland, 2015; Zhuang *et al.*, 2019).

A different study in mice also showed that maternal separation can result in long lasting anxiety and depression like behaviours which have also been associated with compositional changes of the gut microbiota, the presence of which, compared to germ-free mice, had a significant effect on hippocampal serotonin levels (De Palma *et al.*, 2015).

In humans and rodents, links have been made between ELS and IBS (Bradford *et al.*, 2012; Collins, 2020) which can be the result of microbial dysregulation at a gut level (Chey and Menees, 2018). In addition, it has been shown that GF mice accumulate natural killer T-cells in the lungs and intestine, which also increases susceptibility to IBD and asthma (Olszak *et al.*, 2012).

In farm animals, early life stress is not so widely explored in terms of how the microbiome is affected in terms of behaviour although studies highlight shifts in the microbiome, although is

more likely due to a significant diet change and correlations with stress cannot be easily made. For example in pigs, early weaning can lead to intestinal inflammation and impairment of mucosal immune responses (McLamb *et al.*, 2013; Guevarra *et al.*, 2019) and is associated with the host responding via production of nitric oxide that is rapidly converted to nitrate (NO_3^-) when released in the intestinal lumen (Zeng, Inohara and Nuñez, 2017). This environment has been shown to favour the growth of enteropathogenic bacteria such as *Enterobacteriaceae* (McLamb *et al.*, 2013; Winter *et al.*, 2013). Lambs weaned early demonstrated a significant and lasting increase in diversity and relative abundance of several bacterial taxa of the ileal microbiota and early weaning was shown to impact expression levels of genes related to intestinal barrier function (Li *et al*, 2018a). The effect of stress cannot be disentangled from the effect of diet at these stages, and long-term effects of early life stress on the microbiome have not been reported in farm animal species.

1.4.3 Acute and chronic stress

1.4.3.1 Effects on physiology, metabolism, immune system and behaviour

Acute stress, as described previously, consists of a short exposure to a stressor, which has a physiological effect preparing the animal for "fight-or-flight", and can then be subdued while the animal returns to a status of homeostasis. Acute stress, despite mobilising the CNS, ANS and HPA axis, as well as the endocrine and cardiovascular system, should not have lasting effects. Despite this, intense acute stress has been associated with feeding suppression and reduced liveweight gain due to CRH and catecholamine effects on the liver and adipose tissues (Rabasa and Dickson, 2016). Acute stress has even been shown to assist differentiation of stem cells into new nerve cells, improving cognitive performance in rats (Kirby *et al.*, 2013).

On the other hand, exposure to repeated and/or cumulative stressors can lead to chronic stress. In animals, chronic stress can also often be induced by an inability to adapt to environmental stressors and challenges posed by the management system they are in, not allowing animals to exhibit natural behaviours. Such factors can lead to increased disease susceptibility, undernutrition, endocrinal, metabolic and behaviour changes (aggression, stereotypies, changes in activity patterns etc.) (Dwyer and Bornett, 2004). Crowding, tail pinching, unpleasant handling and restraint can result in suppressed growth, not only due to reduced feed intake but stress-induced decrease in feed conversion efficiency (Moberg and Mench, 2000)). Due to inflammation, protein metabolism can be affected resulting in long-term metabolic and reproductive issues.

Chronic stress is linked to numerous and lasting impairments in mood, cognition and memory (Qiao et al., 2016) and may play a major role in the development of inflammation (Tian *et al.*, 2014), immunodeficiency via lymphocyte reduction (Maydych *et al.*, 2017), autoimmune disorders, cardiovascular dysfunction, diabetes, Alzheimer's disease, Parkinson's disease, gastric ulceration, and cancer (Thaker *et al.*, 2007; Mariotti, 2015; Rohleder, 2016). Chronic stress can also lead to over-consumption of food, leading to increased visceral fat and weight gain, which can be partially explained by a chronic release of glucocorticoids and NY (Rabasa and Dickson, 2016). Furthermore, in humans, chronic stress had been linked with an increased likelihood to develop depression and anxiety disorders. The mechanisms of this rely on endocrine dysregulation leading to alterations in the SNS, brain synapses and brain function (Breslau and Davis, 1986; S. Checkley, 1996; Yang *et al*, 2015; Zhang *et al*, 2019; Sheline, Liston and McEwen, 2019).

The way in which chronic stress can affect the immune system is by altering cytokine production. Usually glucocorticoids downregulate proinflammatory cytokine production, but persistent exposure to high levels of cortisol during chronic stress can lead to a downregulation of the glucocorticoid receptors on immune cells (Glaser and Kiecolt-Glaser, 2005; Gouin, 2011). Due to glucocorticoid resistance, immune cells can become unresponsive to cortisol which then leads to low grade chronic inflammation. This can lead to longer healing times from injury and higher susceptibility to disease (Glaser and Kiecolt-Glaser, 2005).

In a study conducted on pigs that were chronically stressed, animals submitted to heat stress, crowding, and social mixing had lower average daily gain and cortisol values compared to control animals. In addition, lipopolysaccharide-induced proliferation and natural killer cytotoxicity were greater in stressed pigs compared to controls indicating low-grade inflammation. Chronically stressed quails, selected for higher levels of reactivity exhibited significantly decreased basal corticosterone levels post-stress compared to non-stressed birds (Calandreau *et al.*, 2011). A study conducted with sheep investigating the relationship between stress, temperament and the presence of a protective antigen against GI nematodes showed that stress and temperament did in fact affect immunological aspects (Sutherland *et al.*, 2019).

Heart rate (HR) and heart rate variability (HRV) are also affected by chronic stress. There are consistent results in rodent and human studies describing the effect of chronic emotional stress in baseline HR values and autonomic activity (Inagaki, Kuwahara and Tsubone, 2004; Wood, 2014; Crestani, 2016). In dairy cows chronically stressed due to lameness, heart rate and heart rate variability were examined as indicators of autonomic nervous system activity. Heart rate and all HRV measures were lower in lame cows compared to non-lame ones, suggesting that

chronic stress affected cardiac function (Kovács *et al.*, 2015). Previously, Harlow *et al.*, (1987) had found significant correlations between heart rate and cortisol values in sheep tested to graded stressors, and had also suggested that heart rate could be a potential tool for predicting stress-induced changes in animal production systems. Long-term exposure of female lambs to uncontrollable stressors led to treated lambs having lower leukocyte counts, plasma cortisol levels and HR compared to control lambs (Destrez *et al.*, 2013).

Stress affects animal behaviour, and this can be manifested as changes in vocalizations, motor activity or in the expression of stereotypic behaviours, which can lead to negative effective states in animals, such as anxiety and depression and can pose a risk to animal welfare. Adaptations of attention bias tests are used to evaluate this (Crump, Arnott and Bethell, 2018; Monk *et al.*, 2018). These tests have shown that in humans, anxious individuals are more aware of threats compared to non-anxious individuals (Cisler and Koster, 2010; Beard, 2011).

In sheep for example, the use of this test allowed Monk *et al.* (2018) to distinguish behavioural differences between medically-induced states of anxiety and depression in sheep. The depressed animals had increased temperatures compared to controls and anxious animals during the tests, while both anxious and depressed animals showed increased signs of fear as indicated by higher vigilance and urination frequency. Previously, using a 9 week model of unpredictable mild chronic stress, Destrez *et al.* (2013) demonstrated that treated lambs had lower scores in a learning test and were also more reluctant to approach a familiar object (bucket) when placed in unfamiliar locations, thus demonstrating pessimistic-like judgment biases.

Investigation of physiological and behavioural parameters allows us to gain a better and more complete understanding of the effects that management systems and human intervention have on animal stress response, and the mechanisms used for them to cope and overcome adversity. This information can be applied to improve animal welfare and productivity.

1.4.3.2 Stress effects on the microbiome

Acute stress, via the activation of endocrinological and neurological pathways potentially affects the microbiome, but these changes would be transient due to the robustness of this virtual organ and difficult to separate from other shifts occurring because of animal physiology, diet and environmental influences, which may have a more pronounced effect.

Stress in animals, as previously described, involves a bi-directional communication between the brain and peripheral organs, and is mediated by a number of hormones and neuroactive

factors. When stressors are perceived, the CNS is activated triggering the secretion of various hormones and immune-related compounds from the endocrine system. These are most notably glucocorticoids and catecholamines (Mostl and Palme, 2002), in the presence of which iron is liberated from lactoferrin and transferrin, thus increasing the growth capacity of gram-negative bacteria (Freestone *et al.*, 2002).

This bidirectional communication has been explored in many species, where the focus has mainly been on the gut microbiome, as in this location microorganisms are particularly abundant, and research has proven a multitude of ways in which the communication takes place.

In general, the mechanisms via which stress is hypothesised to disrupt the microbial communities present in the gut, and potentially the rumen, are: Stress via hormonal and immune pathways can damage epithelial cells and disrupt the integrity of epithelial barriers, potentially increasing permeability in the gut. This can lead to "leaky gut", systemic inflammation and various other disorders (Lyte, Vulchanova and Brown, 2011; Le*nnon et al*, 2013; Kelly *et al*, 2015a; Obrenovich, 2018). Hormonal and immune deregulation can lead to issues with microbial neuroendocrine functions, for example by affecting serotonin production (Galland, 2014; Kelly *et al.*, 2015; Martin *et al.*, 2018).

The induction of low-grade inflammation by affecting the commensal bacterial populations and balance between microbiota species, which potentially allows for pathogen proliferation (Pickard *et al.*, 2017; Lazar *et al.*, 2018). Furthermore, pathogen survival and translocation may be facilitated, increasing inflammation (Kelly *et al*, 2015a; Pickard *et al*, 2017). The effect of corticosteroids can disrupt absorption of nutrients and minerals, both from the host and the microbiota, resulting in metabolic disorders (Pickard *et al.*, 2017).

One of the first studies exploring the relationship between the HPA axis and the microbiome was conducted by (Sudo *et al.*, 2004) on mice. Corticosterone and ACTH levels were higher in GF mice compared to mice that were specific pathogen free *(E. coli*) in response to restraint stress, indicating a more sensitive HPA response in GF animals. In a mouse model of chronic depression, elevated central CRH expression were observed at the same time-point as changes occurred in the gut microbiota (Park *et al.*, 2013). Using a model of social stress on mice, Bailey et al. (2011) demonstrated a shift in microbiota composition and this was accompanied by an increase in circulating interleukin-6 (IL-6), suggesting the induction of a proinflammatory microbiota community, such as a higher presence of *Bacteroides* and an increase in the *Clostridium* genus.

Similarly, chronic social defeat induced behavioural changes in mice, which were associated with reduced richness in the gut microbial community and shifts at a phylum OTU level (Bharwani, M. Firoz Mian, *et al.*, 2016), particularly *Lactobacillus*. In this study, chronic stress also altered immune function, as defeated mice exhibited higher serum IL-6 levels five days after the last defeat test had taken place (Bharwani, M. Firoz Mian, *et al.*, 2016).

Anxiety-like behaviours were also exacerbated in mice when infected with *Campylobacter jejeni* or *Citrobacter rodentium,* and this appeared to be a direct effect of bacterial activity on neural pathways (Lyte, Varcoe and Bailey, 1998; Lyte *et al.*, 2006). Marin *et al*. (2017) observed that chronically stressed mice exhibiting despair behaviour, had significantly reduced *Lactobacillus* and increased kynurenine levels. Kynurenine is a key metabolite playing an important role in the kynurenine pathway of tryptophan, identified as a novel communication pathway between the gut microbiome and the immune system (Van der Leek, Yanishevsky and Kozyrskyj, 2017) and potentially the brain (Kennedy *et al.*, 2017).

In mice following psychological stress, small intestine transit was slower, while a decrease in the relative proportion of *Lactobacilli* and *E. coli* was also noted (Wang and Wu, 2005). In general, in human and rodent studies investigating disruption of the microbiome due to antibiotic administration, a shift at the phylum level in the *Firmicutes/ Bacteroidetes* ratio can act as indication of disruption and metabolic disorders (Mariat *et al.*, 2009; Verdam *et al.*, 2013; Fransen *et al.*, 2017; Koliada *et al.*, 2017; Méndez-Salazar *et al.*, 2018; Mir *et al.*, 2019; Rinninella *et al.*, 2019).

In other species and in particularly farm animal species, the investigation of the microbiome is usually linked to productivity parameters and immune function. Many studies also explore the effects of heat stress, which is an important factor in management systems, but is considered a physiological stressor due to immediate effects on metabolism and immunity (Sohail *et al.*, 2015; S. Chen *et al.*, 2018; Jun He *et al.*, 2019).

In dairy calves, dehorning and castration stress resulted in a significant decrease in Shannon diversity index in faecal samples, particularly in lighter calves (Mir *et al.*, 2019). On day 3 after castration, heavier calves had higher abundance of *Aerococcaceae* and *Bacillaceae,* while lighter calves had higher *Prevotellaceae* and *Pseudomonadaceae* abundance. In addition, on day 3 after dehorning, heavier calves had higher relative abundance of *Elucimicrobiaceae* and *Turibacteriaceae,* while lighter calves had higher abundance of *Erysipelotricheae* and *Verrucomicrobiaceae*. Finally, the *Firmicutes/ Bacteroidetes* ratio was significantly decreased in lighter calves (Mir *et al.*, 2019).

This type of stress, as it is based on the painful effects of the treatments applied, is both physical and psychological, and the two cannot be separated, despite administration of anti-inflammatory medication. This is because the procedures followed were invasive in the sense that they initiated an immune response, but also because pain signals are identified by the prefrontal cortex, while chronic pain has been linked to a hyperactive prefrontal cortex (Yang and Chang, 2019). The prefrontal cortex regulates stress-induced fear and anxiety-like behaviours via inhibitory effects on the amygdala (Banks *et al.*, 2007; Hoon, 2012). As described previously, this could lead to a cascade of endocrinological and immunological reactions affecting the microbiota community structure of these calves.

Behavioural changes such as reduced feeding, increased running bouts and increased distance from the rest of the flock have been observed in chickens several days or even weeks after infection with *Salmonella enteritidis*, even if the animals are considered asymptomatic (Toscano *et al.,* 2010). GF quails have also shown behavioural changes compared to quails with a colonised gut. GF animals spent less time in tonic immobility (a test used to assess bird reactivity). These birds travelled shorter distances at a slower pace during a social separation test, and also spent more time close to a familiar object at the start in a novel object test (Kraimi *et al.*, 2018). Similar findings had been observed by Campos *et al*. (2016), where GF quails exhibited lower emotional reactivity, but no difference was observed in weight or other physiology parameters.

As above, many studies focus on the influence bacteria exert on the HPA axis and behaviour. However, a few studies have recently emerged on the effects of psychological stress on the gut (and rumen) microbiome in farm animals. In pigs differing in terms of feed efficiency, sanitary stress had a minor influence on the overall faecal microbiota composition, although *Helicobacter* abundance increased in LRFI pigs after stress was applied (Kubasova *et al.*, 2018).

Finally an experiment conducted on goats demonstrated that chronic exposure to dexamethasone (Dex), which is an corticosteroid frequently used to simulate stress conditions in non-ruminant and ruminant animals, did not result in significant changes in the rumen, caecum or colonic microbiota diversity or abundance metrics (Hua *et al.*, 2018b). This indicates that potentially the bacterial communities in ruminants are resistant to stress, or that the model used was not efficient, as low doses of Dex can lead to hypo-corticoid states in the brain (Karssen *et al.*, 2005). Despite this, Dex exposure did affect body weight and dry matter intake levels negatively, although white blood cells and plasma glucose levels were higher in Dex treated animals. Rumen VFA concentration was not affected (Hua *et al.*, 2018b).

It is evident that the effect of stress and stress hormones is poorly explored in species other than rodents and humans. As recent work in non-ruminants has shown that micro-organisms proliferate in response to stress hormones such as cortisol (Freestone *et al.*, 2008), it was considered of interest to investigate this bi-directional communication within the gut and rumen of sheep. Other studies have shown that exogenous administration of glucocorticoids leads to enhanced populations of aerobic gram negative bacteria in the gut, and influences translocation of these bacteria to distal organs (Kirimlioglu *et al.*, 2006). However the administration of certain doses of Dex (5 and 10 mg/kg) increases the numbers of total anaerobic bacteria (lactobacilli) in rats (Unsal and Balkay, 2012). Norepinephrine and ACTH have been shown to increase cecal and colonic adherence of *E. coli* 0157:H7 through interactions with α-2 adrenergic receptors in pigs (Green *et al.*, 2003 and Schreiber and Brown, 2005). It is unknown if and which hormones have the ability to reach the rumen, and whether they can affect the bacteria present within the rumen.

The rumen has metabolic functions similar to endocrine organs such as the liver (Xiang *et al.*, 2016) but its multifunctional role, as well as the potential effects of behavioural and physiological stress on the microbiome, have not been investigated. Therefore, one of the focuses of this project was to explore at an initial stage whether correlations could be made between the stress hormone cortisol and the neurotransmitter serotonin, and the presence or absence of particular rumen and faecal bacteria in sheep.

As bacteria are in constant communication with the host, all the above can have potential long-term implications on host health, behaviour, and immunity. The communication between host and the microbiome is not explored in this project directly, but to explore initial effects of genetic susceptibility and psychological stress on the ovine rumen and gut, we investigated bacterial taxonomic profiles, relative abundance, and diversity parameters according to correlations between cortisol levels, estimated breeding values and serotonin.

Finally, better knowledge about the links between behaviour, physiology and the gut/rumen microbiome of farm animals would aid in the detection of preclinical infections and increase biosecurity. Lower levels of animal stress could potentially be linked to lower inflammation and consequentially lower antibiotic use, which would reduce the potential for antimicrobial resistance and potentially also improve animal welfare.

1.5 Objectives

The main objective of this project was to investigate the effect of different expressions of stress on the ovine faecal and rumen microbiome, and secondarily on behaviour and factors related to stress response (such as stress hormones, heart rate) and some production parameters such as liveweight gain.

In order to do this, three experiments aimed to complement one another by investigating the influence of 1) genetics via predetermined temperament models on sheep stress hormone profiles and rumen/gut bacterial profiles; 2) the effect of prenatal stress (induced using conditions reflected in normal husbandry practices on sheep farms in the UK) on the developed rumen bacterial community structure in lambs; and finally 3) effects of repeated mild stressors, which have shown to induce mild chronic stress affecting lambs' behaviour, physiology and rumen/gut bacteria.

In more detail, the aim of the first experiment was to investigate the likelihood that genetic differences in ewes, already proven to differ in terms of stress responsiveness via genetic selection, could lead to divergent stress hormone levels and bacterial profiles present in the rumen and faecal samples (used as proxies for the gut bacterial community structure).

The second experiment focused on investigating mid- to long-term differences in prenatally stressed animals expressed in terms of rumen bacterial community structure at the age of 8 months when the rumen is considered to have developed, and the bacterial community present can be an indication of the future mature microbiome community. The gestating ewes whose lambs were sampled at slaughter for their rumen content, had been repeatedly exposed to a dog and an unpredictable feeding schedule and different diet during the last third of pregnancy, for the purposes of another project.

In the third experiment, the aim was to investigate the effects of unpredictable and uncontrollable events known to provoke stress in sheep, on the behaviour, stress response mechanisms (such as stress hormone levels and heart rate), physiology (i.e., growth rate) and rumen/gut microbial profiles of young growing lambs. Many stressors were applied (exposure to a dog, social mixing, wet bedding, confined space leading to higher stocking density, unpredictable or restricted feeding, lights during the night, exposure to noisy humans or objects, rough handling and more) in a repeated and uncontrollable, for the lambs, manner. As studies have rarely looked at the influence of behavioural stress on the ovine microbiome, this study provides a unique outlook on the subject.

Knowing how stress can affect animal behaviour and physiology as well as influence the microbiome, will provide knowledge on whether selecting for more phenotypically resilient animals and minimizing sources stress can improve lamb welfare and productivity.

1.6 Hypotheses

As previously described, this project consisted of three major experiments, which included different hypotheses:

1.6.1 Experiment 1

- The two genetic lines of Romane sheep, tested and selected on the basis of social reactivity differ in basal cortisol levels due to prior stress exposure and susceptibility to stress.
- The genetics of the two lines will influence rumen and faecal microbiome profiles indicating that responsiveness to stress is linked with the microbiome.
- Cortisol can be used as a predictor of the presence/absence or abundance of certain bacterial phyla, orders or genera.

1.6.2 Experiment 2

- Three different levels of maternal gestational stress (Stressed: dog exposure, unpredictable feeding, high stocking density; Non-stressed: no dog exposure, predictable feeding, high stocking density; Alternative: no dog exposure, *ad libitum* feed, low stocking density) will differentially affect basal cortisol levels of female and male lambs, as investigated in later life (8 months of age).
- Three different levels of maternal gestational stress (Stressed: dog exposure, unpredictable feeding, high stocking density; Non-stressed: no dog exposure, predictable feeding, high stocking density; Alternative: no dog exposure, *ad libitum* feed, low stocking density) will differentially affect rumen microbiota profiles of male and female lambs at 7 months of age.
- Two different early life treatments will affect basal cortisol levels in basal cortisol levels of female and male lambs, as investigated in later life (8 months of age).
- Two different early life treatments will differentially affect rumen microbiota profiles in male and female lambs investigated at 7 months of age.

- Cortisol can be used as a predictor of the presence/absence or abundance of certain bacterial phyla, orders or genera.

1.6.3 Experiment 3

- Repeated unpredictable mild stressors for a duration of 6 weeks can lead to behavioural (time spent performing certain activities, synchronisation between animals) and physiological changes (e.g., cortisol, glucose, serotonin, heart rate, weight gain) in female lambs.
- Stressed animals respond to acute stressors during the 6 weeks, meaning cortisol, glucose and heart rate values increase compared to NS animals.
- Stressed animals respond differently to a suddenness test, when compared to NS animals tested under the same conditions (differences in behaviour and heart rate).
- Repeated unpredictable mild stressors for a duration of 6 weeks will result in altered rumen and faecal profiles between treatment groups (MCS animals and NS animals) post-trial.
- Cortisol and serotonin levels can act as predictors of the presence/absence or abundance of certain bacterial phyla, orders or genera.

Chapter 2

Interactions between stress and microbiota profiles: effects of genetic susceptibility to stress

2 Interactions between stress and microbiota profiles: effects of genetic susceptibility to stress

2.1 Personal contribution

This experiment was conceived in collaboration with my supervisors Diego Morgavi, Alain Boissy, Marie Madeleine Mialon-Richard, Milka Popova and Richard Dewhurst. Blood, rumen and faecal sampling was conducted by myself, Stephane Andanson, Milka Popova and Diego Morgavi. Analysis of the cortisol samples was conducted in the INRAE biomarkers' lab by me and Christine Ravel. Protozoan counts, faecal and rumen sample crushing, and subsequent DNA extraction, 16S rRNA gene amplicon sequencing and library preparation were conducted by me. I performed data processing and all statistical analyses, after receiving advice from David Ewing at Bioss.

2.2 Introduction

2.2.1 Influence of genetics on stress susceptibility

Stress, defined as a state of dysregulation and disturbed homeostasis occurs as a necessary and natural response to environmental, psychological and physiological stressors. Evidence of heritability of stress response factors has been achieved through a number of studies investigating various genetic factors such as breeds (Boissy *et al.*, 2007; Blanco, Casasú and Palacio, 2009; Hough *et al.*, 2013; Haskell, Simm and Turner, 2014), genetic lines (A. Boissy *et al.*, 2005; Hazard *et al.*, 2016a, 2020), gene effects (e.g. polymorphisms or differences in temperament) (Lesch, 2004; Uhart *et al.*, 2004; Hazard *et al.*, 2014b, 2016b, 2020; Qiu, Martin and Liu Xiaoyan Qiu, 2015) and sex (Kendler and Greenspan, 2006; Balhara, Verma and Gupta, 2012) . Advances in molecular genetics and immunogenetics have offered an important understanding of the means by which genetic factors influence the mechanisms participating in the biology of a normal reaction to stressors or the presence of an impaired immune response (Ising and Holsboer, 2006). This is because knowledge of the genes or specific genetic variations involved in basic but intricate processes such as the stress response or the immune response allows us to investigate overlapping loci, or allow the explanation of stress-related disorders, such as autoimmune disorders (Segerstrom and Miller, 2004; Casey, 2017; Dantzer *et al.*, 2018; Song *et al.*, 2018) or neurological disorders. Additionally, the pathways and moderators implicated in both systems can be explored allowing deeper understanding of the biology of normal and altered stress response.

Stress response, as mentioned in the General Introduction, is triggered and regulated by the HPA axis. The intensity with which it is expressed is relevant to the structure and function of neurobiological pathways expressing brain signalling molecules (i.e., dopamine or serotonin), which are involved in the sympathetic and hypothalamic-pituitary-adrenocortical (HPA) axis or related to inflammation and immune response. These in turn are heavily influenced by genetic factors.

Many stress-related disorders have been attributed high to moderate heritability. Examples include cardiovascular disorders like hypertension and coronary artery disease, as well as psychological and psychiatric disorders (bipolar disorder, depression, Post-Traumatic Stress Disorder -PTSD- and anxiety). Further genetic investigation identified single nucleotide polymorphisms (SNPs) in genes of the stress hormone signaling pathway (examples being FKBP5, NR3C1, and CRHR1) associated with depressive symptoms in humans and mice (Gillespie *et al.*, 2009).

Generally, in livestock species involved in production systems stress response in relation to the psychological parameters involved and how these may lead to psychosocial disorders is not frequently explored as it not economically important and not easily quantifiable. The focus evolves around genes involved in the response to environmental stressors (e.g., heat stress) or stressors related to the physiology and immune system of the animal (e.g., oxidative stress).

In recent years, however, stress responsiveness has been investigated not only in relation to parameters associated with the physiology, biochemistry and immunological or anatomical profile of the animal, but also in relation to behavioural parameters which have come to be considered as "temperament".

Temperament in psychology refers to characteristics and aspects of personality which can be genetically defined prior to birth, and which can be influenced thereafter by the environment. In animal ethology, temperament is defined as variability in behaviour between individuals that is consistently displayed when tested under similar situations (Boissy *et al.*, 2005; Hausberger *et al.*, 2004; Zetner and Shiner, 2012).

As temperament evidently consists of multiple traits, a number of those most easily discerned whilst observing the animal's behaviour have been studied in order to assess how they are linked with physiological, immunological and other traits of importance to animal performance and health, as well as to assess heritability. Some first observations made in mice by Scott and Fredericson (1951) resulted in the description of animals which tried to escape or avoid human contact during the tests as "wild," whereas animals that did not react aversely to human contact as "tame".

Temperament profiles are known to be heritable, and more relevant to this study. Temperaments related to high and low stress reactivity in sheep have been reported to exhibit medium to high heritability (0.20 to 0.49) (Wolf *et al.*, 2008; Boissy *et al.*, 2009). Hazard *et al.* (2016) performed a series of behavioural tests (an arena test, a corridor test, an isolation box test and a shearing test, as previously described by Boissy *et al.*, 2009) on Romane female and male lambs, and identified five main QTL regions on sheep chromosomes (Ovis Aries Region, OAR) 12, 16, 19, 21 and 23 which among numerous other QTLs appeared to have small to moderate effect. Regarding social reactivity, the QTLs on OAR12, 16 and 21 exhibited the highest effect, whereas QTLs on OAR19 and 23 were associated with reactivity to humans.

From the above, it becomes evident that temperament and specific behaviours (e.g., high and low frequency of bleating) are inherited and can therefore act as useful selection tools. This allows selection for more resistant animals within breeds, or even for specific breeds, permitting sheep to cope with management stressors encountered in modern farming systems. As a result, losses due to poor health because of extreme stress responses and chronic stress could be avoided, while handling and adaptation of the animals in novel environments would be facilitated.

2.2.2 Influence of genetics and stress susceptibility on the microbiome

Genetics is considered to play an important role in the formation and evolution of the microbial structure in many body locations in humans (Blekhman *et al.*, 2015). These host-microbial interactions have been explored in great depth in the gastrointestinal tract of humans, due to their impact on host health (Dąbrowska and Witkiewicz, 2016). The same is true for murine species often used as study models (McKnite *et al.*, 2012; Davenport, 2016).

The rumen has also been explored, as it presents a complex system of vital importance for the productivity of ruminant livestock. The core bacterial species have been investigated (Jami and Mizrahi, 2012), metagenomic analysis has provided information on function (F. Li, Hitch, *et al.*, 2019) particularly in relation to improving feed efficiency and methane production, and proof of host genetic influence has been reported (Hernandez-Sanabria *et al.*, 2013*;* Henderson *et al.*, 2015*;* Roehe *et al.*, 2016; Li *et al.*, 2019b). Most of these studies have been performed on bovine species, with fewer studies using ovine species.

Stressful events affect the microbiome in many species (Househam *et al.*, 2017; Marin *et al.*, 2017a; Karl *et al.*, 2018), and the mechanisms by which this occurs are still being explored.

Bacteria communicate with the endocrine system by receiving “messages” through receptors or via the production of peptides and other molecules that in turn can signal information to the nervous system and the brain (Neuman, Justine W. Debelius, *et al.*, 2015).

One of these mechanisms recently explored involves cortisol, as researchers have found predictive relationships between the faecal microbiota and cortisol. Using pigs as a model, Mudd et al., (2017) observed that bacterial genera *Bacteroides* and *Clostridium* predicted higher concentrations of myoinsitol, *Butyricimonas* positively predicted n-acetylaspartate (NAA), and *Bacteroides* also predicted higher levels of total creatinine in the brain. They also observed that when *Ruminococcus* was more abundant in faecal samples NAA concentrations in the brain were lower. This study then used these 4 bacterial genera to predict blood plasma concentrations of cortisol and serotonin, via mediation analyses. As this was successful, cortisol and serotonin pose interesting targets for further exploration.

On the other hand, studies investigating relationships between temperament profiles and the microbiota community structure of the gut are scarce. In human studies, Aatsinki *et al.*, (2019) and Christian *et al.* (2015) investigated differences in infant faecal microbiota communities according to temperament profiles or, more broadly, how certain bacteria related to temperament profiles. Healthy adult temperament scores were correlated with faecal microbiome profiles in a pilot study by Kim and Park (2017), while stress profiles during pregnancy in women were explored in relation to differences expressed in the faecal microbiota (Hechler *et al.*, 2019).

In murine species, the relationships investigated were mainly to assess the influence of specific treatments rather than explore temperaments (e.g. Marin *et al.*, 2017), while an interesting study exploring temperaments and correlations with the microbiome in farm animals was conducted by Kraimi *et al.* (2019a). This study used germ-free chicks from a quail line selected for a high emotional reactivity (E+) and germ-free chicks from a line with low emotional reactivity (E−). Germ-free quail chicks from the E+ line were inoculated with faecal samples from either an “E+” quail or an “E-” quail and were reared in different isolators. Quails that received feces from the E- line expressed a lower emotional reactivity at the age of two weeks compared to the quails inoculated with faecal bacteria of the “E+” line. This result was reversed two weeks later. This proves both the influence of the host, attempting to reverse the effects of inoculation, and also that a relationship between temperament and certain bacteria exists. Yet again, little is known in terms of ruminant temperament and relationships with bacteria present both in the rumen and the gut.

The genetic background of an organism can influence and guide the structure of the microbial community in many organs and particularly in the gut. The same has been observed for the microbial community of the rumen and gut in ruminant species, which may be heavily influenced by diet but still tends to steer towards particular genetically defined balances. Furthermore, there are indications that temperament is linked to some aspects of the microbiota, although more research is needed. Taking all the above into account, exploration of the two genetic lines of Romane sheep, selected to differ in their reactivity to social isolation and human contact would potentially highlight differences in the structure of the rumen and faecal microbial community, due to the genetic background of these sheep and whatever physiological consequences this implies.

2.3 Study hypotheses and objectives

The Romane sheep selected for exhibiting differences in reactivity when socially isolated or in the presence of humans have two reactivity temperaments (B+ and B-), as measured by Estimated Breeding Values presented by lAlain Boissy *et al.*, 2005 ; Ligout *et al.*, 2011 and Hazard *et al.*, 2014.

This would suggest that over their lifetime these sheep react in different ways to events which may have led to or be a result of discrepancies between the two lines when exploring certain physiological parameters related to the stress response. Hence blood cortisol samples were taken to evaluate whether the animals had different baseline levels.

Additionally, it was hypothesised, that temperament may be correlated with a certain microbiome profile as has been the case in other species. As two generations of animals were also included in the study, it was considered prudent to include this factor in the analyses.

Therefore, rumen and faecal communities were explored on the basis of genetic line and generation. Finally, EBVs and cortisol and rumen protozoa were explored in relation to the rumen/faecal archaeal and bacterial microbiota community to investigate potential correlations between these markers and the relevant abundance at a phylum, order and genus level. If these markers investigated whether had the ability to act as predictors of abundance, this would allow further exploration into the mechanisms implicated.

- The two genetic lines of Romane sheep, tested and selected on the basis of social reactivity differ in basal cortisol levels due to prior stress exposure and susceptibility to stress.

- The genetics of the two lines will influence rumen and faecal microbiome profiles indicating that responsiveness to stress is linked with the microbiome.
- Cortisol can be used as a predictor of the presence/absence or abundance of certain bacterial phyla, orders or genera.

2.4 Materials and methods

All experimental procedures were performed in accordance with the French Ministry of Agriculture guidelines for animal research and all applicable European guidelines and regulations on animal experimentation. The experiments were approved by the Toulouse Regional Ethics Committee for Animal Experimentation, approval number A312031 (or AE 32-2017 for UK).

The ewes from the two stress-susceptible lines were sampled *in situ* at the farm (Unité Expérimentale 321, Domaine expérimental de La Fage, Roquefort, France) for blood, rumen content and faeces.

2.4.1 The animals

For the purposes of this experiment, 57 INRA401 ewes (now classed as the Romane breed), a fixed crossbreed between Romanov × Berrichon du-Cher (Ricordeau *et al.*, 2001) were sampled in September 2017, after the last lambing in spring. Sampling took place on one occasion for blood plasma, rumen digesta and faecal matter. The animals sampled were located at the INRAE experimental farm of La Fage, which acts as an experimental and breeding facility. The ewes were kept on alternating pasture grounds in a single flock throughout the year and during lambing. They grazed on these pastures all year round, received some roughage in the winter, and higher quality forage and some concentrate at the end of pregnancy. The animals selected were healthy and had only received anthelminthic treatment (Ivermectin) in June of that year as a precautionary measure.

These animals, born between 2012 and 2015, were part of an ongoing research project where male and female sheep are selected according to their behavioural reactivity towards a temporary separation from congeners on the basis of bouts of high bleats, with High reactivity classed as B+ and Low reactivity classed as B-. These behavioural tests took place shortly after weaning and the test procedures were described by Ligout *et al.* (2011). In addition to this information, pedigree and behavioural phenotypes were then used to calculate individual

Estimated Breeding values (EBVs) for each lamb using a linear mixed model and the Best Linear Unbiased Prediction method (BLUP) on the ASREML software (Butler *et al.*, 2018). For this study, this procedure was conducted by D. Hazard.

From this process, extreme animals for each reactivity character are chosen each year according to their high or low EBV for social reactivity and used to produce the next generation of animals. Specifically, for the purposes of this study, the criteria by which the animals were selected, in decreasing order of importance, were:

1) The sires had to be genotyped. This is indispensable for assessing heritability as it allows investigation of the combination of alleles passed down from each parent, allowing potential future exploration of the effects the haplotypes have on behavioural QTLs
2) The mothers were also descendants of the same genetic line as the sire (i.e. B+ ewe from B+ sire). This increases the chances that the ewes used in this experiment are carriers of haplotypes of interest
3) The ewes were both primiparous and multiparous born between 2012 and 2015, as it was impossible to have 30 animals per genetic line of the same age

The number of ewes sampled was balanced for sire and EBVs. The ewes from under-represented sires were excluded. According to these criteria, a list of 70 animals, selected based on Phenotypical Variability (related to number of High and Low Bleating), was made available and a subset of 60 animals was selected for sampling. Two generations of animals, G0 and G1, were chosen for sampling (G0, n = 17; G1, n = 43), in order to acquire the same number of animals from each genetic line (n = 30). After one generation of selection, the difference between the two lines was 1.05 phenotypic standard deviation and 1.5 genetic standard deviation.

Sampling took place in September 2017, after the last lambing had taken place the previous spring. All animals were healthy, however, upon arrival at the farm, some animals on the sampling list could not be found, and therefore, 57 animals in total were sampled (B-, B+) for blood plasma, faecal and rumen digesta. Due to human error, only 51 samples were analysed for protozoal counts. The combination of Genetic Line and Generation resulted in four groups: B-G0 (n = 8); B-G1 (n = 20); B+G0 (n = 6); B+G1 (n = 24).

2.4.2 Sample collection

2.4.2.1 Rumen and faecal sample collection for 16S rRNA gene amplicon sequencing and protozoa counts

Rumen samples were acquired via intubation. Each ewe was immobilised in a crush. The animal's mouth was gently opened and a wooden mouth guard, pierced to allow the passage of a tube, was inserted. The use of the guard allowed insertion of the tube without forcing the animal's mouth open and causing abrasions, but also restrained the tongue to avoid damage or choking. A flexible, plastic tube (diameter approx. 1.50 cm and total length approx. 1m) attached to a pump and leading to a collection DURAN® bottle was then inserted down the immobilised animal's oesophagus. As soon as the tube reached the rumen, the sampler would start pumping in order to aspirate the rumen liquid.

Approximately 50 ml was collected from each animal. The rumen content was directly aliquoted in 15 ml Falcon tubes and dipped in liquid nitrogen for subsequent DNA extraction. Additionally, 1 ml was aliquoted into a 2 ml tube containing 1 ml Methyl-Formaldehyde Solution (MFS) for protozoa counting. These samples were placed in a container to avoid exposure to light. After collection of rumen content from each animal, the tube and DURAN® were rinsed with 70% ethanol to avoid cross-contamination.

Faecal samples were collected directly from the rectum. To avoid inflicting pain or discomfort as much as possible, gloves were lubricated before sampling. Five to ten grams of faeces were collected from each animal and placed in dry ice containers.

All samples were transported to the INRAE at Clermont Ferrand on dry ice or secure liquid nitrogen carrier tanks (journey of approximately 4h) and placed for long-term storage at -80°C. Samples for protozoal counts were stored in a dark storage area at room temperature.

2.4.2.2 Blood sampling for plasma cortisol

As the sampling process was performed in one day, we had to ensure that the blood samples for cortisol analysis were taken in a uniform way with the minimum time elapsing between the first and last animal sampled. As cortisol levels can rise within 20 min after exposure to a stressor, the animals were placed in pens of 10 the previous evening, and blood sampling was performed first. Two samplers and two helpers were present and sampled animals in parallel in two different pens at a time. All samples from all sheep were collected within 40 min, between 9h00 and 10h00.

Each animal was individually contained in the home pen, amongst pen-mates, by the trained helper. The animal was locally shorn on the neck area to facilitate access and identification of

the jugular vein. The sampler gently directed the animal's head into the correct position so as to not cause distress, and proceeded to identify the jugular vein, inserting the needle for blood collection. Three 10 ml EDTA vacutainers were collected from each animal. All equipment used was age and species appropriate (20 G, 1-inch needles).

Blood samples were immediately centrifuged for 10 min at 2500 *g*. Plasma was aliquoted in 1.00 ml Eppendorf tubes, and placed on ice for transportation back to INRAE where they were stored at -20°C until analyses took place. A subset of the aliquots was stored at -80°C for long-term storage.

2.4.3 Sample processing

2.4.3.1 DNA extraction and library preparation from rumen and faecal samples at INRAE

Rumen and faecal samples were crushed using liquid nitrogen in an "A11 basic Analytical mill" (IKA, Staufen, Germany) to ensure homogeneity of each sample. 0.30 g of the crushed rumen sample or 0.03 g of the crushed faecal sample was added to a 2 ml screw-cap tube containing 0.4 g of sterile zirconia beads (0.3 g of 0.10 mm and 0.10 g of 0.50 mm beads). Microbial DNA extraction was carried out using the method described by Yu and Morrison (2004). Purification, as well as RNA and protein removal, were carried out using the QIAamp DNA stool Mini Kit (Qiagen, Lyon, France), according to manufacturer's instructions. DNA samples were tested for yield and quality using a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Scientific, France). Throughout processing, a "no-template" control went through all the DNA extraction and purification steps.

PCR was performed on ABI 2700/2720 thermal cycler (SEQGEN, France), using custom Primers for the V3-V4 region of the bacterial 16S rRNA gene: PCR1F_460bp:

5' CTTTCCCTACACGACGCTCTTCCGATCTACGGRAGGCAGCAG 3' (Liu *et al.*, 2007)

PCR1R_460bp:

5'GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT 3' (Andersson *et al.*, 2008)

Samples were prepared in 50 µl volumes and contained 0.50 µl MTP Taq DNA polymerase (5.00 U/µl SIGMA, ref: D7442 protocol; France), 5 µl 10x MTP Taq Buffer (SIGMA, ref D7442 protocol, France), 1 µl dNTP mix (10mM), 1.25 µl of each primer (20 mM) (Thermo Scientific, France) and 1 µl nuclease free water (Thermo Scientific, France), with the addition of 1 µl DNA for each sample.

After an initial denaturation at 94°C for 2 min, 30 cycles of PCR were performed (94°C for 60 s, 65°C for 40 s, and 72°C for 30 min), and a final extension step at 72°C for 10 min. Negative controls, containing all the reagents except DNA template, were included in each PCR performed.

The samples were processed in a random order (regardless of genetic line, generation or sample type, i.e., faecal or rumen). 1µl of each PCR reaction/ product was run on a FlashGel DNA cassette (Lonza, Levallois, France) to confirm that amplification had taken place successfully (no smear would suggest that the amplification was specific, as required). The samples were placed in 96 well plates and sent to the Genotool GeT platform for NextGen sequencing in Toulouse (INRAE, Castanet Tolosan, Cedex, France) for library preparation and 16S rRNA gene amplicon sequencing.

The libraries were sequenced on a 250 paired-ends MiSeq run and generated 167023 raw reads for the faecal samples and 120231 raw reads for the rumen samples.

2.4.3.2 16S rRNA gene amplicon sequencing read processing

Data was analysed on an INRAE-specific Galaxy-based graphic user interface for QIIME (QIIME 2 2018.10; Caporaso *et al.*, 2010) using tools from mothur (Schloss *et al*., 2009), as described by Popova *et al.* (2018).

Briefly, upon receipt of the sequence data, these were imported into Galaxy along with a file containing sample information necessary to de-multiplex the sequences. Forward and reverse reads of the overlapping sequences were merged using the "make.contigs" command of mothur (Schloss *et al.*, 2009) with default parameters (match bonus = 1, mismatch penalty = −1, gap penalty = −2, gap extend penalty = −1, insert quality ≥ 20, mismatch quality difference ≥ 6). Sequences of poor quality (presence of ambiguous base pairs, homopolymers greater than 8, length divergent to 200 – 460bp) were discarded. To reduce sequencing error, sequences with 3 or fewer differences were pre-clustered. Chimeric sequences were removed using UCHIME (Edgar *et al.*, 2011).

Sequences were then aligned to the Greengenes 16S rRNA gene reference alignment database (DeSantis *et al.*, 2006, Greengenes 13.8 Version). All sequences were grouped into 99% operational taxonomic units (OTUs) by uncorrected pairwise distances and average neighbour clustering in mothur. The most abundant sequence in each OTU was used as a representative sequence for taxonomy affiliation. Singletons were removed to facilitate further analyses. Rarefication curves were computed, offering a graphical representation of coverage. Faecal

and rumen samples were analysed separately, and separate taxonomy and OTU output files were created.

2.4.3.3 Cortisol

A sheep specific competitive ELISA was used for the quantification of cortisol in blood plasma in the CARAIBE biomarkers' lab (Analyses conducted by Stephane Andanson and Christine Ravel). The protocol followed was as described by Andanson *et al.*, (2018), using antibodies described by Boissy and Bouissou (1994). All samples were tested in duplicate, and an average measurement was recorded. Inter (within assay plates) and Intra (between assay plates). Coefficient of Variance (CV) was ensured as <15%. This threshold was based on validation assays performed by Andanson *et al*. (2018). Analysis was repeated as necessary.

2.4.3.4 Protozoa counts

Protozoa counting was performed according to the protocol by Ogimoto and Imai (1981) using Neubaeur microscope slides. The rumen sample had been aliquoted in a 2.00 ml tube already containing 1.00 ml Methyl-Formaldehyde Solution (MFS), which was made up to a volume of 1.00 L and is comprised of 35.00% formaldehyde (100 ml), distilled water (900 ml), Methyl Green (0.60 g) and NaCl (8.00 g). The MFS allows fixation of protozoa, meaning the sample can then be stored for up to three years. The samples were stored in ambient temperature, in the dark, for approximately a month and were then diluted in a PBS solution. The Neubaeur plaque chambers were filled with 15.00 µl and the protozoa were counted under a standard scientific microscope (ALPHATEC ASTREO® 300, Alphatec Scientific, Peru).

The method of counting was as follows:

1. The first step was to count the *Entodiniomorphs* smaller than 100 µm in the 4 large 1.00 mm^2 squares of the Neubaeur plaque. The volume of each square is 0.1 µl (mm^3), thus the total volume is 0.40 µl (mm^3).

2. The second step was to count the larger *Entodiniomorphs* (>100 µm), and then the *Isotricha* and *Dasytricha* present on the entire surface of the plaque (total volume: 15.00 µl). The result is expressed as the number of protozoa/ml and the equation used is:

$$\frac{\text{Protozoa counted in 4 squares x Dilution Factor x Conversion Factor}}{\text{Volume of the four squares}}$$

- Dilution Factor = 2 (one dilution of ½)
- Volume of chamber = 4 x 1.00 mm^2 (surface) x 0.10 mm (depth) = 0.40 µl for the small *Entodionomorphs* and 15 µl for the large *Entodionomorphs*, *Isotricha* and *Dasytricha*.
- Conversion factor = 1000 (µl to ml)

2.5 Statistical analyses

2.5.1 Cortisol

Following a square root transformation (SRT) of the data, a generalised linear model (GLM) was applied for further analysis, using the "glm" core R function (Dobson and Barnett, 2018) with an assumed Gaussian distribution. The model used to determine differences in ewe cortisol levels between Generations, Genetic Lines and possible Interactions of the two was:

*Model: f(y) = Generation + Genetic Line + Genetic Line *Generation;* $p < 0.05$.

2.5.2 Protozoa

As protozoan counts represent discrete variables, a generalized linear model (GLM) with negative binomial distribution was performed in R ("glm.nb" function in MASS package; Venables and Ripley, 2002) for the Total Count of protozoa with Generation, Genetic Line as predictors, while an Interaction effect was also explored. In order to define the model needed, a Pearson chi-squared dispersion statistic was employed (using the function "chisq.test"; Hope, 1968) to examine whether the variance was equal to the mean, in which case the dispersion statistic would be equal to 1. In this case, it was much larger than 1 (363.40, $p < 0.01$) and therefore the negative binomial model was chosen. Using the "Anova" function from the "car." package in R (Fox and Weisberg, 2019), an analysis of deviance allowed the acquisition of F- and p-values, with the assumption that the theta was fixed.

*Model: f(y) = Generation + Genetic Line + Genetic Line * Generation;* $p < 0.05$.

Similarly, for individual genera counted, namely *Dasytricha*, *Isotricha* as well as Large and Small *Entodiniomorphs*, the appropriate model (negative binomial distribution analysis) was selected and performed following the process described above.

2.5.3 Microbiota

2.5.3.1 Downstream analysis with R and phyloseq

OTU tables were imported in "phyloseq" (Callahan *et al.*, 2016; McMurdie and Holmes, 2014, 2013) for R (R version 3.6.1). By importing a "metadata" file which included the important information accompanying the OTU table (Animal Ids, Genetic Line, Generation etc.) and the Taxonomy table, a "phyloseq" object was created. Taxa where ambiguous taxonomy was classed as "None" were removed. Taxa with 0 readswere removed. Archaea and bacteria were treated separately.

2.5.3.2 Taxonomy

The different taxonomic levels of the rumen bacteria and archaea samples, as well as the faecal bacteria and archaea samples, were explored separately using "phyloseq" and other R packages, as deemed appropriate.

2.5.3.3 Diversity

Alpha diversity is the diversity (either measured in terms of a synthetic diversity index or species richness) within a community. Several diversity indices were computed, plotted and explored for statistical significance between groups. Indices explored included Observed diversity, Shannon and Simpson, Inverse Simpson, Fisher, Chao1 and Abundance Based Coverage estimators (ACE).

Observed Species diversity was used to explore Species diversity, which gives the count of unique OTUs within a sample and within a group and the Chao1 and ACE indices. These estimate the diversity present in a sample with added importance to the rare OTUs. Further information and formulas can be found in Hughes *et al*. (2001).

To explore within sample diversity, the Simpson, Shannon, Inverse Simpson and Fisher's Indices were used. Simpson's Index is a measure of biodiversity that takes into account richness and evenness. It measures the probability that two individuals randomly selected from a sample will belong to the same species (in the context of this study, it suggests that randomly selected OTUs will belong to the same species). Shannon diversity measures the order observed within a particular system. As with Simpson's index, Shannon's index accounts for both abundance and evenness of the species present. Inverse Simpson explores the effective number of species present, and Fisher's Index measures the evenness by which individuals are

divided among the taxa present. Calculations for the diversity Indices can be found in Simpson, (1949) Kim *et al.* (2017).

Beta diversity is the rate of change in species composition from one community to another along gradients. Sample distances that can be used to explore diversity between samples and bacterial communities can be visualised using Nonmetric Multidimensional scaling (NMDs), or Principal Coordinate Analysis (PCoA). Both methods are widely used for visual exploration of the data. In PCoA, the solution is found using eigen decomposition of the transformed dissimilarity matrix, while in NMDS the solution is found by an iterative approximation algorithm, attempting to show ordinal distances between samples, as accurately as possible, in two dimensions. Both were calculated, but for the purposes of this study only PCoAs will be presented. Further statistical analyses regarding alpha and beta-diversity, as well as investigation of potential relationships between cortisol and Estimated Breeding values (EBVs) with bacterial OTUs were performed using R, version 3.6.1.

2.5.3.4 Taxonomy

Calculations for RA and the percentage of the most abundant phyla, classes, etc. overall and per Grouping Genetic Lines and Generations directly (B+G0, B+G1, B-G0, B-G1) were carried out using QIIME on Galaxy. From the subsequent RA tables produced for archaea and bacteria together, once again by creating 4 subgroups (B+G0, B+G1, B-G0, B-G1), further statistical analysis was performed on each taxonomic level, but for the purposes of this study, phylum, order and genus level will be discussed.

Shapiro Wilk tests were used to assess normality. When normality assumptions were met, a Two-way ANOVA was performed to compare the subgroups (B+G0, B+G1, B-G0, B-G1). When normality assumptions were not met, a nonparametric test (Kruskal-Wallis) was used. When differences in the later test were significant, pairwise Wilcoxon Rank Sum tests were applied with Bonferroni correction.

2.5.3.5 Microbial diversity analyses

Faecal and rumen OTU tables were acquired separately from QIIME, Galaxy and therefore processed separately. These were imported into “phyloseq” R, along with a file containing the taxonomic ranking related to each OTU and a “metadata” file that had all the information related to the animal (Genetic Line, Generation, etc.).

Unidentified reads at a Kingdom Level, labelled as “None” or “Other”, were removed. archaeal and bacterial diversity was investigated separately. For the rumen samples, 12423 bacterial OTUs and 66 archaeal OTUs were acquired. As a means of normalisation, a Hellinger transformation was applied (square root transformations of relative abundances, given at the scale [0,1]).

2.5.3.6 Alpha diversity

Alpha diversity is a means of exploring the variance within a particular sample. For this purpose, boxplots were created for the different alpha diversity Indices (Shannon, Simson, Inverse Simpson, Chao1, ACE and Observed diversity) using. These Indices were computed for each sample through “phyloseq” and differences between groups B-G0, B+G0, B-G1 and B+G1 were explored.

Non-Parametric testing was applied on rarefied data, as a means of normalisation. For rumen samples, the lowest number of bacterial sequences was 3198, and the maximum number of reads was 13435. For faecal samples, the lowest number of sequences per sample was 3183 and the highest number was 10116. Reads were rarefied to the minimum number of reads present. The V3-V4 region selected for amplification in this study underrepresents the archaeal population and is not as accurate as a separate analysis of the archaeal and bacterial domain would be (McGovern *et al*., 2018). The presence of a maximum of 135 reads and 20 minimum reads for the faecal archaea, and maximum 110 and minimum 28 reads for the rumen archaeal reads means that these data can only provide a limited, though nonetheless useful, overview of the archaeal community structure. However, publications have used this region to explore the archaeal population with a low number of reads (Wang *et al*., 2016). Despite this, using the V6–V8 16S rRNA gene for future studies would be more efficient and accurate (Snelling *et al*., 2014). As normality assumptions were not met after Shapiro-Wilk testing, a Kruskal-Wallis test was performed for the effect of Generation and Genetic Line. Post-hoc tests for pairwise multiple comparisons (pairwise Wilcoxon Rank Sum tests) were applied with a Bonferroni correction (“PMCMRplus” library) when necessary.

2.5.3.7 Beta diversity

A homogeneity of dispersion test, using the “betadisper()” function in “vegan”, was performed, confirming that the samples within treatment groups had the same dispersion.

Unconstrained ordination in the form of Principal Coordinate Analysis (PCoA) with Bray-Curtis dissimilarity was used on the "Hellinger" transformed OTU filtered table to investigate beta diversity by exploring patterns in the bacterial and archaeal composition of the rumen and faecal samples. PCoA is a multi-dimensional scaling method used to explore and visualize similarities or dissimilarities of data.

In this case, a dissimilarity matrix was computed, and a location was assigned for each sample, in a low-dimensional space, by calculating a series of eigenvalues and eigenvectors. Each eigenvector corresponds to an eigenvalue, and their number is equal to sample number in the initial matrix. Through these eigenvectors an initial distance matrix was visualised. This resulted in a rotated data matrix which does not change the relative positioning between samples but alters the coordinated system. Through visualisation using PCoA, individual and group differences were explored.

Differences in beta-diversity of the Hellinger transformed data were tested with permutation analysis of variance (PERMANOVA, adonis) using "vegan" (Oksanen *et al.*, 2020) within "phyloseq" (McMurdie and Holmes, 2013b). A maximum of 999 permutations was applied, comparing groups B-G0, B+G0, B-G1 and B+G1 into account. A PERMANOVA allows determining whether centroids of the cluster of samples differ between groups and is used with PCoA and NMDs to distinguish inter-sample/sample differences. PERMANOVA is not performed on the actual ordination but on the underlying distance matrices (i.e., Bray-Curtis in this case).

2.5.3.8 Correlation analyses

A Spearman-Rho correlation test was conducted (library "ggpubr"; visualisation of plots "ggplot2" Kassambara, 2019) on raw cortisol and EBV values, as the latter could not be normalised, to assess whether animals that belonged to different genetic lines were likely to have higher or lower cortisol profiles due to their overall exposures to stress and their different reactivity throughout their life.The variables were transformed to ranks through the "cor()" base function in R. As a means of exploring potential relationships between cortisol levels in blood plasma and the number of protozoa present in the rumen digesta, correlations between them were explored using Pearson correlation coefficient or Spearman-rho ranked correlation analysis, depending on normality of the data.Constrained ordination methods are routinely used to investigate how environmental variables are associated with changes in community composition (Anderson and Willis, 2003). For the purposes of this study, cortisol values and the EBVs attributed to the animals were explored in relation to changes in the bacterial and

archaeal composition at an OTU level, using Canonical Analysis of Principal coordinates (CAP). Canonical Analysis of Principal coordinates (CAP), on the basis of Bray-Curtis dissimilarity distances, was used on Hellinger transformed data. First, the ordination axes were constrained to linear combinations of our selected variables (cortisol and EBVs) and then the variable scores were plotted onto the ordination. This allowed us to estimate how much variation can be explained specifically by our factors. Arrow length, thickness and direction display the drive of each variable.

Partial Least Squares regression (PLS) has been suggested as an alternative means of exploring the relationship between variables in large datasets in ecology (Carrascal, Galván and Gordo, 2009) and has recently been used in exploring microbiota profiles and their relationship to a large number of study variables in human and mice studies (Moen *et al.*, 2016; Hechler *et al.*, 2019).

A PLS structural equation model is a composite model composed by the measurement model, which represents the relationship between observed data and factors for exploration, and the structural model, which represents the relationships between the factors to be explored. The structural equation model is solved by estimating the factor variables, using the measurement and structural model in alternating steps. The measurement model estimates the factor variables as a weighted sum of its predicted variables. The structural model uses the factor variables computed by the measurement model to estimate differences between them by means of simple or multiple linear regression. This algorithmic process was repeated until convergence was achieved. Comparison of groups by genetic line and generation did not yield significant differences in terms of alpha and beta diversity. Using OTU levels was not considered efficient for PLS investigation, therefore all PLS analyses were conducted on a phylum, order and genus level for the rumen microbiota community and the faecal microbiota community, as a whole.

After identifying important phyla, orders and genera from the PLS, the direction of the relationship between Relative Abundance (RA) and cortisol and EBVs was explored via Correlation Matrices.

Therefore, PLS modelling from the “mix0mics” package in R (Lê Cao, González and Déjean, 2009; Rohart *et al.*, 2017). PLS is a component-based estimation approach that fits a composite model, rather than attempting to fit a common factor model to the data (Sarstedt *et al.*, 2016). This allows the maximum amount of variance to be explained.

The average of the squared VIP scores equals 1 and therefore, the "greater than 1" rule is generally accepted and used. This is not statistically justified and there are arguments against the use of it (Akarachantachote *et al.*, 2014; Farrés *et al.*, 2015), as VIP calculation is very sensitive to the presence of non-relevant information pertaining to the "x-axis" variables of the model and to large sample sizes. As such, after initial exploration of the data, a VIP score >1.00 was used to select phyla and orders for further correlation analyses. Due to the large number of Genera, and also due to the large number of genera related to cortisol in the output of the PLS analysis, a VIP score of over 1.50 was selected as a more stringent cut-off threshold, attempting to increase confidence in the relationship explained.

To explore the relationship of the different Taxonomic levels of faecal and rumen samples with the EBVs and cortisol data, normality was tested, and assumptions not met. A correlation matrix was visualised in the form of a heatplot to explore potential co-variations between phyla, orders and genera selected and the variables of interest, using a Pearson correlation in R, [library("Hmisc"), ("Hmisc package | R Documentation" 2019); library("corrplot"), (Taiyun Wei *et al.*, 2017)].

2.5.3.9 Presentation of results

Means, standard deviations (SD) as well as and the degrees of freedom (DF) were presented where appropriate. F-values, chi-squared values, the Mean of Squared Error (SSE) are also presented where relevant. Where F(x, y) = M is presented, x = SD, y = n and M = F-value. Significance was considered ≤ 0.05. Where $p < 0.01$, is due to the p-values being smaller than the cut-off threshold. For the correlations performed, R (the correlation coefficient) and p-values are reported. Variable Importance in Projection (VIP) scores estimating the importance of each variable in PLS analyses were used for variable selection. A variable (phylum, order, and genus) with a VIP score greater than 1.00 or 1.50 was considered important.

2.6 Results

2.6.1 Blood plasma cortisol

A Generalised Linear model (GLM) was conducted on the influence of the two independent variables (Generation and Genetic Line) on SRT cortisol levels in blood plasma, and effects of interaction were explored. Generation included two levels (G0 and G1), as did Genetic Line (B+, B-). No significant difference was observed between Generations ($F = 0.25$; $p = 0.62$) or Genetic Lines ($F = 0.95$; $p = 0.33$), and the interaction was also not statistically significant [$F(1, 53) = 0.01$, $p = 0.92$] (**Figure 2.1**).

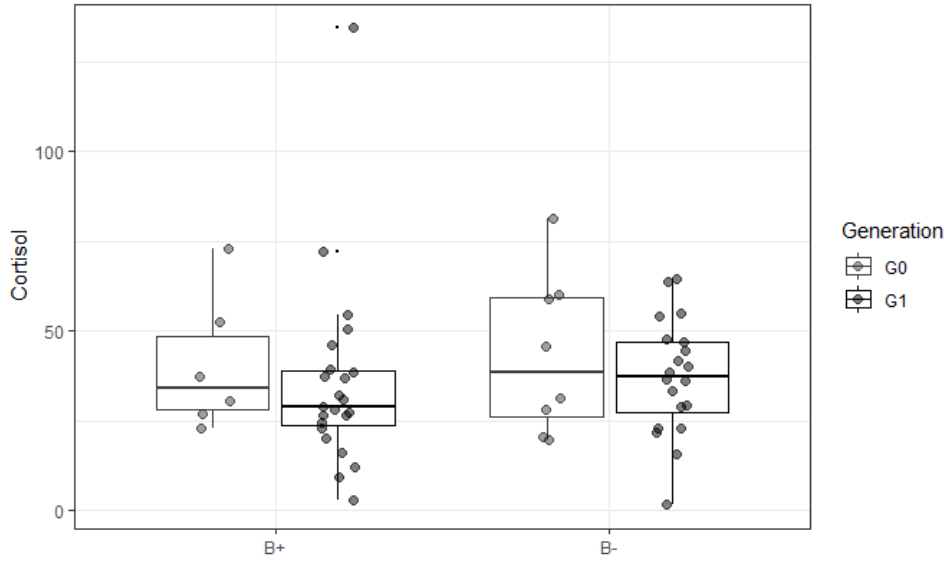


Figure 2.1 Box plots representing cortisol values (ng/ml) according to Genetic Line (B+, B-) and Generation (G0, G1). The central line shown in each box plot indicates the median of the data, the box represents the interquartile range, and whiskers extend to cover the whole range.

2.6.2 Rumen samples

2.6.2.1 Protozoa

For the total protozoa counts, a generalised linear model with negative binomial regression (GLMnb) was applied to compare the effect of two Genetic Lines (B+, mean: 60.20 ±20.00 and B-, mean: 52.50 ±20.30), the two generations (G0, mean:53.70 ± 21.50 and G1, mean: 57.80 ±20.00) and their Interaction (B+G0, mean: 69.20 ±15.10; B-G0, mean: 42.10 ±18.30; B+G1, mean: 57.70 ±20.80; B-G1, mean: 58.00 ±19.60).

There was no significant difference in total protozoa counts for Generation and Genetic Line. Interaction was statistically significant (p = 0.03) with B+G0 significantly higher compared to all other groups and B+G1 and B-G1 significantly higher compared to B-G0. Similarly, a GLMnb model was used to explore the effects of Genetic Lines and Generation, as well as Interaction on the concentration of Small *Entodiniomorphs*, Large *Entodiniomorphs*, *Dasytricha* and *Isotricha* genera. The only statistically significant effect was observed for Interaction between Genetic Line and Generation on the concentration of Small *Entodiniomorphs* (p <0.01), as B+G0 (mean: 30.30 ±4.68) was significantly higher compared to groups B-G0 (mean: 17.10 ±9.11); B+G1 (mean: 24.00 ±8.41), whilst B+G1 and B-G1 (mean: 25.90 ±10.80) were also significantly higher compared to B-G0. Further details are reported in **Table 2.1** below.

Table 2.1: GLMnb results. Mean concentration per ml and SD is presented for each protozoal genera counted (Dasytricha, Isotricha, Small and Large Entodiniomorphs), as well as for the total number present in the samples. Chi-squared and p-value obtained from the model are also reported. Statistically significant results are noted with the presence of a "*" symbol.

GLM with negative binomial regression results			
	Factor	**mean ±SD /ml**	**P-value**
Total Protozoa counts	**Genetic Line**	**B+** 60.20 ± 20.00	Pr (>chisq) = 0.56
		B- 52.5 ± 20.30	
	Generation	**G0** 57.80 ± 20.00	Pr (>chisq) = 0.19
		G1 53.70 ± 23.50	
	Interaction		Pr (>chisq) = 0.03*
Dasytricha	**Genetic Line**	**B+** 21.20± 11.90	Pr (>chisq) = 0.44
		B- 18.9 ±10.40	
	Generation	**G0** 21.80 ± 14.00	Pr (>chisq) = 0.54
		G1 19.6 ± 10.20	
	Interaction		Pr (>chisq) = 0.29
Isotricha	**Genetic Line**	**B+** 1.40 ± 1.40	Pr (>chisq) = 0.36
		B- 1.61 ± 1.78	
	Generation	**G0** 1.21 ± 1.42	Pr (>chisq) = 0.66
		G1 1.65 ± 1.62	
	Interaction		Pr (>chisq) = 0.30
Small *Entodiniomorphs*	**Genetic Line**	**B+** 25.4 ± 8.13	Pr (>chisq) = 0.46
		B- 22.9 ± 10.90	

	Generation	**G0** 22.80 ± 9.96	Pr (>chisq) = 0.34
		G1 24.2± 9.36	
	Interaction		Pr (>chisq) <0.01*
Large *Entodiniomorphs*	**Genetic Line**	**B+** 12.10± 7.51	Pr (>chisq) = 0.07
		B- 9.09 ±7.15	
	Generation	**G0** 7.93 ± 6.6	Pr (>chisq) = 0.16
		G1 11.8± 7.53	
	Interaction		Pr (>chisq) = 0.30

2.6.2.2 Microbiota analyses

2.6.2.2.1 Taxonomy analyses

2.6.2.2.1.1 Phyla

The overall rumen community, as derived from the sequence data and analysed by QIIME on Galaxy, was comprised 98.00% of bacteria (range: 95.10% to 99.00%), 0.90% of archaea (range: 0.20% to 1.60%) and 1.20% was Unclassified. At the phylum level, the most abundant bacteria were *Bacteroidetes* representing 64.90% of the total population (range: 51.20% to 75.10%) and *Firmicutes* 25.30% (range: 16.50% to 39.70%). *Synergistetes* made up for 1.70% (range: 0.00% to 4.70%) of the overall population, *Cyanobacteria* 1.50% (range: 0.20% to 3.30%) and finally *Fibrobacteres* made up 1.20% (range: 0.30% to 3.80%) of the total population. The only phylum identified were *Euryarcheota* representing 0.90% (range: 0.20% to 1.60%) of the overall population. Other phyla were present but represented less than 1.00% of the overall population (*Proteobacteria, Spirochaetes, Lentisphaerae, Tenericutes, Elusimicrobia, Plantomycetes, Actinobacteria, Chloroflexi, and Verrucomicrobia*).

At a phylum level, exploring the data by the groups the animals were categorised in according to their Genetic Line and Generation the barplot below depicts most abundant phyla (**Figure 2.2**).

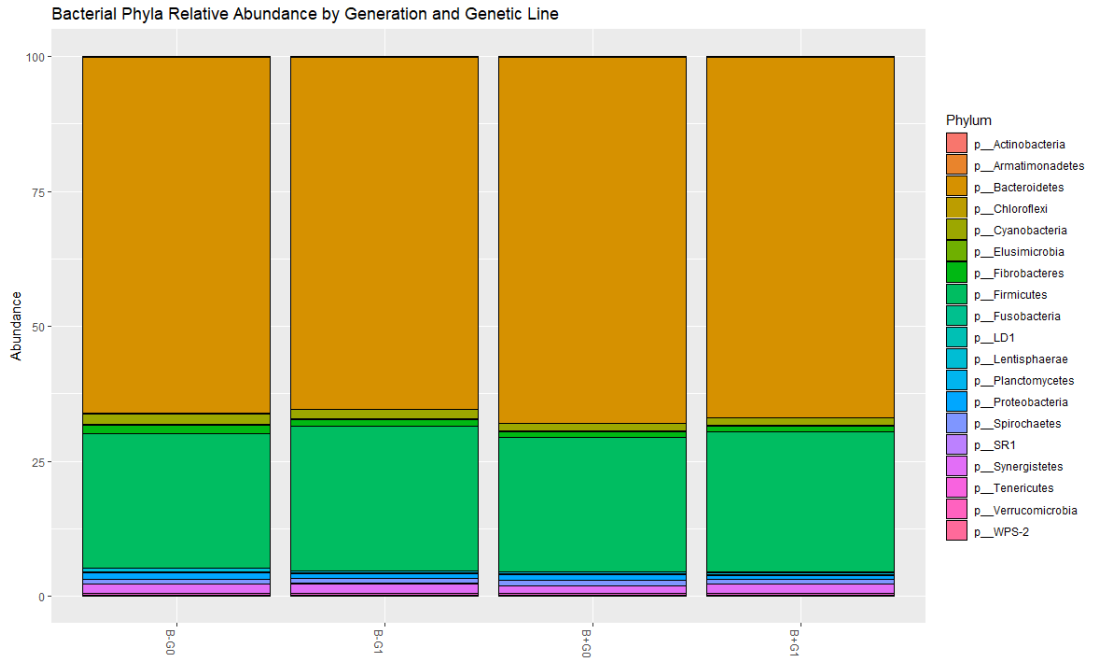


Figure 2.2 Bacterial phyla average abundance by Genetic Line (B-, B+) and Generation (G0, G1). Most abundant bacteria were: B-G0: *Bacteroidetes* (64.30%), *Firmicutes* (24.30%), *Synergistetes* (1.60%), *Cyanobacteria* (1.80%), *Fibrobacteres* (1.60%) and *Proteobacteria* (1.2%); B-G1: *Bacteroidetes* (64.90%), *Firmicutes* (25.50%), *Synergistetes* (1.50%), *Cyanobacteria* (1.80%), *Fibrobacteres* (1.40%) and *Proteobacteria* (1.20%); B+G0: *Bacteroidetes* (66.10%), *Firmicutes* (24.40%), *Synergistetes* (1.40%), *Cyanobacteria* (1.30%), *Fibrobacteres* (1.00%) and *Proteobacteria* (1.00%);B+G1: *Bacteroidetes* (65.20%), *Firmicutes* (25.50%), *Synergistetes* (1.70%), *Cyanobacteria* (1.30%), *Fibrobacteres* (1.00%) and *Proteobacteria* (0.80%).

Statistical tests at the phylum level were conducted using all phyla present (n = 21) after SRT of the RA. Non-parametric Kruskal Wallis, followed by Wilcoxon test with Bonferroni correction, indicated that there was a significant difference between Groups B+G1 >B-G1 for the phylum *Fibrobacteres* (chi-squared = 7.83, DF = 3, $p < 0.01$), between Groups B+G1- B-G1 and B+G1 - B+G0 (chi-squared = 12.72, DF= 3, $p < 0.01$).

2.6.2.2.1.2 Order

At the order level, the most abundant bacteria were *Bacteroidales* with 64.90% (range: 51.20% to 75.10%), *Clostridiales* with 24.90% (range: 15.40% to 39.20%). The next most abundant bacteria were present at much lower levels, i.e., *Synergistales* at 1.70% (range: 0.00% to 4.50%), *YS2* at 1.40% (range: 0.20% to 3.30%), *Fibrobacterales* 1.20% (range: 0.40% to 3.80%). The most abundant archaea were *Methanobacteriales* (range: 0.10% to 1.00%) and

E2 (Class *Thermoplasmata*) (range: 0.00% to 1.10%). *Methanomicrobiale*s and *Methanosarcinales* were mainly present in B+ animals, in very low abundances.Exploring abundance by the groups the animals were categorised (Genetic Line and Generation) the barplot below depicts most abundant orders **(Figure 2.3, Figure 2.4**).

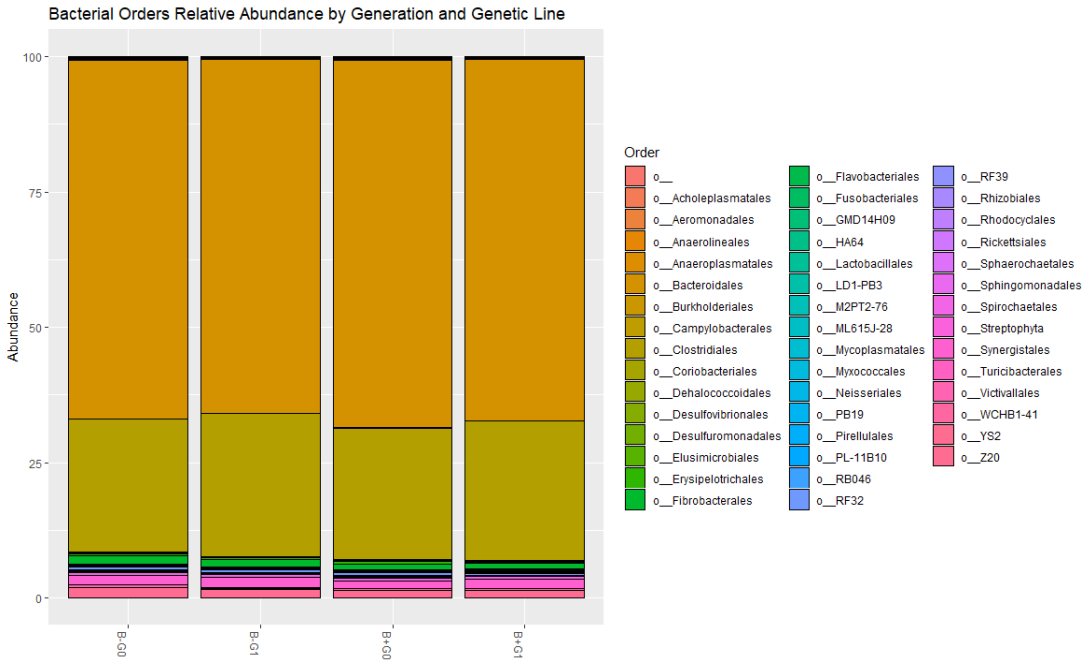


Figure 2.3 Bacterial order average abundance by Genetic Line (B-, B+) and Generation (G0, G1). Most abundant bacteria by Genetic line and Generation were: B-G0: *Bacteroidales* (64.30%), *Clostridiales* (23.90%), *YS2* (1.70%), *Fibrobacterales* (1.40%); B-G1: *Bacteroidales* (64.90%), *Clostridiales* (25.10%), *YS2* (1.40%), *Fibrobacterales* (1.40%); B+G0: *Bacteroidales* (66.00%), *Clostridiales* (23.90%), *YS2* (1.30%), *Fibrobacterales* (1.00%). B+G1: *Bacteroidales* (65.20%), *Clostridiales* (25.10%), *YS2* (1.30%), *Fibrobacterales* (1.00%).

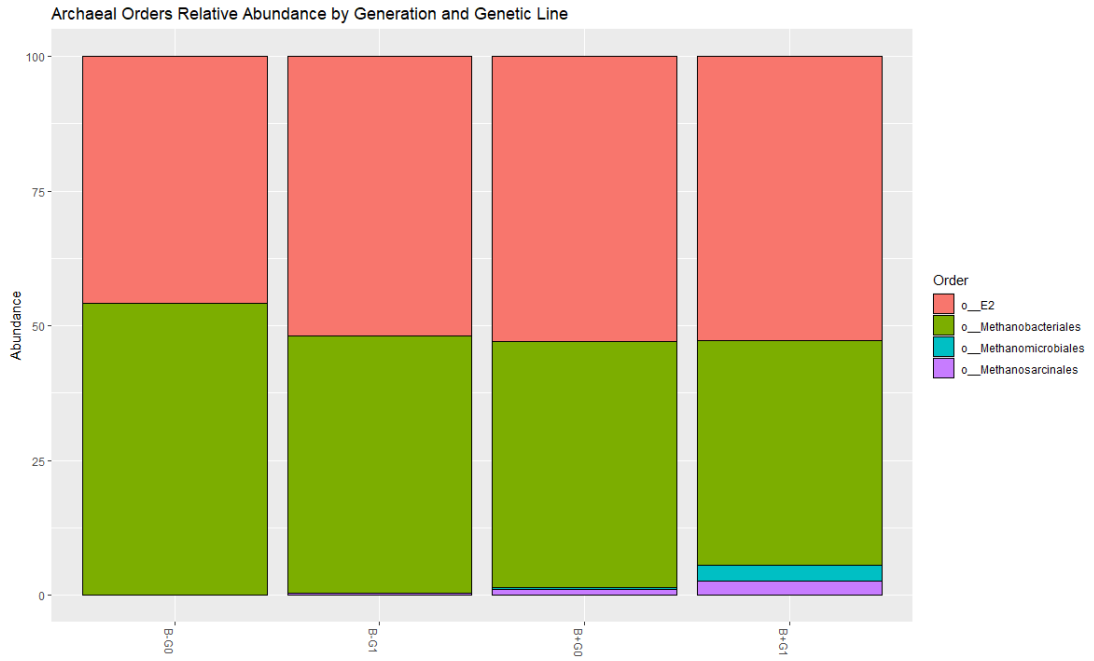


Figure 2.4 Archaeal order average abundance by Genetic Line (B-, B+) and Generation (G0, G1). Most abundant arcahea by Genetic line and Generation were: *Methanobacteriales* and *E2*. Percentages the archaea were present by Group were: Order *Methanobacteriales*: B-G0 54.00%, B-G1 48.00%, B+G0 42.50%, B+G1 37.8%; order *E2* B-G0 46.00%, B-G1:51.80%, B+G0 52.50%, B+G1 52.00%; order *Methanomicrobiales* B+G0 0.20%, B+G1 4.50%; order *Methanosarcinales* B-G1:0.02%, B+G0 2.80%, B+G1 5.70%.

Statistical tests at the order level were conducted between Groups, taking into account RA over 0.1 (order, n = 37). The order *Fibrobacterales* was statistically significant between Groups B+G1 - B-G1 (chi-squared = 7.83, DF= 3, p = 0.02), as was an unidentified member of the *LD1* order which differed between Groups B+G0 - B-G1 and B+G1 - B+G0 (chi-squared = 12.72, DF = 3, p <0.01). *Fibrobacterales* was more abundant in the B-G1 group and that *LD1* was more abundant overall in the G0 group, and therefore B+G0 > B-G1 and B+G0 > B+G1. As *Methanomicrobiales* was only present in B+ animals, RA was significantly higher compared to B- animals. Furthermore, B+G1> B+G0 (p <0.01); order *Methanosarcinales* was mainly present in the B+animals too and therefore was significantly higher in relative abundance compared to the B- animals (p<<0.01), while B+G0 <B+G1 5.70% (p<0.01).

2.6.2.2.1.3 Genera

At the genus level, the most abundant bacteria were: *Prevotella* 29.80% (range: 17.70% to 59.70%), unidentified (order *Bacteroidales*) 10.30% (range: 3.50% to 18.70%), unidentified (family *RF16*, order *Bacteroidales*) 7.40% (range: 2.40% to 15.50%), unidentified (order

Clostridiales) 5.90% (range: 1.90% to 8.60%), unidentified (family *Ruminococcaceae*) 5.50% (range: 0.70% to 13.50%), *CF231* (family *Paraprevotellaceae*) 4.30% (range: 0.80% to 8.00%), unidentified (family *BS11*, order *Bacteroidales*) 3.10% (range: 0.00% to 9.50%), unidentified (family *Prevotellaceae*) 2.80% (range: 0.50% to 4.90%), unidentified (family *Veillonellaceae*) 2.00% (range: 0.50% to 6.30%). *Rumminococcus* was present at a 1.70% level (range: 0.80% to 4.40%) and *Fibrobacter* at a 1.20% level (range: 0.30% to 3.80%).

The most abundant bacteria and archaea by group at the genus level are presented in the barplots below **(Figure 2.5, 2.6).**

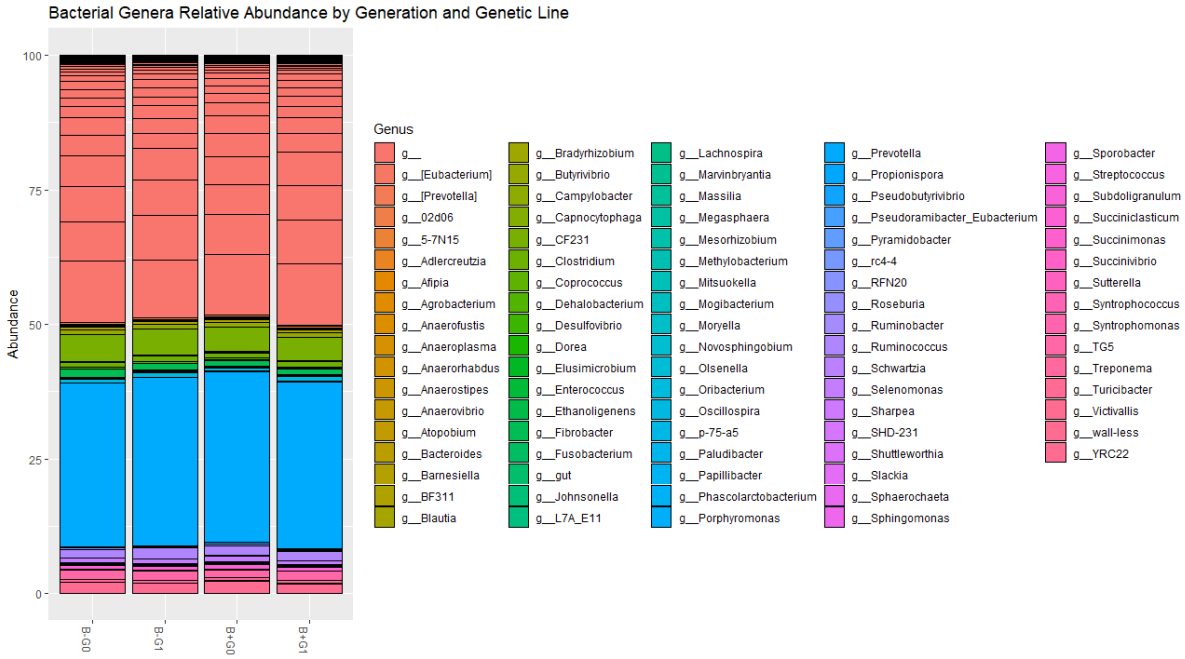


Figure 2.5 Bacterial genus average abundance by Genetic Line (B-, B+) and Generation (G0, G1). Most abundant genera by Genetic line and Generation were: B-G0: *Prevotella* (28.20%), unidentified member of *Bacteroidales* order (10.40%), unidentified member of *RF16* family (order *Bacteroidales*) (7.50%), unidentified member of *Clostridiales* order (6.20%) and an unidentified member of the *Ruminococcaceae* family (5.20%). B-G1: *Prevotella* (31.60%), unidentified member of *Bacteroidales* order (9.70%), unidentified member of *RF16* family (order *Bacteroidales*) (7.70%), unidentified member of *Clostridiales* order (5.90%) and an unidentified member of the *Ruminococcaceae* family (5.20%).;B+G0: *Prevotella* (29.70%), unidentified member of *Bacteroidales* order (10.60%), unidentified member of *RF16* family (order *Bacteroidales*) (6.80%), unidentified member of *Clostridiales* order (5.20%) and an unidentified member of the *Ruminococcaceae* family (4.80%).;B+G1: *Prevotella* (29.10%), unidentified member of *Bacteroidales* order (10.80%); unidentified member of *RF16* family (order *Bacteroidales*) (7.60%); unidentified member of *Clostridiales* order (5.80%) and an unidentified member of the *Ruminococcaceae* family (6.10%). All other genera accounted for less than 5% of the total abundance.

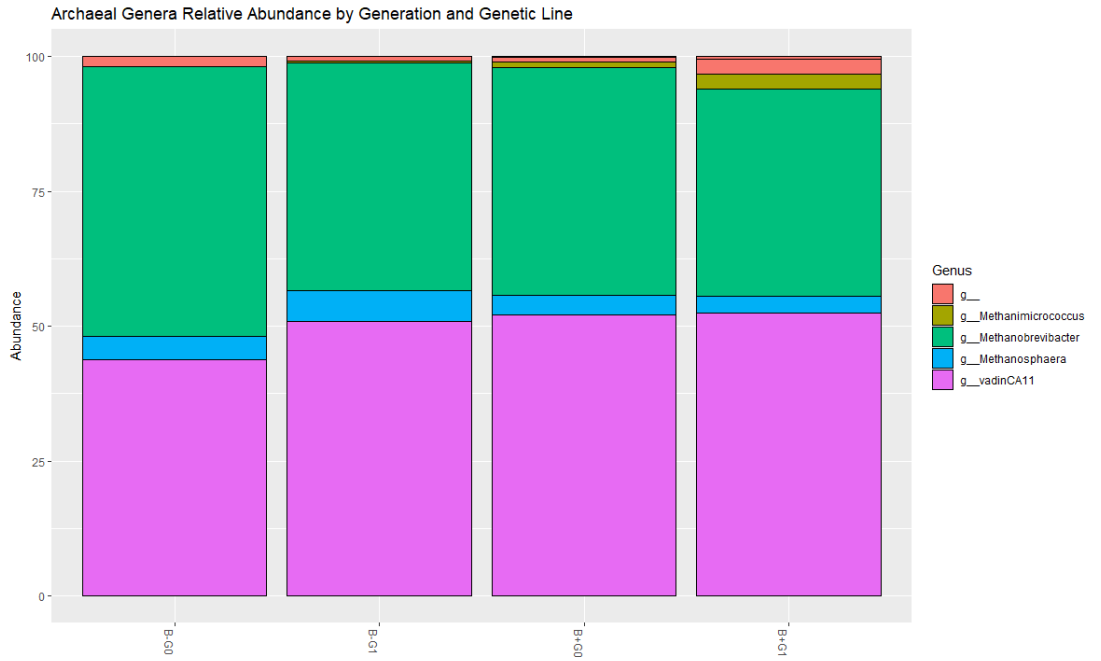


Figure 2.6 Archaeal genus average abundance by Genetic Line (B-, B+) and Generation (G0, G1). Most abundant genera by Genetic line and Generation were *vadinCA11*, *Methanobrevibacter* and *Methanosphaera*. Percentages the archaea were present by group were: genus *Unknown*: B-G0 2.00%, B-G1 1.30%, B+G0 1.50%, B+G1 4.20%; genus *Methanomicrococcus* B-G0 0.50%, B-G1: 2.10%, B+G0 1.80%, B+G1 4.00%; genus *Methanobrevibacter* B-G0 49.00%, B-G1 35.60%, B+G0 37.00%, B+G1 32.00%; genus *Methanosphaera* B-G0 8.40%, B-G1 9.20%, B+G0 7.50%, B+G1 6.20%; genus *vadinCA11* B-G0 40.10%, B-G1 51.80%, B+G0 52.20%, B+G1 53.60%.

Statistical tests at the genus level were conducted for RA over 0.5 (n = 20). An unidentified member of the *Lachnospiraceae* family was significantly different after non-parametric Kruskal –Wallis testing, followed by a Wilcoxon post-hoc analysis with Bonferroni correction (chi-squared = 0.78, DF = 3, p = 0.02) between groups: B+G1 > B-G1. *Methanomicrococcus* was significantly higher in B+ animals and specifically for B+G1 animals. *Methanosphaera* was significantly higher in abundance in the B-G1 animals compared to the other groups (p=0.03) whereas *Methanobrevibacter* was significantly higher in group B-G0 (p<0.01). Finally, *vadinCA11* was significantly lower in B-G0 animals too (p<0.01).

2.6.2.2.2 Rumen bacterial and archaeal diversity exploration

Boxplots (**Figures 2.7, 2.8**) were created in "phyloseq" on rarefied reads to visually investigate potential differences in the different diversity Indices calculated for the bacterial and archaeal OTUs. Normality assumptions were not met, therefore a Kruskal-Wallis test was conducted

on rarefied data to explore differences between groups B+G0 (n = 6), B+G1 (n = 24), B-G0 (n = 8) and B-G1 (n = 20). No differences were found.

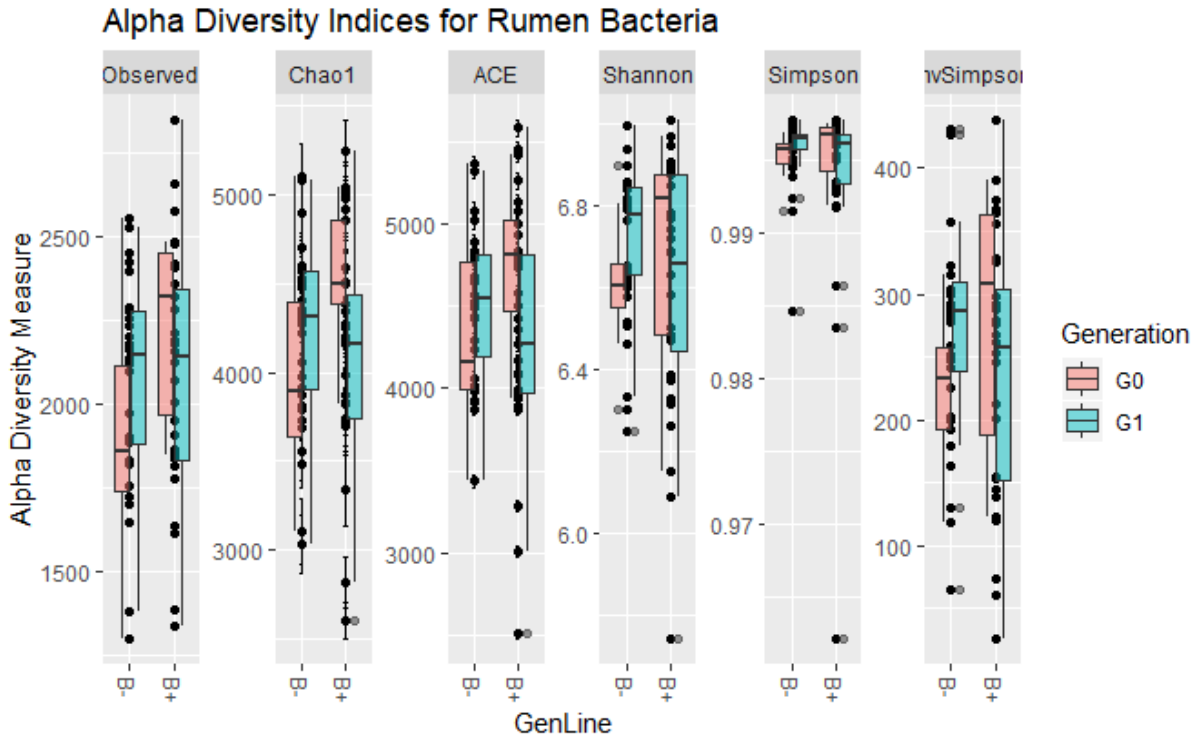


Figure 2.7 Boxplot of the diversity Indices (Observed, Chao1, ACE, Shannon, Simpson and Inverse Simpson) calculated in "phyloseq", to examine differences in rumen bacterial diversity between Genetic Lines (B+, B-) and Generations (G0, G1). Means, IQR and individual values are also presented. No statistically significant differences were observed.

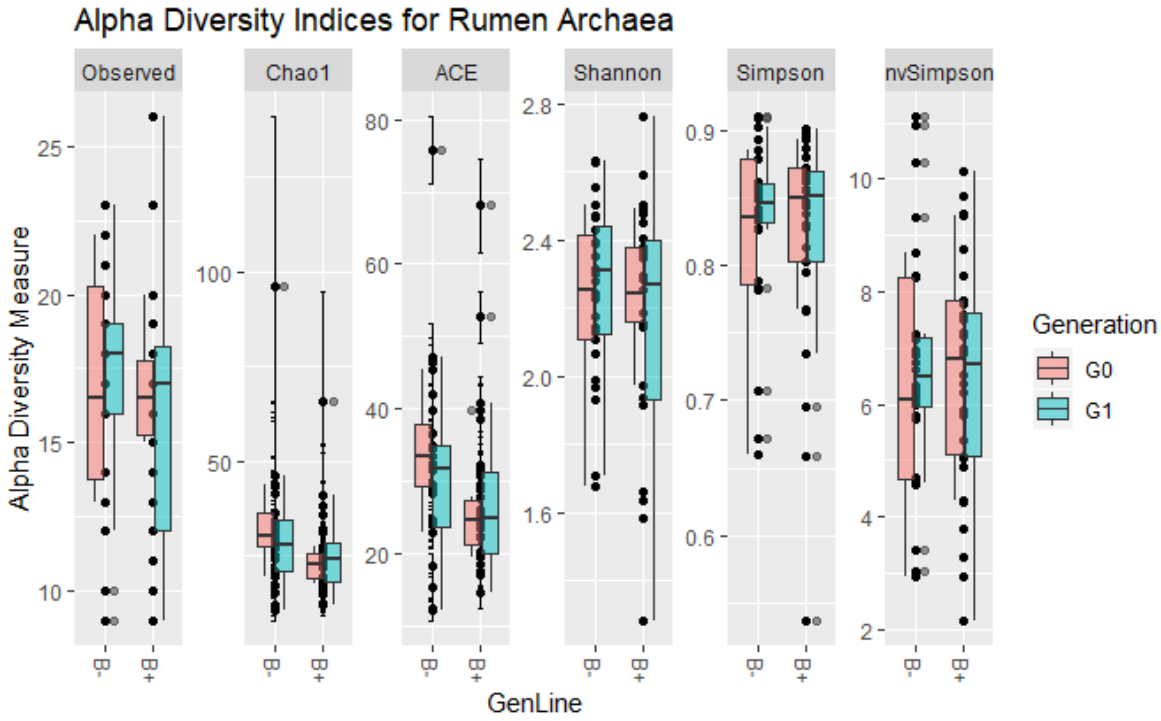


Figure 2.8 Boxplot of the diversity Indices (Observed, Chao1, Ace, Shannon, Simpson and Inverse Simpson) calculated via "phyloseq", to examine differences in rumen archaeal diversity between Genetic Lines (B+, B-) and Generations (G0, G1). Means, IQR and individual values are also presented. No statistically significant differences were observed.

2.6.2.2.2.1 Beta diversity bacteria

A permutation test for homogeneity of multivariate dispersions was applied to investigate the dispersion of the samples within theg groups [B+G0 (n = 6), B+G1 (n = 24), B-G0 (n = 8) and B-G1 (n = 20)] for Generation and Genetic Line. The number of permutations was set at 999. No significant difference was observed for the groups regarding Generation [MSE= 0.002, F (1, 56) = 2.81, p = 0.09] or Genetic Line [MSE= 0.0006, F (1, 56) = 0.84, p = 0.35].

2.6.2.2.2.2 Beta diversity archaea

A permutation test for homogeneity of multivariate dispersions was applied to investigate the dispersion of the samples within the groups [B+G0 (n = 6), B+G1 (n = 24), B-G0 (n = 8) and B-G1 (n = 20)] for Generation and Genetic Line. The number of permutations was set at 999. No difference was observed for the groups regarding Generation [MSE = 0.000003, F (1, 56) = $9e^{-04}$, p = 0.97] or Genetic Line [MSE= 0.002, F (1, 56) = 0.56, p = 0.46].

A multivariate analysis of variance test (Adonis) was performed on the basis of Bray-Curtis distances calculated from the "Hellinger" transformed OTU table, with 9999 permutations. The model took into account Generation, Genetic Line and potential interactions. No significant effect was observed for Generation [F (1, 57) = 0.52, R^2 = 0.01, p = 0.97], Genetic Line [F (1, 57) = 1.18, R^2 = 0.02, p = 0.24] and their interaction [F(1, 57) = 1.12 , R^2 = 0.02, p = 0.32].

2.6.2.2.3 PCoA bacteria

Rumen bacteria did not evidently cluster by Genetic Line (B+, B-) or Generation (G0, G1) when inspecting the PCoA plot. The percentage of total variation explained by the PCoA axes was low (4.9% Axis 1; 3.2% Axis 2) (**Figure 2.9**).

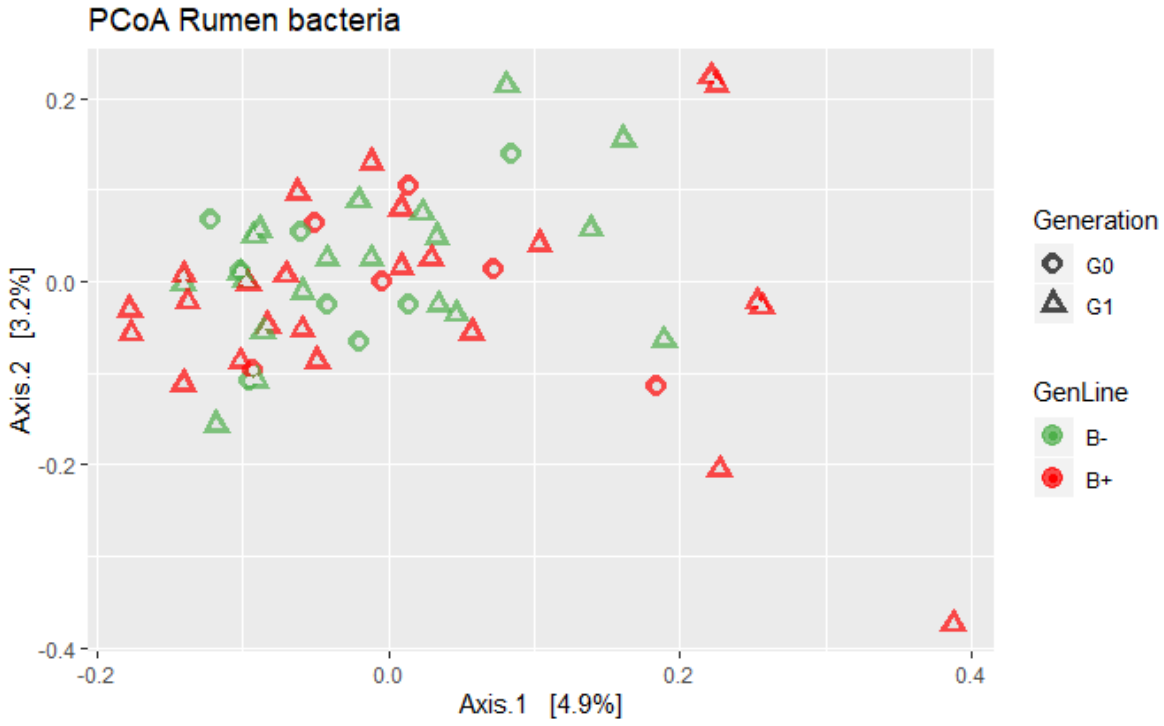


Figure 2.9 Principal coordinate analysis (PCoA) of the rumen bacterial community based on the SRT RA data of OTUs. The relative distances of all points represent the relative dissimilarities of the samples according to the Bray-Curtis index. Genetic Lines are colour coded and indicated as B+ and B- and Generations (G0 and G1) are distinguished by shape. The percentage of total variation explained by each PCoA axis is shown in the brackets.

2.6.2.2.4 PCoA archaea

No separation or grouping was visually evident according to Genetic Line (B+, B-) and Generation (G0, G1) in the rumen archaea. The percentage of total variation explained by the PCoA axes was low (10.30% Axis 1; 8.90% Axis 2-**Figure 2.10**).

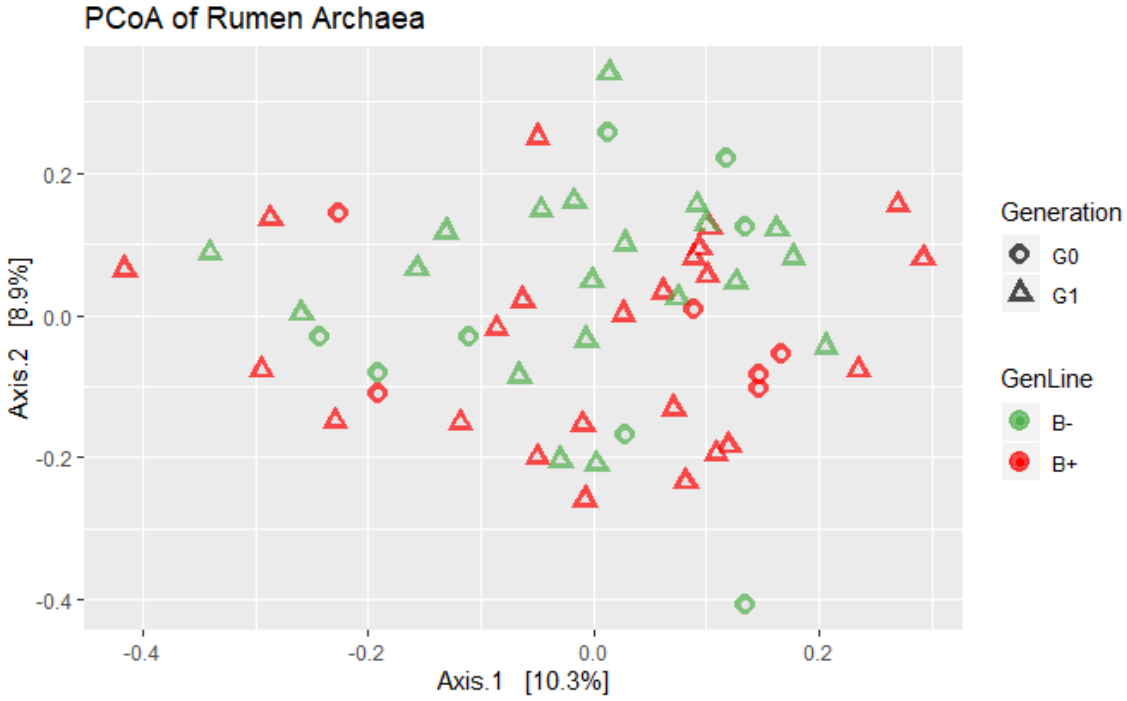


Figure 2.10 Principal coordinate analysis (PCoA) of the rumen archaeal community based on the SRT RA data of OTUs. The relative distances of all points represent the relative dissimilarities of the samples according to the Bray-Curtis index. Genetic Lines are colour coded and indicated as B+ and B- and Generations (G0 and G1) are distinguished by shape. The percentage of total variation explained by each PCoA axis is shown in the brackets.

2.6.3 Faecal samples

2.6.3.1 Microbiota analyses

2.6.3.1.1 Taxonomy analyses

The overall faecal community, as derived from the sequence data and analysed by QIIME on Galaxy, was comprised 98.10% of bacteria (range: 95.10% to 99.00%), 0.90% of archaea (range: 0.20% to 1.60%) and 1.20% were Unclassified. At the phylum level, the most abundant bacteria were *Firmicutes* representing 48.00% of the total population (range: 43.10% to 56.60%) and *Bacteroidetes* 42.80%% (range: 35.90% to 51.90%). *Spirochaetes* made up for 1.80% (range: 0.20% to 3.70%) of the overall population, *Cyanobacteria* 0.9% (range: 0.2% to 1.90%), *Lentisphaerae* 0.80% (range: 0.20% to 1.40%), *Tenericutes* 0.80% (range: 0.00%

to 1.20%), *Fibrobacteres* 0.50% (range: 0.00% to 1.80%) and finally *Proteobacteria* made up 0.50% (range: 0.10% to 0.90%) of the total population. "Unknowns" represented 1.00% of the population.

The most abundant archaea were *Euryarcheota* representing 1.00% (range: 0.20% to 1.80%) of the overall population. Other archaeal and bacteria phyla were present but made up for less than 0.50% of the overall population (*Actinobacteria, Plantomycetes, Unclassified, Elusimicrobia, Chloroflexi, Deferribacteres, LD1*, *Synergistetes* and *WPS-2*).

2.6.3.1.1.1 Phylum level

At the phylum level, exploring the data by the groups the animals were categorised in according to their Genetic Line and Generation, the most abundant phyla are presented in the boxplot below (**Figure 2.11)**

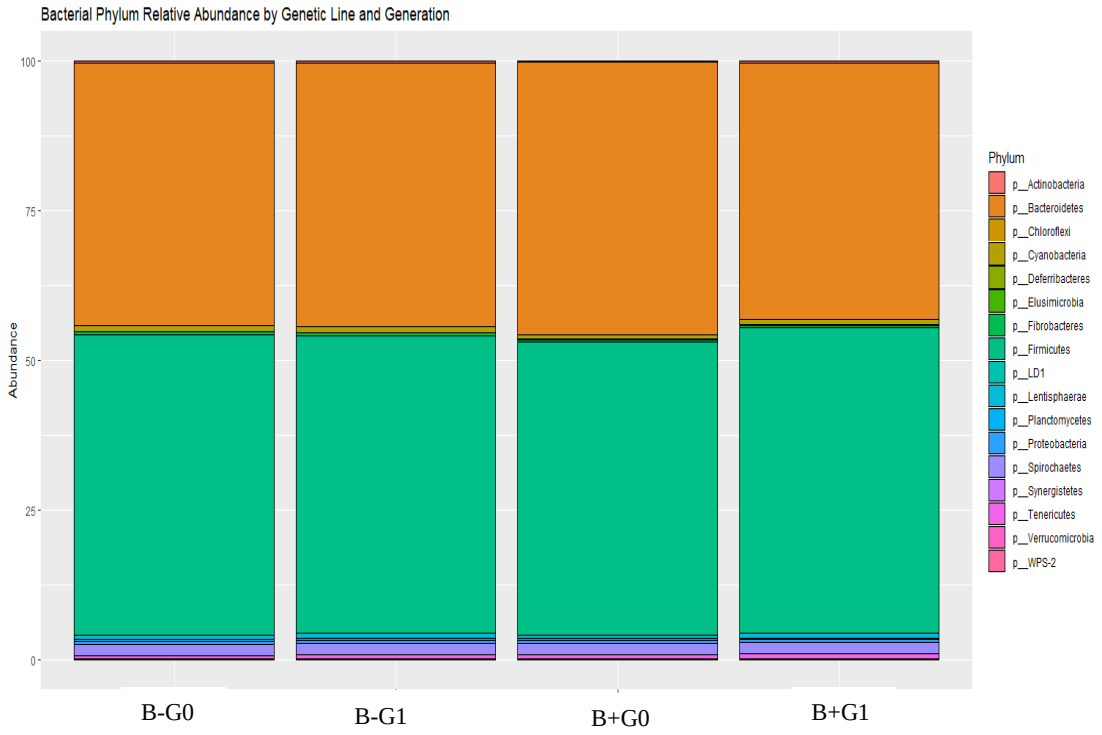


Figure 2.11 Faecal bacterial phylum average abundance by Genetic Line (B-, B+) and Generation (G0, G1). Most abundant bacteria were: B-G0: *Firmicutes* (49.30%), *Bacteroidetes* (43.30%) and *Spirochaetes* (1.9%), while the rest were present in total abundances <0.01; B-G1: *Firmicutes* (48.40%), *Bacteroidetes* (43.40%) and *Spirochaetes* (1.80%); B+G0: *Firmicutes* (48.40%) *Bacteroidetes* (44.50%) and *Spirochaetes* (1.70%); B+G1: *Firmicutes* (50.10%) *Bacteroidetes* (42.10%), and *Spirochaetes* (1.80%)

All archaea belonged to the same phylum, *Euryarcheota*, and for each group the percentage in which it was present in the entire population was: B-G0: 0.70%, B-G1: 1.00%, B+G0: 0.9% and B+G1: 1.00%.

Statistical tests at the phylum level were conducted to explore differences in abundances between Groups (B-G0, B-G1, B_G0, B+G1). All SRT RA at a phylum level present after removal of unidentified Taxa (n = 19) were explored. The only phylum where a significant difference was observed was *Verrucomicrobia* between B+G1 < B-G1 (chi-squared = 13.08, DF = 3, p = 0.02).

2.6.3.1.1.2 Order level

At the order level, the most abundant bacteria belonged to the *Clostridiales* order with 48.70% (range: 43.10% to 53.90%) and *Bacteroidales* with 42.40% (range: 38.20% to 51.80%). The next most abundant bacteria were present at much lower levels, i.e., *Spirochaetales* at 1.70% (range: 0.20% to 3.60%), *YS2* at 0.90% (range: 0.10% to 1.70%), *Victivallales* 0.70% (range: 0.10% to 1.40%), *Methanomicrobiales* 0.90% (range: 0.00% to 1.50%), *Fibrobacterales* 0.50% (range: 0.00% to 1.80%) and *ML615J-28* 0.50% (range: 0.00% to 1.00%). The rest were under 0.50% (**Figure 2.12)**.

The most abundant archaea were *Methanobacteriales* 0.30% (range: 0.00% to 1.50%) and *E2* (Class *Thermoplasmata*) 0.10% (range: 0.00% to 0.30%) (**Figure 2.13).**

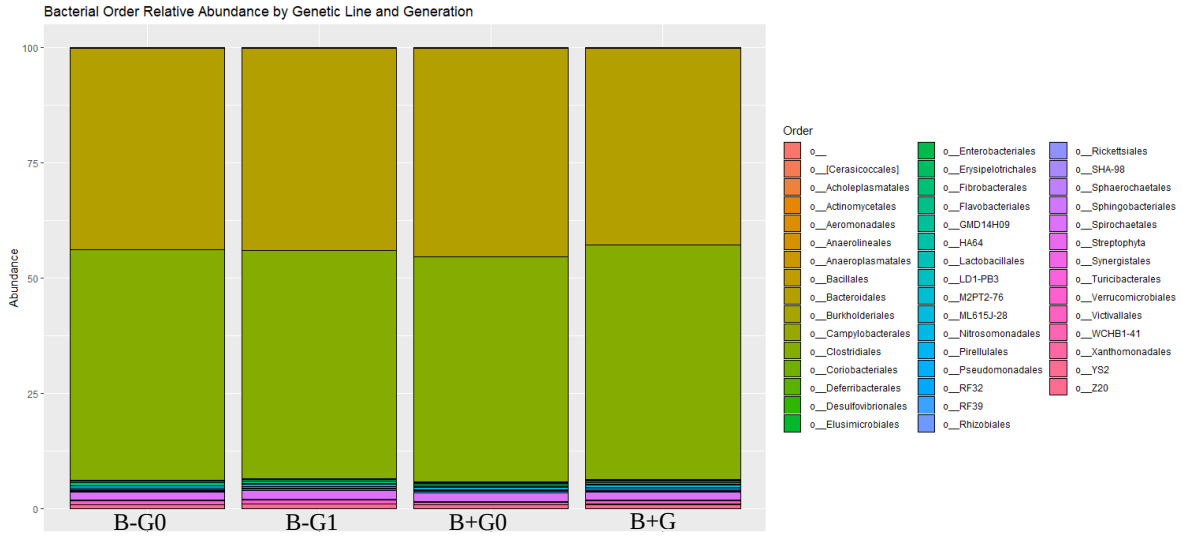


Figure 2.12 Faecal bacterial order average abundance by Genetic Line (B-, B+) and Generation (G0, G1). Most abundant orders were: B-G0: *Clostridiales* (48.90%), *Bacteroidales* (42.80%),

Spirochaetales (1.70%); B-G1: *Clostridiales* (47.90%), *Bacteroidales* (42.90%), *Spirochaetales* (1.80%); B+G0: *Clostridiales* (48.10%), *Bacteroidales* (44.10%), *Spirochaetales* (1.60%); B+G1 *Clostridiales* (49.50%), *Bacteroidales* (41.60%), *Spirochaetales* (1.70%).

Statistical tests at the order level were conducted using all orders with a RA higher than 0.01 prior to square root transformation. SRT RA for order *Z20* (class *Lentisphaeria*) was significantly different (chi-squared = 7.82, DF = 3, p = 0.04) between groups B+G1 - B-G1 (higher in B+G0) and B+G1 - B+G0 (higher in B+G0).

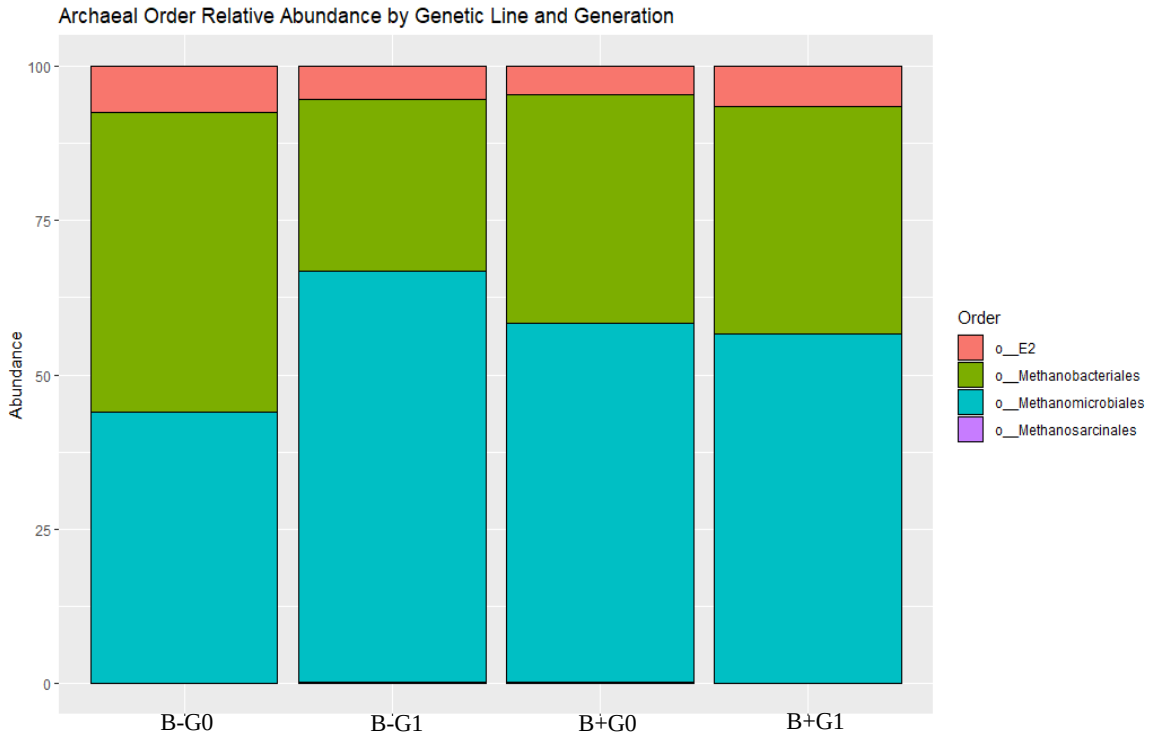


Figure 2.13 Faecal archaeal order abundance barplots by Generation and Genetic Line. *Methanomicrobiales* and *Methanobacteriales* made up for the following percentages in each group: B-G0: 39.80% and 42.50% respectively, B-G1: 69.00% and 25.00% respectively, B+G0: 56.00% and 0.37% respectively, B+G1: 53.00% and 38.00% respectively. E2 was present in the following abundances B-G0: 17.70%, B-G1: 8.00%, B+G0:7.00%, B+G1:9.00%

E2 order abundance (class *Thermoplasmata*) was significantly different (chi-squared = 7.60, DF = 3, p = 0.01) between groups B+G0 <B-G0 and B+G1 > B+G0. *Methanomicrobiales* SRT abundance was also significantly different (chi-squared = 6.90, DF = 3, p = 0.03) between groups B+G0>B-G0 and B-G1 >B-G0, and Methanobacteriales abundances was higher in B-G0 compared to all other groups and significantly lower in B-G0 compared to all other groups (p<0.01 for both)The SRT abundance of *Elusimicrobiales* significantly differed (chi-squared

= 8.02, DF = 3, p = 0.04) between B+G1 < B-G1 groups. Finally, an unclassified member of the *Endomicrobia* class had significant differences in SRT abundance between groups B+G0 < B-G0 and B+G1 < B+G0 (chi-squared = 8.04, DF = 3, p = 0.04).

2.6.3.1.1.3 Genus level

At the genus level, the most abundant bacteria were an unidentified member of the *Ruminococcaceae* family 24.50% (range: 17.60% to 31.10%); unidentified (order *Bacteroidales*) 12.10% (range: 9.10% to 14.70%); unidentified member of the order *Clostridiales* 5.60% (range: 4.40% to 8.10%); unidentified member of the family *Bacteroidaceae* 5.50% (range: 2.80% to 9.60%) and *5-7N15* genus of the *Bacteroidaceae* Family 5.40% (range: 4% to 7.10%).

Genera representing less than 5.00% of the overall population were: unidentified (family *Rikenellaceae*) 3.60% (range: 2.70% to 6.00%), *Ruminococcus* 3.30% (range: 1.40% to 5.10%), *CF231* (family *Paraprevotellaceae*) 3.00% (range: 1.10% to 11.30%), *Oscillospira* 3.00% (range: 2.30% to 4.00%), unidentified (family *RF16*, order *Bacteroidales*) 2.60% (range: 0.60% to 4.50%), unidentified (family *BS11*, order *Bacteroidales*) 1.90% (range: 1.10% to 2.80%), unidentified (family *Lachnospiraceae*) 1.80% (range: 1.00% to 2.50%), Other *Ruminococcaceae* 1.60% (range:1% to 2.1%), *Treponema* 1.60% (range: 0.20% to 3.50%), *BF311* (family *Bacteroidaceae* 1.40% (range: 0.00% to 3.00%), unidentified member of the *Clostridiales* order 1.40% (range: 0.80% to 1.80%), *Bacteroides* 1.30% (range: 0.70% to 2.50%) and *Paludibacter* 1.20% (range: 0.30% to 2.20%). More genera were present at a lower than 1.00% threshold.

The most abundant genera by group (p >0.05), at the genus level are reported in **Figure 2.14**.

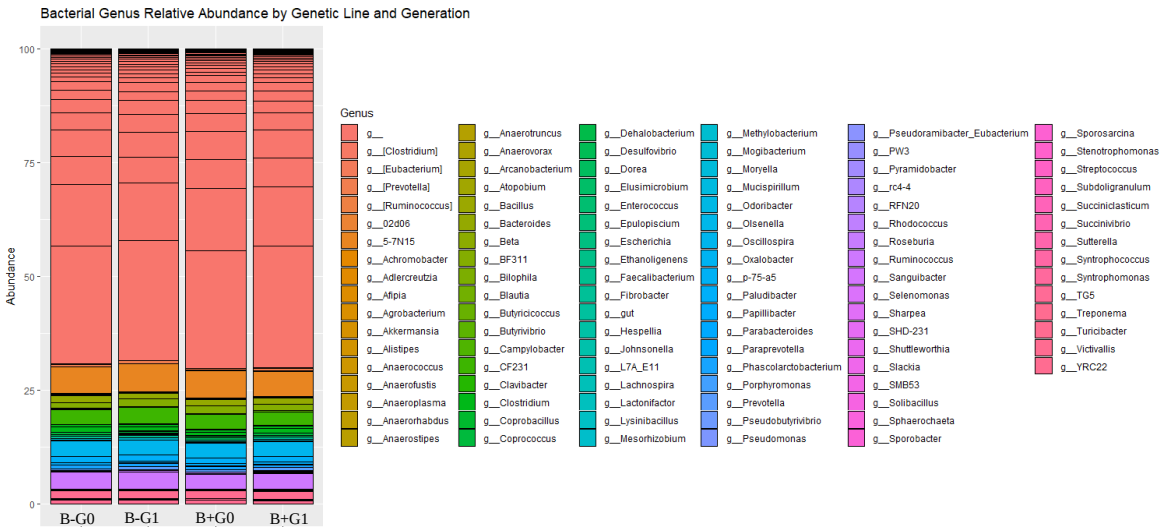


Figure 2.14 Faecal bacterial genus average abundance by Genetic Line (B-, B+) and Generation (G0, G1). Most abundant genera were: B-G0: Unidentified member of the *Ruminococcaceae* family (24.10%),an unidentified member of the *Bacteroidetes* order (11.70%), genus *5-7N15* of the *Bacteroidaceae* family (5.60%), unidentified member of the *Clostridiales* order (5.00%), and an unidentified member of the *Bacteroidaceae* family (5.30%); B-G1: unidentified member of the *Ruminococcaceae* family (24.30%), unidentified member of the *Bacteroidetes* order (12.70%), genus *5-7N15* of the *Bacteroidaceae* family (5.50%), unidentified member of the *Clostridiales* order (5.60%), and an unidentified member of the *Bacteroidaceae* family (5.40%);B+G0: unidentified member of the *Ruminococcaceae* family (25.00%), unidentified member of the *Bacteroidetes* order (12.70%), genus *5-7N15* of the *Bacteroidaceae* family (5.70%), unidentified member of the *Clostridiales* order (5.90%), and an unidentified member of the *Bacteroidaceae* family (5.50%);B+G1: unidentified member of the *Ruminococcaceae* family (24.30%), unidentified member of the *Bacteroidetes* order (12.10%), genus *5-7N15* of the *Bacteroidaceae* family (5.70%), unidentified member of the *Clostridiales* order (5.80%) and an unidentified member of the *Bacteroidaceae* family (5.90%).

Regarding the archaeal genera, *Methanocorpusculaceae* was highest in abundance in the groups representing 0.70% of the overall diversity in the B-G1 group, 0.30% in the B-G0 group, 0.60% in the B+G0 group and 0.50% in the B+G1 group. *Methanobrevibacter* was the second highest in abundance representing 0.30% of the overall population in the B-G1 group, 0.30% in the B-G0 group, 0.30% in the B+G0 group and 0.40% in the B+G1 group.

Statistical analyses performed on SRT RA at the genus level aimed to explore the effect of Generation and Genetic Line on the microbial composition. The genera with a RA >0.05, before transformation, were explored further. The groups explored were once again B-G0 (n = 8), B-G1 (n = 20), B+G0 (n = 5) and B+G1 (n = 23). The RA of an unidentified member of

the *BS11* family (order *Bacteroidales*) differed between groups B+G1> B-G0 and B+G1 > B+G0 (chi-squared = 7.39, DF =3, p <0.01). The RA of the genus *Paludibacter* differed between groups B-G1 > B-G0 and B+G1 < B-G0 (chi-squared = 6.90, DF =3, p = 0.01).

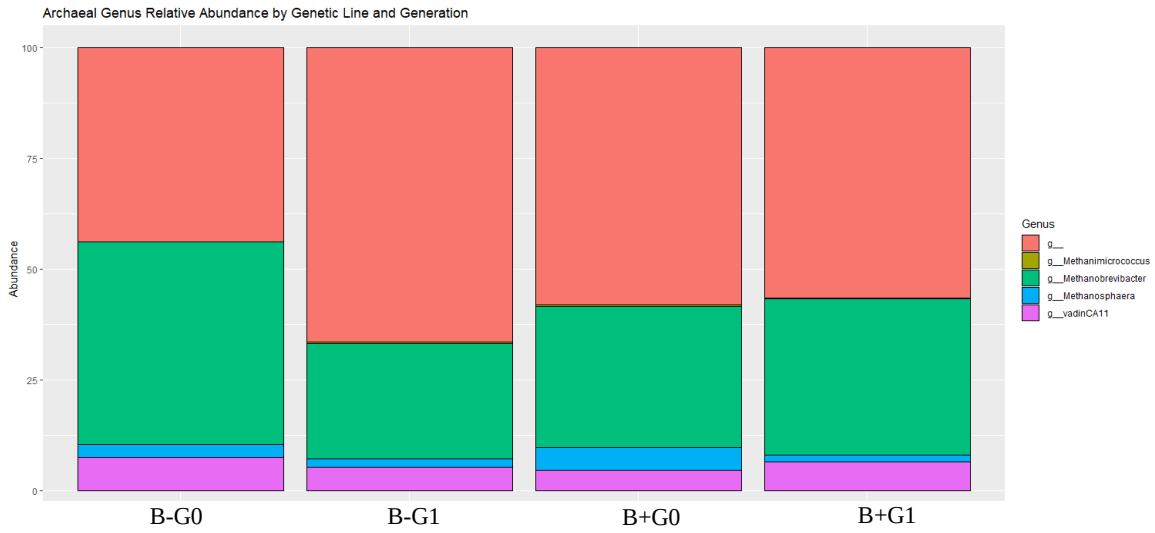


Figure 2.15 Faecal archaeal genus abundance barplots by Generation and Genetic Line*Methanobrevibacter* and and Unknown genus made up for the following percentages in each group: B-G0: 50.00% and 39.50% respectively, B-G1: 25.00% and 67.50% respectively, B+G0: 30.00% and 59.00% respectively, B+G1: 34.00% and 57.00% respectively. *Methanosphaera* was present in the following abundances B-G0: 3.50%, B-G1: 2.00%, B+G0:5.00%, B+G1:2.00%, and *vadinCA11*: B-G0: 7.00%, B-G1: 5.50%, B+G0:6.00%, B+G1:7.00%. *Methanomicrococcus* was present in less than 1.00% abundance.

Statistical analyses performed on SRT RA at the genus level aimed to explore the effect of Generation and Genetic Line on the archaeal composition. The groups explored were once again B-G0 (n = 8), B-G1 (n = 20), B+G0 (n = 5) and B+G1 (n = 23). *Methanobrevibacter* was significantly more abundant in B-G0 animals compared to all other groups (p<0.01) and lower in B-G1 animals (p<0.01). Similarly, the unknowm archaeal genus was significantly higher in abundance in the B-G1 animals anD lower in B-G0 animals (p<0.01 for both). *Methanosphaera* was significantly higher in relative abundance for the B+G0 animals (p=0.02).

2.6.3.1.2 Faecal bacterial and archaeal diversity exploration

2.6.3.1.2.1 Alpha diversity

Boxplots (**Figure 2.16**, **Figure 2.17**) were created in "phyloseq" from the rarefied OTU data, to visually investigate potential differences in the different diversity indices calculated for the bacterial and archaeal communities.

The Kruskal-Wallis test was conducted on rarefied OTU counts to explore differences between groups B+G0 (n = 6), B+G1 (n = 24), B-G0 (n = 8) and B-G1 (n = 20) and did not result in any statistically significant differences.

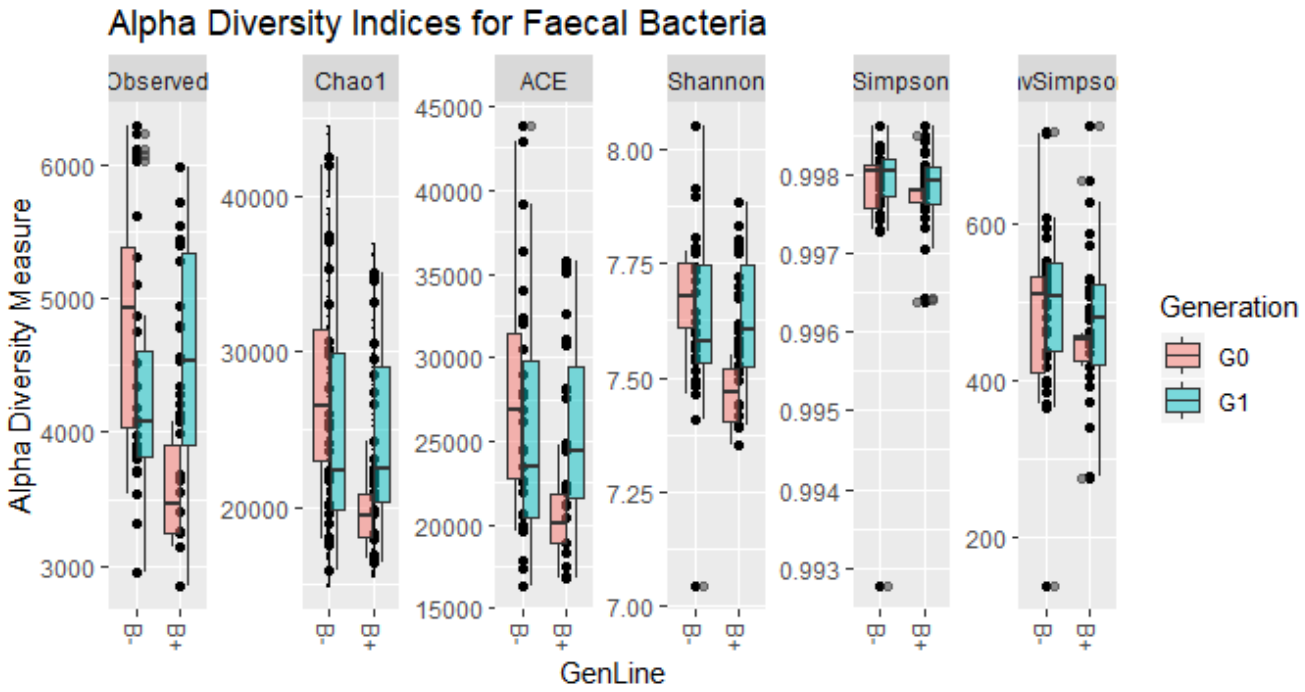


Figure 2.16 Boxplot of the diversity indices (Observed, Chao1, ACE, Shannon, Simpson and Inverse Simpson) calculated in "phyloseq", to examine differences in faecal bacterial diversity between Genetic Lines (B+, B-) and Generations (G0, G1). Means, IQR and individual values are also presented. No statistically significant differences were observed.

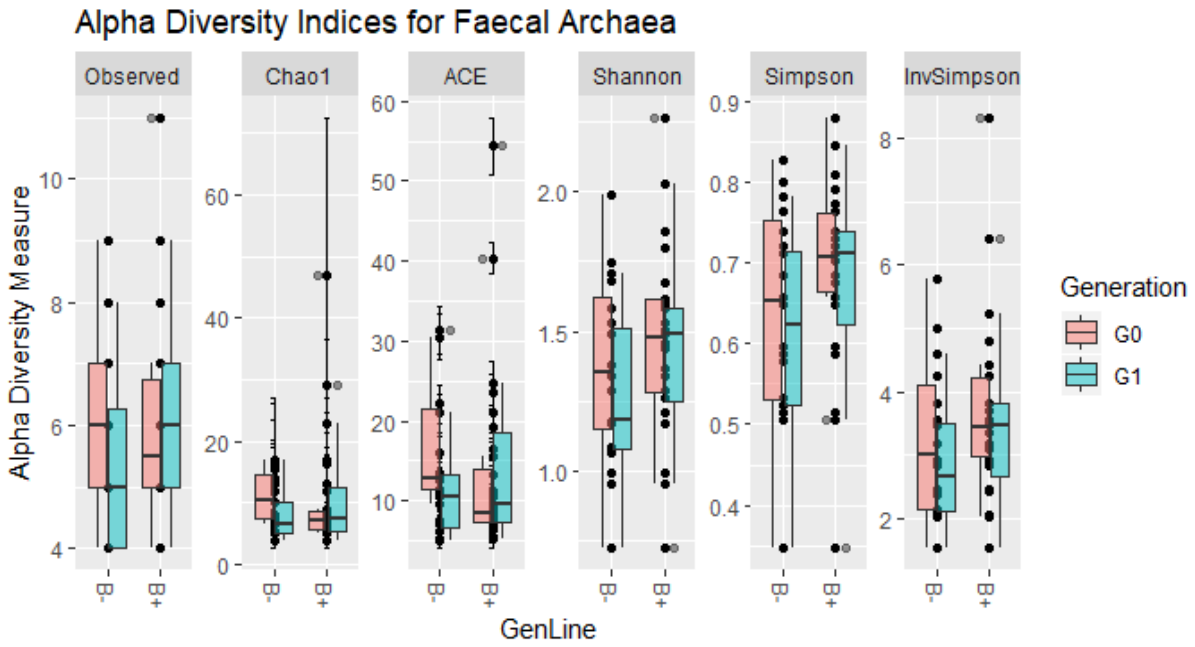


Figure 2.17 Boxplot of the diversity indices (Observed, Chao1, Ace, Shannon, Simpson and Inverse Simpson) calculated in "phyloseq", to examine differences in faecal archaeal diversity between Genetic Lines (B+, B-) and Generations (G0, G1). Means, IQR and individual values are also presented. No statistically significant differences were observed.

2.6.3.1.3 Beta diversity bacteria

A permutation test for homogeneity of multivariate dispersions was applied to investigate the dispersion of the samples within the groups [B+G0 (n = 6), B+G1 (n = 24), B-G0 (n = 8) and B-G1 (n = 20)] for Generation and Genetic Line. The number of permutations was set at 999. A statistically significant difference was observed for Generation [MSE = $2.65e^{-03}$, F (1, 55) = 56.45, p <0.01] and the difference in dispersion can be observed in **Figure 2.18** below. No difference was observed with regards to Genetic Line [MSE = $2.70e^{-08}$, F (1, 55) <0.01, p = 0.98].

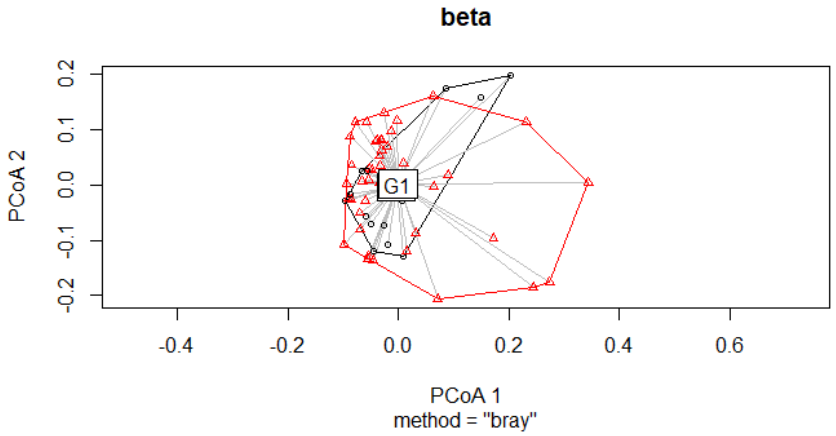


Figure 2.18 Beta dispersion plot indicating the difference in dispersion between Generation G0 (black points and line) and G1 (red points and line) as calculating using Bray-Curtis distances on "Hellinger" transformed OTU data.

A Multivariate Analysis of Variance test (Adonis) was performed on the basis of Bray-Curtis distances calculated from the "Hellinger" transformed OTU table, with 999 permutations. The model took into account Generation, Genetic Line and potential interactions. No significant effect was observed for Generation [F (1, 56) = 0.99, R^2 = 0.02, p = 0.56], Genetic Line [F (1, 56) = 1.01, R^2 = 0.02, p = 0.25] and their interaction [F (1, 56) = 1.01, R^2 = 0.02, p = 0.10].

2.6.3.1.4 Beta diversity archaea

A permutation test for homogeneity of multivariate dispersions was applied to investigate the dispersion of the samples within the groups [B+G0 (n = 6), B+G1 (n = 24), B-G0 (n = 8) and B-G1 (n = 20)] for Generation and Genetic Line. The number of permutations was set at 999. No difference was observed for Generation [MSE = 0.004, F (1, 55) = 2.04, p = 0.17] or Genetic Line [MSE = 0.002, F (1, 55) = 0.83, p = 0.36].

A Multivariate Analysis of Variance test (Adonis) was performed on the basis of Bray-Curtis distances calculated from the Hellinger transformed OTU table, with 999 permutations. The model took into account Generation, Genetic Line and potential interactions. No significant effect was observed for Generation [F (1, 56) = 1.03, R^2 = 0.02, p = 0.45], Genetic Line [F (1, 56) = 0.93, R^2 = 0.01, p = 0.70] and their interaction [F (1, 56) = 1.02, R^2 = 0.02, p = 0.40].

2.6.3.2 PCoA bacteria and archaea

No separation or grouping was observed according to Genetic Line (B+, B-) and Generation (G0, G1) in the faecal bacteria. The percentage of total variation explained by the PCoA axes was low (2.20% Axis 1; 2.10% Axis 2, **Figure 2.19**).

No separation or grouping was observed according to Genetic Line (B+, B-) and Generation (G0, G1) in the faecal archaea. The percentage of total variation explained by the PCoA axes was low (7.10% Axis 1; 4.80% Axis 2, **Figure 2.20**).

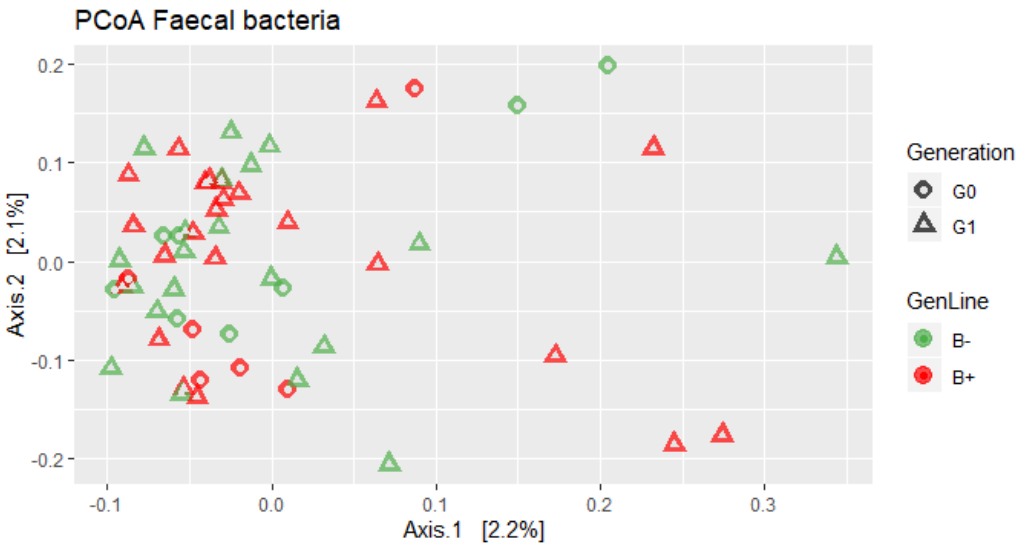


Figure 2.19 Principal coordinate analysis (PCoA) of the faecal bacteria community, based on the SRT RA data of OTUs. The relative distances of all points represent the relative dissimilarities of the samples according to the Bray-Curtis index. Genetic Lines are colour coded and indicated as B+ and B- and Generations (G0 and G1) are distinguished by shape. The percentage of total variation explained by each PCoA axis is shown in the brackets.

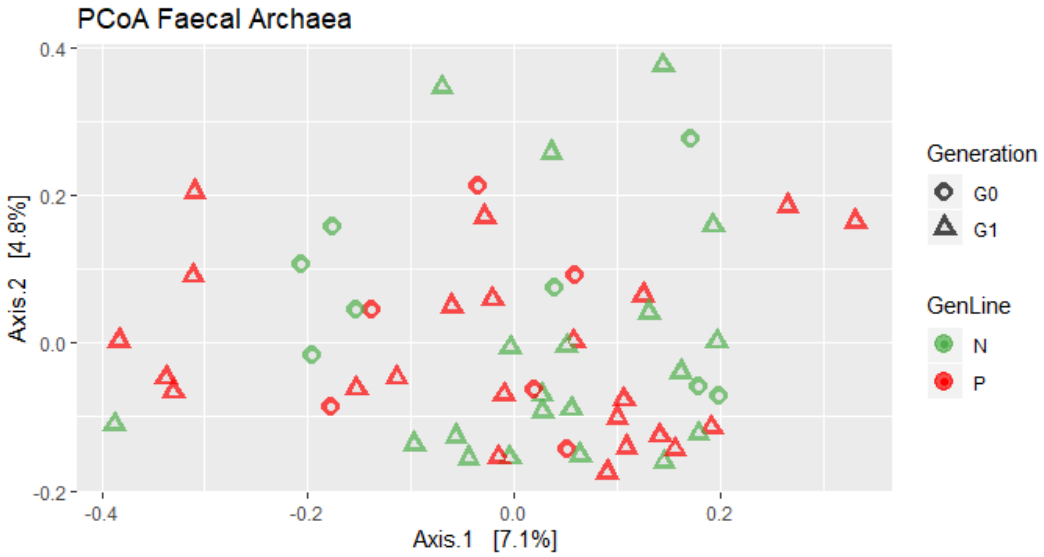


Figure 2.20 Principal coordinate analysis (PCoA) of the faecal archaeal community based on the SRT RA data of OTUs. The relative distances of all points represent the relative dissimilarities of the samples according to the Bray-Curtis index. Genetic Lines are colour coded and indicated as B+ and B- and Generations (G0 and G1) are distinguished by shape. The percentage of total variation explained by each PCoA axis is shown in the brackets.

2.6.3.3 Correlation analyses and PLS

2.6.3.3.1 Correlation between estimated breeding values and cortisol concentration

Values that were not available were removed and a ranked Spearman-Rho correlation was applied. No significant relationship was observed between the SRT cortisol values and the EBVs (R = -0.16, p = 0.25).

2.6.3.3.2 Correlation between cortisol and protozoa

The correlation coefficient between cortisol and the total protozoan counts was R = -0.04 (p = 0.78) indicating a very poor correlation. Pearson correlation coefficient between cortisol and *Dasytricha* resulted in R = 0.10 (p = 0.49), indicating the relationship between the two was poor.

Similarly, Pearson correlation coefficient between cortisol and small *Entodiniomorphs* showed no significant relationship between the two (R = -0.0, p = 0.85). Spearman-rho ranked correlation for cortisol- *Isotricha* and cortisol-large-*Entodiniomorphs* did not indicate the presence of any statistically significant relationship between variables (R = 0.06, p = 0.65 and R = -0.17, p = 0.23 respectively).

2.6.3.3.3 Canonical analysis of principal coordinates

On an OTU level, a plot of the canonical analysis of principal coordinates (CAP) was used to visualise potential differences in bacterial and archaeal communities in the rumen and faecal samples studied separately according to Genetic Line and Generation of animals, and the potential effect of cortisol and EBVs, based on Bray–Curtis dissimilarity of Hellinger transformed data.

2.6.3.3.3.1 Rumen bacteria OTU level

The CAP plot produced using Bray Curtis distances calculated on the Hellinger transformed OTU data for rumen bacteria is depicted in **Figure 2.21**. The first two principal component axes (containing the most variability) were used for this plot, but the amount of variability explained still remained low. A CAP plot aids in the visualisation of differences in the location or relative dispersion between *a priori* set groups. Here (**Figure 2.21**) we see a clear separation of groups B+ and B-, less so for Generations G0 and G1. Cortisol and EBVs appear to be unrelated as they are facing in opposite directions. The EBVs appear to be more related with the B+ genetic line, but for cortisol this is not so evident.

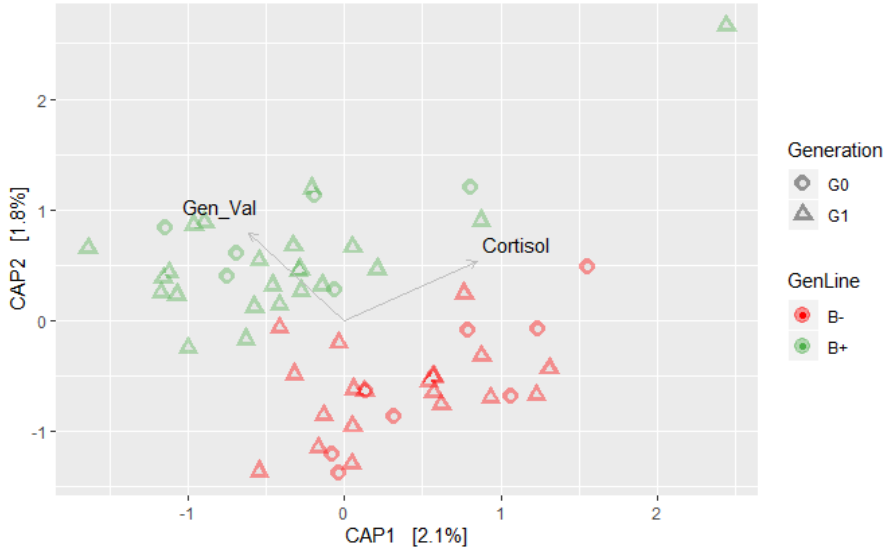


Figure 2.21 Canonical analysis of principal coordinates (CAP) ordination plot (based on Bray-Curtis of SRT bacterial OTU abundances) showing canonical axes that best discriminate the bacterial community assemblages across Genetic Line (Red and Green) and Generations (circle vs triangle). Cortisol and EBVs have been overlaid on the plot as vectors. Vector length corresponds to the strength of the correlation.

2.6.3.3.3.2 Rumen archaea OTU level

The CAP plot produced using Bray-Curtis distances calculated on the "Hellinger" transformed OTU data for rumen archaea is depicted below (**Figure 2.22**). The first two principal component axes (containing the most variability) were used for this plot, but the variability explained remained low (**Figure 2.22**).

The separation between Genetic Lines B+ and B- and Generations G0, G1 is not defined. Cortisol and EBVs appear to be unrelated as they are facing in opposite directions. The EBVs appear to be more related with the B+ Genetic Line, but for cortisol it is not so evident.

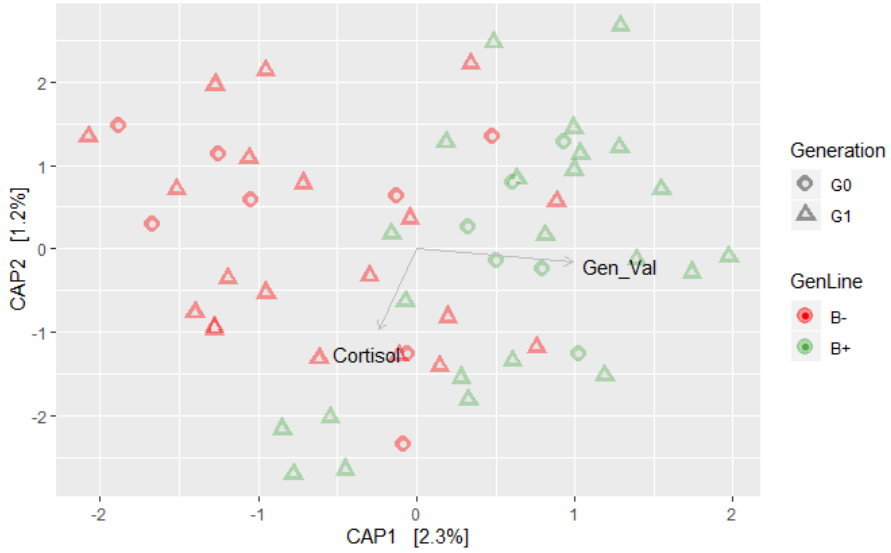


Figure 2.22 Canonical analysis of principal coordinates (CAP) ordination plot (based on Bray-Curtis of SRT archaeal OTU abundances) showing canonical axes that best discriminate the archaeal community assemblages across Genetic Line (Red and Green) and Generations (circle vs triangle). Cortisol and EBVs have been overlaid on the plot as vectors. Vector length corresponds to the strength of the correlation**.**

2.6.3.3.3.3 Faecal bacteria OTU level

The CAP plot produced using Bray-Curtis distances calculated on the Hellinger transformed OTU data for faecal bacteria is depicted below (**Figure 2.23**). The first two principal component axes (containing the most variability) were used for this plot, but the variability explained remained low. Here we see a clear separation of groups B+ and B-, less so for Generation G0 and G1. Cortisol and EBVs appear to be unrelated as they are facing in opposite

directions. The EBVs appear to be more related with the B+ genetic line, but for cortisol it is not so evident.

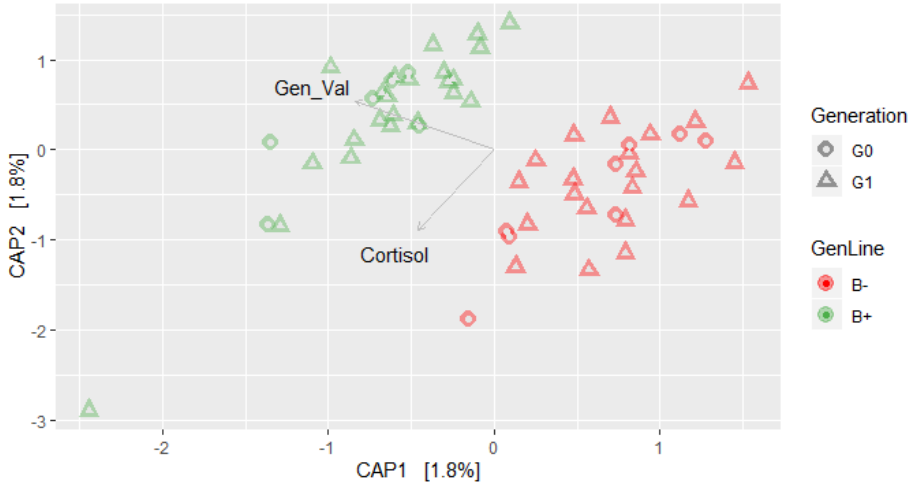


Figure 2.23 Canonical analysis of principal coordinates (CAP) ordination plot (based on Bray-Curtis of SRT faecal bacterial OTU abundances) showing canonical axes that best discriminate the bacterial community assemblages across Genetic Line (Red and Green) and Generations (circle vs triangle). Cortisol and EBVs have been overlaid on the plot as vectors. Vector length corresponds to the strength of the correlation**.**

2.6.3.3.3.4 Faecal archaea OTU level

The CAP plot produced using Bray-Curtis distances calculated on the Hellinger transformed OTU data for faecal archaea is depicted below (**Figure 2.24**). The first two principal component axes (containing the most variability) were used for this plot, but variability explained remained low.

Groups B+ and B- were relatively well distinguished with a few members of each group overlapping. For Generations G0 and G1 the separation was less clear. Cortisol and EBVs appear to be unrelated as they are facing in opposite directions. The EBVs appear to be more related with the B+ genetic line, but for cortisol it is not so evident.

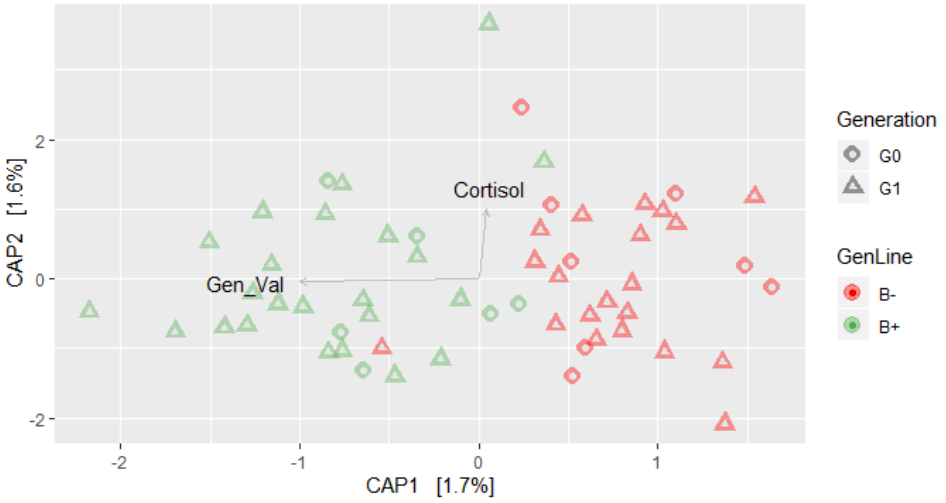


Figure 2.24 Canonical analysis of principal coordinates (CAP) ordination plot (based on Bray-Curtis of SRT faecal archaeal OTU abundances) showing canonical axes that best discriminate the archaeal community assemblages across Genetic Line (Red and Green) and Generations (circle vs triangle). Cortisol and EBVs have been overlaid on the plot as vectors. Vector length corresponds to the strength of the correlation.

2.6.4 PLS regression analyses between cortisol, estimated breeding values and phylum level taxa

2.6.4.1 Rumen samples

2.6.4.1.1 Phylum level

2.6.4.1.1.1 Phyla-cortisol

A PLS regression between SRT RA of all rumen phyla with SRT cortisol values was carried out. We observed that 69.70% of the observed variability in cortisol explained 22.54% of the variability of abundances at a phylum level. The phyla with VIP >1.00 were: *Synergistetes* (VIP: 2.07), *Actinobacteria* (VIP: 1.17), *Elusimicrobia* (VIP: 1.13), *Spirochaetes* (VIP: 1.09), *Cyanobacteria* (VIP: 1.07), *SR1* (VIP: 1.06) and *Firmicutes* (VIP: 1.07). In this instance all of the phyla (n = 20), apart from the unidentified "Other", were used for further analyses by creating a correlation matrix between their SRT abundances and SRT cortisol values.

From the table below (**Table 2.2**), we can see the relationship the phyla with VIP >1.00 have with the cortisol levels. The phylum exhibiting the strongest positive correlation with cortisol was *Actinobacteria* (A3) (R = 0.31, p = 0.02 and the phylum with the strongest negative correlation was *Synergistetes* (A18) (R = -0.23, p = 0.08). Correlograms exploring the relationship between cortisol and taxonomic order RA, as well as relationships between the

bacterial and archaeal abundances with VIP >1 are reported in the Appendix **Figure 7.1** and **Table 7.1**.

Table 2.2. Rumen phyla relationship with SRT cortisol values. VIPs from the PLS analysis, relative abundance, direction of correlation, correlation coefficients (R) and p-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix in the Appendix **Figure 7.1**.

	Phylum	**VIP**	**Mean RA**	**Direction**	**R**	**p-value**
A1	*Euryarchaeota*	0.87	0.51	Positive	0.16	0.24
A3	*Actinobacteria*	1.17	0.08	Positive	0.31	0.02
A4	*Armatimonadetes*	0.94	0.01	Positive	0.02	0.88
A5	*Bacteroidetes*	0.92	38.94	Positive	0.07	0.61
A6	*Chloroflexi*	0.86	0.05	Positive	0.05	0.72
A7	*Cyanobacteria*	1.08	0.87	Negative	-0.06	0.67
A8	*Elusimicrobia*	1.13	0.12	Positive	0.13	0.32
A9	*Fibrobacteres*	0.95	0.71	Positive	0.02	0.89
A10	*Firmicutes*	1.02	15.18	Negative	-0.11	0.40
A11	*Fusobacteria*	0.70	0.00	Positive	0.04	0.77
A12	*LD1*	0.81	0.02	Positive	0.13	0.34
A13	*Lentisphaerae*	0.73	0.23	Positive	0.10	0.46
A14	*Planctomycetes*	0.99	0.10	Positive	0.12	0.38
A15	*Proteobacteria*	0.89	0.54	Positive	0.15	0.25
A16	*SR1*	1.07	0.01	Positive	0.15	0.26
A17	*Spirochaetes*	1.09	0.50	Positive	0.14	0.30
A18	*Synergistetes*	2.07	1.00	Negative	-0.23	0.08
A19	*Tenericutes*	0.79	0.23	Positive	0.05	0.69
A20	*Verrucomicrobia*	0.77	0.06	Positive	0.16	0.24
A21	*WPS-2*	0.77	0.01	Positive	0.10	0.45

2.6.4.1.1.2 Phyla-estimated breeding values

PLS regression between SRT RA of all rumen phyla with the animals' EBVs was carried out. We observed that 71.25% of the observed variability in EBVs explained 34.99% of the variability of abundances at a phylum level. The phyla with VIP >1.00 were: *Elusimicrobia* (VIP: 2.26; p = 0.02), *Fusobacteria* (VIP: 1.87; p = 0.51), *Planctomycetes* (VIP: 1.12; p = 0.79), *Tenericutes* (VIP: 1.12; p = 0.74) and *Bacteroidetes* (VIP: 1.00; p = 0.15) (**Table 2.3**).

In this case all of the phyla (n = 20), apart from the unidentified "Other", were used for further analyses by creating a correlation matrix between their SRT RA and EBVs. Correlograms exploring the relationship between EBVs and taxonomic order RA, as well as relationships between the bacterial and archaeal abundances with VIP >1 are reported in the Appendix **Figure 7.2** and **Table 7.2**.

Table 2.4 Rumen phyla relationship with EBVs. VIPs from the PLS analysis, RA, direction of correlation, correlation coefficients (R) and p-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix in the Appendix **Figure 7.2**.

	Phylum	**VIP**	**Mean RA**	**Direction**	**R**	**p-values**
A1	*Euryarchaeota*	0.57	0.51	Negative	-0.12	0.36
A3	*Actinobacteria*	0.84	0.08	Negative	-0.29	0.03
A4	*Armatimonadetes*	0.90	0.01	Negative	-0.08	0.57
A5	*Bacteroidetes*	1.00	38.94	Positive	0.19	0.15
A6	*Chloroflexi*	0.90	0.05	Negative	-0.07	0.61
A7	*Cyanobacteria*	0.73	0.87	Negative	-0.25	0.06
A8	*Elusimicrobia*	2.26	0.12	Negative	-0.31	0.02
A9	*Fibrobacteres*	0.98	0.71	Negative	-0.29	0.03
A10	*Firmicutes*	0.70	15.18	Negative	-0.11	0.43
A11	*Fusobacteria*	1.87	0.00	Positive	0.09	0.51
A12	*LD1*	0.49	0.02	Negative	-0.06	0.64
A13	*Lentisphaerae*	0.58	0.23	Negative	-0.15	0.28
A14	*Planctomycetes*	1.12	0.10	Negative	-0.04	0.79
A15	*Proteobacteria*	0.70	0.54	Negative	-0.35	0.01
A16	*SR1*	0.86	0.01	Negative	-0.09	0.48

A17	*Spirochaetes*	0.51	0.50	Negative	-0.14	0.30
A18	*Synergistetes*	0.45	1.00	Negative	0.00	0.99
A19	*Tenericutes*	1.12	0.23	Positive	0.05	0.74
A20	*Verrucomicrobia*	0.76	0.06	Negative	-0.14	0.31
A21	*WPS-2*	0.64	0.01	Positive	0.02	0.86

2.6.4.2 Order level

2.6.4.2.1 Orders-cortisol

PLS regression conducted for the rumen SRT RA at an order level and cortisol values suggested that 15.43% of the variability present in the cortisol values explains 51.98% of the variability present in the RA. Orders with VIP >1.00 are presented in below (**Table 2.4**).

Table 2.5 Orders with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT order relative abundances and SRT cortisol values. RA, direction of correlation, correlation coefficient (R) and p-values from the correlation analysis are also reported. Order level as well as higher levels (if order was ambiguous or unidentified) are reported. Coding to relate to the correlation matrix is found on the left.

	Orders	**VIP**	**Mean RA**	**Direction**	**R**	**p-values**
A28	*Lactobacillales*	2.03	0.02	Positive	0.35	0.01
A19	*Coriobacteriales*	1.75	0.08	Positive	0.31	0.02
A15	*p_Spirochaetes; c_MVP-15;o_PL-11B10*	1.74	0.09	Positive	0.30	0.02
A31	*Myxococcales*	1.52	0.02	Positive	0.26	0.05
A26	*c_[Lentisphaeria] ;o_Z20*	1.41	0.04	Positive	0.25	0.06
A3	*Synergistales*	1.41	0.98	Negative	-0.23	0.08
A22	*p_Tenericutes;c_RF3;o_ ML615J-28*	1.36	0.06	Positive	0.22	0.10

A25	*c_Verruco-5; o_LD1-PB3*	1.28	0.04	Positive	0.15	0.28
A8	*Methanobacteriales*	1.15	0.23	Positive	0.20	0.13
A4	*p_Cyanobacteria; c_4C0d-2;o_YS2*	1.14	0.85	Negative	-0.06	0.63
A11	*Erysipelotrichales*	1.06	0.18	Positive	0.19	0.17
A35	*p_Firmicutes;Other; Other*	1.03	0.01	Negative	-0.17	0.20
A9	*c_Alphaproteobacteria o_RF32*	1.00	0.22	Positive	0.15	0.28

These orders were used for further analyses by creating a correlation matrix between their SRT RA and the cortisol values. The order exhibiting a strong positive correlation with cortisol was the order *Lactobacillales* (A28) (R = 0.35, p = 0.01) and the order with the strongest negative correlation was *Synergistales* (A3) (R = -0.23, p = 0.08). Correlograms exploring the relationship between cortisol and taxonomic order RA, as well as relationships between the bacterial and archaeal abundances with VIP >1 are reported in the Appendix **Figure 7.3** and **Table 7.3.**

2.6.4.2.1.1 Orders-estimated breeding values

PLS regression conducted for the rumen SRT RA at an order level and the EBVs, suggested that 15.90% of the variability present in the EBVs explains 39.30% of the variability present in the RA. Orders with VIP >1.00 are presented in (**Table 2.5**) below.

Table 2.6 Orders with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT Order Mean RA and EBVs. Relative abundance, direction of correlation, correlation coefficient (R) and p-values from the correlation analysis are also reported. Order level as well as higher levels (if order was ambiguous or unidentified) are reported. Coding to relate to the correlation matrix is found on the left.

	Order	**VIP**	**mean RA**	**Direction**	**R**	**p-value**
A5	*Fibrobacterales*	1.94	0.66	Negative	-0.29	0.03
A34	*p_Planctomycetes;c_vadinHA49;Other*	1.76	0.01	Negative	-0.30	0.02
A19	*Coriobacteriales*	1.58	0.08	Negative	-0.29	0.03
A16	*Elusimicrobiales*	1.56	0.09	Negative	-0.29	0.03
A4	*p_Cyanobacteria;c_4C0d-2;o_YS2*	1.45	0.85	Negative	-0.26	0.05
A8	*Methanobacteriales*	1.43	0.23	Negative	-0.26	0.05
A9	*c_Alphaproteobacteria;o_RF32*	1.40	0.22	Negative	-0.26	0.05
A23	*c_Anaerolineae;o_Anaerolineales*	1.31	0.05	Negative	-0.06	0.65
A15	*p_Spirochaetes;c_MVP-15; o_PL-11B10*	1.31	0.09	Negative	-0.24	0.08
A12	*c_Mollicutes;o_RF39*	1.25	0.12	Negative	-0.12	0.37
A21	*c_Alphaproteobacteria;Other*	1.21	0.07	Negative	-0.22	0.10
A24	*c_Mollicutes;o_Anaeroplasmatales*	1.17	0.04	Positive	0.22	0.11
A26	*c_[Lentisphaeria];o_Z20*	1.15	0.04	Negative	-0.18	0.17
A10	*Victivallales*	1.12	0.19	Negative	-0.13	0.33
A1	*Bacteroidales*	1.05	37.54	Positive	0.19	0.15

These orders were used for further analyses by creating a correlation matrix between their SRT abundances and the EBVs. Correlograms exploring the relationship between EBVs and taxonomic order RA, as well as relationships between the bacterial and archaeal abundances

with VIP >1 are reported in the Appendix **(Figure 7.4)**. The order exhibiting a strong positive correlation with the EBVs was the order *Anaeroplasmatales* (A24) (R = 0.22, p = 0.11) and the order with the strongest negative correlation was an unidentified member of the *Planctomycetes* phylum (A34) (R = -0.29, p = 0.03).

2.6.4.2.2 Genus level

2.6.4.2.2.1 Genera- cortisol

PLS regression conducted for the rumen SRT RA at a genus level and cortisol values suggested that 19.29% of the variability present in the cortisol values explains 53.02% of the variability present in the RA. Genera with VIP >1.50 are presented in (**Table 2.6**) below.

Table 2.7 Genera with VIP scores higher than 1.5, as reported by the PLS regression used to explore the relationship of SRT Genera relative abundances and cortisol. Genera are presented along with Family and higher taxonomic level if necessary, for recognition purposes. The VIP score from the PLS analysis, overall relative abundance of each genus, the direction of correlation with cortisol as well as the correlation coefficient (R) and p-values as reported from the correlation analyses.

	Genera	**VIP**	**mean RA**	**Direction**	**R**	**p- value**
A130	*Afipia*	2.30	0.200	Negative	-0.31	0.02
A168	*f_Dethiosulfovibrionaceae;g_TG5*	2.01	0.001	Negative	-0.19	0.17
A118	*f_Erysipelotrichaceae;g_p-75-a5_ Unkniwn*	1.86	0.045	Positive	0.10	0.45
A36	*f_[Paraprevotellaceae];Other*	1.85	0.026	Positive	0.03	0.83
A38	*f_[Paraprevotellaceae];g_CF231*	1.83	0.008	Positive	0.08	0.53
A100	*f_Veillonellaceae;Unknown*	1.83	0.008	Negative	-0.11	0.41
A80	*Pseudobutyrivibrio*	1.82	0.004	Negative	-0.23	0.08
A84	*f_Peptococcaceae;Unknown_*	1.81	0.020	Negative	-0.18	0.17
A9	*f_Coriobacteriaceae;Other*	1.73	0.008	Positive	0.34	0.01
A55	*p_Firmicutes;Other;Other; Other;Other*	1.72	0.007	Negative	-0.20	0.13
A39	*f_[Paraprevotellaceae];g_YRC22*	1.68	0.008	Positive	0.05	0.70

A68	*f_Lachnospiraceae;g_*	1.67	0.009	Positive	0.09	0.53
A150	*o_Myxococcales;f_0319-6G20;Unknown_*	1.66	0.015	Positive	0.31	0.02
A111	*f_Erysipelotrichaceae;Other*	1.57	0.001	Negative	-0.20	0.15
A65	*Anaerofustis*	1.56	0.001	Negative	-0.25	0.06
A11	*Adlercreutzia*	1.52	0.000	Positive	0.30	0.02
A5	*f_[Methanomassiliicoccaceae];Other*	1.51	0.214	Negative	-0.22	0.10

The genera above were used for further analyses to create a correlation matrix between their SRT abundances and the SRT of the cortisol values. Correlograms exploring the relationship between cortisol and taxonomic order RA, as well as relationships between the bacterial and archaeal abundances with VIP >1.5 are reported in the Appendix **Figure 7.4** and **Table 7.4**.

The genus exhibiting a strong positive correlation with cortisol was an unidentified member of the *Ruminococcaceae* Family (A30) ($R = 0.38$, $p < 0.01$) and the genus with the strongest negative relationship from the correlation analysis was *Methylobacterium* ($R = -0.44$, $p < 0.01$).

2.6.4.2.2.2 Genera- estimated breeding values

PLS regression conducted for the rumen SRT RA at a genus level and EBVs, suggested that 15.90% of the variability present in the EBVs explains 30.30% of the variability observed in the relative abundances. Orders with VIP >1.00 are presented in **Table 2.7** below.

Table 2.8 Genera with VIP scores higher than 1.5, as reported by the PLS regression used to explore the relationship of SRT Genera relative abundances and Estimated Breeding values (EBVs). Genera are presented along with family and higher taxonomic level, if necessary, for recognition purposes. The VIP score from the PLS analysis, overall relative abundance of each genus, the direction of correlation with EBVs as well as the correlation coefficient (R) and p-values as reported from the correlation analyses.

	Genera	**VIP**	**mean Ra**	**Direction**	**R**	**p-value**
A113	*Anaerorhabdus*	1.96	0.004	Positive	0.24	0.07
A116	*Sharpea*	1.64	0.01	Negative	-0.20	0.13
A126	*p_Proteobacteria;Other;*	1.79	0.004	Positive	0.22	0.11

	Other;Other;Other					
A134	*Agrobacterium*	1.57	0.0003	Positive	0.19	0.15
A136	*Novosphingobium*	1.94	0.0004	Positive	0.24	0.07
A137	*Sphingomonas*	1.78	0.001	Positive	0.22	0.10
A142	*f_Neisseriaceae;Other*	1.42	0.0004	Negative	-0.18	0.19
A146	*f_Desulfovibrionaceae;g_*	1.97	0.001	Negative	-0.23	0.09
A147	*Desulfovibrio*	1.32	0.002	Positive	0.16	0.23
A148	*o_Desulfuromonadales; Other;Other*	1.53	0.006	Positive	0.19	0.16
A15	*Slackia*	1.85	0.001	Negative	-0.23	0.09
A151	*c_Deltaproteobacteria; o_PB19;f_;g_*	2.01	0.003	Negative	-0.25	0.06
A155	*Ruminobacter*	1.77	0.005	Positive	0.22	0.10
A180	*c_Verruco-5; o_LD1-PB3;f_;g_*	1.60	0.04	Positive	0.19	0.15
A2	*Methanosphaera*	1.82	0.02	Positive	0.22	0.10
A37	*f_[Paraprevotellaceae];g_*	1.98	0.95	Negative	-0.24	0.08
A4	*Methanimicrococcus*	1.62	0.01	Positive	0.20	0.14
A47	*o_Streptophyta;f_;g_*	1.71	0.01	Positive	0.21	0.12
A62	*f_Clostridiaceae;g_02d06*	1.83	0.20	Negative	-0.22	0.09
A76	*Lachnospira*	2.13	0.01	Positive	0.26	0.05
A82	*Shuttleworthia*	1.84	0.07	Negative	-0.22	0.10
A83	*Syntrophococcus*	2.54	0.01	Negative	-0.31	0.02
A93	*Oscillospira*	1.57	0.39	Negative	-0.18	0.18
A96	*Sporobacter*	2.14	0.01	Negative	-0.26	0.05

The genera above were used for further analyses to create a correlation matrix between their SRT abundances and the EBVs. Correlograms exploring the relationship between cortisol and taxonomic order RA, as well as relationships between the bacterial and archaeal abundances

with VIP >1.5 are reported in the Appendix **Figure 7.5** and **Table 7.5**. The genus exhibiting a strong positive correlation with EBVs was *Lachnospira* (A76) (R = 0.26, p = 0.05) and the genus with the strongest negative correlation was and *Syntrophococcus* (A83) (R = -0.31, p = 0.05).

2.6.4.3 Faecal samples

2.6.4.3.1 Phylum level

2.6.4.3.1.1 Phyla- cortisol

By conducting a PLS regression between SRT relative abundances of all faecal phyla with SRT cortisol values, we observed that 34.70% of the observed variability in cortisol explained 23.64% of the variability of abundances at a phylum level. The phyla with VIP >1.00 were: WPS2 (VIP: 1.90), *Plantomycetes* (VIP: 1.60), *Elusimicrobia* (VIP: 1.5), *Synergistetes* (VIP: 1.20), *Cyanobacteria* (VIP: 1.00). In this instance, all the phyla (n = 19) were used for further analyses by creating a correlation matrix between their SRT abundances and SRT cortisol values. Phyla exhibiting a strong positive correlation were *Cyanobacteria* (A6) with *Proteobacteria* (A14) (R = 0.72, p <0.01), whereas those exhibiting a strong negative correlation were *Bacteroidetes* (A4) with *Firmicutes* (A10) (R = -0.84, p <0.01) (**Table 2.8**).

Table 2.9 Faecal phyla relationship with cortisol. VIPs from the PLS analysis, relative abundance, direction of correlation, correlation coefficients (R) and p-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix in the Appendix **Figure 7.8**.

	Phylum	VIP	mean RA	Direction	R	p-value
A19	*WPS-2*	1.90	0.00	Positive	0.26	0.05
A13	*Planctomycetes*	1.60	0.14	Negative	-0.20	0.13
A8	*Elusimicrobia*	1.54	0.03	Positive	0.22	0.10
A17	*Tenericutes*	1.22	0.43	Negative	-0.17	0.22
A6	*Cyanobacteria*	1.00	0.53	Negative	-0.11	0.40
A1	*Euryarchaeota*	0.97	0.55	Positive	0.12	0.38
A3	*Actinobacteria*	0.93	0.13	Positive	0.07	0.63
A9	*Fibrobacteres*	0.93	0.26	Negative	-0.11	0.42

A5	*Chloroflexi*	0.90	0.01	Positive	0.08	0.54
A15	*Spirochaetes*	0.82	1.04	Negative	-0.01	0.93
A16	*Synergistetes*	0.81	0.00	Positive	0.04	0.75
A14	*Proteobacteria*	0.76	0.31	Negative	-0.08	0.57
A12	*Lentisphaerae*	0.74	0.44	Negative	-0.03	0.81
A11	*LD1*	0.68	0.00	Positive	0.05	0.72
A4	*Bacteroidetes*	0.53	24.42	Negative	-0.03	0.85
A7	*Deferribacteres*	0.49	0.01	Positive	0.01	0.93
A10	*Firmicutes*	0.36	28.05	Positive	0.05	0.70
A18	*Verrucomicrobia*	0.35	0.05	Negative	-0.04	0.77

2.6.4.3.1.2 Phyla- estimated breeding values

PLS regression between SRT relative abundances of all faecal phyla with EBVs indicated that 36.76% of the observed variability in EBVs explained 37.72% of the variability of abundances at a phylum level. The phyla with VIP >1.00 were: *Fibrobacteres* (VIP: 1.50), *WPS2* (VIP: 1.40), *Chloroflexi* (VIP: 1.30), *Actinobacteria* (VIP: 1.30), *Firmicutes* (VIP: 1.20), *Cyanobacteria* (VIP: 1.20) and *Synergistetes* (VIP: 1.10) (**Table 2.9,** Appendix **Figure 7.6**).

Table 2.10 Faecal phyla relationship with EBVs. VIPs from the PLS analysis, relative abundance, direction of correlation, correlation coefficients (R) and p-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix in the Appendix. Phylum and kingdom are presented for recognition purposes.

	Phyla	**VIP**	**mean RA**	**Direction**	**R**	**p-values**
A13	*Planctomycetes*	0.38	0.14	Negative	-0.04	0.76
A18	*Verrucomicrobia*	0.46	0.05	Positive	0.08	0.56
A14	*Proteobacteria*	0.61	0.31	Negative	-0.03	0.81
A7	*Deferribacteres*	0.73	0.01	Positive	0.10	0.46
A4	*Bacteroidetes*	0.77	24.42	Negative	-0.13	0.32

A11	*LD1*	0.83	0.00	Positive	0.13	0.35
A3	*Actinobacteria*	0.86	0.13	Positive	0.01	0.95
A15	*Spirochaetes*	0.87	1.04	Negative	-0.03	0.84
A17	*Tenericutes*	0.87	0.43	Positive	0.06	0.67
A8	*Elusimicrobia*	0.94	0.03	Positive	0.16	0.23
A16	*Synergistetes*	1.07	0.00	Positive	0.18	0.19
A6	*Cyanobacteria*	1.18	0.53	Negative	-0.19	0.15
A10	*Firmicutes*	1.23	28.05	Positive	0.21	0.12
A12	*Lentisphaerae*	1.31	0.44	Negative	-0.23	0.09
A5	*Chloroflexi*	1.33	0.01	Negative	-0.15	0.28
A19	*WPS-2*	1.40	0.00	Negative	-0.24	0.08
A9	*Fibrobacteres*	1.47	0.26	Negative	-0.25	0.06

In this instance all the phyla (n = 19) were used for further analyses by creating a correlation matrix between their SRT abundances and EBVs. The relative correlograms can be found in the Appendix (**Figure 7.7**).

2.6.4.3.2 Order level

2.6.4.3.2.1 Orders-cortisol

Carrying out PLS regression between SRT relative abundances of all faecal orders with SRT cortisol values, 15.50% of the observed variability in cortisol explained 53.80% of the variability of abundances at an order level. The orders with VIP >1.00 are presented in **Table 2.10** below. Coding is available to relate with **Figure 7.7** and **Table 7.7** in the Appendix.

Table 2.11 Faecal order relationship with cortisol. VIPs from the PLS analysis, relative abundance, direction of correlation, correlation coefficients (R) and p-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix in the Appendix **Figure 7.8.** Order and higher taxonomic levels (if necessary) are presented for recognition purposes.

	Order	VIP	mean RA	Direction	R	p-value
A53	*Acholeplasmatales*	2.80	0.0006	Negative	-0.37	0.01
A62	*p__WPS-2;c_; Unidentified*	1.94	0.0005	Positive	0.26	0.05
A37	*c_Betaproteobacteria;Other*	1.86	0.004	Negative	-0.25	0.06
A49	*Sphaerochaetales*	1.78	0.03	Negative	-0.31	0.02
A16	*Elusimicrobiales*	1.71	0.03	Positive	0.21	0.11
A30	*Pirellulales*	1.66	0.14	Negative	-0.20	0.13
A48	*c_Spirochaetes;o_M2PT2-76*	1.41	0.04	Negative	-0.18	0.18
A14	*Streptophyta*	1.37	0.04	Negative	-0.18	0.17
A17	*c_Endomicrobia;Unidentified*	1.32	0.001	Positive	0.13	0.32
A61	*Verrucomicrobiales*	1.29	0.02	Negative	-0.18	0.17
A1	*Methanobacteriales*	1.27	0.18	Negative	-0.08	0.55
A2	*Methanomicrobiales*	1.26	0.33	Positive	0.10	0.44
A44	*Aeromonadales*	1.22	0.003	Positive	0.11	0.40
A45	*Enterobacteriales*	1.15	0.0005	Positive	0.13	0.32
A55	*c_Mollicutes;o_RF39*	1.15	0.12	Negative	-0.13	0.33
A29	*c_[Lentisphaeria];o_Z20*	1.13	0.02	Positive	0.19	0.16
A39	*Nitrosomonadales*	1.09	0.002	Positive	0.15	0.26
A54	*Anaeroplasmatales*	1.07	0.005	Negative	-0.13	0.32
A51	*Synergistales*	1.04	0.004	Positive	0.04	0.75
A59	*c_Verruco-5;o_LD1-PB3*	1.02	0.003	Positive	0.09	0.52

Twenty orders presented VIP >1.00 and were subsequently selected for further analyses by creating a correlation matrix between their SRT abundances and SRT cortisol values. The matrix is presented in the Appendix (**Figure** and **Table 7.8**). Orders with VIP >1.00 had moderate to low correlation scores with cortisol.

The order with the highest positive correlation coefficient to cortisol was an unidentified WPS-2 (R = 0.27), while that with the highest negative correlation coefficient was *Acholeplasmatales* (R = -0.37).

2.6.4.3.2.2 Orders-estimated breeding values

PLS regression between SRT relative abundances of all faecal orders with EBVs indicated that 24.50% of the observed variability in EBVs explained 51.40% of the variability of abundances at an order level. The orders with VIP >1.00 are presented in **Table 2.11** below.

Table 2.12 Faecal order relationship with EBVs. VIPs from the PLS analysis, relative abundance, direction of correlation, correlation coefficients (R) and p-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix in the Appendix. Higher taxonomic levels are presented, where necessary, for recognition purposes.

	Order	VIP	mean RA	Direction	R	p-value
A20	*Bacillales*	2.10	0.02	Negative	-0.22	0.10
A12	*Anaerolineales*	1.97	0.006	Positive	0.01	0.95
A57	*Opitutae;o_HA64*	1.76	0.001	Negative	-0.07	0.59
A42	*Campylobacterales*	1.58	0.01	Positive	0.25	0.06
A24	*Clostridiales*	1.49	27.76	Negative	-0.16	0.23
A27	*k_Bacteria;p_LD1;c_;Unidentified*	1.47	0.003	Positive	0.20	0.14
A6	*Actinomycetales*	1.44	0.005	Positive	0.06	0.67
A23	*c_Clostridia;Other*	1.39	0.01	Positive	0.20	0.14
A8	*p_Bacteroidetes;Other;Other*	1.37	0.21	Positive	0.18	0.19
A35	*Rhizobiales*	1.36	0.005	Positive	0.27	0.04
A47	*Xanthomonadales*	1.34	0.001	Positive	0.08	0.58

A9	*_Bacteroidales*	1.28	24.16	Negative	-0.24	0.08
A28	*Victivallales*	1.27	0.42	Negative	-0.30	0.02
A19	*p_Firmicutes;Other;Other*	1.26	0.01	Positive	0.17	0.20
A13	*p_Cyanobacteria;c_4C0d-2;o_YS2*	1.12	0.49	Negative	-0.26	0.05
A7	*Coriobacteriales*	1.11	0.12	Negative	-0.02	0.89
A62	*k_Bacteria;p_WPS-2;c_; Unidentified*	1.08	0.0005	Negative	-0.24	0.08
A56	*p_Tenericutes;c_RF3;o_ML615J-28*	1.07	0.30	Negative	-0.09	0.53
A18	*Fibrobacterales*	1.07	0.26	Negative	-0.24	0.07
A25	*c_Clostridia;o_SHA-98*	1.05	0.005	Positive	0.15	0.26
A55	*c_Mollicutes;o_RF39*	1.04	0.12	Positive	0.13	0.33
A61	*Verrucomicrobiales*	1.03	0.02	Positive	0.05	0.69
A50	*Spirochaetales*	1.02	0.97	Negative	-0.10	0.46
A3	*Methanosarcinales*	1.01	0.001	Positive	0.14	0.29
A40	*Desulfovibrionales*	1.01	0.01	Positive	0.10	0.46

Twenty-seven orders with VIP >1.00 were explored further by creating a correlation matrix between their SRT abundances and EBVs. Correlograms exploring the relationship between EBVs and taxonomic order RA, as well as relationships between the bacterial and archaeal abundances with VIP >1 are reported in the Appendix **Figure** and **Table 7.9**. The strongest positive correlation between EBVs and orders was observed for order *Rhizobiales* (A35) (R = 0.27, p = 0.04) and the strongest negative correlation for order *Victivallales* (A28) (R = -0.29, p = 0.02).

2.6.4.3.3 Genus level

2.6.4.3.3.1 Genera-cortisol

By conducting a PLS regression between SRT relative abundances of all faecal Genera with SRT cortisol values, 14.20% of the observed variability in cortisol explained 82.33% of the variability of abundances at a genus level. Genera with a VIP score >1.50 were used for further analyses by creating a correlation matrix between their SRT abundances and SRT cortisol values. The genera with VIP >1.50 can be seen in **Table 2.12** below.

Table 2.13 Faecal genera relationship with cortisol. VIPs from the PLS analysis, relative abundance, direction of correlation, correlation coefficients (R) and p-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix in the Appendix. Order and higher taxonomic levels (if necessary) are presented for recognition purposes.

	Genera	VIP	mean RA	Direction	R	p-value
A177	*o_Acholeplasmatales;Unidentified*	2.66	0.001	Negative	-0.52	<0.01
A133	*Coprobacillus*	2.55	0.02	Negative	-0.40	<0.01
A110	*Clostridium*	2.43	0.28	Negative	-0.52	<0.01
A171	*Sphaerochaeta*	2.20	0.03	Negative	-0.16	0.24
A28	*Paludibacter*	2.12	0.67	Positive	0.02	0.88
A30	*Porphyromonas*	2.03	0.01	Negative	-0.06	0.67
A112	*Faecalibacterium*	1.87	0.01	Negative	-0.48	<0.01
A188	*p_WPS-2;c_;o_;f_;Unidentified*	1.87	0.00	Negative	-0.37	<0.01
A42	*f_[Paraprevotellaceae]; Unidentified*	1.81	0.04	Negative	-0.40	<0.01
A104	*f_Peptostreptococcaceae; g_[Clostridium]*	1.78	0.01	Negative	-0.16	0.25
A155	*c_Betaproteobacteria;Other; Other;Other*	1.77	0.004	Negative	-0.19	0.15
A175	*f_Dethiosulfovibrionaceae; g_TG5*	1.73	0.001	Negative	-0.19	0.16
A172	*f_Spirochaetaceae; Unidentified*	1.72	0.04	Positive	0.35	0.01
A144	*f_Pirellulaceae; Unidentified*	1.56	0.14	Positive	0.13	0.34
A117	*Subdoligranulum*	1.55	0.002	Negative	-0.31	0.02
A16	*Olsenella*	1.52	0.004	Positive	0.23	0.08

Genera with the strongest relationship to cortisol were: an Unidentified member of the *Spirochaetaceae* family (R = 0.35, p <0.01) and the genus *Olsenella* (R = 0.23, p =0.08), whereas those exhibiting the strongest negative correlation were *Clostridium* (R = -0.53, p

<0.01) and an unidentified member of the order *Acholeplasmatales* (R = -0.51, p <0.01). Further information can be found in Appendix **Figure** and **Table 7.10**.

2.6.4.3.3.2 Genera- estimated breeding values

PLS regression between SRT relative abundances of all faecal genera with EBVs indicated that 14.94% of the observed variability in EBVs explained 77.37% of the variability of abundances at a genus level. Genera with VIP >1.00 are presented in **Table 2.13**.

Table 2.14 Faecal genera relationship with EBVs. VIPs from the PLS analysis, Mean RA, direction of correlation, correlation coefficients (R) and p-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix. Order and higher taxonomic levels (if necessary) are presented for recognition purposes.

	Genera	**VIP**	**mean RA**	**Direction**	**R**	**p-value**
A68	*o_Clostridiales;Unidentified*	2.52	3.17	Positive	0.42	<0.01
A152	*Agrobacterium*	2.15	0.002	Positive	0.33	0.01
A63	*Sporosarcina*	2.14	0.004	Negative	-0.35	0.01
A41	*f_[Paraprevotellaceae];Other*	2.01	0.006	Negative	-0.31	0.02
A94	*Pseudobutyrivibrio*	2.00	0.007	Positive	0.32	0.01
A121	*Phascolarctobacterium*	1.97	0.35	Negative	-0.27	0.04
A98	*f_Lachnospiraceae;g_[Ruminococcus]*	1.93	0.01	Negative	-0.32	0.02
A174	*Pyramidobacter*	1.87	0.003	Positive	0.26	0.05
A38	*o_Bacteroidales;f_S24-7; Unidentified*	1.79	0.19	Negative	-0.29	0.03
A10	*Sanguibacter*	1.69	0.001	Negative	-0.28	0.04
A182	*c_Opitutae;o_HA64;Unidentified*	1.62	0.001	Negative	-0.26	0.05
A118	*Syntrophomonas*	1.62	0.002	Positive	0.27	0.05
A102	*f_Peptostreptococcaceae; Unidentified*	1.61	0.26	Negative	-0.25	0.06
A184	*c_Verruco-5;o_LD1-PB3; Unidentified*	1.60	0.003	Positive	0.21	0.11
A142	*Victivallis*	1.60	0.13	Negative	-0.26	0.06

A57	*Fibrobacter*	1.59	0.26	Negative	-0.25	0.06
A70	*f_Christensenellaceae; Unidentified*	1.58	0.36	Positive	0.26	0.05
A104	*f_Peptostreptococcaceae ;g_[Clostridium]*	1.58	0.01	Negative	-0.24	0.07
A188	*k_Bacteria;p__WPS-2; Unidentified*	1.55	0.0005	Negative	-0.24	0.08
A32	*Prevotella*	1.55	0.32	Positive	0.25	0.06
A24	*f_Bacteroidaceae;g_5-7N15*	1.54	3.07	Negative	-0.25	0.06
A72	*f_Clostridiaceae; Unidentified*	1.53	0.003	Negative	-0.25	0.06
A90	*Johnsonella*	1.52	0.004	Positive	0.23	0.08

The genera with VIP >1.50 were selected to perform correlation analyses. The matrix presented in the Appendix (**Figure** and **Table 7.11**) shows the relationship the genera with VIP >1.50 have with the EBVs. Genera with the highest positive correlation values with EBVs were: an unidentified member of the *Clostridiales* order (A68) (R = 0.42, p >0.01), the genus *Agrobacterium* (A152) (R = 0.33, p = 0.01) and the genus *Pseudobutyrivibrio* (A94) (R = 0.32, p = 0.01). Genera with the highest negative correlation with EBVs were: *Sporosarcina* (A63) (R = -0.35, p <0.01), a *Ruminococcus* like member of the *Lachnospiraceae* Family (A98) (R = -0.32, p = 0.02) and an unidentified member of the *Paraprevotellaceae Family* (A41) (R = -0.31, p = 0.02).

2.7 Discussion

This chapter used two genetic lines of Romane sheep, selected to differ in their reactivity to social isolation and human contact, to assess whether there were measureable differences in their rumen and faecal microbiota that might be related to their differing stress responses. Despite extensive analyses of the microbiota at the phylum, order and genus level, there were only minor significant differences in the rumen and faecal microbiota, even when including cortisol levels or EBVs in the analyses.

2.7.1 Cortisol results

Stress susceptibility, defined as exhibiting extreme responses to stressful events and increased occurrences/maintenance of pathologies such as cardiovascular, metabolic and immunological diseases in humans, mice and other species, as well as stress-related psychopathologies (depression, anxiety and pessimistic tendencies). It is also closely linked to altered output of hormones that interact, regulate the hypothalamic-pituitary-adrenal (HPA) axis, the autonomic nervous system (ANS), and act as mediators of the immune and metabolic systems. Cortisol is one such example and was therefore regarded as a good candidate to explore in terms of identifying differences in the two lines of sheep (B+ and B-). No difference was observed between the lines or between generations (G0 and G1) for the levels of cortisol measured in blood plasma. It is possible that the animals would exhibit different cortisol levels if sampled after exposure to a stressor, but this was not part of the experimental design where the sampling was done in a calm environment without disturbing the animals.

Furthermore, it could be argued that serum cortisol may not be an ideal marker for assessing chronic stress or long term systemic exposure as, due to its protein-binding capacity it becomes difficult to evaluate (Lee, Kim and Choi, 2015). In addition, cortisol was not correlated with the EBVs, therefore suggesting that it is not an adequate marker for distinguishing differences at a genetic level, at least in the absence of a stressor, which could have led the animals to express a more or less stress-reactive and vocal phenotype.

2.7.2 Rumen and faecal microbiota profiles

2.7.2.1 Alpha and beta diversity

Exploration of the microbial profiles in the rumen and faecal samples was carried out at an OTU level by exploring rarefied RA for alpha diversity measures and SRT data for beta diversity indices. No differences arose from the non-parametric testing for the rumen or faecal samples in the measures used to assess richness and evenness intra- and inter-groups (defined as B+G0, B-G0, B+G1 and B-G1) used to account for the Genetic Lines and the two Generations of animals.

Dispersion of microbial communities between samples from Generations G0 and G1 differed significantly for the faecal samples, but this is likely due to an age effect. It is well documented that bacterial community structure is affected by age, with the bacterial communities of older animals or humans becoming less variable and more similar to each other (Hopkins, Sharp and Macfarlane, 2001; Saraswati and Sitaraman, 2014; Aleman and Valenzano, 2019; de la Cuesta-Zuluaga *et al.*, 2019). As all of the animals in G0 were born in 2012 and all others in

G1 born between 2013 and 2015, graphical inspection verified that they all differed according to age, with the animals born in 2015 demonstrating larger dispersion compared to those born in 2012, thus explaining the results.

Visual exploration at an OTU level using PCoA analysis did not reveal any patterns between groups.

2.7.2.2 Rumen and Faecal Taxonomy

Comparison of the RA between groups for three different taxonomy levels (phylum, order and genus) in the rumen and faecal samples indicated several differences. For the rumen samples, the phylum *Fibrobacteres* within Generation G1 was higher in abundance in the B- line, indicating a potential genetic influence. As *Fibrobacteres* consists of cellulose degrading bacteria (Ransom-Jones, Jones and Mcdonald, 2016), differences in abundances could play a significant role in how the two lines process fibre. The same applied at the order level for *Fibrobacterales*. An unidentified member of the *LD1* order was also significantly more abundant in group B+G0 compared to B+G1 and B-G0, in the first case indicating a generation difference and in the second a potential genetic line influence. This order is frequently observed in studies related to the rumen microbiome, but little is known about its role. At the genus level, an unidentified member of the *Lachnospira* family within Generation G1 was more abundant in the B+ line. Members of the *Lachnospira* family produce butyric acid that plays a protective role against colon cancer (Daniel *et al.*, 2017) whilst in a restraint stress study involving mice, *Lachnospiraceae* was found to increase in abundance over the course of the study (S. Li *et al.*, 2017), and as B+ animals were more stress susceptible, potentially this family may increase in stress conditions.

Regarding the faecal microbiota, the *Verrucomicrobia* order was more abundant in the B- line within Generation G1. *Verrucomicrobia* has been reported to be present in lower abundances in people with post-traumatic stress disorder (PTSD) (Hemmings *et al.*, 2017), B- animals are less reactive so potentially higher abundance of this bacterium is linked with temperament.

The *Elusimicrobiales* order differed significantly between generations within the B+ line (B+G1 < B+G0) and was more abundant in the B- line, within G1. This order is found in low abundances in rumen and faecal samples of ruminants, but literature is lacking about its role.

Two genera differed: *BS11* (*Bacteroidales* order), with G1 in abundances higher compared to G0 in the B+ line; genus *Paludibacter* was also more abundant in G1 animals compared to G0 animals in the B- line. Increased *Paludibacter* abundance in faecal samples, and decreased

Lachnospiraceae abundance were linked to higher susceptibility to parasite infections in ponies (Clark *et al.*, 2018), indicating that this genus may play a role in parasite susceptibility/resilience. Older animals (G0) may have developed immunity against parasites, and this could be reflected in the RA of this genus.

What is of particular interest is that many significant differences were observed in the archaeal abundances at an order and genus level for both the rumen and faecal samples. Of particular interest, in the faecal samples *Methanobrevibacter* was significantly more abundant in B-G0 animals compared to all other groups ($p<0.01$) and lower in B-G1 animals ($p<0.01$). Therefore, we observe a generation by genetic line effect which may be due to an age effect. *Methanobrevibacter* species are the most abundant archaeal genus in the rumen and have been shown to removes the end-product H_2 from bacterial fermentation, therefore promoting fermentation rate and steering colonic energy production in the form of SCFAs. This could potentially have effect on production and product composition in these animals making the G0 animals of the lower stress susceptibility genetic line more efficient. Similarly, *Methanosphaera*, generally the second most abundant archaea, swere significantly higher in relative abundance for the B+G0 animals ($p=0.02$). Once again, these differences may indicate that G0 animals, i.e., younger animals have a different methane production profile. In cattle shifts have been identified based on animal age (Liu *et al.*, 2017), but otherwise literature on the topic is very scarce.

2.7.3 Relationship with cortisol and estimated breeding values

As no difference was observed exploring groups at an OTU level, the relationship between cortisol and EBVs with the microbiota profiles was further explored at a phylum, order and genus level for the rumen samples and faecal samples, using PLS regression and correlation analyses. The PLS was conducted on SRT data (not considering groups at this stage), with the aim of exploring general tendencies between the RA data and cortisol or EBVS. The correlation analyses that followed the PLS were performed on the phyla, orders and genera with VIP > 1.

PLS considers the variability of all samples when exploring the relationship between the variables and can partial out shared variance. It is therefore more robust compared to correlation analysis, as such, the correlations were conducted to give an indication of the direction of the correlation, and supplementary information about the potential relationships between bacteria involved.

The PLS regression analysis for rumen samples demonstrated variability in cortisol levels, and best explained the variability present in RA at the order level (15.43% of the variability in cortisol explained 51.9% of the variability at an order level). For the faecal samples, variability in cortisol best explained the variability present at a genus level (14.2% of the variability in cortisol explains 82.33% of the variability in RA at a genus level). Correlation between bacterial abundances and cortisol are tentative, and more likely explain covariations than cause-effect situations. This relationship becomes more ambiguous when relating EBVs to RA. For the rumen samples, the EBVs best explained the variability in abundances at an order level (15.9% variability in EBVs explained 39.30% variability in order RA), whereas for the faecal samples 14.94% of the variability explained 73.37% of the variability at a genus level.

Of all the bacteria presented with VIP scores higher than 1 or 1.5 for the genus level, those which when cross-referenced against literature findings appeared to have relevant roles in stress response mechanisms and were correlated with cortisol, stress biomarkers in general, depression and immune issues as well as temperament are discussed below.

A bacterial order of interest in the rumen samples is *Lactobacillales*, which exhibited the highest VIP score and a positive correlation coefficient with cortisol. *Lactobacillales* RA was increased in more stress-susceptible rats in a study by Zhang *et al.* (2019), when they were exposed to inescapable electric shocks. Although cortisol measurements were not reported, it would be likely that hormones relevant to the stress response would be affected. This bacterium could therefore be a biomarker of interest for investigating stress.

The orders *Coriobacteriales* and *Erysipelotrichales*, positively related to cortisol in the rumen samples, could also be of interest, as in a study examining the results of Partial Sleep Deprivation (PSD) on humans, after two nights of disturbed sleep, the families *Coriobacteriaceae* and *Erysipleotrichaceae* were observed in significantly higher abundances (Benedict *et al.*, 2016). In addition, BALB mice exposed to grid floors and tested for their behavioural reaction to different conditions (Tripletest, Burrowing, Tail Suspension Test) were found to have increased RA of the phylum *Coriobacteriaceae* (Bangsgaard *et al.*, 2012). Again, assuming that cortisol is an indicator of stress and that significant correlations with bacteria that appear to be in highe abundance in animals that have been subjected to stressful interventions may indicate a potential pathway for exploration.

Interestingly, a member of the order *Synergistales* (VIP : 1.41 and positive correlation with cortisol, in the present study), *P. gingivalis*, grew significantly in the presence of cortisol under *in vitro* conditions, despite this growth being unrelated to cortisol concentration (Akcali *et al.*, 2014). This could mean that this bacterium proliferated in the presence of cortisol and could

act as a good indicator of stress. Furthermore, members of the *Myxococacceae* Family (*Myxococcales* order in this study had a VIP score : 1.52, very low RA and demonstrated a positive correlation with cortisol) play a role in the production of diverse secondary metabolites acting as antimicrobials, antiparasitics, antivirals, cytotoxins, and anti-blood coagulants (Garcia and Müller, 2013). They have also been reported in higher abundances in the rumen of bloated sheep compared to healthy sheep, which had higher abundances of *Fibrobacter* and *Ruminococcus* (Azad *et al.*, 2019). It may be hypothesised that the presence of these bacteria is once again influenced by cortisol which also stimulates the immune system, so depending on cortisol exposure effects may vary.

The *Pseudobutyrivibrio* genus (VIP: 1.82, negative correlation with cortisol, faecal samples) was observed in decreased abundance in mice exposed to a social stressor i.e. social disruption (SDR) and was correlated to increased circulating levels of IL-6 and MCP-1 in blood plasma (Bailey *et al.*, 2011a).

The intestinal bacterial genera *Faecalibacterium, Roseburia, Dorea, Clostridium, Coprobacillus* have also been reported to be strongly associated with chronic fatigue syndrome/myalgic encephalomyelitis (ME/CFS) (Nagy-Szakal *et al.*, 2017). All of the above, with the exception of *Roseburia*, were related to cortisol levels in the faecal samples (*Faecalibacterium* VIP: 1.87, negative; *Dorea* VIP: 1.10, positive; *Coprobacillus* VIP: 2.55, negative correlation; *Clostridium* VIP: 2.43, positive correlation. Interestingly, *Roseburia* was negatively correlated with the EBVs in the rumen samples (VIP: 1.31) meaning that animals of a more reactive temperament were likely to have lower RA of this genus.

Finally, most of the bacteria mentioned above were present in low abundances, suggesting that interplay between the microbiome and the immune/ nervous system occurs at a lower level via complex mechanisms that are not yet fully understood. Regardless, members of the order *Bacteroidales*, which, along with *Firmicutes*, were the most abundant bacteria, were found in relation to cortisol and EBVs in both rumen and faecal samples (i.e., VIP: 1.38, positive correlation with cortisol in faecal samples; VIP: 1.00, negative correlation with EBVs in the rumen samples; VIP: 1.28, negative correlation with EBVs in faecal samples). Members of this order consistently appear in the human studies literature related to depression (Imhann *et al.*, no date; Naseribafrouei *et al.*, 2014; Jiang *et al.*, 2015) although at lower levels (genera and Species), the relationship with stress and depression becomes more complex due to the large number of bacteria included in this order.

The importance of investigating shifts in microbial profiles according to temperament using EBV scores and cortisol as proxies is a first step towards investigating how the genetic profile

of these animals can influence their microbiome. Further exploration using larger numbers of animals incorporating full genotype analyses and metabolomics would allow us to distinguish interaction pathways more accurately.

Investigating genetic differences that regulate the stress response could allow us to distinguish small alterations in the physiology and immune system linked to microbiome-genetic (temperament) crosstalk. Small disruptions in the microbial communities could potentially facilitate the expansion of opportunistic pathogens, therefore influencing the animals' health, metabolism and productivity.

2.7.4 Examination of hypotheses

- The genetic line did not affect rumen and faecal micobial profiles, which means either that stress susceptibility and microbiota heritability and not necessarily correlated, or that to express differences in the microbial structure, their differences in stress response have to be illicidated by means of stressors/ Generation had an effect on Alpha and Beta diversity. This could be due to an age effect and the fact that the rumen continues to progress and mature throughout life, or simply due to fewer number sampled from these two groups
- The most interesting observation relevant to RA between groups was that for the rumen samples, the phylum *Fibrobacteres* within Generation G1 was higher in abundance in the B- line, indicating a potential genetic influence. Suchdifferences in abundance could indicate differences in fiber metabolism
- Cortisol did not differ between Generations and Genetic Lines. Particularly in reference to the genetic lines it may be due to the fact that in order to express variability linked to stress susceptibility genetic, some stress needs to be applied, as the overall effect of normal management conditions may be buffering these differences
- Cortisol was positively correlated with several bacterial phyla, orders and genera and these findings appeared to be consistent with literature findings, indicating that despite having low correlation scores, the VIP scores may allow distinction of potential bacteria that could act as biomarkers of stress. Due to low numbers and low correlation values, further investigation would be advantageous

Chapter 3

Residual effects of prenatal and neonatal stress on the rumen microbial composition of lambs

3 Residual effects of prenatal and neonatal stress on the rumen microbial composition of lambs

3.1 Personal contribution

This experiment was conceived in collaboration with my supervisors, Richard Dewhurst, Alastair Macrae and Cathy Dwyer. Rumen and blood samples had already been collected prior to my involvement in the project, by animal technicians at SRUC for other projects. Prenatal stress and Neonatal treatments were conducted for two separate PhD degrees, the first conducted by Nadiah Yusof and the second by Leonor Valente. I had no role in the collection or management of the data. I worked with Christine Ravel to analyse the cortisol samples in the INRAE biomarkers' lab. Rumen DNA extraction, 16S rRNA gene amplicon sequencing and library preparation were conducted by me at SRUC. I performed data processing and all statistical analyses, after receiving advice from David Ewing at Bioss.

3.2 Introduction

3.2.1 Prenatal stress

Prenatal stress (PNS) can be defined as factors with a biological impact on pregnant mothers that affect the development of offspring (Braastad, 1998). The PNS literature is vast, and numerous studies focus on the distinct and long-lasting effects that this type of stress can have on the offspring. The majority of the research has been conducted on rats, although there is also some focus around humans, with very little work done on non-human primates (Kofman, 2002). The effects of PNS on farm animals (i.e. livestock) has progressively been researched more intensively in a variety of farmed species (sheep, pigs, cattle, poultry and fish) (Merlot *et al.*, 2008; Rutherford *et al.*, 2012). Prenatal stress, which is stress experienced by the pregnant mother, has a long-lasting 'programming' effect on some aspects of lamb stress reactivity and behaviour (Sinclair *et al.*, 2016). Prenatally stressed lambs have shown permanently altered stress responsiveness in later life following a period of stress experienced by their mothers (Roussel *et al.*, 2004; Brunton, 2013; Coulon *et al.*, 2015; Rooke *et al.*, 2015). Previously, feed competition was found to be the largest source of social stress in pregnant

ewes, and the design of the Maternal Stress Treatments aimed to increase or reduce social stress relative to normal husbandry.

3.2.1.1 Effects of PNS on offspring

The concept of foetal programming, or prenatal programming, suggests that certain events occurring during critical points of pregnancy may cause permanent effects on the foetus and the infant long after birth. This hypothesis was first presented by David Barker in 1995 and hence it is known as "Barker's hypothesis" (Barker, 1995; Barker and Clark, 1997). This hypothesis states that undernutrition in the womb during middle to late pregnancy causes improper fetal growth, which may lead to predisposition to certain diseases in adulthood. Since then, a vast amount of research has been conducted exploring the effects of diet, but also other forms of physical, environmental and psychological stress may have on the foetus or infant short-, mid- and long-term. This is based on the basis that genes may be expressed in different ways due to external influences, without changes to the DNA, meaning that the genes are inherited intact, but function may vary (De Boo and Harding, 2006; Calkins and Devaskar, 2011; Kwon and Kim, 2017). PNS can alter stress-coping ability and behaviour in aversive situations. Exploratory, social, sexual and maternal behaviour, learning ability and motor development can also be altered and impaired (Braastad, 1998).

Negative effects of PNS on the offspring might occur as pathologies due to hormonal, immune or neurodevelopmental deregulation (Jašarević *et al.*, 2015; Kofman, 2002; Maccari *et al.*, 2003; Peters, 1990). However, in other cases, they may represent early life adaptations/trade-offs or long-term adaptive strategies that went amiss (Agrawal *et al.et al.*, 2010; Nettle and Bateson, 2015). From a behavioural-evolutionary ecology perspective, it has been proposed that communicating the presence of external stressors to the foetus could have an adaptive role (Gluckman *et al.*, 2005).

Studies of particular interest that demonstrate these misadaptations are those conducted on exploring the longterm effects of the Duch famine of 1944-1945 (Bengtsson and Lindström, 2000; Kauhanen *et al.*, 2006; Heijmans *et al.*, 2008; Painter *et al.*, 2008; Stein *et al.*, 2009; Schulz, 2010; Roseboom, 2017). Many studies have found links between undernutrition and offspring health and epigenetic influences (Bengtsson and Lindström, 2000; Kauhanen *et al.*, 2006; Heijmans *et al.*, 2008), while others don't find direct relationships, despite mothers reporting lower mental health (Painter *et al.*, 2008; Stein *et al.*, 2009). This may mean that the effects on offspring are not purely physiological via metabolism pathways but may also be influenced by endocrinal factors related to stress response and mental health.

Similarly, after the terrorist attacks of September 11, 2001, a specific cohort of children were exposed to increased maternal psychological stress in utero. The precise timing of the event and the large amount of data that was possible to be collected, allowed insights into the health effects of exposure to maternal psychological stress across gestation(Berkowitz *et al*, 2003; Engel *et al*, 2005; Endara *et al*, 2009; Currie, 2016; Brown, 2020). Results suggest that children exposed in utero were born significantly smaller and earlier. This could be due to the impact of the stress itself, or due to toxic elements found in the air after the attacks (Currie, 2016).

The current adaptive theories propose that the effects of adverse conditions may benefit the offspring either in the short (developmental constraints) or in the long term (predictive adaptive responses), in the expectation of future adaptive advantages (Berghänel *et al.*, 2016). Whether the effects of prenatal stress appear to be short-term or long-term, they appear to come at a cost, as other skills and features such as growth rate, learning speed, immune function and adequate maturation for reproduction are negatively affected (Belsky.*et al.*, 2015; Coall and Chisholm, 2003; Metcalfe and Monaghan, 2001).

Overall, dynamic modulation of fetal programming is significantly involved in shaping health throughout life, possibly by influencing metabolic parameters including insulin action, hypothalamic-pituitary-adrenal activity and immune function. Stress has been shown to modify the vaginal microbiome, so initial colonisation of the infant gut may also be impacted in adverse ways.

More specifically, studies exploring the effects of prenatal stress in sheep have also shown many diverse outcomes in the offspring of stressed mothers. According to Dodic *et al.* (1998), foetal exposure to maternal stress glucocorticoids leads to cardiovascular disorders i.e. hypertension. In this study, two groups of ewes were administered dexamethasone every 48h between the 22nd and 29th day of pregnancy (Group 1) or the 59th and 66th day of pregnancy (Group 2), whereas a third group was used as a Control group.

Offspring of Group 1 were found to have higher arterial pressure compared to the Controls when assessed at approximately 4, 10 and 19 months after birth. Other physiological aspects affected in offspring due to PNS are sperm quality in early puberty, with an observed increase in sperm defects (Henrique *et al.*, 2019) and reduced birthweight in female offspring, due to prenatal exposure to dexamethasone (Long, Ford and Nathanielsz, 2013). Female offspring of the first and second generations in the latter study had increased baseline (but reduced stimulated) HPA activity. In general, animal research investigates prenatal stress focused on animal models for humans. Farm animals have more specific and environmental/ management

challenges which may impact progeny, such as lead to lower birthweight (Merlot, Couret and Otten, 2008; Amugongo and Hlusko, 2014; Khanal and Nielsen, 2017; Coloma-García *et al.*, 2020).

In lambs, PNS not only alters physiology but also can affect cognitive and behavioural aspects. According to Coulon *et al.*, (2015), stressors applied during the final third of gestation in the form of restraint, unpredictable social mixing and transport, resulted in offspring of the PNS group underperforming in a maze test compared to Control animals. Furthermore, when submitted to a "human presence" test, animals of the PNS group were more fearful, and in a novel object test were more reluctant to approach the object and explore. Overall, they were characterised by pessimistic-like judgment, as assessed via a cognitive bias test.

Undernutrition, used as a stressor during pregnancy, has also been shown to affect offspring behaviour (Erhard *et al.* 2004). Feeding ewes half of the of estimated metabolisable energy (ME) required for maintenance purposes from day 1 to day 95 of pregnancy, led to changes in the emotional reactivity of male and female offspring, and impaired cognitive flexibility in male offspring. Lambs were tested at 18 months of age under different situations such as reaction to restraint, to a sudden stimulus or in a maze. Males from the Undernutrition Diet (UD) were more active during restraint, approached novel stimuli more slowly and appeared to have neutral limb side preference (laterality). They also failed to improve learning speed. Females, on the other hand, had higher reactivity levels during exposure to the sudden stimulus, which then reverted to immobility. Laterality shifted to a left bias.

Overall, sheep seem to be sensitive to prenatal exposure to stress, making management adjustments during gestation an important factor that should be considered in experimental planning and general animal management.

3.2.1.2 Effects of maternal stress on vaginal and placenta microbiome

In females, persistent exposure to psychosocial stress, stimulation of the hypothalamic-pituitary-adrenal (HPA) and sympathetic-adrenal-medullary (SAM) axes, as well as overproduction and exposure to associated hormones, pose a risk factor for genitourinary tract infections (Amabebe and Anumba, 2018). According to the authors, this could be due to a dysregulated immune response, or related to a cortisol-induced inhibition of vaginal glycogen deposition.

Oestrogen-related increased vaginal glycogen and epithelial maturation are required for the maintenance of the vaginal environment exhibiting a *Lactobacillus* dominance. Cortisol has

the potential to disrupt these processes, as demonstrated in rats by Wrenn *et al.* (1968). This would offer an explanation regarding the pathway via which *Lactobacillus* concentrations and the overall vaginal microbial community is modified. Reduced breakdown of glycogen by α-amylase could subsequently lead to low lactic acid production, causing an increase in vaginal pH, thus hindering *Lactobacillus* growth.

This phenomenon may be exacerbated when local production of corticotropin-releasing hormone (CRH) from the decidua, foetal membranes and placenta are increased during pregnancy (Jones, Brooks and Challis, 1989). In combination with externally induced stress, severe dysregulations of the vaginal microbiome could be grounds for future disruptions of the microbiome in progeny.

Another means by which the uterine/placenta/vaginal microbiome could be altered, is via stress–induced inflammation in the relevant tissues and by promoting cytokine proliferation. There is ample evidence of cytokine increases in many tissues in studies conducted in humans and mice, including the brain and the placenta (Bronson and Bale, 2014; Gur *et al.*, 2017; Johnson *et al.*, 2019). Furthermore, changes in the gut microbiome of mice and humans have been associated with increases in inflammatory cytokines (Schirmer *et al.*, 2016; H. L. Li *et al.*, 2017). Gur *et al.* (2017) reported an IL-1β increase in the placentas of stressed female mice and foetal brains, coupled with changes in the dam faecal microbiota composition. Placenta microbiota composition was also compared, but despite tendencies, statistical significance was not achieved.

These mechanisms, whether they involve corticoid-driven moderation or cytokine influence on the microbiome, are potentially complementary, and assist in explaining the means by which the maternal microbiome can be affected by stress and how these changes can potentially affect progeny.

3.2.1.3 PNS effects on foetal, early life and long-term development of the microbiome

There is undoubtedly significant interplay between intrauterine growth factors, hormones, the immune system and the microbiome. As mentioned above, distress and hunger may be implicated in endocrinological influences from the mother to the embryo or epigenetic manipulation of genes implicated in immune and stress response, thus affecting metabolism, altering hypothalamic neuropeptide production and increasing hyperphagia in offspring, or changes in adipose tissue storage (Parlee and MacDougald, 2014). Because of hormonal influences and the impact diet has on the microbiome, these could be factors that contribute to

microbial diversity (Giraudo *et al.*, 2010; Sommer and Bäckhed, 2013; Li, 2018). If exposure to stress also has the ability to influence the microbiota composition of the placenta and vagina via mechanisms described above, perhaps offspring subjected to antenatal stress are exposed to altered populations of microbes in utero and during delivery. These changes may subsequently contribute to the formation of "abnormal" commensal communities, leading to long term dysregulations and potential dysbiosis.

Changes in the vaginal microbiome linked to PNS (psychological, physical and dietary) have recently been associated with effects on offspring gut microbiota and on the developing brain (Jašarević *et al.*, 2015; Codagnone *et al.*, 2019; Hechler *et al.*, 2019).

Gur *et al.* (2017) reported increased proliferation of IL-1β in mice foetuses whose mothers had been subject to stress. They also observed significantly reduced concentrations of a compound supporting neuronal survival and growth, the Brain Derived Neurotrophic Factor, in placentas of female foetuses and in the amygdala of female offspring when adults. Furthermore, the study outlined differences in the maternal faecal microbiota community, but differences were also observed in the faecal community structure of female offspring. More specifically, at a phylum level, the Relative Abundance (RA) of *Firmicutes* and *Bacteroidetes* was significantly different compared to control mice, and at a family level, abundances of *Bifidobacteriaceae* and *Rikenellaceae* differed compared to controls. Finally, RA of *Rikenellaceae* in adult female offspring significantly correlated with RA of *Rikenellaceae* in their dams.

Additionally, by examining outcomes of early prenatal stress on a mouse model, Jašarević *et al.* (2017) identified long-term shifts in maternal faecal communities across pregnancy influenced by stress. Vaginal bacterial community structure and composition also exhibited a lasting disruption following stress exposure, with a notable reduction in *Lactobacillus* concentrations in the Stressed group. Maternal vaginal microbiota showed the greatest overlap with offspring gut profiles at the neonatal stage, whereas maternal gut bacterial profiles were more closely related to offspring gut microbiota profiles at the weaning stage. The structure of the bacterial community was also significantly different in a sex and age specific manner, with many differences in abundances at a phylum, family and OTU level.

No study has so far reported differences in the vagina or placenta microbiome in sheep due to stress, so we can only hypothesise that the mechanisms are the same. Furthermore, despite existing evidence on how prenatal stress can affect the gut microbiota in offspring, this has not been shown in sheep, even less so regarding the rumen.

3.2.1.4 Neonatal stress effects on the microbiome

The mechanisms linking microbiome development and early life experience are still poorly understood. Caesarean delivery, use of antibiotics, lack of breast-feeding, and exposure to environmental toxicants have all been shown to contribute to the occurrence of dysbiosis in the infantile gut (Berrington *et al.*, 2014). Moreover, exposure to early life physiological and psychosocial stressors such as pain and separation can lead to oxidative stress, which may affect bacterial colonisation in preterm infants (Arboleya *et al.*, 2013).

Furthermore, exposure to maternal separation stress in new-born mice significantly increased ACTH levels and altered the gut microbiota, with an overgrowth of total aerobes and anaerobes, and particularly potentially pathogenic bacteria such as *E. coli*, enterococci and clostridia (Barouei, Moussavi and Hodgson, 2012; O'Mahony *et al.*, 2015).

According to Cong *et al.* (2015), changes in neonatal gut microbiota could be explained by the fact that the composition of the postnatal developing microbiota is very susceptible to environmental factors, as during this time the enteric neuro-immune system suppresses bacterial growth, and at some later point, cytokine proliferation reduces, allowing bacterial colonization.

Li *et al.* (2018) observed that early weaning in lambs led to changes in the ileal microbiota on the 42nd day of life compared to lambs that were not weaned. These effects persisted to the 84th day of life. Specifically, increases were noted for the RA of *Bacteroidetes*, *Proteobacteria*, *Euryarchaeota*, and *Cyanobacteria*, while the RA of *Firmicutes* and *Chlamydiae* decreased. Their findings also highlighted early weaning impacts on expression levels of genes related to intestinal barrier function.

Despite findings linking early life events with microbiome modulation in various tissues of various species, the full effects of early life stress on modulating the gut microbiome, GI tract function, and neurodevelopment remain largely unexplored. To date, no evidence exists on how early life stress can affect the rumen microbiota in sheep. In the present study, the lambs whose mothers had been prenatally exposed to social and diet stressors were also exposed to a variety of stressors and tests in the first days of life, which could potentially lead to long-term effects on the microbiota structure of the rumen.

3.3 Study hypotheses and objectives

- Three different levels of maternal gestational stress (Stressed: dog exposure, unpredictable feeding, high stocking density; Non-stressed: no dog exposure, predictable feeding, high stocking density; Alternative: no dog exposure, *ad libitum* feed, low stocking density) will differentially affect basal cortisol levels of female and male lambs, as investigated in later life (7 months of age).
- Two different early life treatments will differentially affect rumen microbiota profiles in male and female lambs investigated at approximately 33 weeks.
- Cortisol can be used as a predictor of the presence/absence or abundance of certain bacterial phyla, orders, or genera and/or these bacterial abundances may act as indicators of stress.

3.4 Materials and methods

All experimental procedures were performed in a research farm setting at SRUC, Edinburgh, with approval from the ethics committee (SRUC Animal research ED RP 04-2011, AE 4-2015) which encompassed the two PhD projects the data analysis was based on.

For the purposes of a PhD project carried out by Yusof (2018), stressors relevant to normal husbandry (stocking density, feeder space and competition for feed, exposure to predator stimuli) were chosen as being representative of on farm conditions and applied to pregnant ewes in the last trimester of gestation. Eighty-four primiparous, twin–bearing Scottish Mule ewes (pregnant for the first time: n = 43 ewes) and multiparous ewes (second pregnancy: n = 41 ewes), which were served by Suffolk rams, were selected to be studied. The animals were expected to start lambing in the first week of April 2015. The ewes were observed for a total of 8 weeks, between weeks 11 -18 of pregnancy, during which they were separated into 3 treatment groups; four pens were allocated for each treatment group, with 7 ewes in each, balanced for parity, expected lambing date and temperament (previously assessed, not considered or explored further in the present study). Layout of the experimental shed is presented in **Figure 3.1**, and is copied from Yusof, 2018.

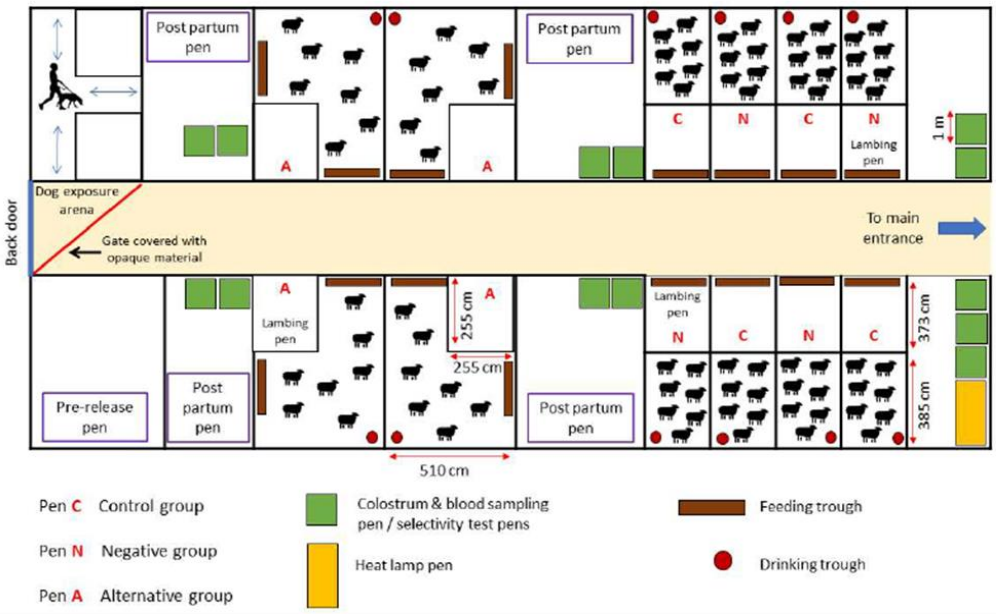


Figure 3.1 Layout of experimental shed and presentation of treatment groups

3.4.1 Treatment groups

Twenty-eight ewes were chosen for the Control, Alternate and Negative group. The animals were kept in pens of seven animals under a stocking density of 1.28 m^2/ewe and allowed 33 cm feed-face per ewe. The animals were fed *ad libitum* hay, plus supplementary concentrate feed (20% crude protein, Davidsons Super Ewe Nuts, Davidsons, Scotland) from week 14 of gestation onwards, adjusted weekly to meet the ewes' daily nutrition requirements (approximately 250 g/day/ewe). The ewes remained on this diet until leaving the shed.

A feeding/lambing pen (8.65 m^2) was also available, adjacent to the experimental pen, where feed troughs were located. Access to the feeding/lambing pen was only allowed during feeding times or when a ewe started lambing. The Alternative groups also had a smaller pen (6.5 m^2), inside the experimental pen, which was only used when a ewe started lambing.

Control animals were fed at 9h00 after an auditory cue (radio) every day, whereas Negative animals were under an unpredictable feeding schedule (between the 14th and 19th week of gestation). These ewes were either fed at the same time as the Controls or feeding was delayed or made inaccessible by 15, 30- or 60-min. Delays were applied randomly, according to a pre-determined schedule.

The Alternative group's diet consisted of grass silage (Dry Matter (DM) 251 g/kg; Protein 11.4 g/kg DM; ME: 11.4 MJ/kg DM) *ad libitum*, from the beginning of the experiment. Silage

was formulated to meet the ewes' dietary requirements and was supplemented with approximately 14 g/ewe/day of vitamins and minerals (Norvite Farm Minerals for Cattle and Sheep, Norvite, Aberdeenshire). From week 14 until the end of gestation, they were also offered 100 g/ewe/day of soya bean meal (Sopralin: 46.5% crude protein, Trouw Nutrition, Ireland). Troughs were refilled twice a day without entering the pens. Differences in treatment are presented in **Table 3.1** below.

All twin pairs born from these ewes were used for further tests in the context of two different PhD projects, and a subset of the rumen samples collected at slaughter was chosen for the purposes of the present study.

Table 3.1 Overview of experimental conditions for the treatment groups

Treatment	**Diet**	**Feeding schedule**	**Pen Space**	**Stressors**
Control (n=28)	*ad libitum* hay, plus supplementary concentrate feed	9h00 after an auditory cue (radio)	1.28 m^2/ewe and allowed 33 cm feed-face per ewe	None
Alternative (n=28)	*ad libitum* grass silage 100 g/ewe/day of soya bean meal	9h00 after an auditory cue (radio)	5.52 m^2/ewe and allowed 66 cm feed-face per ewe	None
Negative (n=28)	*ad libitum* hay	unpredictable feeding schedule (between the 14th and 19th week of gestation)	1.28 m^2/ewe and allowed 33 cm feed-face per ewe	Exposure to different dog once per week (six times in total), between weeks 12 and 17 of gestation

3.4.2 Tests and observations on pregnant ewes

Body condition as well as bodyweight were measured to ensure the animals were fit to participate in Yusof's study. During the experiment, the animals were weighed weekly to determine whether body weight and body condition scores were affected by stress.

Behavioural observations included actions at the feed-face in order to observe agonistic behaviour during feeding, as well as scan sampling of postures and behaviours. A Qualitative Behaviour Assessment (QBA) was performed using ewe body language to investigate their emotional state. Hormonal measurements throughout pregnancy included faecal corticoids (to measure glucocorticoid metabolites as an indicator of stress) and plasma levels of oestradiol, progesterone and beta-hydroxy-butyrate (BHB).

3.4.3 Tests performed on the lambs

The Early Life Stress performed on the lambs was part of the work that another PhD student (Leonor Valente) conducted, and provided the following information directly relevant to this study:

Lambs born in twin pairs were kept on the study for further testing. Lamb behaviour was recorded for 2h after birth to assess latency to stand and suck and to count vocalizations for the first 30 min. Rectal temperatures were taken at 30 min and 2h after birth. Lambs were weighed and had their crown rump length measured at 6h. In order to test a lamb's ability to discriminate its mother from another parturient ewe (alien ewe) at a distance and at close quarters, a maternal recognition test (Nowak *et al.*, 1987) was conducted at 6h for all animals. Each twin was then allocated to one of two sampling regimes: The Isolation lambs and the Recognition lambs.

Isolation lambs

Isolation lambs were sampled for blood plasma IgG and rectal temperature was taken prior to an Isolation Test carried out at 24h. The isolation pen, measuring $1m^2$ had a straw covered floor and was located in a different shed, visually and acoustically isolated from the experimental shed. Isolation lasted for 5 min and was video recorded for analysis of activity (number of squares entered), attempts to escape (lamb tries to or fits its head through the bars of the pen walls) and number of vocalizations using The Observer XT 11.5 software (Noldus Information technology, Netherlands). Upon finishing the test, all lambs were re-united with their dams and returned to the post-partum pens. At 37h they were tail docked and rectal temperature was retaken at 48h.

Recognition lambs

For Red lambs, blood plasma for IgG measurements was sampled and rectal temperature was recorded at 24h prior to a Recognition Test, which took place while their Blue sibling was being assessed in the Isolation test. For the purposes of this test, the Recognition lambs were moved from the post-partum pen to the testing arena with their dams (**Figure 3.2,** taken from Valente, 2017). The dam of a lamb to be tested was placed in one of the holding pens, and another ewe with a similar lambing time (max of 24h between lambing) was placed in the other, without her own lambs. The side of the holding pen where the dam of the test lamb was placed was chosen randomly. The tested lamb was placed in the waiting pen immediately after being separated from its own dam, facing the ewes in a standing position. The test started as soon as the holding pen door was opened and lasted for a 3 min.

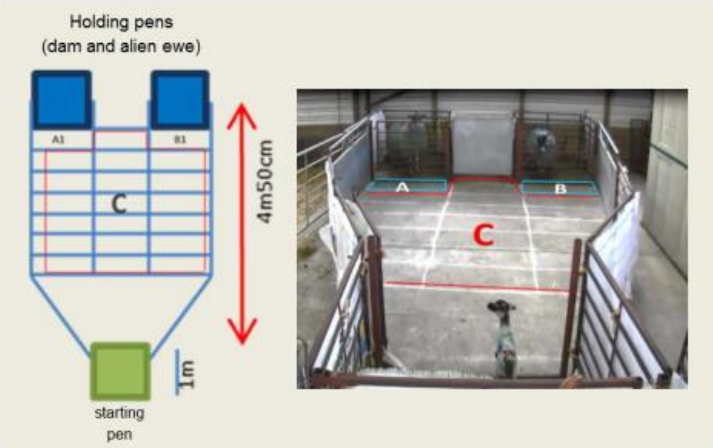


Figure 3.2 Recognition test arena layout. Image by Valente, 2017.

Behaviours recorded were: lamb latency to go to its own dam, first choice (mother or alien ewe), time spent close to each ewe, time spent in the neutral zone and activity (number of squares crossed during a test -a lamb was considered to have crossed a square when both forelegs had stepped over the white stripes on the floor). Observer XT 11.5 software (Noldus, Information Technology, Netherlands) was used for recording. More information about the procedures and the results can be found in the thesis of Valente (2017).

These animals were also involved in fear potentiation tests at 9, 12 and 16 weeks of age, but this aspect was not known at the time, and not taken into consideration in the selection of the lambs. As a consequence, details of the tests will not be presented, and effects of these treatments have not been explored further. More information on the procedures can be found in Valente's thesis (2017). After this test, the animals were released onto pasture and managed as a single flock until slaughter in December 2015.

3.4.4 Sample collection

3.4.4.1 Liquid Digesta

Liquid digesta was collected from the rumen of male and female lambs slaughtered at approximately 7 months of age using procedures based on the protocol by Fliegerova *et al.* (2014). The females were fed approximately 100g/ lamb concentrate (18% crude protein, Harbro, UK) and *ad libitum* hay for nearly 2 weeks prior to slaughter, whereas the males had been on pasture [grass diet with straw *ad libitum* and concentrate (18% crude protein, Harbro, UK)] until the day prior to slaughter. Digesta (10g) was collected directly from the rumen and stored at -30 ^{o}C until DNA extraction.

3.4.4.2 Blood samples for plasma cortisol

Blood samples were collected from the jugular vein of the male and female lambs the morning prior to slaughter in plastic EDTA vials and centrifuged for 10 min at 2500 g. Plasma was aliquoted (1ml) and stored at -80 °C until analysis took place. The samples were shipped to France and analysed by the Biomarker's lab at INRAE, Theix, in order to standardise the methodology used for all measurements of cortisol involved in this project.

3.4.5 Sample processing

3.4.5.1 Microbial DNA extraction

Thirty six samples were selected on the basis of the treatment group the lambs belonged to: 12 lambs whose mothers belonged to the Control group, 12 whose mothers were submitted to the Alternative Treatment and 12 whose mothers belonged to Negative Treatment group; 6 females and 6 males were selected from each group. The subgroups the animals had been divided in ("Recognition" and "Isolation" lambs) were also included as variables for further analysis.

Microbial DNA extraction was carried out following the Yu and Morrison (2004) protocol. Purification and RNA and protein removal used QIAamp DNA stool Mini Kits (Qiagen). DNA samples were tested for yield and quality using a NanoDrop spectrophotometer (Thermo Scientific, Loughborough, UK), and gel electrophoresis procedures. A no template control went through all DNA extraction and purification steps, as a means of assessing potential contamination.

3.4.5.2 Illumina Sequencing Preparation

PCR and sequencing were performed using the Caporaso *et al.* (2010) method with one round of PCR, using 40 different reverse primers and 1 forward, as described in the Supplementary Materials of that paper. The samples were purified using a Qiagen PCR purification kit and pooled to ensure equal volumes and DNA concentration for each sample. In total, 10 µl of each sample were added to the pool.

The DNA pooled samples were subject to gel electrophoresis and products were visualised using an ultraviolet imager (Gel Doc XR+ System, Bio-Rad, UK). The band including the DNA was cut out and purified using a Qiagen gel extraction kit and then concentrated to 20 µl using a Qiagen PCR purification kit. Concentration and quality of the libraries was assessed on the NanoDrop spectrophotometer (Thermo Scientific, Loughborough, UK) and Qubit (Thermo Scientific, Loughborough, UK).

Double stranded DNA was quantified using a fluorometric kit (Quant-iT PicoGreen, Invitrogen, UK). Readings from this assay were used to create two 3.5 nM pools, using equimolar concentrations of each library and were submitted to the sequencing centre (Edinburgh Genomics, UK). Sequencing was carried out using the Illumina MiSeq platform (Illumina, CA, USA) using V2 chemistry and producing 250 bp paired-end reads.

3.4.5.3 Sequence processing

Sequences were processed using the Galaxy Platform as described in **Chapter 2** (**Section 4.2.3**). OTU tables were constructed using the entire data set and also by separating females and males to retrieve individual tables. Taxonomic tables, providing RA, were acquired at the overall level (males and females together).

3.4.5.4 Cortisol ELISA

Plasma cortisol levels were assessed using the direct ELISA assay recently developed at INRAE as described by Anderson *et al.*, 2018 and previously mentioned in **Chapter 2** (**Section 2.4.3.6**). Coefficient of Variance (CV) was ensured as <15%. This threshold was based on validation assays performed by Andanson *et al*. (2018).

3.4.6 Grouping of treatments explored

Given the novel aspects of this study, there was no previous data to conduct a proper power calculation, particularly in terms of the microbiota and PLS analyses. Sample size was estimated based on sample size from previous microbiota studies, but as the neonatal data was also made available at a later time and due to a limited availability of rumen samples, it was not possible to increase experimental numbers, particularly when considering all factors together (Sex, Pre and Neo-natal Treatment).

Due to problems in sequencing, 16S rRNA gene amplicon sequencing data was not available for one of the males. Furthermore, the cortisol ELISA was not successful for 2 samples, 1 male and 1 female, despite multiple repetitions of the analysis. The final groups and number of animals used for further exploration were as described below:

Microbiota analysis (n = 35):

Sex: Males, n = 17; Females, n = 18

Prenatal Treatment:

Control, n = 12; Alternative, n = 12; Negative, n = 11

Neonatal Treatment: Isolation, n = 15; Recognition, n = 19

Group1: Sex * Prenatal Treatment:

Males Control (MC), n = 6; Males Alternative (MA) n = 6; Males Negative (MN) n = 5; Females Control (FC), n = 6; Females Alternative (FA) n = 6; Females Negative (FN) n = 6

Group 2: Sex * Neonatal Treatment:

Males Isolation (MI), n = 7; Males Recognition (MR) n = 10; Females Isolation (FI) n = 9; Females Recognition (FR) n = 9

Group 3: Prenatal Treatment * Neonatal Treatment:

Control Isolation (CI), n = 5; Control Recognition (CR), n = 6; Alternative Isolation (AI), n = 5; Alternative Recognition (AR), n = 7; Negative Isolation (NI), n = 6; Negative Recognition (NR), n = 5

Group4: Sex * Prenatal Treatment * Neonatal Treatment:

Females Control Isolation (FCI), n = 3; Females Control Recognition (FCR), n = 3; Females Alternative Isolation (FAI), n = 3; Females Alternative Recognition (FAR), n = 3; Females Negative Isolation (FNI), n = 3; Females Negative Recognition (FNR), n = 3

Males Control Isolation (MCI), n = 2; Males Control Recognition (MCR), n = 3; Males Alternative Isolation (MAI), n = 2; Males Alternative Recognition (MAR), n = 4; Males Negative Isolation (MNI), n = 3; Males Negative Recognition (MNR), n = 2

Cortisol Analysis and PLS (n = 33):

Sex: Males, n = 16; Females, n = 17

Prenatal Treatment: Control, n = 12; Alternative, n = 11; Negative, n = 10

Neonatal Treatment: Isolation, n = 16; Recognition, n = 17

Group 1: Sex * Prenatal Treatment:

Males Control (MC), n = 6; Males Alternative (MA) n = 5; Males Negative (MN) n = 5; Females Control (FC), n = 6; Females Alternative (FA) n = 6; Females Negative (FN) n = 5

Group 2: Sex * Neonatal Treatment:

Males Isolation (MI), n = 7; Males Recognition (MR) n = 9; Females Isolation (FI) n = 9; Females Recognition (FR) n = 8

Group 3: Prenatal Treatment * Neonatal Treatment:

Control Isolation (CI), n = 5; Control Recognition (CR), n = 6; Alternative Isolation (AI), n = 5; Alternative Recognition (AR), n = 6; Negative Isolation (NI), n = 6; Negative Recognition (NR), n = 4 **Group 4: Sex * Prenatal Treatment * Neonatal Treatment:**

Females Control Isolation (FCI), n = 3; Females Control Recognition (FCR), n = 3; Females Alternative Isolation (FAI), n = 3; Females Alternative Recognition (FAR), n = 2; Females Negative Isolation (FNI), n = 3; Females Negative Recognition (FNR), n = 3

Males Control Isolation (MCI), n = 2; Males Control Recognition (MCR), n = 3; Males Alternative Isolation (MAI), n = 2; Males Alternative Recognition (MAR), n = 3; Males Negative Isolation (MNI), n = 3; Males Negative Recognition (MNR), n = 2.

3.4.7 Statistical analyses

3.4.7.1 Cortisol

Following a "log(x+1)" transformation of the data, an F/Bartlett's test was used to investigate the variances for Sex (Males, Females), Prenatal Treatment (Control, Alternative, Negative) and Neonatal Treatment (Isolation, Recognition). To investigate the effect of Sex on cortisol, a t-test was applied; for the effects of Prenatal Treatment a two-way ANOVA was used; a Wilcoxon rank-sum test was used to compare Neonatal Treatment effects.

A GLM was applied ("glm" core R function with an assumed Gaussian distribution) to determine differences in cortisol levels between Sex, Prenatal Treatment, Neonatal Treatment and Interactions among variables. The model used was: *f(y) = Sex +Prenatal Treatment +Neonatal Treatment +Sex *Prenatal Treatment +Prenatal Treatment * Neonatal Treatment + Sex * Neonatal Treatment.*

3.4.7.2 16S rRNA gene amplicon sequencing files and OTU table processing

The rumen 16S rRNA gene amplicon sequencing fasta files were imported in Galaxy and processed as described in Chapter 2 (**Sections 2.2.3.2- 2.2.3.5**). All further analyses were carried out in R (Version 6.3.1) using different packages and functions cited in the appropriate sections.

3.4.7.3 Taxonomy

Calculation of RA and percentages of the most abundant phyla, classes etc. were carried out using QIIME. The RA tables produced for archaea and bacteria were used for further statistical analysis at each taxonomic level, adopting squared root transformation. This analysis was performed for all phyla, for orders with RA over 0.1% and genera with RA over 0.5 %.

Shapiro Wilk tests were used to assess normality. As normality assumptions were not met for most of the phyla, orders and genera explored, a nonparametric test (Kruskal-Wallis) was used on grouped variables. When differences in the later test were significant, pairwise Wilcoxon Rank Sum tests were applied with Bonferroni correction.

3.4.7.4 Microbial diversity analyses

An OTU table for all samples was acquired from QIIME, Galaxy. These were imported into "phyloseq" R, along with a file containing the taxonomic ranking related to each OTU and a

"metadata" file that had all the information related to the animal (Genetic Line, generation, etc.).

Unidentified reads at a kingdom level, labelled as "None" or "Other", were removed. Archaeal and bacterial diversity were investigated separately. For the rumen samples 1999 bacterial OTUs (max reads: 132.586) and 16 archaeal OTUs (max reads: 8.140) were acquired. Alpha diversity metrics were explored as described in Chapter 2 (**Section 2.3.3.2, 2.3.3.3**) (Shannon, Simson, Inverse Simpson, Chao1, ACE and Observed diversity).

Tests were conducted using rarefied data, a method used as a means of normalisation. Reads were rarefied to the minimum number present 21.682 for the bacteria and 510 for the archaea. This was done by randomly selecting reads from each sample to match the lowest count of reads present in the dataset. As normality assumptions were met after Shapiro-Wilk testing, ANOVA was applied to explore differences between Sexes, Prenatal Treatment , Neonatal Treatment and Grouping as described above: "Group1": Sex * Prenatal Treatment, "Group2": Sex * Neonatal Treatment, "Group3": Prenatal Treatment * Neonatal Treatment and "Group4" Sex * Prenatal Treatment * Neonatal Treatment. Tukey's honestly significant difference test (Tukey HSD) for multiple comparisons of means was used for Post-Hoc analysis.

Beta diversity was calculated on Hellinger transformed data for Sex, Prenatal, Neonatal Treatment and combined variables using PERMANOVA on R, as described previously in Chapter 2 (**Section 2.3.3.4**), after exploring dispersion using the "betadisper()" function in "vegan". Similarly, PCoA plots were created for visual exploration of the data.

3.4.7.5 Partial Least Squares modeling

PLS modelling was used to investigate the relationship between "log(x+1)" transformed values of cortisol and SRT RA of different taxonomic levels (phylum, order and genus) of the rumen microbiota sequences, using the "mix0mics" package in R (Lê Cao, González and Déjean, 2009; Rohart *et al.*, 2017).

As the influence of Sex and Neonatal Stress on the rumen microbiota structure were significant, PLS models explored the relationship between cortisol and Sexes, "Group1": Sex * Prenatal Treatment and "Group2": Sex * Neonatal Treatment. PLS exploring the relationship between cortisol levels, Prenatal Treatment, Neonatal Treatment and "Group3": Prenatal Treatment * Neonatal Treatment will not be reported, as in the Taxonomy and diversity analyses these factors and combination of factors had no/ limited influence on the microbiota community structure and further investigation was considered redundant. Exploration of

relationships of all variables with cortisol was not possible due to low sample numbers available for the combinations, resulting in error messages of non-convergence when applying the PLS model.

A further step in exploring the relationship of the different Taxonomic levels within each defined group with cortisol data was conducted by correlating the phyla with VIP >1.0 and orders and genera presenting VIP values >1.50 from the PLS analysis with each of the variables. As discussed in **Chapter 2, (Section 2.4.3.8**), the average of the squared VIP scores equals 1 and therefore, the "greater than 1" rule is generally accepted and used. This though is arbitrary and greatly depends on the number of samples and variables. As such, due to the large number of orders and genera, and due to the large number of orders and genera related to cortisol in the output of the PLS analysis, a VIP score of over 1.50 was selected as a more stringent cut-off threshold, attempting to increase confidence in the relationship explained.

Normality was tested and assumptions not met. A correlation matrix was visualised in the form of a heatplot to explore potential covariations between phyla, orders and genera selected and the variables of interest, using Pearson's correlation in R [library("Hmisc"), ("Hmisc package | R Documentation" 2019); library("corrplot"), (Taiyun Wei *et al.*, 2017)].

3.5 Results

3.5.1 Stress treatment on treatment on ewes

According to Yuosof's findings (2018), Treatment had little effect on the parameters studied. Of mention, it affected a few blood markers (red blood cell count, neutrophil to lymphocyte ratio and eosinophil counts) and more importantly ADG, as ewes in the Alternative Treatment had significantly lower ADG compared both to the Negative and Control groups ($p < 0.01$).

There were no effects on body condition scores or hormonal levels. Overall Treatment appears to have had a mild effect mainly regarding the Alterative group which is most likely linked to the diet offered. The results from ewe weights in that study suggest that the feeding system may not have been adequate for the ewes' needs.

3.5.2 Prenatal and neonatal treatment effects on lambs

Briefly, according to Valente (2017), regarding the aspects of her study of interest to this project: Prenatal Treatment had some effects on lamb behaviour and physiology. More specifically, lambs of the Alternative Treatment had lower body weight compared to the two other treatment groups, when weighed 6h after birth ($p < 0.01$). Furthermore, IgG levels were lower in the Alternative group, presenting a statistically significant difference compared to the Negative group levels ($p < 0.01$). Finally, Treatment affected the rate of escape attempts ($p = 0.02$), with Alternative group lambs attempting to escape at a significantly lower rate compared to the Negative group lambs.

Sex affected the number and frequency of bleats during the first 30 min of life, with the females bleating more frequently compared to males ($p = 0.03$). Regarding lambs' temperatures at 24h, only the females of the Control group had higher temperatures compared to males ($p = 0.04$).

The lambs derived from the Alternative treatment group appear to have been most affected by the Treatment. This is likely because nutritional requirements may not have been met for the Alternative ewes towards the end of gestation. Observed differences in bodyweight, temperature and IgG levels may be caused by influence of the Alternative Treatment and the diet the ewes received (undernutrition), in the heat production mechanism of the lambs and the quality and quantity of colostrum produced from the ewes.

3.5.3 Cortisol results

A generalised Linear model (GLM) was conducted on the influence of the independent variables (Sex, Pre and Neo-natal Treatment Groups) on "log(x+1)" transformed cortisol levels in blood plasma, and effects of Interactions were explored. A significant difference was observed between Females (mean: 61.40 ± 37.30 ng/ml) and Males (mean: 40.40 ± 13.30 ng/ml) [F (1, 35) = 4.36, p = 0.04](**Figure 3.3**), but no other variables differed significantly.

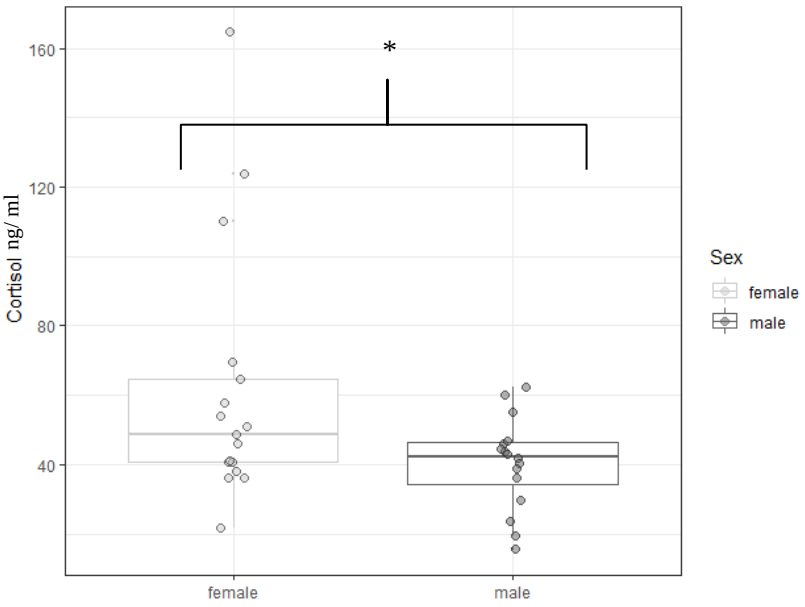


Figure 3.3 Untransformed cortisol levels, as obtained by the sheep specific ELISA, are presented (ng /ml) for each sex (females, males). The points represent individual values by sex and the boxplots include the median and SD. Sex had a significant effect (p = 0.04).

3.5.4 Rumen microbiota

3.5.4.1 Taxonomy analyses

3.5.4.1.1 Phyla

The overall rumen community, as derived from the sequence data and analysed by QIIME on Galaxy, was comprised 94.20% of bacteria (range: 90.60% to 97.20%), 4.60% of archaea (range: 0.00% to 9.00%) and 1.20% was Unclassified.

At a phylum level the most abundant bacteria were: *Firmicutes* representing 43.30% of the total population (range: 29.20% to 65.50%), followed by *Bacteroidetes* 35.30% (range: 17.10% to 56.30%). *Verrucomicrobia* made up for 3.40% (range: 0.10% to 11.60%) of the overall population, *Proteobacteria* 3.00% (range: 0.30% to 7.30%), *Fibrobacteres* 2.00% (range: 0.00% to 6.30%) *Synergistetes* 1.80% (range: 0.20% to 4.80%), *Tenericutes* 1.10%

(range 0.23% to 5.30%) and finally, *Spirochaetes* 1.10% (range 0.30% to 1.90%). "Unknowns" represented 1.20% of the population. The only archaea were *Euryarcheota* representing 4.60% (range: 0.70% to 10.90%) of the overall population. Other phyla were present but represented less than 1.00% of the overall population (*Actinobacteria*, *Armatimonadates*, *Chloroflexi*, *Cyanobacteria*, *Elusimicrobia*, *LD1*, *Lentispherae*, *Plantomycetes*, *SR1*, *WPS-2*).

At a phylum level, exploring the data by Sex, Prenatal and Neonatal Treatment Groups the most abundant prokaryote phyla are reported in **Figures 3.4 -3.6**.

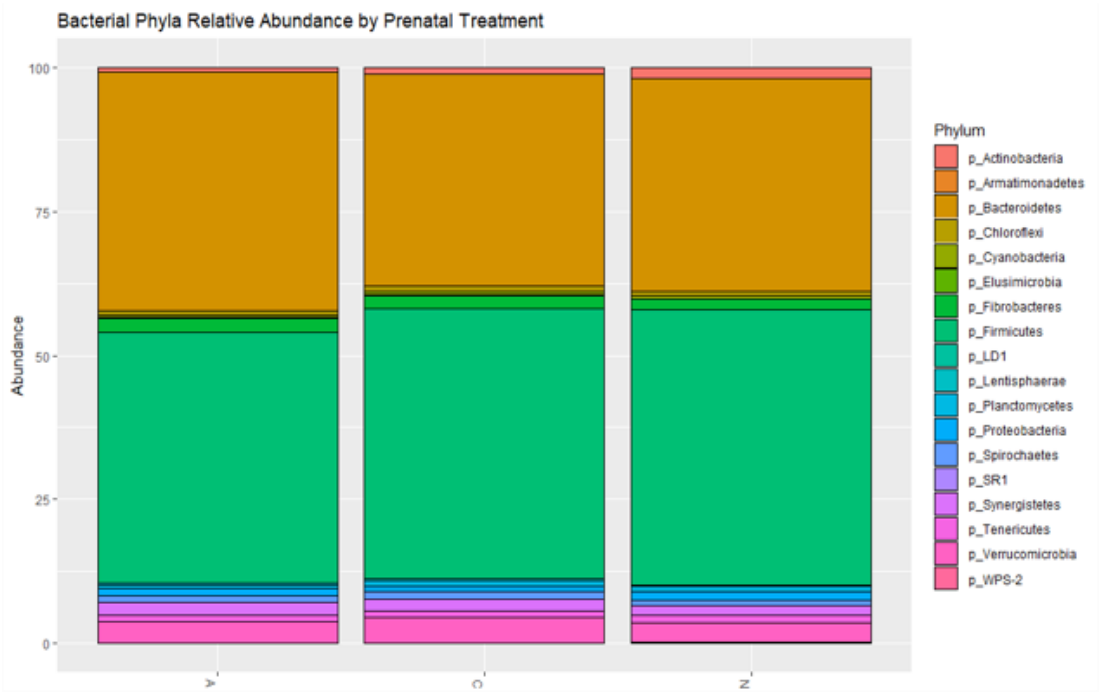


Figure 3.4 Barplot representing the average phylum abundances of the rumen bacteria by Prenatal treatment. Most abundant phyla were: Control- *Firmicutes* (44.30%), *Bacteroidetes* (34.90%), *Verrucomicrobia* (4.10%), *Fibrobacteres* (2.10%), *Synergistetes* (1.84%), *Spirochaetes* (1.20%), *Proteobacteria* (1.00%). *Euryarcheota* were present at 3.30% of the total abundance; Alternative: *Bacteroidetes* (39.20%), *Firmicutes* (41.00%), *Verrucomicrobia* (3.40%), *Fibrobacteres* (2.40%), *Synergistetes* (2.20%), *Spirochaetes* (1.10%), *Proteobacteria* (1.20%). *Euryarcheota* were present at 4.50% of the total abundance; Negative: *Firmicutes* (44.81%), *Bacteroidetes* (34.70%), *Verrucomicrobia* (3.10%), *Fibrobacteres* (1.70%), *Synergistetes* (1.50%) and *Proteobacteria* (1.40%) *Spirochaetes* (1.50%). *Euryarcheota* were present at 5.23% of the total abundance.

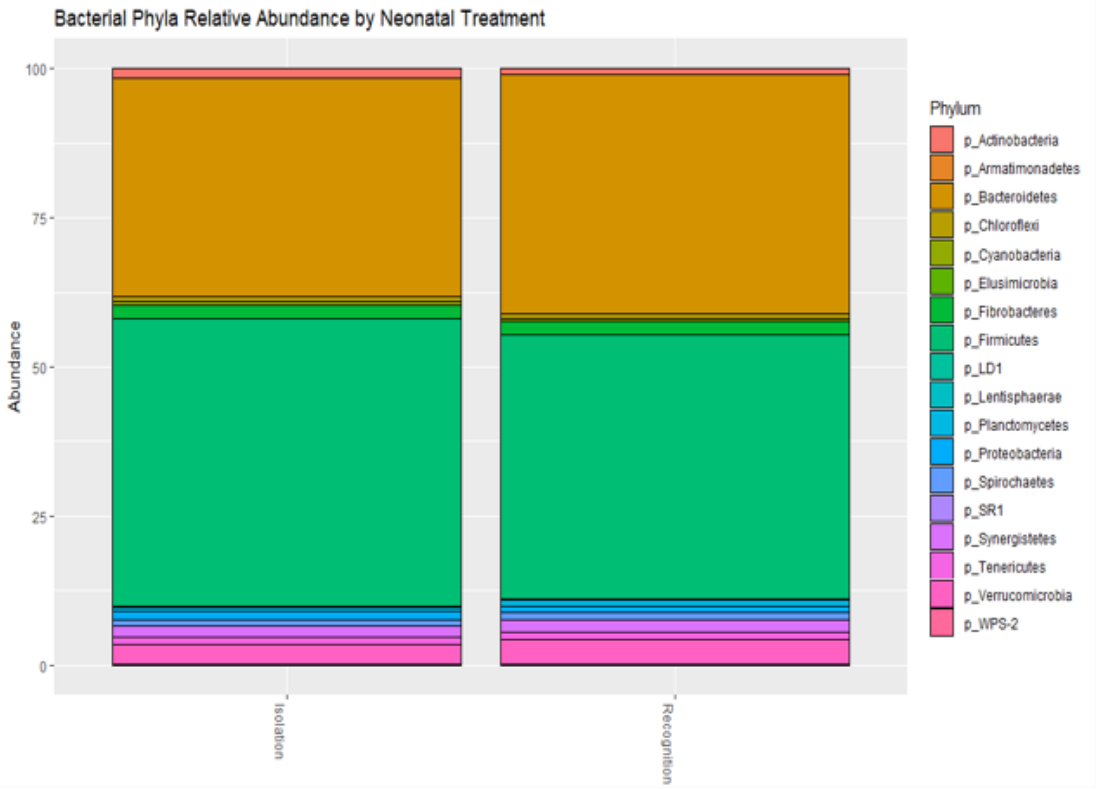


Figure 3.5 Barplot representing the average phylum abundances of the rumen bacteria by Neonatal treatment. Most abundant phyla were: Recognition lambs - *Firmicutes* (41.70%), *Bacteroidetes* (37.80%), *Verrucomicrobia* (3.90%), *Fibrobacteres* (2.20%), *Synergistetes* (1.90%) and *Spirochaetes* (1.90%); Isolation lambs: *Firmicutes* (45.30%), *Bacteroidetes* (34.50%), *Verrucomicrobia* (3.10%), *Fibrobacteres* (2.00%), *Synergistetes* (1.80%) and *Proteobacteria* (1.50%) and *Spirochaetes* (1.80%).

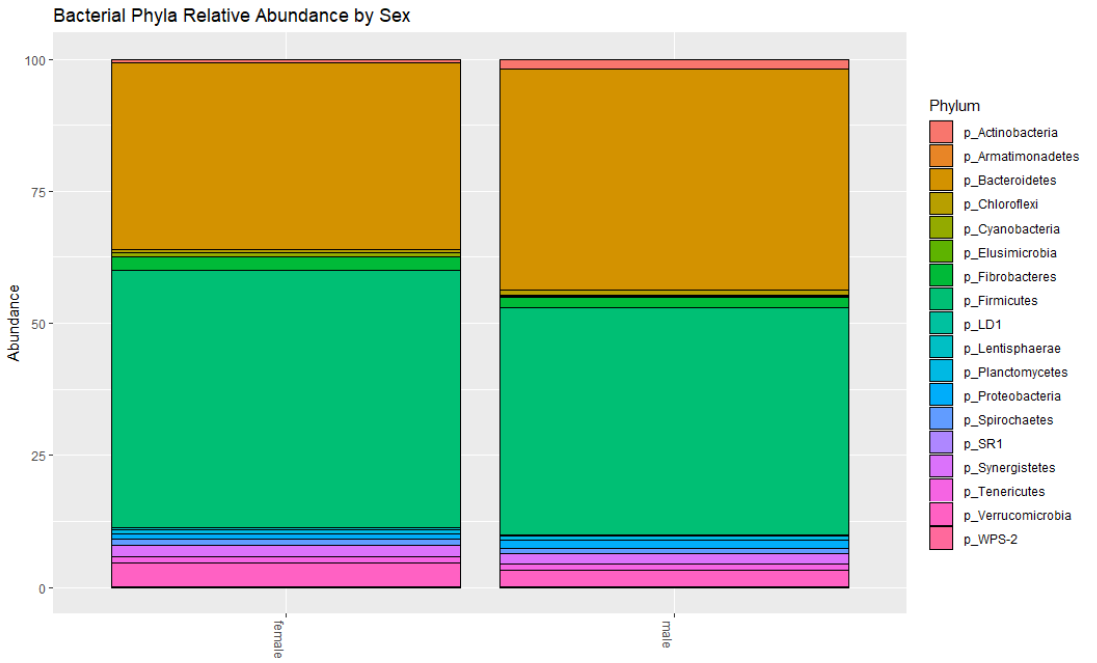


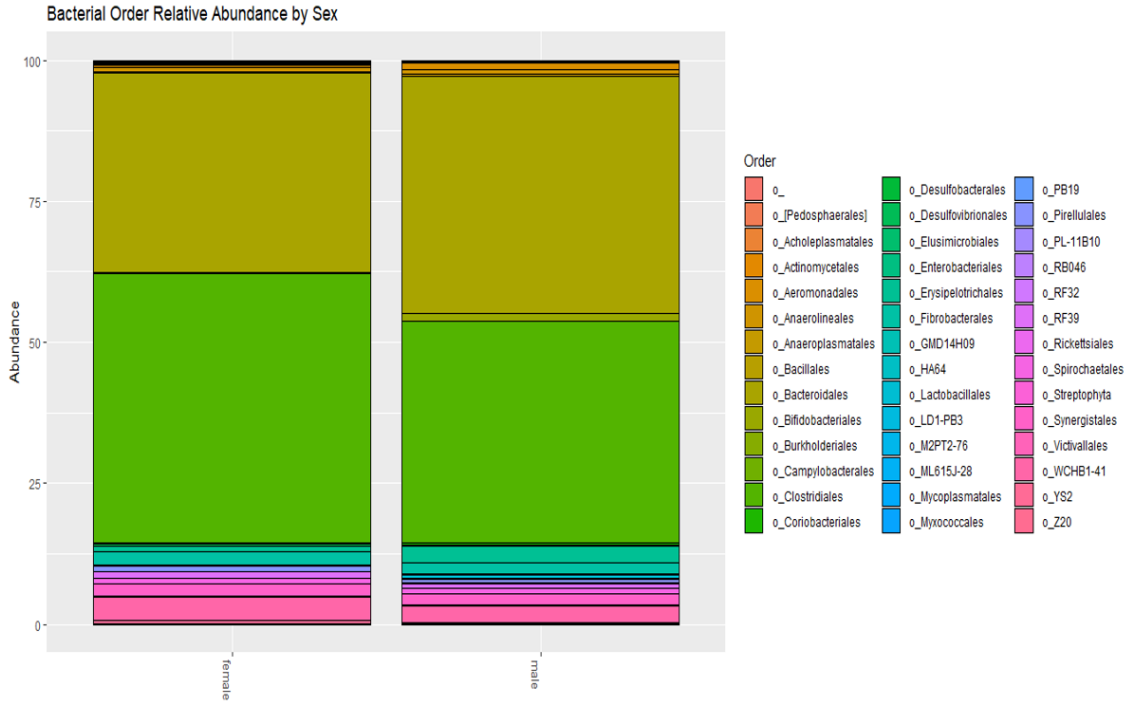
Figure 3.6 Barplot representing the average phylum abundances of the rumen bacteria by Neonatal treatment. Most abundant phyla were: Females: *Firmicutes* (46.00%), *Bacteroidetes* (33.30%), *Verrucomicrobia* (4.10%), *Fibrobacteres* (2.30%), *Synergistetes* (1.90%) and *Spirochaetes* (1.20%).. The rest were at low abundances, under 1.00%; Males: *Bacteroidetes* (39.50%), *Firmicutes* (40.50%), *Verrucomicrobia* (3.00%), *Fibrobacteres* (1.90%), *Synergistetes* (1.80%), *Proteobacteria* (1.60%).

Statistical tests at a phylum level were conducted on all phyla present (n = 20) after square root transformation (SRT) of the RA. Non-parametric Kruskal Wallis, followed by Wilcoxon test, with Bonferroni correction, or ANOVA when normality assumptions were met, indicated that there was no significant difference between Sexes or Prenatal Treatment groups when investigated independently.

Phyla that differed between Neonatal Treatment groups were: *Armatimonadetes* (chi-squared = 5.04, p = 0.03) with higher RA observed for the Recognition lambs (n = 19, mean = 1.8 $x10^{-5}$ ± 2.5 $x10^{-5}$) compared to the Isolation lambs (n = 16, mean = 1.5 $x10^{-5}$ ± 1.5 $x10^{-5}$); *Lentispherae* (chi-squared = 6.44, p = 0.01) with higher RA observed for the Recognition lambs (n = 19, mean = 0.004 ± 0.002) compared to the Isolation lambs (n = 16, mean: 0.003 ± 0.002) and *Verrucomicrobia* (chi-squared = 5.03, p = 0.03) with higher RA observed for the Recognition lambs (n = 19, mean = 0.04 ± 0.03) compared to the Isolation lambs (n = 16, mean = 0.03 ± 0.02). There were no significantly different phyla when exploring the effect of Group1, Group2 or Group3. The effect of all variables together (Group4) was explored despite having low sample numbers available per group. No significant difference was observed.

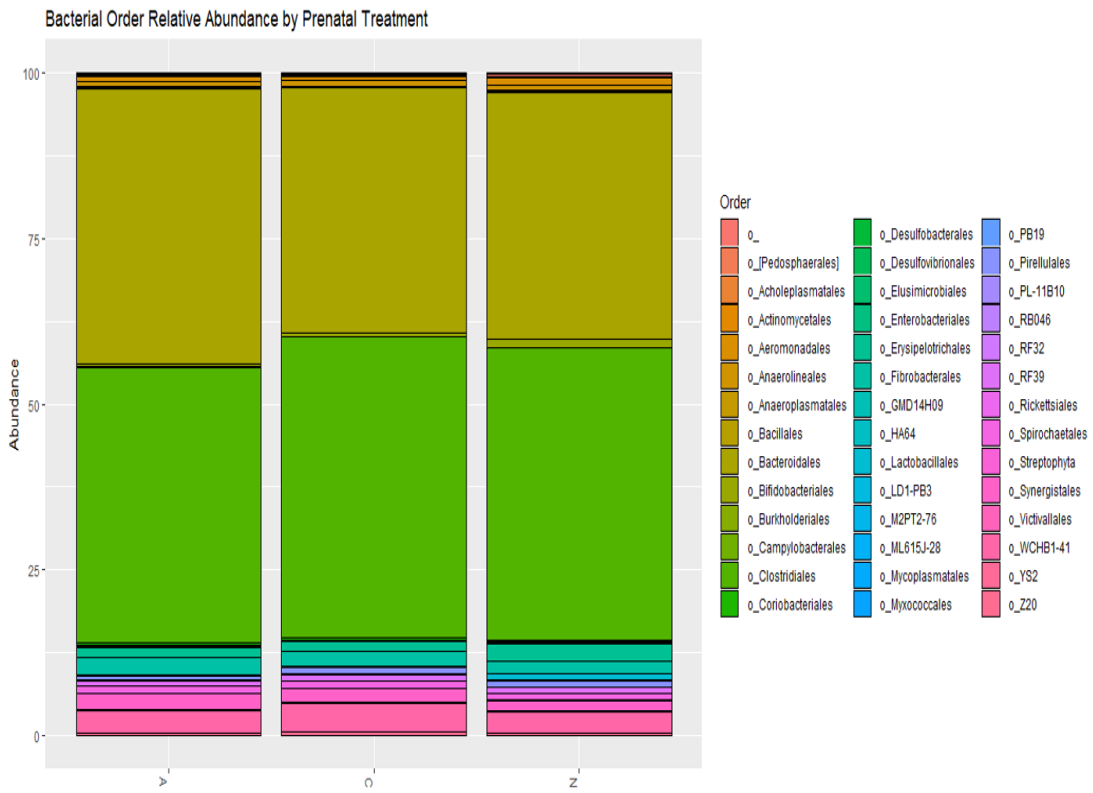
3.5.4.1.2 Order

At an order level the most abundant bacteria were *Clostridiales* at 41.00% (range: 26.30% to 64.70%) and *Bacteroidales* at 35.30% of total prokaryotes (range: 17.10% to 56.30%). The next most abundant bacteria were present in much lower percentages i.e. *WCHB1-41* (Class: *Verruco-5*) at 3.20% (range: 0.10% to 11.10%), *Aeromonadales* at 2.50% (range: 0.00% to 7.20%), *Fibrobacterales* 2.00% (range: 0.00% to 6.30%), *Synergistales* 1.80% (range: 0.20% to 4.80%), *Erysipelochaetales* 1.70% (range: 0.30% to 13.50%) and *Spirochaetales* 1.00% (range: 0.20% to 1.80%). The most abundant archaea were *Methanobacteriales* at 4.40% (range: 0.60% to 10.70%). Bacteria and archaea by Treatment group or Grouping, at an order level are presented in **Figures 3.7-3.9**.



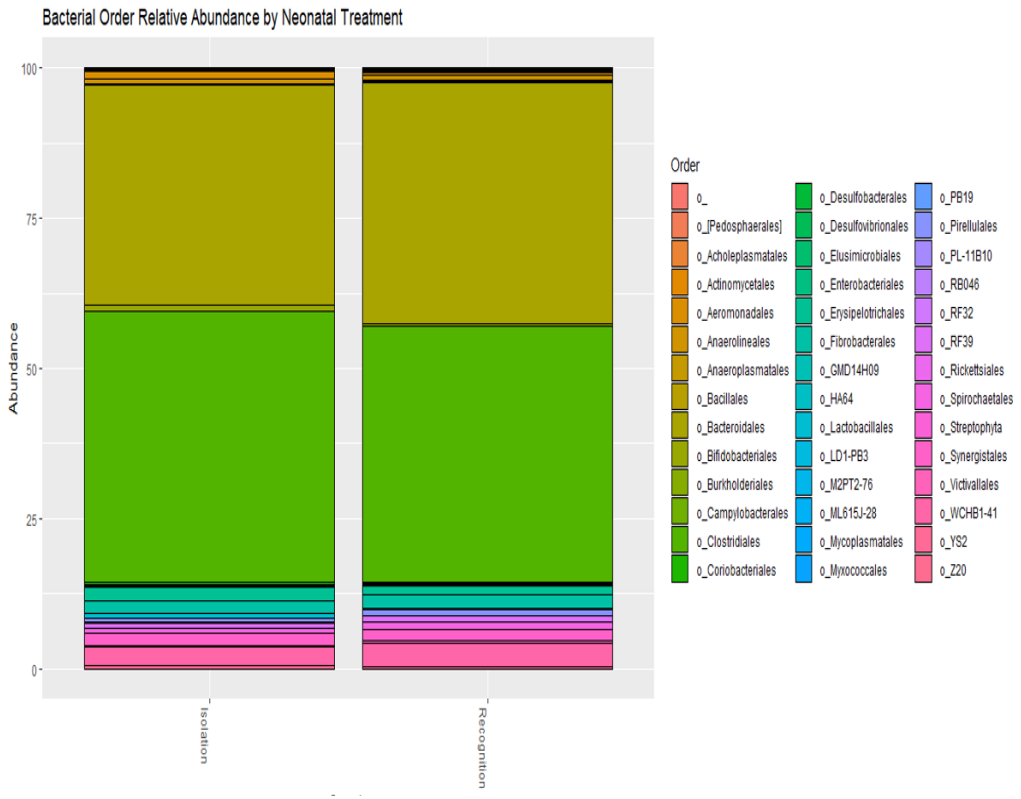
Order	Females		Males	
	% overall	Range (%)	% overall	Range (%)
Clostridiales	44.90	28.60 – 64.70	37.00	26.30 – 56.10
Bacteroidales	33.30	17.10 - 56.30	39.40	26.90 – 49.50
Methanobacteriales	4.30	0.60 – 8.70	4.80	1.70 – 9.00
Verruco_5_WCHB1-41	3.90	1.50 – 11.10	2.90	0.10 – 5.10
Aeromonadales	0.40	0.00- 1.30	1.10	0.20 – 7.20
Fibrobacterales	2.30	0.90 – 4.60	1.90	0.00 – 6.30
Synergistales	1.90	0.20 – 4.00	1.80	0.20 – 4.80
Erysipelochaetales	0.90	0.50 – 2.40	2.80	0.30 – 13.50
Spirochaetales	1.10	0.20 – 1.80	0.90	0.40 – 1.50

Figure 3.7 Barplot with average bacterial order abundance by sex and table with perspective percentages and range of most abundant archaeal and bacterial orders (> 1%) in the overall community structure of each Sex.



Order	Control		Alternative		Negative	
	% overall	Range (%)	%overall	Range (%)	%overall	Range (%)
Clostridiales	42.60	31.20– 56.10	39.20	26.30- 48.60	41.10	27.50 – 64.50
Bacteroidales	34.90	22.40 -49.50	39.20	20.80 – 56.30	34.70	17.10 – 42.80
Methanobacteriales	4.20	0.60 –10.70	4.40	2.00 – 8.70	5.10	1.70 – 9.00
Verruco_5_ WCHB1-41	4.00	1.80 –11.10	3.20	1.20 – 8.00	2.90	0.10 – 6.70
Aeromonadales	0.50	0.10 – 1.40	0.70	0.1 – 3.10	1.00	0.00 – 7.20
Fibrobacterales	2.10	1.10 – 4.70	2.40	0.70 – 6.30	1.70	0.00 – 2.80
Synergistales	1.80	0.80 –3.07	2.20	0.20 – 4.80	1.50	0.20 – 3.10
Erysipelochaetales	1.40	0.3 – 5.00	1.50	0.50 – 7.20	2.60	0.50 – 13.50
Spirochaetales	1.10	0.50 – 1.60	1.00	0.20 – 1.60	0.90	0.40 – 1.80

Figure 3.8 Barplot with average bacterial order abundance by Prenatal treatment group and table with perspective percentages and range of most abundant archaeal and bacterial orders (> 1%) in the overall community structure of each Prenatal Treatment.



Archaeal and bacterial orders in abundances higher than 1.00%, Neonatal Groups

Order	Recognition		Isolation	
	% overall	Range (%)	% overall	Range (%)
Clostridiales	40.00	28.60 –56.10	42.20	26.30 –56.10
Bacteroidales	37.80	22.3 -56.30	34.50	17.10 -49.50
Methanobacteriales	4.40	0.60 –8.70	4.70	1.70 –10.70
Verruco_5_WCHB1-41	3.80	1.10 –11.10	3.00	0.10 –5.80
Aeromonadales	0.50	0.00 -3.10	1.10	0.10 -7.20
Fibrobacterales	2.20	0.70 –4.70	2.00	0.00 -6.30
Synergistales	1.90	0.20 –3.40	1.80	0.20 -4.80
Erysipelochaetales	1.40	0.50 –4.50	2.30	0.50 -13.50
Spirochaetales	1.10	0.40 –1.60	0.80	0.20 -1.80

Figure 3.9 Barplot with average bacterial order abundance by Neonatal treatment group and table with perspective percentages and range of most abundant archaeal and bacterial orders (> 1%) in the overall community structure of each Neonatal Treatment.

Statistical tests at an order level were conducted between groups, taking into account RA over 0.1 (order, n = 22). Orders with significantly different RA by Sex, Neonatal Treatment group, "Group1" and "Group2" are presented in the table below (**Table 3.2**). No differences were observed for Neonatal Treatment, "Group3" or "Group4".

Table 3.2 Kruskal Wallis and Wilcoxon Post-Hoc Test results for bacterial and archaeal orders (RA >0.1). Orders, the variable demonstrating a significant effect, p-values, chi-squared values and Degrees of Freedom (DF) are reported. P-values derived from the Wilcoxon test, as well as means, Standard Deviation (SD) of the variables found to be significantly different, are also reported. N.D. indicates no significant difference.

Order	**Variable**	**Kruskal Wallis results**			**Variables and Groupings Mean ± SD**		**Wilcoxon p-value**
		p-value	**chi-squared**	**DF**			
Cyanobacteria_YS2	Sex	<0.01	10.03	1	**Females**	**Males**	NA
					0.07 ± 0.03	0.04 ± 0.03	
	Grouping1	<0.01	15.26	5	N.D.		
	Grouping2	<0.01	14.64	3	N.D.		
Bacteroidales	Sex	0.03	4.74	1	**Females**	**Males**	NA
					0.62 ± 0.05	0.57 ± 0.08	
	Grouping2	0.02	9.25	3	**FR**	**MI**	0.02
					0.5 ± 0.07	0.6 ± 0.05	
Clostridiales	Sex	0.02	5.18	1	**Females**	**Males**	NA
					0.6 ± 0.08	0.6 ± 0.05	
	Grouping2	0.01	10.79	3	N.D.		
Thermoplasmata_E2	Sex	0.05	5.74	1	**Females**	**Males**	NA
					0.04 ± 0.009	0.3 ± 0.008	

	Grouping2	0.02	9.17	3	N.D.		
Bifidobacteriales	Sex	<0.01	8.19	1	**Females**	**Males**	NA
					0.01± 0.02	0.08 ± 0.08	
	Grouping1	0.04	11.61	5	N.D.		
	Grouping2	0.01	11.44	3	N.D.		
Bacillales	Sex	<0.01	10.98	1	**Females**	**Males**	
					0.02 ± 0.02	0.01 ± 0.01	
	Grouping1	0.04	11.75	5	N.D.		
	Grouping2	<0.01	14.60	3	N.D.		
[Pedosphaerales]	Sex	<0.01	10.70	1	**Females**	**Males**	NA
					0.04 ± 0.02	0.01 ±0.02	
	Grouping1	0.03	12.21	5	N.D.		
	Grouping2	0.01	10.74	3	N.D.		
Actinomycetales	Sex	<0.01	10.03	1	**Females**	**Males**	NA
					0.04 ± 0.04	0.01 ± 0.01	
	Grouping2	<0.01	13.66	3	**FI**	**MI**	<0.01
					0.04±0.007	0.007 ±0.001	
					FR	**MI**	<0.01
					0.04 ± 0.05	0.007 ±0.003	
	Sex	0.01	5.74	1	**Females**	**MalES**	NA
Verruco-5					0.01 ± 0.01	0.008 ± 0.01	
LD1-PB3	Grouping2	0.01	10.71	3	**FR**	**MI**	0.02
					0.003 ±0.004	0.008 ±0.006	
Spirochaetes	Grouping2	0.01	11.41	3	**MI**	**FI**	0.02
PL-11B10					0.01±0.006	0.02 ± 0.01	
Spirochaetales		0.03	4.49	1	**Recognition**	**Isolation**	NA

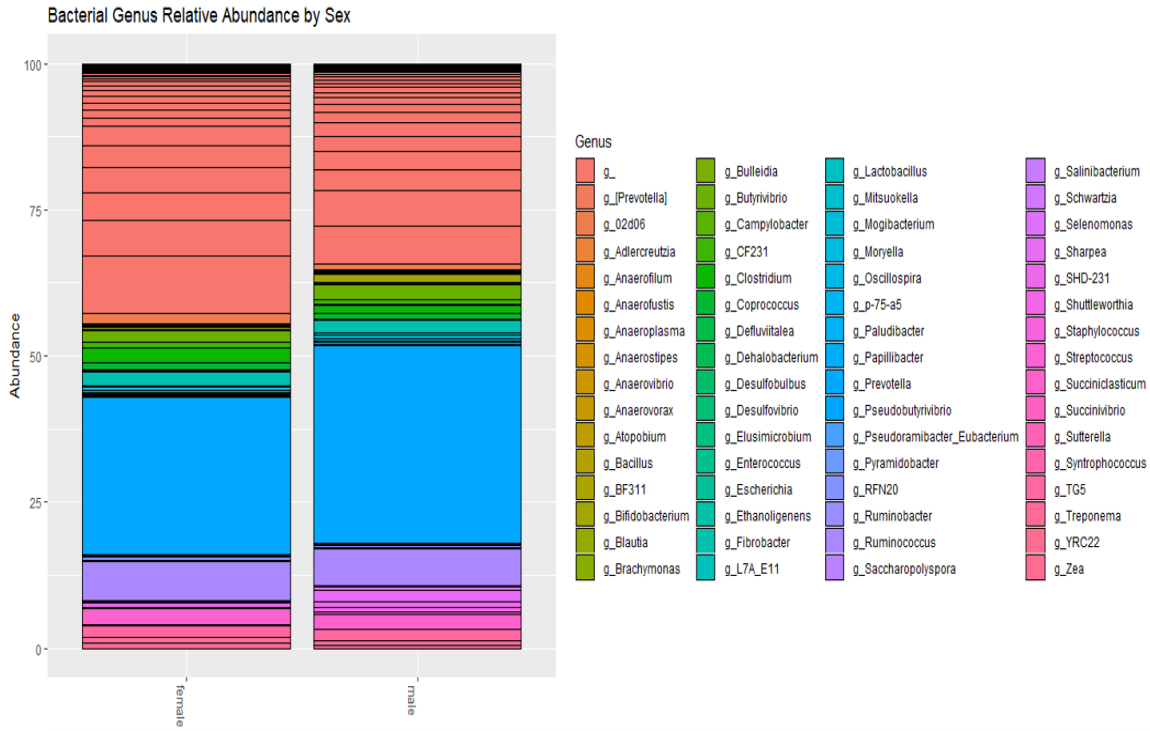
	Neonatal Treatment				0.08 ± 0.02	0.1 ± 0.02	

3.5.4.1.3 Genera

At a genus level, the most abundant bacteria in the overall community and the range is presented in **Table 3.3.** Barplots below present the mean relative abundance by Sex, Neonatal and Prenatal treatment group. Percentages of the most abundant bacteria and archaea in the overall community and the range they were reported in, are presented in **Figures 3.10-3.12**.

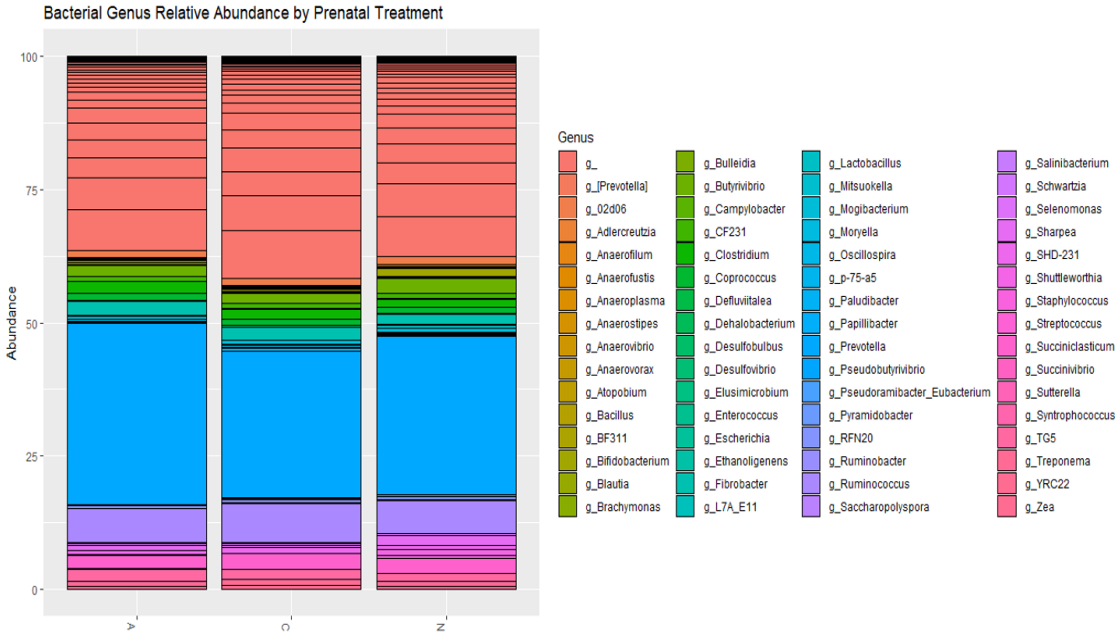
Table 3.3 Percentages and range of most abundant archaeal and bacterial genera (>2%) in the overall community structure.

Genus	% in overall community	Range (%)
Prevotella	26.90	13.20 - 47.80
Clostridiales_Unidentified	6.90	0.80 – 16.30
Ruminococcus	5.70	1.20 – 11.80
Veillonellacea_Unidentified	5.30	0.30 – 17.40
Methanobrevibacter	4.20	0.60 - 10.30
Lachnospiraceae_Unidentified	3.70	0.30 – 7.40
Bacteroidales_Unidentified	3.40	0.40 - 9.40
RFP12_Unidentified	2.60	0.00 – 7.70
Ruminococcacea_Unidentified	2.60	0.30 – 4.60
Succiniclasticum	2.60	1.20 – 4.30
Succinivibrionacea_Unidentified	2.30	0.00 – 6.90
Fibrobacter	2.00	0.00 – 6.30
Butyrivibrio	2.00	0.80 – 6.30
Mogibacteriaceae_Unidentified	2.00	0.40 – 1.80



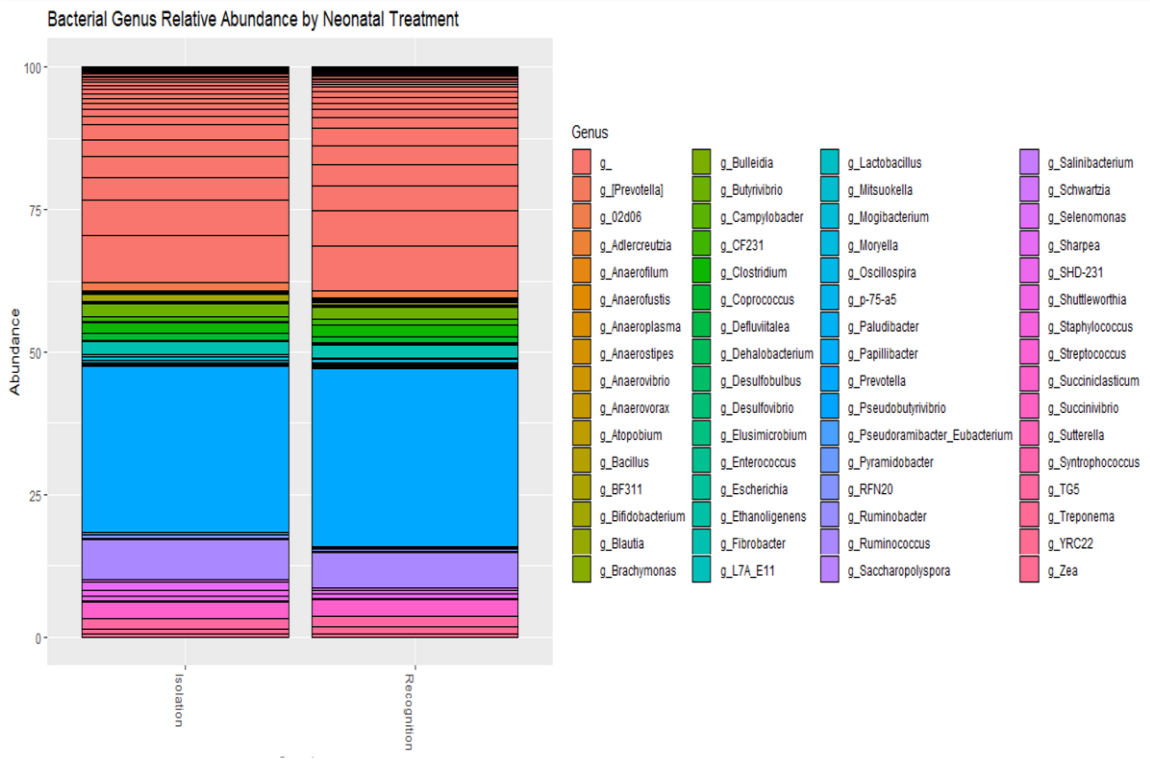
Genus	Females		Males	
	% overall	Range (%)	% overall	Range (%)
Prevotella	24.40	13.20 - 47.80	30.70	16.50 – 42.30
Clostridiales_Unidentified	8.80	2.50 -16.30	5.50	0.80 – 9.70
Ruminococcus	6.00	2.70 – 11.80	5.80	1.20 – 9.10
Veillonellaceae_Unidentified	5.40	0.80 – 16.20	5.80	0.30 – 17.40
Lachnospiracea_Unidentified	4.20	1.50 – 7.40	2.80	0.30 –7.10
Methanobrevibacter	4.00	0.60 – 10.30	4.60	1.60 – 8.80
Bacteroidales_Unidentified	3.90	1.70 – 9.40	3.20	0.40 – 6.10
Ruminococcaceae_Unidentified	3.30	1.80 –4.60	2.10	0.30 – 4.60
RFP12_Unidentified	3.00	1.20 –7.70	2.40	0.00 – 4.10
Succiniclasticum	2.60	1.80 – 4.30	2.40	1.20 – 3.00
Fibrobacter	2.30	0.90 – 4.60	<2.00	-
Butyrivibrio	< 2.00	-	2.20	0.90 – 6.30

Figure 3.10 Barplot with average bacterial genus abundance by Sex and table with perspective percentages and range of most abundant archaeal and bacterial genera (> 2%) in the overall community structure of each sex.



Genus	Control		Alternative		Negative	
	% overall	Range (%)	% overall	Range (%)	% overall	Range (%)
Prevotella	24.90	16.50– 36.50	30.70	16.90– 47.80	26.50	32.00–42.00
Clostridiales-Unidentified	8.00	4.10- 13.00	6.90	3.00– 12.80	6.70	0.80–16.30
Ruminococcus	6.50	1.80–11.80	5.70	2.70– 10.30	5.50	1.20 –8.90
Veillonellaceae- Unidentified	6.00	1.40–17.40	5.50	0.80– 16.20	5.40	0.30–16.30
Bacteroidales- Unidentified	4.10	2.10–9.40	3.40	1.40– 6.20	3.20	0.40 –7.50
Lachnospiraceae-Unidentified	4.00	1.30–7.40	3.10	1.90– 5.40	3.50	0.30 –7.10
Methanobrevibacter	4.00	0.60–10.30	4.20	2.00– 8.60	4.90	1.60 –7.40
RFP12- Unidentified	3.20	1.50–7.70	2.50	0.80– 6.70	2.30	0.00 –5.30
Succiniclasticum	2.60	1.20–4.30	2.30	1.50–3.30	2.60	1.70 –3.10
Ruminococcaceae- Unidentified	2.80	1.30–4.60	2.70	0.90–4.60	2.60	0.30 –4.60
Fibrobacter	2.10	1.10–4.70	2.40	0.70– 6.30	< 2.00	-
Clostridium	<2.00	-	2.00	0.70–5.90	< 2.00	-
Butyrivibrio	<2.00	-	<2.00	-	2.50	0.90 -6.30
Dethiosulfovibrionaceae-TG5	<2.00	-	2.10	0.10–4.80	< 2.00	-

Figure 3.11 Barplot with average bacterial genus abundance by Prenatal treatment (Control, Alternative and Negative) and table with perspective percentages and range of most abundant archaeal and bacterial genera (> 2%) in the overall community structure of each Prenatal treatment.



Genus	Recognition		Isolation	
	% overall	Range (%)	% overall	Range (%)
Prevotella	28.30	16.30 -47.80	26.30	13.20 -42.00
Clostridiales_Unidentified	7.10	2.50 -13.00	7.30	0.80 –16.30
Ruminococcus	5.60	1.80 –8.90	6.20	1.20 –11.80
Veillonellaceae_Unidentified	5.60	0.80 –17.40	5.60	0.30 –16.30
Lachnospiraceae_Unidentified	3.50	1.30 –7.40	3.60	0.30 –7.20
Methanobrevibacter	4.20	0.60 –8.60	4.50	1.60 –10.50
Bacteroidales_Unidentified	3.80	1.40 –9.40	3.30	0.40 –6.20
Ruminococcaceae_Unidentified	2.70	0.90 –4.30	2.70	0.30 –4.60
RFP12_Unidentified	3.00	0.90 –7.70	2.30	0.00 –4.90
Succiniclasticum	2.50	1.40 –3.50	2.40	1.20 –4.30
Fibrobacter	2.20	0.70 –4.70	2.00	0.00 –6.30
Butyrivibrio	<2.00	-	2.10	0.80 –4.60
Lachnospiraceae_Other	<2.00	-	2.20	0.50 –7.20

Figure 3.12 Barplot with average bacterial genus abundance by Neonatal treatment (Recognition, Isolation) and table with perspective percentages and range of most abundant archaeal and bacterial genera (> 2%) in the overall community structure of each Neonatal treatment.

Statistical tests at a genus level were conducted for RA over 0.5% (n = 56). Archaeal and bacterial genera that had significantly different RA for the variables and Treatment groups explored are reported in **Table 3.5** below.

Table 3.4 Kruskal Wallis and Wilcoxon Post-Hoc Test results for bacterial and archaeal genera (RA >0.1). Genera, the variable demonstrating a significant effect, p/ chi-squared values and DF are reported. P-values (Wilcoxon test), means and SD of the variables found to be significantly different, are also reported. "N.D" signifies that although the Kruskal Wallis indicated a significant difference, no difference was observed in the Post-Hoc analysis. "NA" indicates Wilcoxon test was not performed.

Genus	**Variable**	**Kruskal Wallis results**			**Treatment Groups and Groupings** **mean ± SD**		**Wilcoxon** **p-value**
		p-value	**chi-squared**	**DF**			
Lactobacillus	Sex	<0.01	8.01	1	Females	Males	NA
					0.002 ± 0.003	0.02 ± 0.05	
	Sex*Prenatal Treatment	0.02	12.99	5	N.D.		
	Sex*Neonatal Treatment	<0.01	12.31	3	**FI**	**MI**	<0.01
					0.001 ± 0.002	0.04 ± 0.08	
Ruminococacceae	Sex	<0.01	7.53	1	**Females**	**Males**	NA
					0.05 ± 0.009	0.04 ± 0.01	
	Sex*Neonatal Treatment	<0.01	13.44	3	**MR**	**MI**	0.05
					0.04 ± 0.01	0.03 ± 0.01	
					FI	**MI**	0.02
					0.05 ± 0.01	0.03 ± 0.01	

Christinellaceae_ Unidentified	Sex	<0.01	7.50	1	**Females**	**Males**	
					0.06 ± 0.02	0.04 ± 0.01	
	Sex*Neonatal Treatment	<0.01	12.20	3	**MR**	**MI**	0.05
					0.05 ± 0.01	0.03 ± 0.01	
					FI	**MI**	<0.01
					0.06 ± 0.02	0.03 ± 0.01	
Cyanobacteria YS2_ Unidentified	Sex	<0.01	10.15	1	**Females**	**Males**	NA
					0.07 ± 0.03	0.04 ± 0.03	
	Sex*Prenatal Treatment	0.01	15.3	5	N.D.		
	Sex*Neonatal Treatment				**FI**	**MI**	
					0.08 ± 0.01	0.02 ± 0.01	<0.01
[Paraprevotellaceae] YRC22	Sex	<0.01	10.15	1	**Females**	**Males**	
					0.08 ± 0.03	0.05 ± 0.02	NA
					FP	**MP**	
					0.08 ± 0.01	0.05 ± 0.005	0.03
					FR	**MI**	

	Sex*Neonatal Treatment	<0.01	13.04	3	0.08 ± 0.02	0.05 ± 0.01	<0.01
Succinivibrionaceae Unidentified	Sex	<0.01	10.04	1	**Females**	**Males**	NA
					0.03 ± 0.02	0.08 ± 0.06	
	Sex*Prenatal Treatment	0.03	12.37	5	N.D.		
	Sex*Prenatal Treatment	<0.01	11.92	3	**FI**	**MI**	0.04
					0.03 ± 0.02	0.1 ± 0.07	
Bifidobacterium	Sex	<0.01	8.29	1	**Females**	**Males**	NA
					0.01 ± 0.02	0.08 ± 0.08	
	Sex*Prenatal Treatment	0.04	11.61	5	N.D.		
	Sex*Neonatal Treatment	<0.01	11.44	3	**FI**	**MI**	<0.01
					0.01 ± 0.01	0.12 ± 0.09	
	Sex	<0.01	9.51	1	**Females**	**Males**	NA
					0.005 ± 0.007	0.09 ± 0.11	

Sharpea	Sex*Prenatal Treatment	0.03	12.20	5	N.D.		
	Sex*Neonatal Treatment				**FI**	**MI**	
					0.004 ± 0.005	0.12 ± 0.13	<0.01
Clostridium	Sex	0.02	3.47	1	**Females**	**Males**	
					0.14 ±0.04	0.10 ±0.03	NA
	Sex*Neonatal Treatment	0.02	9.22	3	**FI**	**MI**	
					0.14 ±0.05	0.08 ±0.03	0.03
Ruminococcaceae Unidentified	Sex	<0.01	10.47	1	**Females**	**Males**	NA
					0.18 ± 0.02	0.13 ± 0.04	
	Sex*Prenatal Treatment	0.04	11.71	5	N.D.		
	Sex*Neonatal Treatment				**FI**	**MI**	<0.01
					0.2 ± 0.02	0.11 ± 0.03	
Clostridiales_Unidentified	Sex	<0.01	9.13	1	**Females**	**Males**	NA
					0.30 ± 0.05	0.22 ± 0.05	
	Sex*Prenatal Treatment	0.03	9.92	5	N.D.		

	Sex*Neonatal Treatment	<0.01	11.99	3	**FI**	**MI**	<0.01
					0.3 ± 0.05	0.2 ± 0.05	
Lachnospiraceae Unidentified	Sex	<0.01	9.13	1	**Females**	**Males**	NA
					0.30 ± 0.05	0.22 ± 0.05	
	Sex*Neonatal Treatment	0.02	9.22	3	**FI**	**MI**	<0.01
					0.21 ± 0.03	0.13 ± 0.04	
Prevotella	Sex	0.01	6.80	1	**Females**	**Males**	NA
					0.48 ± 0.08	0.55 ± 0.07	
	Sex*Neonatal Treatment	0.02	10.08	3	**FI**	**MI**	NA
					0.46 ± 0.05	0.57 ± 0.06	
Verruco-5_WCHB1-25 Unidentified	Sex	0.04	4.33	1	**Females**	**Males**	NA
					0.08 ± 0.03	0.06 ± 0.02	
Mogibacteriaceae Unidentified	Sex	0.03	4.60	1	**Females**	**Males**	NA
					0.10 ± 0.01	0.08 ± 0.02	
Treponema	Neonatal Treatment	0.04	4.08	1	**Isolation**	**Recognition**	NA
					0.08 ± 0.02	0.10 ± 0.02	
Clostridia_Other		0.02	5.15	1	**Isolation**	**Recognition**	NA

	Neonatal Treatment				0.11 ± 0.02	0.13 ± 0.01	
					0.07 ± 0.11	0.02 ± 0.006	
Selenomonas	Sex*Neonatal Treatment	0.02	9.40	3	**MI**	**MR**	0.03
					0.08 ± 0.02	0.06 ± 0.01	

3.5.4.2 Rumen bacterial and archaeal diversity

3.5.4.2.1 Alpha diversity

A Sex effect was observed for the Shannon, Chao1, Observed, ACE and Fisher diversity indices. Furthermore, Sex * Neonatal Treatment also had an effect on all indices apart from Simpson's. Boxplots (**3.13, 3.14** and **3.15**) were created in "phyloseq" using rarefied reads, to visualise differences in diversity indices calculated for the bacterial OTUs. The boxplots presented here are by Sex and Sex * Neonatal Treatment and for all variables. Significant differences and p-values are reported in the figures. No differences were observed between Sexes, Prenatal or Neonatal Treatment groups and subgroups: Group1 (Sex * Prenatal Treatment), Group2 (Sex * Neonatal Treatment), Group3 (Neonatal x * Prenatal Treatment) for the alpha diversity metrics in the rumen archaeal community.

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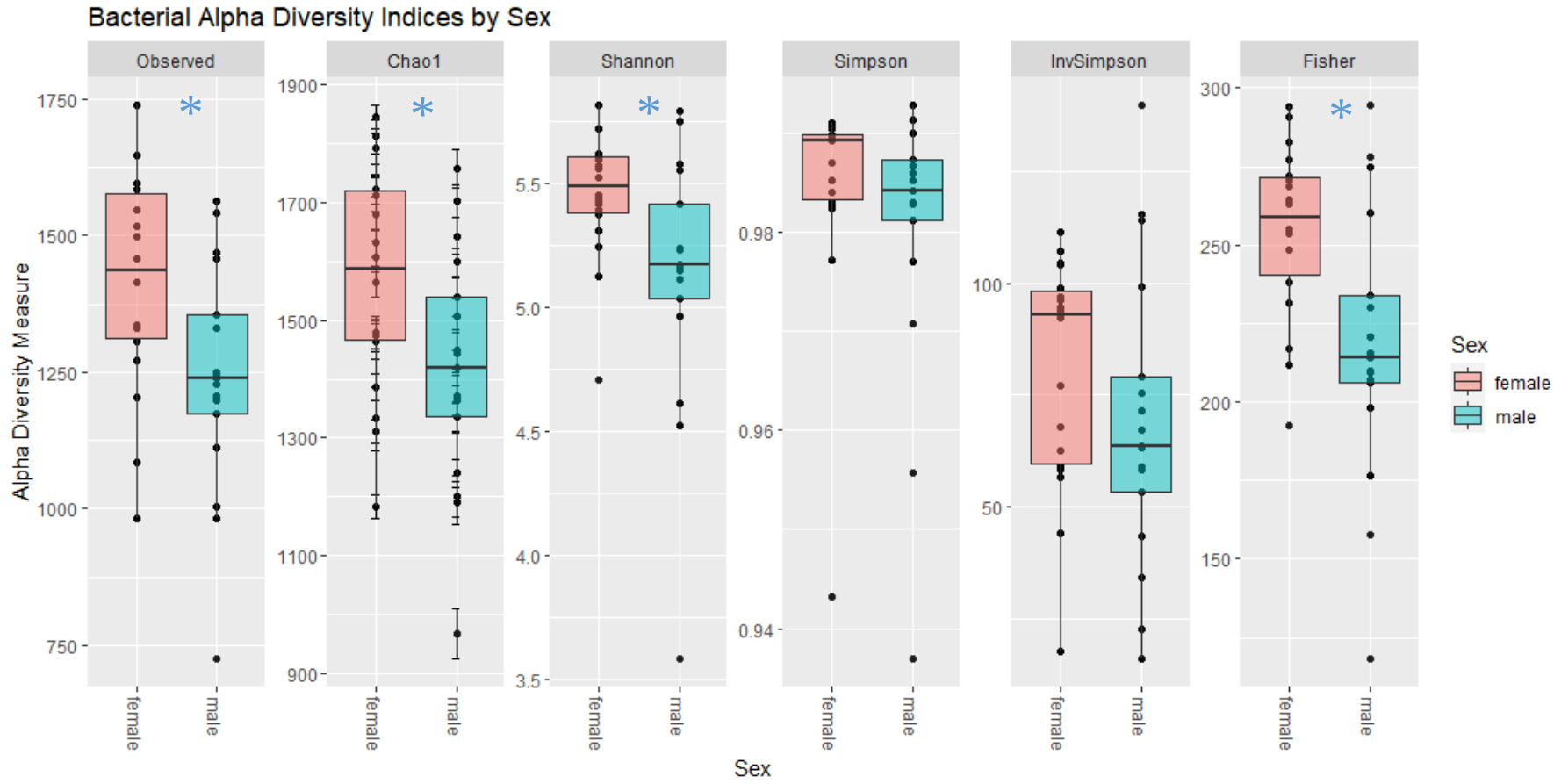

Figure 3.13 Boxplot of the diversity indices (Observed, Chao1, Ace, Shannon, Simpson and Inverse Simpson) calculated using "phyloseq" on rarefied counts, to examine differences in rumen bacterial diversity between females and males, which from the Kruskal Wallis test were significantly different for the Observed ($p = 0.02$), Chao1 ($p = 0.04$), Shannon ($p = 0.02$) and Fisher's ($p < 0.01$) indices. Means, IQR and individual values are presented.

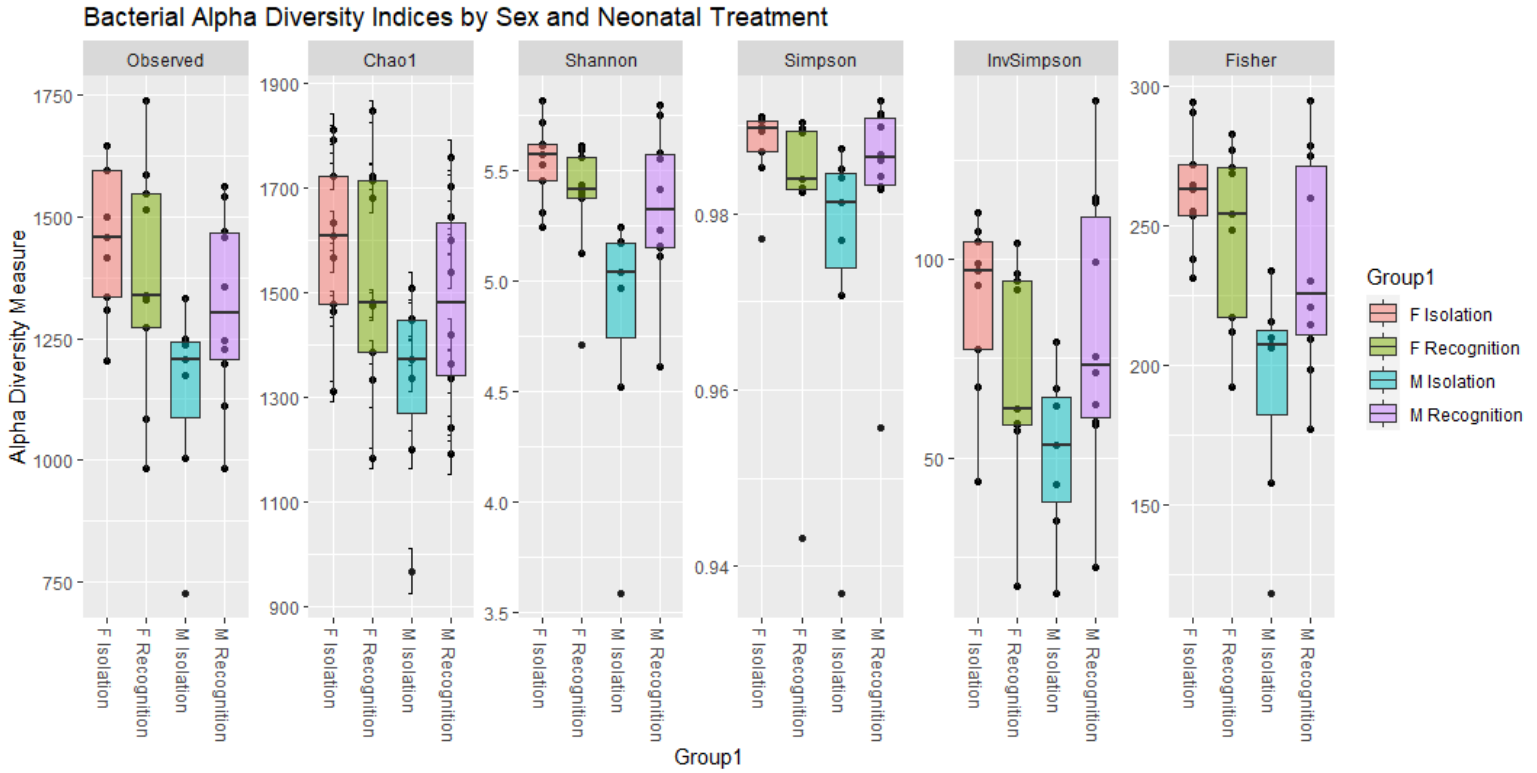

Figure 3.14 Boxplot of the diversity indices (Observed, Chao1, Ace, Shannon, Simpson and Inverse Simpson) calculated via "phyloseq" on rarefied counts, to examine differences in rumen bacterial diversity between females and males and Neonatal treatment groups (F Isolation, F Recognition, M Isolation, M Recognition). Kruskal Wallis tests indicated differences were significantly different between the M Isolation and F Isolation groups for the Observed diversity ($p = 0.02$), Chao1 ($p = 0.04$), Fisher's ($p < 0.01$) and Inverse Simpson ($p = 0.04$) indices. Significant differences were also observed between M Isolation –F Isolation ($p < 0.01$), M Isolation – F Recognition ($p = 0.03$) and M Isolation – M Recognition ($p = 0.03$) for the Shannon Index. Means, IQR and individual values are presented.

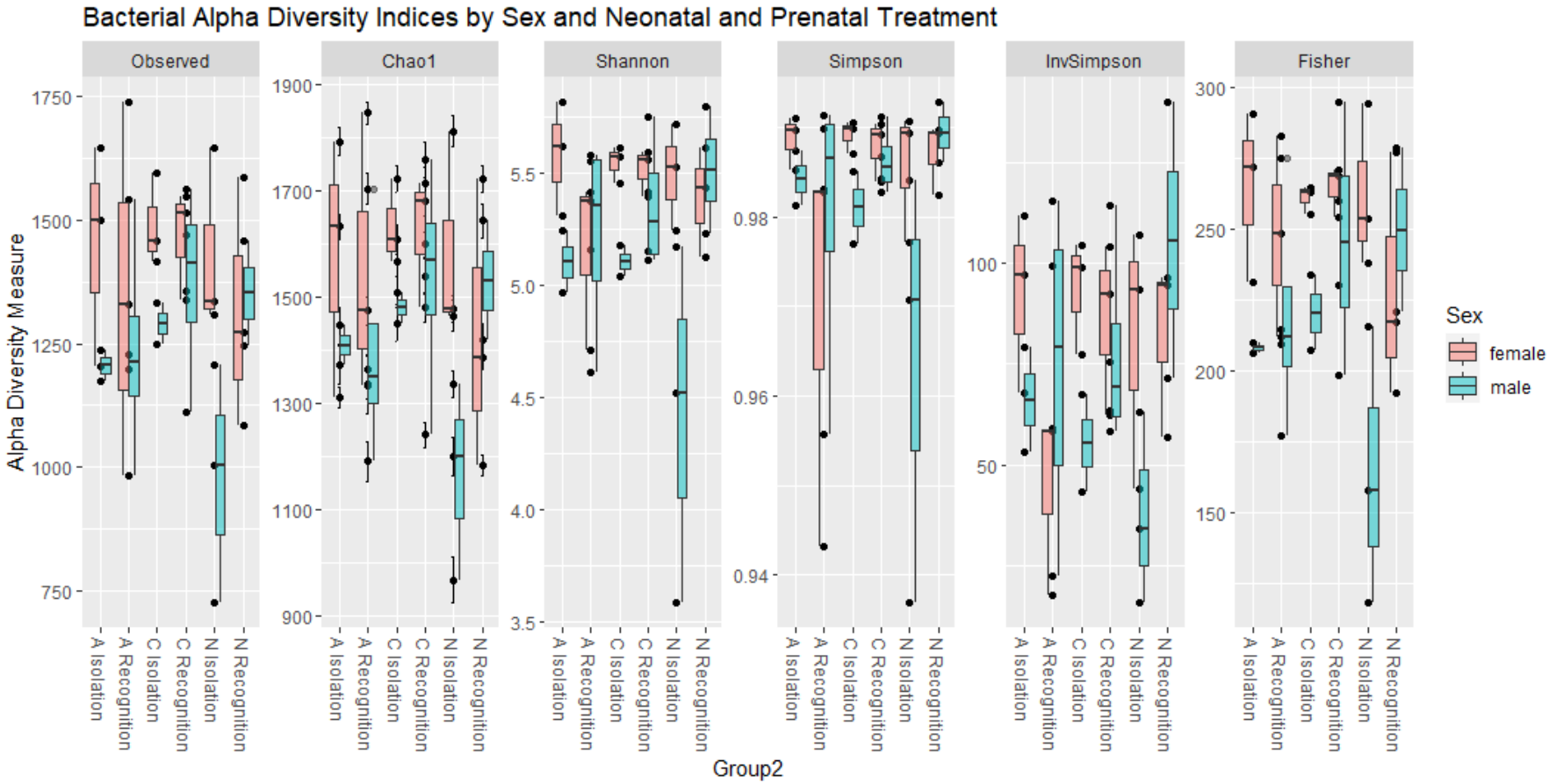

Figure 3.15 Boxplot of the diversity indices (Observed, Chao1, Ace, Shannon, Simpson and Inverse Simpson) calculated via "phyloseq" on rarefied counts, to examine differences in rumen bacterial diversity between females and males, Prenatal (Alternative, Negative and Control) and Neonatal treatment groups (A Isolation, A Recognition, C Isolation, C Recognition, N Isolation, N Recognition. Means, IQR and individual values are presented.

3.5.4.2.2 Beta diversity

A permutation test for homogeneity of multivariate dispersions was applied to investigate the dispersion of archaeal and bacterial samples between Sexes, Pre/Neonatal Treatment groups and for Subgroups: Group1 (Sex * Prenatal Treatment), Group2 (Sex * Neonatal Treatment) and Group3 (Neonatal* Prenatal Treatment). The number of permutations was set at 999. No difference in dispersion was observed.

A Multivariate Analysis of Variance test (Adonis) was performed on the basis of Bray-Curtis distances calculated from the "Hellinger" transformed OTU table, with 9999 permutations. Significant effects were observed for Sex [$F(1, 35) = 4.23$, $R^2 = 0.11$, $p < 0.01$] and Group1 [$F(3, 35) = 2.42$, $R^2 = 0.19$, $p < 0.01$ for the archaea. For the bacteria, significant differences were observed between Sexes [$F(1, 35) = 2.95$, $R^2 = 0.08$, $p < 0.01$], Group1 [$F(1, 35) = 1.36$, $R^2 = 0.19$, <0.01] and Group 2 [$F(1, 35) = 1.98$, $R^2 = 0.16$, $p < 0.01$]. Results are presented in **Table 3.6** below.

Table 3.5 Results from the beta-dispersion and Adonis tests are presented below for each Treatment Group and Grouping of variables. P-values, F-values, Mean Standard Error (MSE) has also been reportedaccordingly. Significance has been noted with the presence of a "*" symbol.

Bacterial β-Dispersion results							
	Sex	**Prenatal Treatment**	**Neonatal Treatment**	**Grouping**			
				1	**2**	**3**	**4**
p-value	0.16	0.14	0.14	0.35	0.31	0.18	0.38
F-value	1.92	2.09	2.09	1.16	1.25	1.64	1.18
MSE	0.01	0.008	0.008	0.005	0.005	0.005	0.004
Bacterial Adonis results							
	Sex	**Prenatal Treatment**	**Neonatal Treatment**	**Grouping**			
				1	**2**	**3**	**4**
p-value	<0.01*	0.52	0.73	0.02*	<0.01*	0.9	0.12
F-value	2.95	0.93	0.8	1.36	1.98	0.82	1.14
MSE	0.08	0.05	0.02	0.12	0.16	0.12	0.35
Archaeal β-Dispersion results							

	Sex	Prenatal Treatment	Neonatal Treatment	Grouping			
				1	2	3	4
p-value	0.17	0.51	0.75	0.60	0.36	0.86	0.88
F-value	0.60	0.67	0.11	0.77	1.11	0.38	0.5
MSE	0.003	0.004	0.0004	0.004	0.005	0.002	0.005
Archaeal Adonis results							
	Sex	**Prenatal Treatment**	**Neonatal Treatment**	**Grouping**			
				1	**2**	**3**	**4**
p-value	<0.01*	0.98	0.58	0.08	<0.01 *	0.95	0.06
F-value	4.23	0.35	0.77	1.43	1.43	0.59	1.39
MSE	0.11	0.02	0.02	0.19	0.19	0.09	0.39

3.5.4.2.3 PCoA plots

PCoA plots of the variables and Treatment Groupings for which significant differences in beta diversity were observed have been presented below (**Figure 3.16**, **Figure 3.17**, **Figure 3.18**, **Figure 3.19**). For the bacteria, the percentage explained by each axis (Axis 1: 24.10%, Axis 2: 8.00%) is moderate. For the archaea, description levels are higher, with Axis 1 explaining: 31.00% and Axis 2: 20.00%. We can see that female and male OTUs cluster separately, both for bacteria and archaea. Distinguishing clusters due to Sex * Prenatal Treatment group or Sex * Neonatal Treatment group are not as evident.

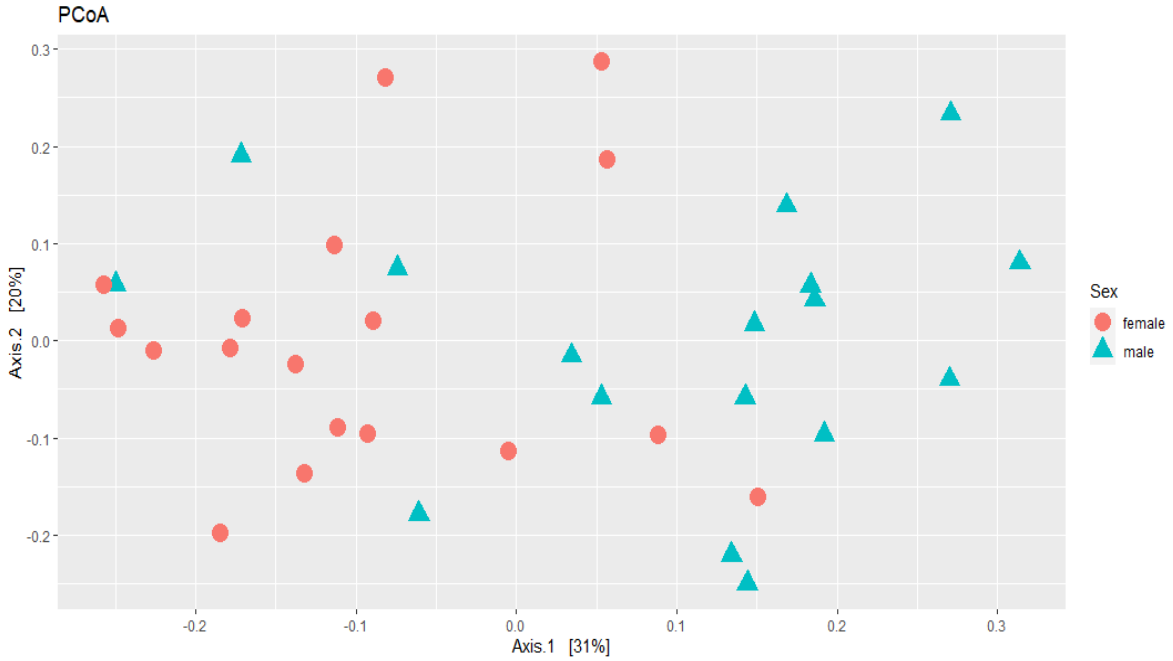

Figure 3.16 Principal coordinate analysis (PCoA) of the rumen bacterial community based on the SRT RA data of OTUs. The relative distances of all points represent the relative dissimilarities of the samples according to the Bray-Curtis index. Females and Males are colour coded and distinguished by shape (circle = females, triangle = males). The percentage of total variation explained by each PCoA axis is shown in the brackets.

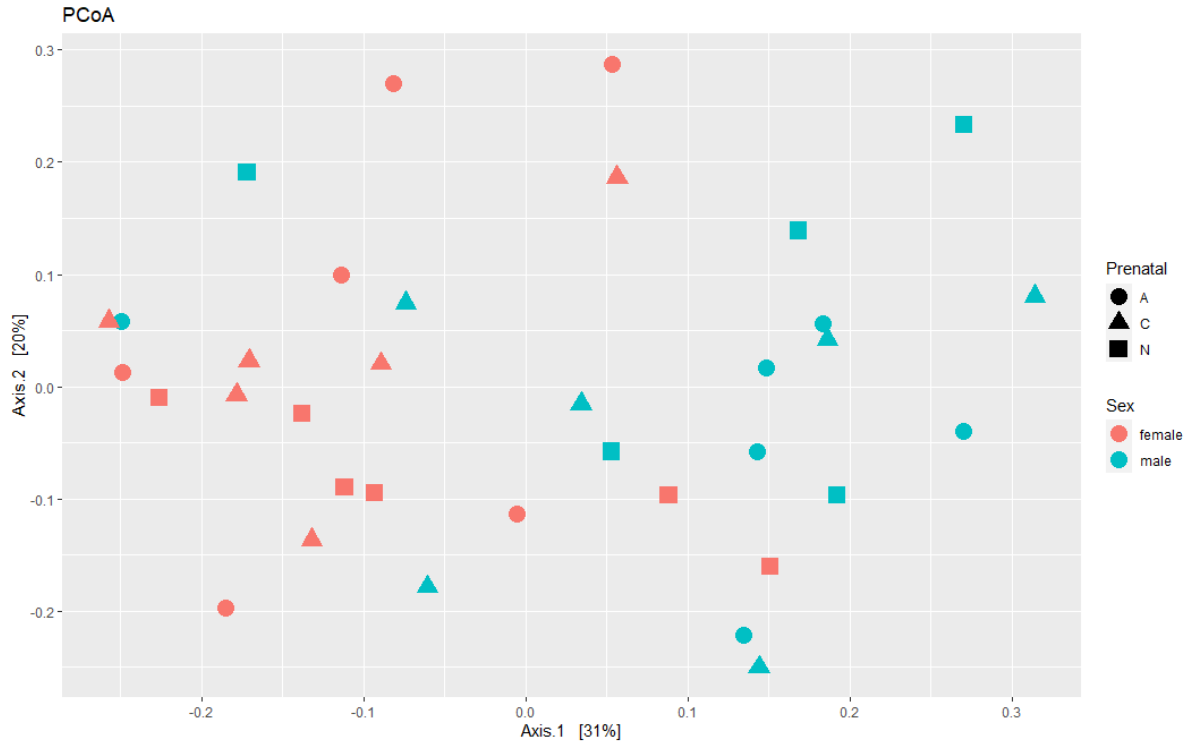

Figure 3.17 Principal coordinate analysis (PCoA) of the rumen bacterial community based on the SRT RA data of OTUs. The relative distances of all points represent the relative dissimilarities of the samples according to the Bray-Curtis index. Sex is is colour coded and Prenatal treatment groups are distinguished by shape (circle = A-Alternative, triangle= C-Control, square= N-Negative). The percentage of total variation explained by each PCoA axis is shown in the brackets.

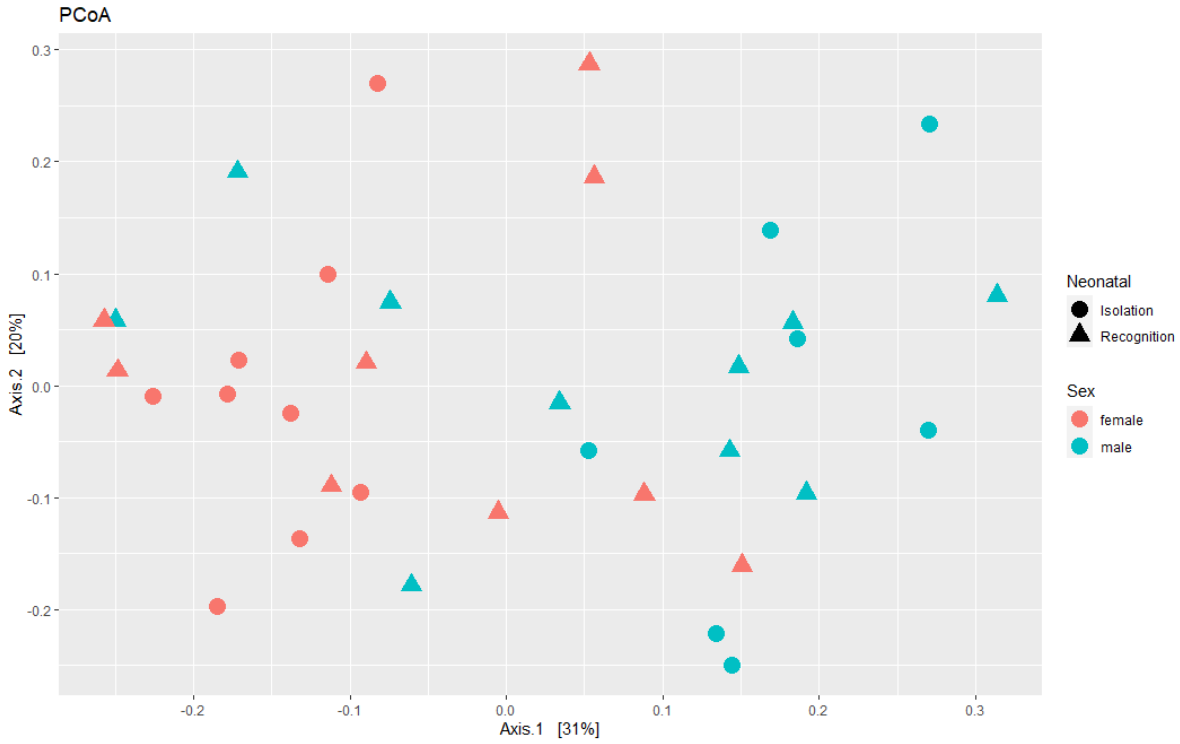

Figure 3.18 Principal coordinate analysis (PCoA) of the rumen bacterial community based on the SRT RA data of OTUs. The relative distances of all points represent the relative dissimilarities of the samples according to the Bray-Curtis index. Sex is colour coded and Neonatal Treatment groups are distinguished by shape (circle = Isolation, triangle = Recognition). The percentage of total variation explained by each PCoA axis is shown in the brackets.

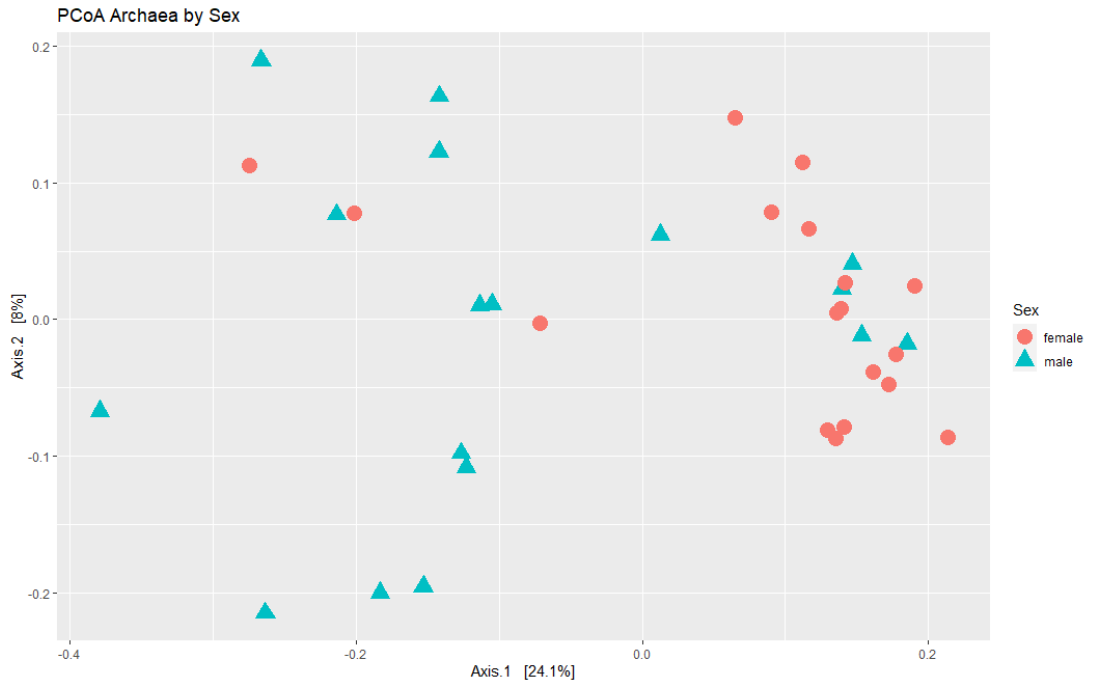

Figure 3.19 Principal coordinate analysis (PCoA) of the rumen archaeal community based on the SRT RA data of OTUs. The relative distances of all points represent the relative dissimilarities of the samples according to the Bray-Curtis index. Sexes are distinguished by colour and shape. The percentage of total variation explained by each PCoA axis is shown in the brackets.

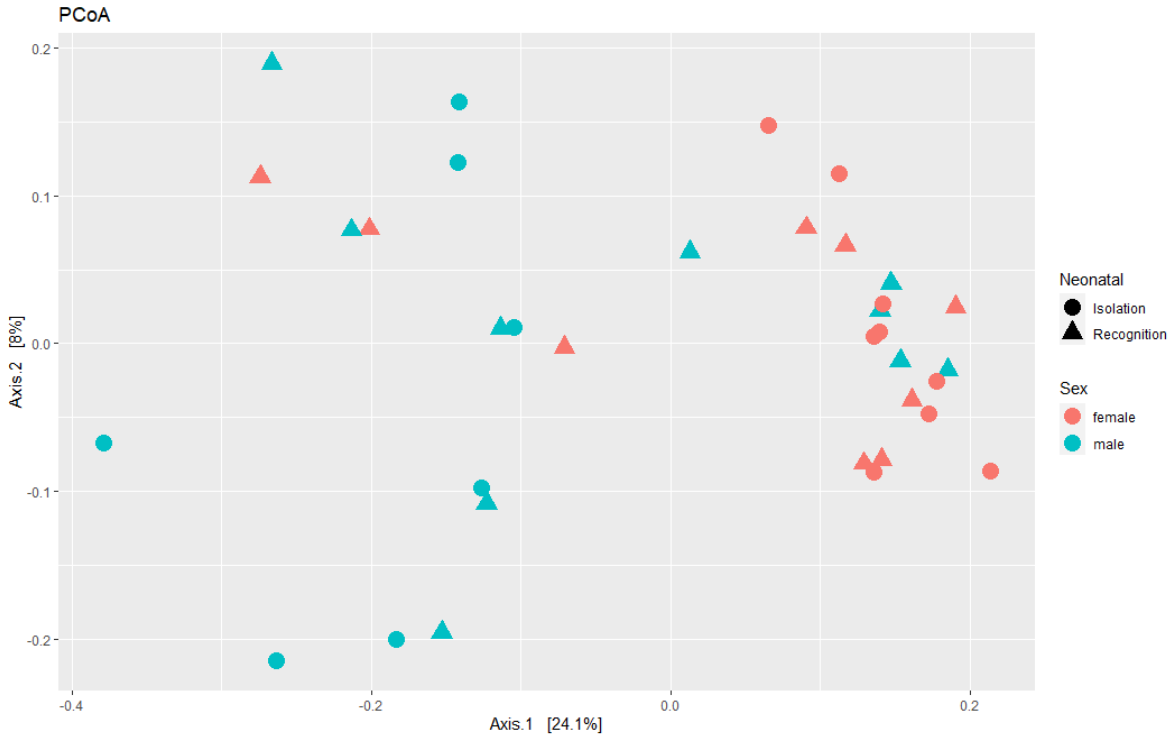

Figure 3.20 Principal coordinate analysis (PCoA) of the rumen archaeal community based on the SRT RA data of OTUs. The relative distances of all points represent the relative dissimilarities of the samples according to the Bray-Curtis index. Sexes are distinguished by colour and Neonatal Treatment groups by shape (circle = Isolation, triangle = Recognition). The percentage of total variation explained by each PCoA axis is shown in the brackets.

3.5.4.3 Partial Least Square Regression analyses

3.5.4.3.1 PLS phylum level

A PLS regression between SRT RA of all rumen phyla with "log(x+1)" transformed cortisol values was carried out for Females, Males, Control Females, Control Males, Alternative Females, Alternative Males, Negative Females and Negative Males. Regarding the Neonatal Treatment, PLS with cortisol was carried out with RA for Isolation Females, Isolation Males, Recognition Females and Recognition Males.

The percentage of Variability in cortisol explaining Variability present in the RA is presented in **Table 3.7** below. Following the PLS regression, Pearson correlation was conducted between phyla that had a VIP score >1.00 in each group and cortisol. The correlation coefficient (R) was mainly used as an indication of the direction (positive or negative) of the relationship.

Occasionally, R was higher than 0.8, or lower than -0.8 indicating that the phyla in question have a particularly strong relationship with cortisol. Phyla with PLS VIP scores >1.00 for each group, their relationship with cortisol and the variables/groups for which the correlation coefficient was significant are reported in **Table 3.8** below.

VIP scores, R values and RA for the phyla with VIP >1.00 for each group can be found in Appendix (**Tables 7.12- 7.14).** Heatplots created on R to explore the relationship between cortisol and phyla by variable are also available in the Appendix (**Figures 7.12 -7.15**).

Table 3.6 Percentage of cortisol variability and percentage of explained variability of RA for each variable. The higher the percentage of variability explained in the RA, the higher our confidence in the relationship and VIP scores reported.

Variable	% Cortisol variability	% Variability explained
Females	36.23	67.94
Males	42.50	41.54
Females*Control	59.06	96.70
Females*Alternative	43.20	93.97
Females*Negative	59.90	96.70
Males*Control	61.53	95.32

Males*Alternative	53.47	94.63
Males*Negative	76.81	99.5
Females*Recognition	46.53	84.80
Females*Isolation	43.54	89.98
Males*Recognition	47.44	97.57
Males*Isolation	37.58	88.46

Table 3.7 Phyla with PLS VIP scores >1 for each Sex (F: Females, M: Males) and Treatment Grouping (**Sex * Prenatal Treatment:** FC: Females Control, FA: Females Alternative, FN: Females Negative; MC: Males Control, MA: Males Alternative, MN: Males Negative and **Sex * Neonatal Treatment**: FR: Female Recognition, FI: Female Isolation, MR: Male Recognition, MI: Male Isolation). The relationship with cortisol is noted with "+" if positive and "-" if negative, as indicated by the Pearson Correlation Analysis. Significant correlations R >0.8 or R <-0.8 are mentioned. These, in combination with the VIP score found in the Appendix suggest a particularly strong relationship with cortisol.

	Phylum	**F**	**M**	**FC**	**FA**	**FN**	**MC**	**MA**	**MN**	**FR**	**FI**	**MR**	**MI**	**R >0.8 or R <-0.8**
A1	*Euryarchaeota*				-									
A2	*Bacteria;Other*					+		-			+			
A3	*Actinobacteria*				-									
A4	*Armatimonadetes*		+						+		-		+	
A5	*Bacteroidetes*	-				-					-			
A6	*Chloroflexi*	+		+	+	+		-		+		-		FN/ FA/ FR
A7	*Cyanobacteria*				-				+		-		+	
A8	*Elusimicrobia*	-	-		-			+		-				
A9	*Fibrobacteres*	-				-	-	-	+	-	-	-		FN /MA
A10	*Firmicutes*	+				+					+			
A11	*LD1*				+		-		+	+		-	+	

A12	*Lentisphaerae*			-		-	-				-		+	
A13	*Planctomycetes*	+	-	+		+	-		+	+	+	-		MC
A14	*Proteobacteria*		-			-			-		-		-	MN/ MI
A15	*SR1*			+						+				FC
A16	*Spirochaetes*		-			-	-			-	-			
A17	*Synergistetes*		+		+		+	-	+		+	+		
A18	*Tenericutes*												-	
A19	*Verrucomicrobia*					-		-						
A20	*WPS-2*	+		+		+		+		+			+	

3.5.4.3.2 PLS order level

Variability in cortisol levels explaining the variability in order RA is presented in **Table 3.9**, below. Following the PLS regression, Pearson correlation was conducted between orders with a VIP score > 1.50 for each variable and cortisol.

Orders with PLS VIP scores >1.50 for each group, their relationship with cortisol and whether the correlation coefficient was significant are reported in **Table 3.10** below. VIP scores, R values and RA for the orders with VIP > 1.50 for each group can be found in Appendix (**Tables 7.15- 7.17**). Heatplots created on R to explore the relationship between cortisol and phyla by variable are also available in the Appendix (**Figures 7.16-7.18**).

Table 3.8 Percentage of cortisol variability and percentage of explained variability of order level RA for each Variable.

Variable	**%** **Cortisol variability**	**%** **Variability explained**
Females	19.50	76.91
Males	38.07	63.13
Females*Control	47.57	98.75
Females*Alternative	38.6	99.69
Females*Negative	36.41	99.81
Males*Control	53.41	98.43
Males*Alternative	34.36	99.96
Males*Negative	66.25	95.7
Females*Recognition	34.64	93.88
Females*Isolation	38.51	93.80
Males*Recognition	43.63	99.90
Males*Isolation	47.02	95.18

Table 3.9 Orders with PLS VIP scores >1.50 for each **Sex** (F: Females, M: Males) and Treatment Grouping (**Sex * Prenatal Treatment:** FC: Females Control, FA: Females Alternative, FN: Females Negative; MC: Males Control, MA: Males Alternative, MN: Males Negative and **Sex * Neonatal Treatment**: FR: Female Recognition, FI: Female Isolation, MR: Male Recognition, MI: Male Isolation). The relationship with cortisol is noted with "+" if positive and "-" if negative, as indicated by the Pearson correlation analysis. Significant correlations R > 0.8 or R <-0.8 are mentioned. These, in combination with the VIP score found in the Appendix suggest a particularly strong relationship with cortisol.

	Order	**F**	**M**	**FC**	**FA**	**FN**	**MC**	**MA**	**MN**	**FR**	**FI**	**MR**	**MI**	**R > 0.8 or R < -0.8**
A2	*Thermoplasmata;o_E2*	+		+	-	+				+				
A4	*Actinomycetales*						-							
A6	*Coriobacteriales*							+						MA
A7	*Armatimonadetes;o_RB046*												+	MI
A9	*Bacteroidales*										-			
A10	*Anaerolineales*	+			+	+				+		-		FA/ FN/ FR
A13	*Elusimicrobiales*		-											
A14	*Endomicrobia;o_Unidentified*		+					+					+	
A15	*Fibrobacterales*							+	-					MA
A16	*Firmicutes;o_Unidentified*						+					+		MC
A18	*Lactobacillales*								+		-			
A19	*Clostridia;Other*		-				-			+		-		

A20	*Clostridiales*							+			+			
A21	*Erysipelotrichales*							+	-					MA/ MN
A22	*LD1_Unidentified*											-	+	
A25	*Pirellulales*	+	-	+			-					-		MC
A26	*vadinHA49;Other*						-							MC
A27	*Proteobacteria;Other*							-		-				MA
A28	*Alphaproteobacteria;Other*				+									
A31	*Rickettsiales*				+		-			-				
A32	*Burkholderiales*										-			FI
A34	*Desulfovibrionales*			-										
A35	*Deltaproteobacteria; o_GMD14H09*										-			FI
A38	*Campylobacterales*								+					
A39	*Gammaproteobacteria;Other*	-	-					+	-					MA/ MN
A40	*Aeromonadales*								-					MN
A41	*Enterobacteriales*	+		+		+				+				FC/ FN
A42	*SR1_Unidentified*			+										FC

A44	*Spirochaetes;o_M2PT2-76*	-				-								
A45	*Spirochaetales*		-			-								
A46	*Synergistales*							-				+		
A48	*Mollicutes;Other*		-		+									
A49	*Mollicutes_Unidentified*												-	
A50	*Acholeplasmatales*										-			
A51	*Anaeroplasmatales*											+	-	
A52	*Mycoplasmatales*			+	+									FA/ FC
A54	*Tenericutes; c_RF3;o_ML615J-28*	-		-										FC
A55	*Verrucomicrobia;Other*					-	+							FN
A57	*Verruco-5;o_LD1-PB3*						-							
A58	*Verruco-5;o_WCHB1-41*							-						
A60	*WPS-2_Unidentified*	+		+		+				+				

3.5.4.3.3 PLS genus level

Variability in cortisol levels explaining the variability in the RA at genus level is presented in **Table 3.11** below. Following the PLS regression, Pearson correlation was conducted between genera that had a VIP score >1.50 in each group and cortisol. The correlation coefficient (R) was mainly used as an indication of the direction (positive or negative) of the relationship.

Orders with PLS VIP scores >1.50 for each group, their relationship with cortisol and whether the correlation coefficient was significant are reported in **Table 3.12** below. When the correlation coefficient was not significant and the genus was found correlated with cortisol only in one group, it was not reported in the table. However, VIP scores, R values and RA for the genera with VIP >1.50 for each group can be found in Appendix **(Tables 7.18-7.22**) Heatplots created in R to explore the relationship between cortisol and genera by variable are also available in the Appendix (**Figures 7.20- 7.24**).

Table 3.10 Percentage of cortisol variability and percentage of explained variability of genus level RA for each variable.

Variable	% Cortisol variability	% Variability explained
Females	21.36	83.65
Males	32.42	73.26
Females*Control	44.80	98.70
Females*Alternative	34.39	99.38
Females*Negative	37.60	100.00
Males*Control	54.43	98.83
Males*Alternative	34.00	99.88
Males*Negative	43.47	99.71
Females*Recognition	36.33	95.88
Females*Isolation	38.51	93.80
Males*Recognition	35.78	102.58
Males*Isolation	47.85	94.83

Table 3.11 Table 3.12 Genera with PLS VIP scores >1.50 for each Sex (F: Females, M: Males) and Treatment Grouping (**Sex * Prenatal Treatment:** FC: Females Control, FA: Females Alternative, FN: Females Negative; MC: Males Control, MA: Males Alternative, MN: Males Negative and **Sex * Neonatal Treatment**: FR: Female Recognition, FI: Female Isolation, MR: Male Recognition, MI: Male Isolation). The relationship with cortisol is noted with "+" if positive and "-" if negative, as indicated by the Pearson Correlation Analysis. Significant correlations R > 0.8 or R <-0.8 are mentioned. These, in combination with the VIP score found in the Appendix suggest a particularly strong relationship with cortisol.

	Genus	**F**	**M**	**FC**	**FA**	**FN**	**MC**	**MA**	**MN**	**FR**	**FI**	**MR**	**MI**	R > 0.8 or R < -0.8
A1	*Methanobacteriaceae_Unidentified*						-					-		
A2	*Methanobrevibacter*					+		-						
A3	*Methanosphaera*					+					+	-		FN
A4	*[Methanomassiliicoccaceae]_ Unidentified*							+						MA
A7	*Actinopolysporaceae_Unidentified*						-			+		-		FA/ FN/ FR
A9	*Salinibacterium*		+					+					+	
A14	*Coriobacteriaceae_Unidentified*							+						MA
A15	*Adlercreutzia*								+	+		-		
A16	*Atopobium*							+			-			
A20	*Bacteroidales;Other*						-	+				-		MR
A21	*Bacteroidales_Unidentified*						-					-		MC

A24	*Paludibacter*	-		-		-				-				FC/ FR
A25	*Prevotellaceae_Unidentified*										-			FI
A27	*Bacteroidales;f_RF16_Unidentified_*	-		-				-		-				FR
A28	*Bacteroidales;f_S24-7_Unidentified*							+						MA
A29	*[Paraprevotellaceae];Other*								+	-		-	+	
A30	*[Paraprevotellaceae]_Unidentified*					-	+							FN
A32	*[Paraprevotellaceae];_YRC22*		-				-					-		MC
A33	*[Paraprevotellaceae];_[Prevotella]*						-				+	-		MC
A34	*Anaerolinaceae;g_SHD-231*	+			+	+				+		-		FN/ FR
A37	*Elusimicrobiaceae_Unidentified*							-					-	
A38	*Elusimicrobium*	-				-								
A41	*Firmicutes;Other*						+	-				+		MC/ MA
A44	*Lactobacillus*			-							-			
A45	*Streptococcus*										-			
A46	*Clostridia;Other*		-				-			+		-		MR
A47	*Clostridiales;Other*		-			+		-						MA
A48	*Clostridiales_Unidentified*		-				-							

A49	*Christensenellaceae_Unidentified*						-							
A50	*Clostridiaceae;g_02d06*	+				+		-		+				FN
A53	*Dehalobacterium*	+						+		+				
A55	*Eubacterium*			-				+				-		MA
A64	*Moryella*	+		+		+			-	+				MN/FN
A65	*Pseudobutyrivibrio*	+								+				
A66	*Shuttleworthia*					+								FN
A67	*Syntrophococcus*		-						-					
A75	*Papillibacter*				+		-					-	+	MI
A77	*Veillonellaceae;Other*		+						+				+	
A79	*Anaerovibrio*		+										+	MI
A81	*Schwartzia*	+					-			+				MC
A83	*Succiniclasticum*						-		+			-		
A84	*[Mogibacteriaceae]_Unidentified*									+			+	
A87	*Erysipelotrichaceae;Other*	-		-	-			+	-	-				MN
A88	*Erysipelotrichaceae_Unidentified*							-					-	MI
A92	*Sharpea*							+	-				-	MA/MN

A93	*Erysipelotrichaceaeg_p-75-a5*							-					-	MA
A94	*LD1_Unidentified*						-					-	+	
A97	*Pirellulaceae_Unidentified*	+		+			-					-		MC
A98	*Planctomycetes;c_vadinHA49;Other*						-							MC
A99	*Proteobacteria;Other*							+		-				MA
A104	*Zea*				+									FA
A105	*Sutterella*										-			FI
A106	*Brachymonas*		+	-	-		+	+			-		+	FI
A110	*c_Deltaproteobacteria* *o_GMD14H09;_Unidentified*										-			FI
A115	*Gammaproteobacteria_Other*	-	-					+	-					MA/ MN
A116	*Succinivibrionaceae_Unidentified*				-				-				-	MN/MI
A117	*Ruminobacter*					-		-	-				-	MN
A119	*Escherichia*	+		+		+				+				FC/ FN
A120	*o_SR1_Unidentified*			+										FC
A124	*Spirochaetaceae;Unidentified*	-		-						-				FC
A128	*Dethiosulfovibrionaceae;g_TG5*							-				+		

A129	*Synergistaceae_Unidentified*				-							+		
A130	*Tenericutes;Other*			-								-		
A131	*Mollicutes;Other*		-		+								-	
A134	*Anaeroplasma*											+	-	
A135	*Mycoplasmataceae_Unidentified*			+	+									FC/FA
A137	*Tenericutes;c_RF3;o_ML615J-28; Unidentified*	-		-									-	
A138	*Verrucomicrobia_Other*					-	+							FN
A143	*Verruco-5;o__WCHB1-41;f__RFP12_ Unidentified*							-						MA
A146	*WPS-2_ Unidentified*	+		+						+				

3.6 Discussion

In this Chapter, 35 lambs from three maternal treatment groups (Control, Negative and Alternative), which were also subject to different Neonatal testing (sub-categorising them into Isolation and Recognition lambs), were used to explore differences due to Prenatal and Neonatal experiences on cortisol levels and the rumen microbiome at 7 months of age.

Significant differences were observed between sexes in terms of cortisol levels on the day of slaughter at approximately 33weeks. The microbial composition of the rumen was also significantly different between sexes in terms of archaeal and bacterial alpha diversity indices and bacterial beta diversity. Furthermore, visual exploration using Bray-Curtis distances indicated clustering of bacterial OTUs by sex. Comparison of RA showed that although phyla did not differ according to sex, males and females had significantly different RA for a number of bacterial and archaeal orders and genera. Sex and Diet are confounded, as females were brought in from pasture approximately two weeks before the males and fed concentrate and hay. Males were left on pasture, and therefore consumed grass, straw and concentrate until the day before slaughter.

Prenatal Treatment, explored independently and in combination with sex, did not have a significant effect on cortisol, or on any of the diversity metrics explored, and no differences were observed in RA at a phylum level. Any differences observed at an order and genus level were subsequently not confirmed by the Post-Hoc analysis.

Neonatal treatment did not have an effect on cortisol levels. Differences were observed in RA at a phylum level. Neonatal stress examined by sex had a significant effect on alpha and beta diversity metrics, on RA at an order level and mainly at a genus level, where Post-Hoc analysis indicated differences mainly between groups FI and MI and between groups MI and MR.

PLS analysis for cortisol and phylum RA indicated that cortisol explained variability in the abundances of females better than in males, and within Females better in the FC and FN groups compared to FA. At an order and genus level, cortisol variability appeared to explain the particularly high levels of variability present. Additionally, many orders and genera exhibiting VIP scores > 1.5 in the subsequent Pearson correlations had correlation coefficients > 0.8 or < -0.8. Despite this, the direction in which cortisol was correlated with the orders and genera was ambiguous, although some relationships of interest did emerge.

3.6.1 Cortisol results

Regarding cortisol levels, “normal” levels for sheep are highly dependent on age and sex (as well as time of day), factors which have also been demonstrated to influence cortisol concentration in humans and mice (Kolbe *et al.*, 2015; Ortiz *et al.*, 2016; Stachowicz and Lebiedzińska, 2016; Adam *et al.*, 2017; Roelfsema *et al.*, 2017) and sheep (McNatty, Cashmore and Young, 1972; McMillen, Thorburn and Walker, 1987; Turner, 2002; van Lier, Pérez-Clariget and Forsberg, 2003; Hucklebridge *et al.*, 2005). Studies investigating sheep cortisol for different purposes, have reported low values of 52.4 ng/ml (Caroprese *et al.*, 2010), 37.50 ng/ml (103.3 - 117 nMol/l) (Okeudo and Moss, 2005) and 30.80 ng/ml (approx. 85.0 nMol/l) for Control animals (Mills-Thompson *et al.*, 2017) and high levels of cortisol ranging anywhere from 60 ng/ml (Okeudo and Moss, 2005) to 120 ng/ml (Caroprese *et al.*, 2010). Differences are mainly reported in terms of proportion of change according to a baseline level or a Control group.

Cortisol levels in this study seem to fall within the limits of “normal” levels, with a few animals, mainly females, exhibiting what could be reported as “high” levels. Although animals were slaughtered on different dates, cortisol was sampled in the morning for both sexes, therefore a diurnal related effect should not be significant. Potentially, day effects may contribute to the difference between females and males.

More importantly though, in many studies, female mice and women have consistently higher levels of cortisol in calm and stressful situations than males (Kurina *et al.*, 2005; Steen *et al.*, 2011; Balhara, Verma and Gupta, 2012; Gong *et al.*, 2015; Reschke-Hernández *et al.*, 2017). This has also been shown in sheep, although comparisons have mainly been done using post-stress tests (Turner, 2002; van Lier, Pérez-Clariget and Forsberg, 2003; Turner *et al.*, 2006; Van Lier, Carriquiry and Meikle, 2014). Therefore, finding higher cortisol levels in females in this study is in agreement with previous findings.

3.6.2 Taxonomy results

Exploring the presence of archaeal and bacterial phyla in the overall community, *Firmicutes* appeared to be more abundant, with *Bacteroidetes* at slightly lower percentages. This is considered normal as *Bacteroidetes* are the first type of bacteria to colonize the rumen in pre-rumination stages and are progressively reduced with the introduction of forages in the diet and the maturation of the rumen. This process leads to the introduction of an array of bacteria and particularly to increased RA of *Firmicutes* (L. Wang *et al.,* 2019) and fluctuation of dominance between *Bacteroidetes* and *Firmicutes*, dependant on genetic predisposition, health issues and diet.

An observation of interest in this study is that *Firmicutes* appear to be present in a higher proportion compared to *Bacteroidetes* in female rumen samples (ratio: 1.32), whereas in males the opposite is true (ratio: 1.02). This discrepancy indicates potential differentiation in rumen maturation between females and males. Since males and females were separated in the last weeks of their life and females offered hay [vs a grass-based diet with concentrate (100g/animal) and straw for the males] prior to slaughter, the effect of diet cannot be overlooked, as it has the potential to drive changes in very short periods of time. Hay is high in fibre and can contribute to higher rumen bacterial diversity (Liu *et al.*, 2016; Klevenhusen *et al.*, 2017), therefore the RA differences are likely to be related to the addition of hay in the females' diet.

Despite this, there were no statistically significant differences between RA for Sexes at a phylum level and therefore it could be hypothesised that the observed inversions are driven by the diet, in combination with sex-dependant factors. The findings here, supporting higher diversity in the females on a hay and concentrate diet are in contrast to what was reported by Belanche *et al.* (2019), where grazing sheep had higher diversity and RA abundance of *Ruminococcus*. *Prevotella*, *Lactobacillus* and *Bifidobacterium* abundances were higher in males in agreement with Belanche's findings for grazing animals.

Furthermore, the offspring of the ewes in the Alternative group had lower birth weight and IgG concentration whilst demonstrating lower effort to escape in the relevant test. In relation to the rumen microbiota structure, this group had a ratio of *Firmicutes: Bacteroidetes* (F: B) of 1.02, whereas lambs from the Negative and Control groups had slightly higher ratios (1.10, 1.25). In mice and human studies this ratio in the gut has been used as an indication of dysregulation and has been linked to metabolism factors such as obesity and diabetes (Mariat *et al.*, 2009; Benedict *et al.*, 2016; H. L. Li *et al.*, 2017; Jun He *et al.*, 2019). In addition, this group had higher *Methanobrevibacter* and *Prevotella* RA compared to the other two groups.

Since Yusof (2018) argued that the energy needs of the Alternative ewes were not met at this crucial stage of pregnancy, this may have had an effect on proteolysis and methane production as expressed by changes in RA for the lambs.

Similarly, Recognition lambs had a ratio F:B of 1.03 compared to Isolation lambs which had a ratio of 1.22. Once again, the fact that females and males were fed differently does not allow firm conclusions, as diet is a significant and confounding driver for these changes.

Significant differences at a phylum level were only observed for RA of Recognition and Isolation lambs. Phyla exhibiting higher RA in the Recognition lambs were the gram-negative *Armatimonadetes, Lentispherae* and *Verrucomicrobia*. In a sleep quality study healthy older adults exhibiting better neuropsychological test performance had higher abundances of *Verrucomicrobia* and *Lentisphaerae* in stool samples (Anderson *et al.*, 2017), which may indicate that the animals that had not been socially isolated but tested in terms of recognsing a familiar ewe, were likely to have better cognitition and be less prone to stress.

Following the pattern reported above, in females, *Clostridiales* (the most dominant *Firmicutes*) was present in a higher proportion in the total population and significantly more abundant compared to males. *Bacteroidales* were proportionately higher in males (considering *Bacteroides* >*Firmicutes* for the males), whereas statistical testing revealed that RA was higher in females. Eight other Orders exhibited differences in RA by Sex: *Cyanobacteria* ($p < 0.01$), *Bacillales* ($p < 0.01$), *Pedosphaerales* ($p < 0.01$), *Actinomycetales* ($p < 0.01$), *LD1-PB3* ($p = 0.01$), *PL-11B10* ($p = 0.01$) and the archaeal order *E2* ($p = 0.05$) were higher for Females and *Bifidobacterales* ($p < 0.01$) which was higher for males. This is likely to be an effect of diet and not necessarily a sex dependant effect.

Differences by Neonatal Treatment were observed for the RA of *Spirochaetales*, which was higher in Isolation lambs compared to Recognition lambs ($p = 0.03$). RA of *PL-11B10* (genus *Spirochaetes)* was also higher in the FI group compared to the MI group ($p = 0.02$). *Spirochaetales* was also significantly higher in FI compared to MI animals, as was the order *Actinomycetales*. In general, most differences of Sex* Neonatal Treatment at a genus level were observed between FI and MI animals, indicating that although diet/sex may have an effect it was more pronounced for the animals of the Isolation neonatal treatment.

Examples of these differences include *Sharpea*, whose RA was higher in males within the Isolation Neonatal Treatment Group (MI > FI). *Sharpea* is a frequently observed microorganism in the rumen and has been linked to low CH_4 yield animals in which rapid heterofermentative growth results in lactate production (Kumar *et al.*, 2018). In the present study males also had higher RA of *lactobacilli* (which may be linked to the higher presence of

lactate), which in combination with higher *Bifidobacterium* RA create a profile of a potentially "healthy" community for the male lambs, since this combination of bacteria is associated in humans with probiotics and health supporting bacteria (Linares *et al.*, 2017).

Furthermore, the negative relationship between cortisol and *Lactobacillus*, although not significant (R <0.8) as expressed from the correlation analysis, is in accordance with literature findings (Kelly *et al.*, 2015; Mudd *et al.*, 2017b; Amabebe and Anumba, 2018) where cortisol affects the presence of this genus via different pathways. Since cortisol levels are higher in females and the RA of *Lactobacilli* is significantly lower for females, we can hypothesise that early life experiences for the females may have influenced this result meaning that a combination of sex (disposition to higher stress responsiveness), early life experiences may have led to unfavourable reduction of *Lactobacilli,* which are generally considered as probiotics and "friendly" bacteria.

Regarding *Ruminococcacea,* as mentioned in the previous chapter, in human studies, this bacterium has been linked to higher anxiety levels girls and higher activity levels in boys. Interestingly, in this study RA were higher in females overall, and in Recognition males compared to Isolation males, as well as Isolation Females compared to Isolation males, which indicated that this bacterium may be a good indicator of sex and stress differences in sheep.

As mentioned above, *Bifidobacterium* RA was higher in males. Literature findings (Sarkar *et al.*, 2016; H. Wang *et al.*, 2019) indicate that several *Bifidobacterium* species, such as *Bifidobacterium longum* 1714 are identified in combination with reduced stress-related behaviors, improved stress responses and cognitive function in mice and healthy volunteers, respectively. However, the mechanisms by which this probiotic influences brain function and human behaviour are unclear, although a hypothesis (Sarkar *et al.*, 2016) that probiotic formulation (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) prevents chronic stress-mediated brain function abnormalities by attenuating the HPA axis response has been put forward making them likely candidates for use as psychobiotics (Sarkar *et al.*, 2016; Kelly *et al.*, 2017). Since males also demonstrated lower levels of cortisol, a stress marker, *Bifidobacterium* RA may act as a good indicator of stress for male sheep.

Additionally, *Prevotella* RA was significantly lower in Female lambs compared to Males and particularly within the Isolation neonatal Treatment group. A previous study (Maslanik *et al.*, 2012), conducted in rats, found that acute stress, although not affecting α- and β- diversity measures, reduced RA of this genus. According to the authors, this indicates that commensal bacteria contribute to acute stress-induced inflammatory protein responses (Maslanik *et al.*, 2012). It is known that *Prevotella* can establish symbiotic relationships with other commensal

bacteria in many locations (vagina, oral cavity) (Pybus and Onderdonk, 1998; Kolenbrander *et al.*, 2002) and in humans and rhesus monkeys has been associated with sociability traits (Gorvitovskaia, Holmes and Huse, 2016; Amaral *et al.*, 2017) and in most species it is implicated in various metabolic pathways (Takahashi and Yamada, 2000; J. N. Kim *et al.*, 2017; Franke and Deppenmeier, 2018; Schären *et al.*, 2018). Again, these findings indicate higher stress levels in females and these bacteria could act for differential assessment of stress response between sexes.

In contrast, *Clostridium* RA was higher in females compared to males and particularly Isolation females compared to Isolation males. There are many species of *Clostridium*, both non-toxinogenic and toxinogenic, the later often related with enterotoxaemia (which can be serious in sheep). Many species also convert lactate to methane mainly via the acetoclastic pathway (Yang and Tang, 1991; Detman *et al.*, 2018). This finding is consistent with the previous results indicating that the female animals may have been distressed and that had less favourable abundance of certain bacteria compared to males.

Finally, potential inflammation-promoting bacteria such as *Helicobacter*, *Peptostreptococcaceae*, *Streptococcus*, and *E. faecalis* (X. Gao, Cao, Cheng, Zhao, Wang, Yang, Wu, You, Wang, Lin, Li, Wang, J.-S. Bian, *et al.*, 2018) were increased in the Negative Prenatal treatment group, meaning that a restrictive diet may have influenced the abundance of these bacteria in offspring, leading to potential higher inflammation susceptibility.

Conclusions we can potentially draw are that diversity was higher overall in females but stress influences (maybe linked to higher cortisol levels or unfavourable diet, or diet change in the last days of their life) on certain bacteria (i.e. *Lactobacilli*) may have affected RA of potential pathogens (*Clostridium, Verruco-5, Selenomonas*) and that females had a microbial profile that was less favourable based on literature findings compared to males; In males the lowest diversity was observed in Isolation males and the majority of differences in RA were observed between sexes in the Isolation Neonatal treatment group and between Recognition and Isolation males. Isolation appeas to have a potential strong and longlasting effect, but low numbers used in this study do not allow further speculation.

3.6.3 Alpha diversity

Sex effects were observed for all bacterial alpha diversity indices, except for the Simpson index. Females consistently had higher diversity, as observed by the statistical tests and Boxplots. Significant differences were also found for all diversity indices between the MI group and FI group, as females of the Isolation Neonatal Treatment group appeared to be more diverse than males, indicating that the events that occurred in early life (isolation etc.) for the Isolation Treatment affected males in a more significant way, suppressing rumen microbial richness, evenness and overall diversity.

If this was a predominantly Diet effect, the Recognition Males should also be significantly different compared to the Recognition and Isolation Females. As this was not the case, we can hypothesise that an alternative mechanism is responsible for this finding. Alpha diversity metrics did not differ when exploring the archaeal rumen population.

3.6.4 Beta diversity metrics

Similar to the results reported for alpha diversity, Sex had an effect on beta diversity. Beta diversity allows us to examine how samples vary against each other. "Group" (Sex and Prenatal Treatment) and "Group 1" (Sex and Neonatal Treatment) also had a significant effect on beta diversity ($p = 0.02$ and $p < 0.01$ respectively). No differences were observed in terms of dispersion.

For the archaeal and bacterial population, the PCoA plotted for Sex indicated clear differences between males and females (**Figures 3.16-3.20**). Similarly, bacterial PCoA plotted for Sex and Neonatal Treatment allowed discrimination between Isolation Males, Isolation Females and Recognition Females (**Figure 3.20**), while from the archaeal PCoA clusters for Isolation Females, Isolation Males and secondarily for Recognition Males, was observed. PCoA plotted for the bacteria using Group1 (Sex and Prenatal Treatment) allowed discrimination of the Control females but otherwise differences were not evident.

3.6.5 Relationship of relative abundances with cortisol

Variability in cortisol levels best explained the variability present at a phylum level for Group 1. Furthermore, cortisol levels best explained the variability present in Females (67.94%) when compared to Males (41.54%) and in Isolation lambs (60.50%) compared to Recognition ones (42.09%). Finally, cortisol variability explained 97.57% of the variability in the Recognition males group.

3.6.5.1 Phylum level

When conducting Pearson correlations between phyla with VIP scores >1.00 and cortisol, several phyla demonstrated significant correlation coefficient values with cortisol (R >0.8 or R <-0.8). In the previous Chapter, no phylum, order or genus exhibited such values and therefore assumptions on that level were not made. In this case, the direction of correlation will be discussed as well as the group for which the significant relationship with cortisol was observed.

Regarding the two most dominant bacteria phyla, *Bacteroidetes* showed a non-significant negative correlation with females, and *Firmicutes* a non-significant positive correlation with females and the Isolation Female group.

Proteobacteria were significantly negatively correlated with the MN, Isolation and MI groups, indicating that cortisol could be a significant driver in RA for this phylum in males. Langgartner *et al*., (2017) observed an increase in the abundance of *Proteobacteria* in a mouse model for chronic psychosocial stress, while also in mice, Jang *et al.* (2018) reported that immobilisation stress led to an increase in *Proteobacteria* abundance. Therefore, it may be possible that Isolation in early life may have affected abundance of this bacterium in a longterm way.

Chloroflexi were positively correlated with cortisol in females, with significant relationships emerging for groups FN, FA and FR. Despite this, the relationship appeared to be negative in males. There is a wide range of bacteria under this phylum, and despite their abundance generally representing <1.00%, they are hypothesised to be implicated in many metabolic pathways (Campbell *et al.*, 2014).

Fibrobacteres exhibited a significant negative relationship with cortisol for groups FN and MI and although R values were not significant, a positive relationship was observed between *Fibrobacteres* and cortisol in the MN group. As this bacterium is associated with cellulose degradation, differences in abundance are potentially reflective of the difference in diet.

The *WPS-2* phylum exhibited non-significant but exclusively positive relationship with cortisol in many groups. These bacteria are uncultured but consistently appear in microbiome studies, without a defined role.

None of the phyla mentioned had significantly different RA between groups.

3.6.5.2 Order and genus level

PLS analysis at an order level indicated very high percentages of variability explained by the variability present in cortisol levels, for all groups. Furthermore, a large number of orders demonstrated significant relationships with cortisol ($R > 0.8$ or $R < -0.8$) in different groups.

In more detail, *Coriobacteriales* was significantly positively correlated with the MA. *Coriobacteriales* is made up of commensal organisms, that are saccharolytic and can metabolise various carbohydrates, producing lactate and other metabolites (Gupta, Nanda and Khadka, 2017). As *Bididobacterium* and *Lactobacilli* (also negatively correlated with Isolation Females) also displayed increased abundance in the males, the presence of this bacterium and its relationship to cortisol, may suggest a pathway or at least an interplay, implicating lactate.

Interestingly, *Firmicutes* were positively correlated with many groupings and significantly so with the MC group. *Biddle et al.* (2018) proposed that these bacteria can be predicted in part by cortisol in horses. Considering that *Firmicutes* were lower in males compared to females, whose cortisol levels were also significantly higher, there appears to be a potential link between the two.

Tenericutes had a non-significant negative relationship with cortisol for female abundances and a significant relationship with FC grouping. In human studies, women with Polycystic Ovary Syndrome, which is characterised by increased cortisol levels in circulation, had lower abundances of these bacteria, rendering the relationship observed here interesting.

Sharpea, whose RA was higher in males within the Isolation Neonatal Treatment Group (MI > FI) was also significantly negatively correlated with cortisol for the Male Negative animals and not significantly for the Isolation Males. As such, it would be of interest to explore potential mechanisms at interplay at this level.

Many orders and genera demonstrated a significant relationship with cortisol, but interpretation should be cautious as this is a first study exploring these aspects in ruminants, while low sample numbers can affect confidence in the PLS and correlations reported and finally, many of the correlations were inconsistent (positive for one group and negative for another). None of the orders mentioned above had significantly different abundances by Sex, Group1 (sex or Group 2.

PLS exploration of cortisol relationship with genera showed that cortisol levels explained very high levels of variability present for all groups studied. Although surprising, variation explained can be >100, as discussed by Fan and Konold. 2010.

Despite being >0.08 none of these were related to changes in abundances which could be an indicator that cortisol concentration is clearly linked with higher or lower RA of bacteria or archaea.

As not many of the bacteria and archaea demonstrated consistently significant positive or negative relationships with cortisol, further assumptions cannot be made about the biological significance of these results. The co-occurrence of high VIP scores and significant correlation coefficients points to covariations in cortisol levels and the proliferation of these bacteria but further exploration in the mechanisms implicated (e.g. via transcriptomic and metabolomic investigation) are needed.

Finally, based on observations from this study, future experiments could focus on identifying changes in the placental and vaginal microbiome due to psychosocial stress in sheep as well as the effects of neonatal stress on the colonisation and progressive formation of the faecal and rumen microbiome in lambs. Further investigation of these aspects could allow to link maternal influences and early life experiences with GI microbiome composition in a more comprehensive and effective way.

Evidently, factors with known large effects such as diet should be considered, since sex has been proven to influence microbiota structure in many species but is still poorly explored in sheep. It would be interesting to explore the potential differences in lactate and lactic acid production between sexes as a result of stress and how this relates to fibre degradation and methane production.

3.6.6 Examination of hypotheses

- Prenatal and Neonatal treatment did not have a pronounced effect on Rumen bacterial and archaeal diversity and composition
- Sex had a significant effect on bacterial and archaeal diversity and composition, which is more likely due to feeding differences introduced the last days of life. Females appeared to hava higher diversity but also higher abundances of potential pathogenic bacteria
- Cortisol did not differ between Prenatal and Neonatal treatment groups, but differed between sexes, with higher levels present for females which is consistent with literature
- Cortisol was correlated with abundances of *Proteobacteria* particularly in males, indicating that cortisol could be a significant driver in RA for this phylum in males.

Proteobacteria abundance has also been reported to increase in stressful conditions in other species. *Fibrobacteres* also exhibited a significant negative relationship with cortisol for groups FN and MI. This bacterium is implicated in cellulose degradation, therefore differences in abundance are potentially reflective of the difference in diet. Cortisol may therefore be an indicator of differences for some bacteria but may be sex dependant

Chapter 4

Mild Chronic Stress Effects on Sheep Behaviour, Physiology and Gastrointestinal Microbiota

4 Mild chronic stress effects on sheep behaviour, physiology and gastrointestinal microbiota

4.1 Personal contribution

This experiment was conceived in collaboration with my supervisors, Alain Boissy, Diego Morgavi, Marie-Madeleine-Mialon Richard and Milka Popova at INRAE and Richard Dewhurst, Alastair Macrae and Cathy Dwyer at SRUC/UoE. I planned the stress trial and managed all decisions relevant to stressors, diet, environment, tests, test dates, data collection points, sample types etc. The members of the Welfare group at INRAE assisted in technical aspects such as building the pens, installing equipment and assisting in handling and sampling the animals based on their experience and relevant licensing. All rumen and faecal samples and subsequent DNA extraction, 16S rRNA gene amplicon sequencing and library preparation were conducted by me at INRAE. I performed the protozoan counts and I assisted in the VFA, BHB, glucose, cortisol, serotonin, NEFA analyses, which were conducted in various specialised labs at INRAE.

Time budgets were done by Eric Delval, a specialised technician, blinded to the treatment groups, whilst I conducted shorted time budget observations at feeders etc. Raw data from ECG equipment was compiled in stepwise files for further analyses by Herve Chandez. I performed all subsequent data processing from the behavioural and heart rate data and all statistical analyses, after receiving advice from David Ewing at Bioss.

4.2 Introduction

4.2.1 Chronic stress and mild chronic stress paradigm

Chronic stress develops as a response to a prolonged exposure to physical and emotional discomfort, during which an individual perceives it has little or no control over events (either continuous exposure to a single unavoidable stressor, or repeated exposure to different stressors). Mild chronic stress paradigms aim to model a chronic depressive-like state that develops gradually over time in response to stress (Willner, 2017b, 2017a).

Mild Chronic Stress (MCS) results from exposing the animals to a series of mild repeated and unpredictable (to them) stressors, which can range from physical (restraint, noise, uncomfortable pen conditions) to social (social mixing or separation). The minimum recorded

duration for a MCS trial in rats is 2 weeks. Many studies conducted on a number of species (Willner, 2017b), including sheep (Destrez *et al.*, 2012, 2017; Coulon *et al.*, 2014) have reported medium- to long-lasting changes in behaviour, neurochemical, neuro-immune and neuro-endocrinology variables (Radley *et al.*, 2015; Destrez *et al.*, 2017;.Gao, *et al.*, 2018). Recent studies have used this model to investigate chronic stress effects on gut function, gastrointestinal health (Gao, *et al.*, 2018), as well as the microbiome (Marin *et al.*, 2017a; Yang *et al.*, 2019).

This model offers good validity (Willner, 1997, 2017a). However, MCS experiments are difficult to carry out due to various constraints, including the fact that they are laborious, there are often space requirements and limitations, special experimental set-up and equipment is required, and they are comparatively long in terms of experimental duration. The procedures can be difficult to establish and differ from species to species, while an important issue is that data is often not easily replicated due to individual variability and circumstantial factors (Willner, 1997).

Regardless of these issues, in order to explore the effects of psychological stress on animals, as models for depression studies, chronic paradigms, such as chronic restraint stress, chronic social defeat stress, and chronic unpredictable mild stress were originally introduced (Willner, 2017). The model of chronic unpredictable mild stress has been validated and is the most commonly applied model for rats (Willner, 2017b; Antoniuk *et al.*, 2019). Adaptations of this model have been successfully applied on sheep in order to study the effects of psychological and social stressors on their behaviour and physiology responses (Przekop *et al.*, 1985; Doyle *et al.*, 2011; A. Destrez *et al.*, 2013a; Alexandra Destrez *et al.*, 2013; Coulon *et al.*, 2014; Petit *et al.*, 2015; Destrez *et al.*, 2017; Frasch *et al.*, 2017).

4.2.2 Effects of mild chronic stress on behaviour, physiology and microbiome

4.2.2.1 Behaviour

Effects of MCS on behaviour have been thoroughly described. In human studies, chronic stress has been associated with depression and anxiety (Stuart Checkley, 1996; Duman and Canli, 2015; Frisbee *et al.*, 2015; Kircanski *et al.*, 2019). In rodents, depressive behaviours are expressed by a decreased sucrose preference and changes in typical behaviours or circadian rhythms. For example, Henningsen *et al.*, (2009) demonstrated that 70% of the rats that were exposed to MCS for 7 weeks showed a gradual reduction in sucrose solution consumption, while cognitive testing showed a decrease in working memory. A different study showed that

socially isolated rats, which were also under MCS, had lower body weight gain and were more active compared to controls (Sequeira-Cordero *et al.*, 2019).

In sheep, behaviour changes observed due to MCS were reported in lambs which were subjected to a chronic stress treatment for 9 weeks (exposure to various unpredictable, uncontrollable and aversive events regularly encountered in ordinary agricultural practices) (Destrez *et al.*, 2013). Before treatment, all the lambs (Stressed and Controls) had been trained to approach or avoid a food bucket depending on its location. After the 9 weeks, the lambs were individually exposed to two tests: a food bucket approach/avoidance test, where the bucket was placed in unfamiliar locations (a judgment bias test); a learning test, where the lambs had to learn to distinguish between two visual stimuli differing in colour and shape in a span of six days and then recall this information two days later. MCS lambs took longer to approach the ambiguous locations of the bucket and had fewer correct choices in the learning and recall tests compared to Controls, meaning that MCS can affect judgment biases and induce learning deficits in sheep (Destrez *et al.*, 2013).

In a second study, Destrez *et al.*, (2013b) subjected lambs to 6 weeks of daily exposure to unpredictable and uncontrollable aversive events, known to occur in farming conditions, such as predatory cues, social mixing and rough handling. After the six-week exposure, MCS lambs interacted less with the novel object, vocalised more in individual tests and approached the human less often compared to Control animals, suggesting that long-term exposure to unpredictable and uncontrollable aversive events can increase fearfulness in sheep (Destrez *et al*., 2013b)

Finally, lambs submitted to a 7 week MCS trial, which were then exposed to a further 4 weeks of positive events (wool brushing, positive contact with humans), demonstrated a positive judgement bias in the bucket approach test and were more likely to approach humans compared to animals that had only been exposed to the unpredictable and repeated to the aversive events (Destrez *et al.*, 2014).

4.2.2.2 Physiology

Whilst MCS typically increases plasma levels of corticosterone in rats, there are also many reports of MCS inducing anhedonia and other depressive-like behaviours without elevated hormone levels. This is likely because cortisol and corticosterone peak in a short time after exposure to an acute stressor and may not reflect the effects of chronic stress accurately. Long-term exposure to stress may lead to higher baseline cortisol, linked to altered metabolic rates, which may potentially be adaptation to the stressful living conditions (Haase, Long and

Gillooly, 2016). If the stressors decline, this could be considered a successful adaptation, although as discussed in the General Introduction (**Chapter 1**), higher corticosterone levels and dysregulation of the HPA axis may have detrimental effects to health.

In general, activation of the HPA axis and increased levels of corticosteroid hormones can have attenuating effect on the immune system (Glaser and Kiecolt-Glaser, 2005; Brown and Vosloo, 2017), as repeated exposure to a stressor can lead to glucocorticoid resistance via changes in glucocorticoid receptors. In contrast, chronic stress can also increase cytokine proliferation and chemokines as increased levels of glucocorticoids play a role in stress-mediated immune activation, which may contribute to a primed immune response of stress susceptible individuals (Niraula *et al.*, 2018). Once again, this may be due to an effort for the individual to adapt to the novel situation and modify aspects of physiology and behaviour in order to achieve a homeostatic state.

In sheep, the effects of chronic stress on the immune system were examined via immunological investigation to a vaccine challenge. MCS sheep had lower haemoglobin concentrations and higher platelet, granulocyte and acute-phase protein concentrations, while antibody response induced by the vaccine strain did not differ between MCS and Control animals stressed and control sheep (Destrez *et al.*, 2017). A potential explanation of this could be that higher cortisol levels can lead to inefficient adherence of granulocytes to blood vessels, which means they are more likely to circulate freely.

4.2.2.3 Microbiome

It has been documented that disruption or absence of the microbiome can impair behaviour responses and cognitive development, leading to increased exploration, decreased apprehension, and impaired social behaviour (Desbonnet *et al.*, 2014; Rogers *et al.*, 2016; Smith *et al.*, 2019; Capuco *et al.*, 2020). Many studies report stress-induced shifts in gut microbial composition, with a frequent finding being reduced *Lactobacillus* abundance and reduced overall diversity (Jašarević *et al.*, 2015, 2017a; Marin *et al.*, 2017a; Amabebe and Anumba, 2018; Jang *et al.*, 2018; Karl *et al.*, 2018; Kubasova *et al.*, 2018; L. Wang *et al.*, 2019; Le Sciellour *et al.*, 2019). Most studies focus on shifts, which express perturbations and changes towards dysbiotic states. It is important however, to consider an alternative hypothesis which has been put forward by Zaneveld *et al*., (2017), referring to the "Anna-Karenina" principle which has found multiple applications in ecology.

The basis of this principle is that "All happy families are alike; each unhappy family is unhappy in its own way" and that this can be applied on a microbiota community level in

animal studies where perturbations are stochastic (Zaneveld *et al.*, 2017). Transitions from stable to unstable community states can also mean that dysbiotic individuals vary more in microbial community composition than healthy individuals, or simply differ in alternative ways (such as inhibited and not necessarily reduced diversification or erratic shifts in successive samplings) (Zaneveld *et al.*, 2017; Sweet *et al.*, 2019; Ma, 2020).

According to previous research (Galland, 2014; Kelly *et al.*, 2015; Karl *et al.*, 2018; Kaur *et al.*, 2019; Silva, Bernardi and Frozza, 2020; Zhu *et al.*, 2020), reporting structural shifts and increased or decreased abundances of certain bacteria, the functional implications for the host are not always clear, but may include psychological impairments influenced by altered tryptophan metabolism, influences on the immune system, altered VFA, hormone and neurotransmitter secretion and altered gut permeability, all leading to potentially increased susceptibility to subsequent stressors.

For example, chronic social defeat induced behavioural changes that were associated with reduced richness and diversity of the gut microbial community, as well as shifts at an OTU level across phyla in male C57BL/6 mice microbiome (Bharwani, M. Firoz Mian, *et al.*, 2016). Additionally, functional diversity was lower, and the pathways involved in synthesis and metabolism of neurotransmitter precursors and VFAs were not as prevalent in defeated mice.

A different study applied a 6 week mild unpredictable stress model on c57bl6/Jax mice to induce despair behaviour (Marin *et al.*, 2017a). Weight and quantity of bacterial DNA in faecal pellets was not affected by stress. In terms of microbiota composition, principal coordinate analysis indicated distinct clustering between samples from naïve and stressed mice, while taxonomic analysis of bacterial types revealed several changes in the microbiota composition (mainly *Firmicutes* i.e., *Bacilli*, *Clostridia*, as well as *Verrucomicrobia*). The study focused on *Lactobacillus* as a potential player in the despair phenotype and demonstrated that chronic stress disturbs the microbiota homeostasis, by decreasing the *Lactobacillus* levels, whilst correlation analysis demonstrated a positive correlation between the relative *Lactobacillus* abundance and escape behaviour.

In ruminants, the only investigation of chronic stress or psychosocial stress effects in general on the gastrointestinal (GI) microbiome has been conducted in goats. A low dosage Dexamethasone (Dex) known to simulate chronic stress, was injected intramuscularly for 21 days in growing goats. Dex-treated goats demonstrated a higher number of white blood cells and blood glucose levels but weighed less, and cortisol concentration was lower compared to saline-injected goats. Dex exposure did not lead to shifts in microbial community structure in the rumen, caecum, or colonic digesta. Abundance of *Prevotella* was increased on days 7 and

14 of Dex treatment but decreased on day 21. Previously, in mice, the use of Dex over a 4-week period had induced shifts in the gut microbiota (Huang *et al.*, 2015), so an explanation may lie in the length of the administration of Dex, or the difference in ruminants may be due to the way that corticosteroids affect the rumen and how this influence is translated in resilience to change. However, Dex simulates the physiological effects of a stress response but may not be directly comparable to emotional and social distress (Starcevic *et al.*, 2016).

4.2.3 Heart rate as an indicator of chronic stress

Stressful or harmful stimuli can induce stress responses in order to maintain homeostasis. Despite this, during chronic stress, hyperactivation of the sympathetic nervous system (SNS) can cause physical, psychological, and behavioural dysregulation.

As mentioned previously, the two main pathways by which psychological stress affects the body are the hypothalamic-pituitary-adrenal (HPA) axis and SNS. Measurement of parasympathetic tone may be a useful index of stress and stress susceptibility (Porges, 1995; Kim *et al.*, 2018; Pinter *et al.*, 2019).

Cardiac rhythmicity is intrinsically regulated by pacemaker cells and tissues, but heart rate (HR) and rhythm are under control of the ANS, as HR depends on the equilibrium between the SNS and PNS (Gordan *et al.*, 2015). Elevated heart rate is linked with increased SNS or lower PNS activity and vice versa. Vagal tone prevails over sympathetic activity during resting phases and vagal dominance occurs when the vagus nerve is more active than sympathetic nerves indicating a dysregulation of the autonomic nervous control of the cardiovascular system, characterised by increased sympathetic and decreased parasympathetic tone activity (Schwartz and De Ferrari, 2011).

As HR intervals are regulated by sympathetic and parasympathetic autonomic activity, the variability reported can provide information about the nervous control on the HR and the heart's ability to respond. Heart rate variability (HRV), the variation over time of the period between consecutive heartbeats, is predominantly dependent on the extrinsic regulation of the HR. Variability is expected, as heart beats are irregular and indicates the heart's ability to efficiently respond to physiological and environmental demands, such as breathing, mental stress, physical activity, temperature changes, metabolic needs and sleep or rest states. HRV can be used as a valuable tool to measure the sympathetic and parasympathetic function and health of the ANS (Kim *et al.*, 2018).

The most frequently reported factor associated with variation in HRV measurements due to stress, according to the meta-analysis conducted by Kim *et al.* (2018), was low parasympathetic activity, characterised by an increase of the Low frequency band and a decrease in the High-frequency band, resulting in a higher ratio of LF/HF. According to the authors HRV may be linked to cortical regions (as observed by neuroimaging studies), which are involved in the assessment of stressful situations.

A study conducted on lame cows, investigated the utility of heart rate (HR) and heart rate variability (HRV) as indicators of chronic stress and demonstrated that heart rate indices can be used as valuable, alternative and non-invasive tool in the assessment of chronic stress (Kovács *et al.*, 2015). Data was recorded during periods of undisturbed lying, for baseline cardiac activity. Amongst their findings, they observed that HR and indices of the sympatho-vagal balance (LF/HF) were lower, while vagal tone parameters (RMSSD and HF) were higher in lame cows. Geometric and non-linear HRV measures were lower in lame cows, suggesting that chronic stress influenced linear and non-linear characteristics of cardiac function. This is in agreement with the principle of stasis, defined as the lack of endogenous variability in peripheral systems under neuronal regulation (such as heart rate) (Porges, 1995).

In sheep, after exposure to various unpredictable and uncontrollable aversive events over 6 weeks, treated lambs in novelty and human interaction tests, which had previously demonstrated an increased reaction to suddenness and novelty, also had significantly lower HR compared to control animals during the tests(Destrez *et al.*, 2013a).

In summation, due to the lack of consistency in behaviour and hormone profiles linked to chronic stress, heart rate indices are promising candidate tools for this purpose as can provide complex information and are not necessarily invasive. Using the equipment on farm animals may have drawbacks linked to ECG signal efficiency and the ease of recording and recovering the data, the cost of the equipment and the need to habituate the animals to the equipment prior to use.

4.2.4 Serotonin and cortisol as predictors of microbiota presence and links with gut-brain axis

The players involved in the bidirectional communication pathways between gut and brain have long been explored, and many candidates have emerged. One of the most prominent appears to be tryptophan and its metabolite 5-hydroxytryptophan (5-HTP), the precursor of serotonin, as well as serotonin itself (Gao *et al.*, 2018; Kaur, Bose and Mande, 2019). Tryptophan is not produced by mammals, and tryptophan depletion can lead to a decrease in neuronal serotonin

release in the brain (Biskup *et al.*, 2012; Jenkins *et al.*, 2016). Tryptophan is also the only essential amino acid which is partially bound to albumin in the plasma which means that the quantity of tryptophan which penetrates cerebral tissues depends on the small proportion of the amino acid still free in the plasma. Therefore, disturbances in the equilibrium between bound and free forms of tryptophan in the plasma can modify the availability of tryptophan in the brain and thus affect the rate of 5-HT synthesis (Jenkins *et al*., 2016).

Evidence to suggest that serotonin (5-hydroxytryptamine, 5-HT) is a mediator for the gut-brain microbiome axis is that during ENS development, serotonergic neurons are first to appear in the ENS, where they impact on neurogenesis and the later development of other neurons, such as those expressing dopamine, gamma-Aminobutyric acid (GABA) and calcitonin gene-related peptide (CGRP) (Israelyan and Margolis, 2019).

Additionally, during the development of the CNS, 5-HT influences neuronal differentiation, migration, myelination and synapse formation (Israelyan and Margolis, 2019). Finally, enteric 5-HT, accounting for >90% of 5-HT reservoir, can be located in the intestinal epithelium, where it is produced by enterochromaffin (EC) cells, and in neurons of the ENS (Israelyan and Margolis, 2019).

The missing link to all of the above is the microbiota, which is known to play a critical role in regulating host 5-HT. It had been suggested that GI bacteria in multiple locations can impact on the development and function of 5-HT-producing cells (Uribe *et al.*, 1994). Yano *et al.* (2015), demonstrated that mouse and human spore-forming bacteria can impact on host physiology by promoting 5-HT biosynthesis from colonic ECs, which supply 5-HT to the mucosa, lumen and circulating platelets. Finally, some species of bacteria grown in vitro have shown the ability to produce 5-HT (Tsavkelova *et al.*, 2006).

From all of the above, it is evident that 5-HT impacts diverse functions such as gut motility, platelet aggregation, immune responses and cardiac function. In combination with its role in the occurrence of brain and intestinal anomalies (Gao *et al*., 2018), this makes it a candidate warranting further exploration, by linking it to various aspects of physiology, behaviour and the microbiome.

In relation to cortisol, given its multifunctional role and its links to almost every system (immune response, heart function, metabolism and growth, stress response, the presence of cortisol-specific receptors on gut bacteria membranes) it automatically serves as a good candidate. In pigs, serum cortisol fully mediated the relationship between faecal *Ruminococcus* and brain N-acetylaspartate (NAA), while *Ruminococcus* negatively predicted NAA and cortisol (Mudd *et al.*, 2017a).

In horses, metabolic factors measured in the blood of obese, normal and lean horses correlated with differences in gut microbiome composition. Obese horses had higher concentrations of leptin, triglycerides, glucose, and cortisol in blood plasma, with higher diversity present in the gut microbiome. Relative abundance (RA) of *Firmicutes* was higher, whereas, RA of *Bacteroidetes* and *Actinobacteria* was lower (Biddle, Tomb and Fan, 2018).

Finally, Duran-Pinedo *et al.* (2018), using a metatranscriptomic approach, demonstrated that cortisol induces shifts in the gene expression profiles of the oral microbiome indicating an influence of cortisol, not only in the management of the bacteria present in different matrices, but also their function.

As such, serotonin and cortisol were considered the most relevant indicators to use to examine correlations with GI bacteria present, to assess the effect that stress may have on these hormones, as well as their potential interactions with gut and rumen bacterial abundance in sheep.

4.3 Study hypotheses and objectives

The hypotheses set out for this project were:

- Stressed animals respond to acute stressors imposed over a 6-week period, meaning cortisol, glucose and heart rate values increase compared to NS animals.
- Repeated unpredictable mild stressors for a duration of 6 weeks can lead to behavioural (time spent performing certain activities, synchronisation between animals) and physiological changes (e.g., cortisol, glucose, serotonin, heart rate, weight gain) in female lambs.
- Stressed animals respond differently to a suddenness test, when compared to NS animals tested under the same conditions (differences in behaviour and heart rate).
- Repeated unpredictable mild stressors for a duration of 6 weeks will result in altered rumen and faecal profiles between treatment groups (MCS animals and NS animals).
- Cortisol and serotonin levels can act as predictors of the presence/absence or abundance of certain bacterial phyla, orders or genera.

In order to investigate these, there were three parts to this study, first to assess the effectiveness of the acute stressors, secondly to explore whether stress had an effect during and after the treatment period and thirdly to assess the presence and effects of chronic stress over time.

4.4 Materials and methods for mild chronic unpredictable stress trial

All experimental procedures were performed in a research farm setting at INRAE (UMR1213 Herbivores) at Theix, Saint-Genès-Champanelle and completed with approval from the regional ethics committee in Auvergne, France and the SRUC Animal Experiments Committee. Animal care was carried out according to UK/ French legislation (SRUC Animal research ED RP 03-2017, AE 31-2017).

4.4.1 Mild chronic stress trial experimental procedures

For the purposes of this project, the Chronic Unpredictable Mild Stress paradigm used, was a modified version of a previous study conducted at the same Experimental Unit at Theix (Destrez *et al.*, 2013b). In Destrez's study, twenty-four lambs were subjected to a chronic stress treatment for 9 weeks (exposure to various unpredictable, uncontrollable and aversive events regularly encountered in ordinary farm settings) while twenty-four different lambs were used as a control group.

During the trial carried out for this project, many of the stressors used were the same as described by Destrez (2013), including several more which allowed automation and more frequent rotation. Twenty-four animals were subjected to MCS for 6 weeks and 24 more were not treated with stressors (NT).

In order to assess the potential influence of chronic stress on the MCS group of animals, we had to ensure that the stressors used would elicit a physiological response. The test which would allow collection of the maximum amount of data in terms of physiology and behaviour was the individual restraint which acts on a dual aspect, by physically restraining the animal and induces the psychological effect of not being in control and separated from the comfort of the flock. This test was repeated on 3 occasions. MCS heart rate (20 min during the test and 4h periods during the night), blood stress biomarkers, and effort to escape were investigated for changes over time. Blood samples were taken from the NT animals on all three occasions, as a means of comparison. Due to human error, blood plasma samples were only available for the third time the stressor was used, close to the end of the trial.

A series of measurements including heart rate variability, suddenness and novelty tests were used to investigate changes over time. Cortisol, serotonin, NEFA, BHB and glucose concentrations at the 6th week of the trial were compared to the plasma concentrations acquired

pre-trial (the 1st day of the trial). Similarly, faecal and rumen microbiota community structure and diversity were examined in relation to pre-trial observations. To identify potential relationships between microbial abundances and serotonin/cortisol, correlation analyses were performed. Behavioural observations in the form of 24h time budgets, weight measurements and heart rate monitoring sessions were performed, in order to assess changes in behaviour and selected physiology parameters throughout the different stages of the experiment. Finally, as a means of assessing the potential influences of the MCS paradigm, a test was developed to compare the response to a sudden event between the two treatment groups. The NT group had not been moved out of their pens or intentionally startled and stressed throughout the entire duration of the experiment and were therefore expected to behave in a different way compared to the MCS group. The test involved measuring heart rate and reactivity parameters (such as latency to approach the novel object, time spent interacting with the novel object and latency to return to lying or eating behaviours) in their home pens (twice) but also in a novel environment (twice per pen).

Briefly, 48 female Romane lambs were separated into two rooms: one where the animals were subjected to mild unpredictable stressors (MCS), and another where the animals were not treated (NT), but included mild enrichment, such as brushes and other objects (**Figure 4.1**).

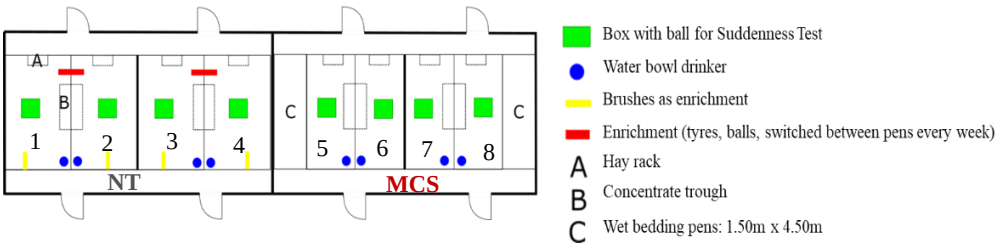

Figure 4.1 Floor plan of the experimental rooms (NT and MCS). The NT room was separated in 4 pens of equal dimensions (1,2,3,4) 3.00m x 4.50m, and the pens included enrichment in the form of tyres, balls and brushes to avoid NT animals developing stress symptoms due to lack of stimulation. MCS pens (5, 6, 7, 8) were: 2.20m x 4.50m and on either side of the room, a Wet bedding pen was constructed to be used for stressing the animals. Between pens 2 and 3 in the NT room and pens 6 and 7 in the MCS room there were wooden panels (dimensions: 4.80m length, 3.00m height) limiting contact between pens. There were 4 doors into each Room, 2 in the front and 2 in the back, which led to external corridors within the building.

4.4.1.1 Animals

Forty-eight female Romane lambs were selected. The sires of the selected lambs were part of an ongoing genetic selection programme, performed by INRAE, focusing on improving feed efficiency and developing two genetically divergent lines for Residual Feed Intake (RFI) with estimated heritability of 0.30. RFI or Net Feed Efficiency (NFE) is defined as the difference between an animal's actual feed intake and its expected feed requirements for maintenance and growth.

However, the dams were not assessed for feed efficiency and the female lambs participating in the MCS trial were not characterised, as individual feeders were not available. Twenty-four offspring (4 animals from 6 sires) from each efficiency line were finally used. Preliminary tests did not indicate behavioural differences, and as feed efficiency and its link with the microbiome was not in the scope of this study, this aspect was not investigated further.

The lambs used in the experiment were born within a span of two weeks (5th to 14th September 2017, since Romane sheep have a good natural capacity for out-of-season lambing) and were kept with the dam until weaning. At birth, 1ml of oxytetracycline (Duphacycline, Zoetis) and 1ml of Sodium Selenite and Tocopherol (Séléphos, Laboratoires BIOVE) were administered for prophylactic purposes (against pneumonia and muscular dystrophy). A vaccine against respiratory infection from *Mannheimia haemolytica* and *Pasteurella trehalosi* (OVILIS® Pastovax, INTERVET, France) was administered and tail docking was performed using rubber rings from the as standard practice on day 21 of age in France.

Weaning was synchronised for all animals and took place on 13th November 2017, at approximately three months of age. The same week the animals were vaccinated against *C. perfringens* (COGLAVAX®, Ceva Santé Animale, France). A week later the animals transported to the experimental farm at INRAE (UMR1213 Herbivores). Vaccinations were deemed as necessary due to the prevalence of these diseases in the area and any changes in microbial community structure were assumed to be reflected in all animals since all were treated.

4.4.1.2 Adaptation period

Upon arrival (November 23rd, 2017), the lambs were weighed and randomly allocated to two identical rooms. In the next two weeks, they were clinically assessed by a vet and were orally administered 17ml Diclazuril (Vecoxan™, Elanco, France), following manufacturer's instructions as an anticoccidial prophylactic measure. Two weeks later (December 7th, 2017), the animals were re-allocated between pens and rooms to form the final Treatment Groups.

Animal allocation took into account sire ID in order that daughters of the same sires were distributed between pens and rooms. Weight from the previous two weeks and average daily gain were also taken into consideration in order to have pens with similar average weight distributions.

For the next 5 weeks, the animals were visited daily to ensure they were in good health and habituated to humans performing various routine management procedures. During this period, the animals were not stressed and enrichment for the NT group was not available. The only interventions were weighing, every second week, and marking the assigned number, for identification purposes on the back of the animals on a weekly basis.

4.4.1.3 Habituation procedures

In order to ensure minimal disruption to the animals from standard sampling procedures, a habituation protocol was set in place for the sampling. Every week, the specific animals assigned to wear heart rate equipment (2 from each pen) were sheared at the location where the electrodes would be attached. Every two weeks, these sheep were fitted with lumbar belts for 24 h, adjusted for each animal's size, but were not equipped with monitors and electrodes. Contention was performed among pen-mates for up to 15 min.

Habituation to the biological sampling procedures, was performed individually by confining each lamb in a limited area of its home pen. Animal order and procedures were the same for all adaptation and actual samplings. Non-Treated animals, were habituated once every two weeks throughout the pre- experimental and experimental period and the MCS group once every three weeks **(Figure 4.2**).

4.4.1.4 Colour of clothing

General management of the animals, such as feed distribution, cleaning the pens and assessment of wellbeing, as well as non-invasive sampling procedures (weighing, behaviour observations etc.) and standard samplings (faecal and rumen sampling pre and post-trial) were carried out by the research team, the lab and animal technicians and trained helpers wearing green overalls.

Stressors that required the presence of a human (individual restraint using the Gambrel restrainer, exposure to a noisy human performing exaggerated gestures and guidance of the dog in the room and pens) were carried out by researchers, lab/animal technicians and trained

helpers dressed in white overalls and lab coats. In these instances, the facial characteristics were hidden by wearing a surgical mask and a green surgical cap.

4.4.1.5 Feed distribution

Time of feeding was standardised: A person dressed in green entered the room and rang a bell at 9h20. At 9h25 two people distributed concentrates in parallel in each room. Hay was distributed at 9h30 in the same order as concentrates. Hay was also offered at 16h00 without using the bell signal.

Feeding was delayed on the days of blood, faecal and rumen sampling, as well as on the test days where the MCS animals were individually restrained (Gambrel restrainer) for blood biomarker stress assessment and heart rate recording. On these days, feed was delivered at 10h00 after the sound of the bell.

Concentrate troughs allowed 46 cm per animal and hay troughs allowed 25 cm per animal. An automatic water dispenser and a mineral supplement block were also available.

4.4.1.6 Diet

From their arrival and until their final reallocation in the treatment groups, the lambs were fed forage *ad lib* and 250 g of concentrate per animal per day. After the initial period of adaptation and re-allocation, the animals were fed concentrate and forage (ratio of 30:70), as is standard practice on the experimental INRAE farm for animals of that age. Concentrate offerings were adjusted according to the average pen weight, aiming at coverage of 80% of animal energy needs to achieve an average daily gain of 200 g/day. Calculations followed guidelines issued by INRAE on small ruminant nutritional needs (INRAE feeding system for ruminants, 2013) and Energy Requirements described as UFV (Feed Unit for maintenance and meat production) were calculated.

The concentrate offered was high in fibre in order to avoid any possibility of rumen acidosis, which could occur in the occasion of offering a high starch diet in combination with exposure to multiple stressors, altering the pH of the rumen. This could affect the bacterial communities, masking differences due to the treatment itself. High fibre diets are also known to enrich the microbial community in the rumen and along the gut (Russell and Rychlik, 2001).

Furthermore, the concentrate formulation the animals had been offered at the breeding farm (Bourges), was used as a guide at Theix in order to make the transition as easy as possible for the lambs. The concentrate was provided by AM FEDA PRESTIGE CS. Energy level,

calculated as UFV/ kg DM (Dry Matter) was 0.88, Protein Content was calculated at 17.00% and Crude Ash at 6.50%, both on a dry matter basis. The final formulation included barley, rapeseed and sunflower meal, beetroot pulp, mixed cereals, triticale, molasses, calcium carbonate and salt. A mixture of vitamins and additives was also included to provide the animals with a balanced diet.

The forage offered was collected at two harvests in June 2015 from the INRAE field of Belin Laschamps, a permanent meadow, mainly made up of a mixture of grasses. Dry matter content was estimated at 87.70% at collection.

4.4.1.7 Housing

During the adaptation period, all animals were housed in two separate rooms with four pens (3 m x 4.5 m). Pen formation was as represented in Error! Reference source not found.. Bars, a llowing contact between animals, separated pens 1 and 2, 3 and 4, 5 and 6 and 7 and 8 from each other. In the middle of each room, between pens 2 and 3 for the NT room and pens 6 and 7 for the MCS room, a wooden panel (dimensions: 4.8 length, 3m height) limited contact between pens.

Upon starting the trial (Trial D0), the MCS treatment pens were reduced in size (2.20 m x 4.50 m) to allow the creation of two smaller pens on each side of the room where the wet bedding was placed (wet bedding pen size: 1.50 m x 4.50 m). NT animals remained in pens of 3 m x 4.5 m. At this point of the experiment, a ball or a tyre were hung in an alternating fashion in pens in the NT room and two brushes were added in each pen as means of enrichment. Pen sizes whether reduced in size or not offered the animals appropriate living space (1.65 m^2 / ewe for the MCS group and 2.25 m^2/ewe for the NT group, adhering to DEFRA regulations). The wet bedding pens allowed 1.125 m^2/animal and did not include feeders or a water source, but this was temporary and used as a stressor.

Woodchips were used as bedding to avoid consumption and therefore any potential interference with microbiota results, as bedding samples were not collected, or included, in the microbiota analyses. The rooms were artificially lit (adhering to DEFRA guidelines for luminosity levels) from 07h00 to 19h00 and the temperature was recorded and monitored, remaining at approximately 15^{o}C throughout the experiment.

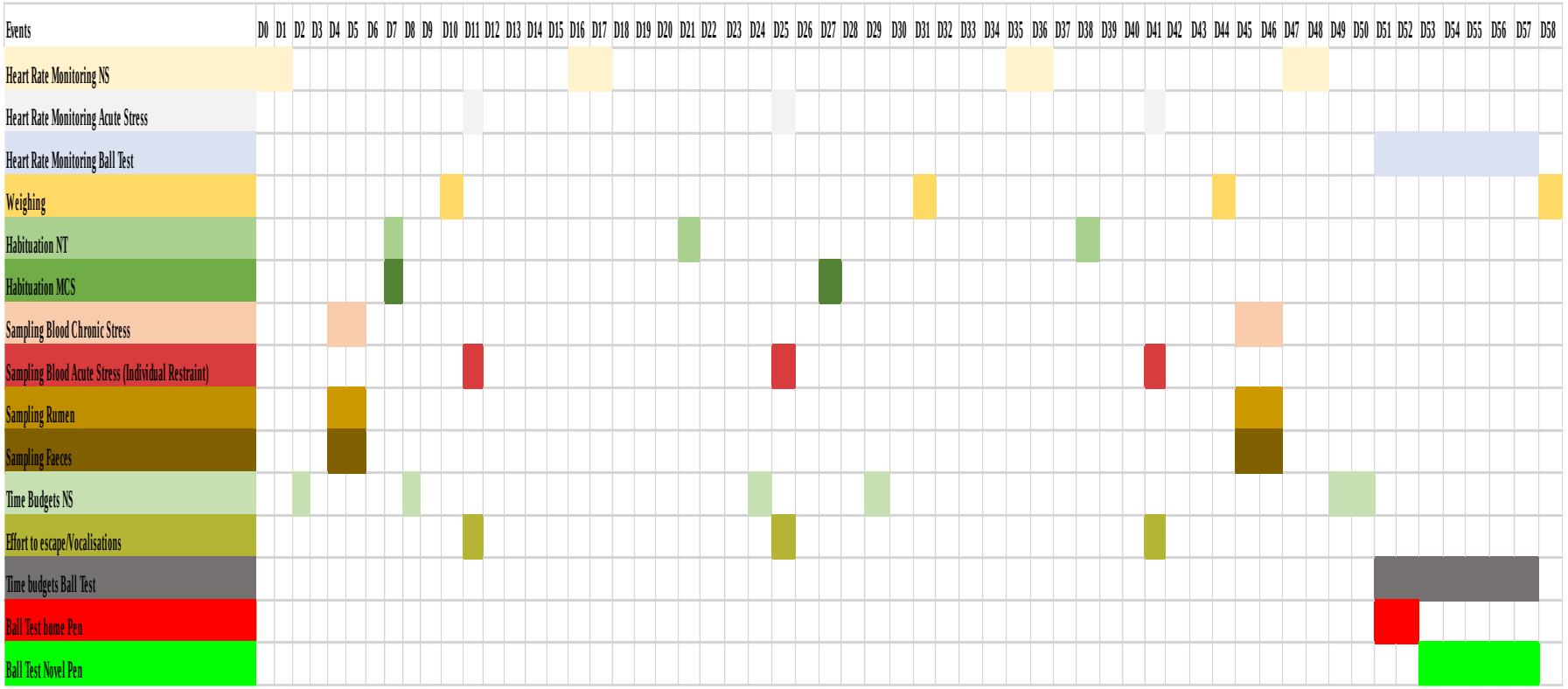

Figure 4.2 Timeline of events during the MCS trial and the following week where ball tests were performed. Days are presented on the top row (D0-D58) and events are coloured coded and denoted in the table.

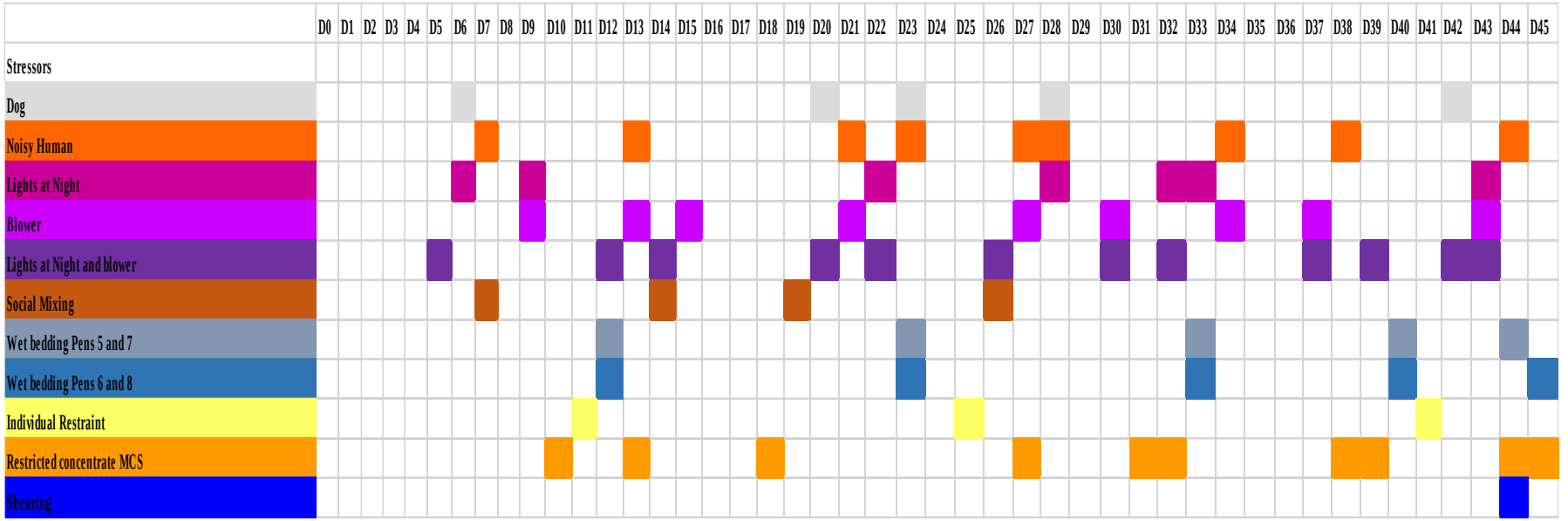

Figure 4.3 Timeline presenting the stressors used during the MCS trial. Days are presented on the top row (D0-D45) and stressors are coloured coded and denoted in the table.

4.4.1.8 Chronic Unpredictable Mild Stress Trial

The stressors used in the current study are presented in **Figure 4.3** above and **Table 4.1** below, whereas detailed descriptions may be found in the Appendix **Section 7.3.1** (**7.3.1.1 -7.3.1.8**) and included anxiety, despair and discomfort-based stimuli. It was important to perform the stressors in an unpredictable manner and include a variety of stressors as to avoid habituation to them.

Table 4.1 Table of Stressors used during the MCS trial and the number of times each stressor was repeated. Further details can be found in the Appendix **Section 7.3.1**.

Stressors		
Stressor	**Times Repeated**	**Comments**
Presence/guidance of/by dog	5	Different dogs used
Noisy human	9	White uniform/ variable means of distressing the animals
Rough handling	3	MCS group when weighed
Blower during the Day	9	Different time of day each time
Lights at Night	7	Different time, duration and different flashing/ sweeping patterns
Lights at Night and Blower	12	Different time, duration and different flashing/ sweeping patterns
Wet bedding	5	Different time and duration
Restricted/Delayed access to concentrate	10	Different time and duration (max 1.5h)
Social mixing	4	Different duration, order of animals and number of animals mixed between pens each time
Shearing	1	Only MCS animals
Individual restraint	3	Description below

4.4.1.9 Individual restraint

This stressor was applied three times to MCS lambs and was considered a stressor but also a means of assessing effectiveness of acute stress (**Figure 4.2**). At 8h00 on the day of the test, two teams consisting of a handler dressed in white an observer wearing green, entered the MCS room simultaneously and worked on two pens in parallel. Three animals were tested at a time in each pen. The handler entered the pen, blocked the animals to be tested, restrained them in a predefined order and then exited the pen. Each animal was restrained for 20 min, using a Gambrel Restrainer that immobilised the head and front legs (Cox Agri, County Durham, UK).

If the animal was in danger of getting hurt the handler repositioned the animal on the ground. Furthermore, if an animal was successful in removing the restrainer, the restrainer was quickly re-adjusted, and the handler re-exited the pen. Individual vocalisations were counted as the number of high and low bleats. As soon as each group of 3 animals had been restrained for 20 minutes, two samplers in green entered the pen, removed the restrainer from one animal at a time.

The duration of this procedure (2 h) meant feeding time on the days of this test would be delayed. The bell was rung at 10h00, distribution of concentrate took place at 10h05 and hay was offered at 10h10 for both the NT and MCS group. The heart rate equipment was removed at 8h00 the following morning. Not all the heart rate information collected from this test was processed and used.

4.4.2 Non-treated group

The animals in room 1 (pens 1, 2, 3 and 4) were not exposed to any of the stressors used with the MCS group. They were visited multiple times during the day by people in green uniform to expose them to human presence at an equal level to the MCS group animals. They were habituated, as mentioned previously, for heart rate and blood/faecal/rumen sampling procedures (**Figure 4.2**).

Blood sampling took place in the 1st of the trial (general pre-trial sampling), but also in the 2nd, 4th and 6th week (in parallel to the MCS group animals as part of the individual restraint test) and in the final week of the trial (general post-trial sampling). Behaviour observations were performed, and ECG data was collected. At the end of the trial, a novelty/startle test was conducted in their home pens twice and in a novel pen (twice for each pen).

4.4.3 Sample and data collection

4.4.3.1 Growth rate, weight gain

Upon arrival to the experimental farm, a month later (Pre1) and every two weeks thereafter (Start, Trial1, Trial2, Trial3 and End) lambs were weighed individually, always in the same order by Pen and Animal ID within Pen, always prior to feed distribution at 08h00.

4.4.3.2 Blood sampling for acute stress response after individual restraint

As soon as each group of 3 animals had been restrained for 20 minutes with the Gambrel restrainer, two samplers wearing the green uniform would enter the pen, remove the Restrainer from one animal at a time and sample two 10ml EDTA vacutainers from each animal. The animals in the NT group were sampled in parallel, in a predefined order (adhered to for the three repetitions of this test). The samples were immediately centrifuged for 10 min at 2500 *g*. Plasma was aliquoted in 1ml Eppendorf tubes and stored at -20ºC.

4.4.3.3 Blood sampling for chronic stress and metabolism indicators

For blood sampling, each animal was individually restrained in the home pen amongst pen-mates by trained personnel. The animal was shorn on the neck area of sample collection to facilitate access and identification of the jugular vein. The sampler gently directed the animal's head in the correct position to not cause distress, and proceeded to identify the jugular vein, inserting the needle for blood collection.

On two occasions, at the start and end of the trial, three 10ml EDTA vacutainers and one 10ml heparin vacutainer were collected for cortisol, glucose, serotonin, beta-hydroxybutyrate (BHB) and non-esterified fatty acids (NEFA) concentration measurements. The samples were immediately centrifuged for 10 min at 2500 *g*. Plasma was aliquoted in 1ml Eppendorf tubes and stored at -20ºC until analyses took place. A subset of the aliquots was kept at -80ºC for long-term storage.

4.4.3.4 Behaviour observations

All videos were coded by a fully trained animal behaviour technician using Observer XT 10.0 behaviour recording software (Noldus 2010), blind to the research hypothesis (INRAE, CARAIBE, Eric Delval).

4.4.3.4.1 Effort to escape Gambrel restrainer test and vocalisations

Each animal was restrained for 20 minutes. During this period, the observers would score "effort to escape", classed from 0-3 every 30 sec. "0" meant the animal was immobile, and performed no visible attempt to escape. "1" was classed as movements specific to the head and neck, where the animal would lift the head or attempt to remove the restrainer. "2" was classified as standing immobile on hind legs. "3" was classed as a vigorous effort to escape, jumping and thrashing.

Vocalisations were counted as the number of high and low bleats on an individual level. Pen order of sampling was the same for all three repetitions of the test: Three animals (two wearing heart rate equipment) from pens 5 and 7 restrained and observed in parallel. The handler and observers would move on to the next three animals (two wearing heart rate equipment) from Pens 6 and 8, continue with the last three animals from pens 6 and 8 and finally restrict and observe the last three animals in pens 5 and 7.

As soon as each group of 3 animals had been restrained for 20 minutes, two samplers wearing the green uniform would enter the pen, remove the restrainer and sample for blood.

4.4.3.4.2 24h Time Budgets

In order to assess activity levels pre-trial, at the start of the trial, mid- and post-trial, we recorded the animals in the NT and MCS Groups for 24h. Scan samples were made at 5 min intervals in order to investigate activity budgets. The Ethogram is presented in **Table 4.2**. From an Excel Macro, programmed by the team at INRAE for previous projects, we calculated the occurrence or frequency of these behaviours (i.e. the number of times these behaviours were observed per animal within 24h), the number of sequences or bouts performed for each behaviour (where a sequence was defined as the animal performing the same activity for two continuous observations), the average length or bouts, and the average synchronisation for each behaviour (the ratio between the percentage of animals performing the same behaviour on average on the day of observation, and the number of scans counted for this activity per animal per day day).

4.4.3.4.3 Suddenness Test Procedures

4.4.3.4.3.1 Home-Pen Ball Test

At the end of the 7 weeks of the mild chronic stress (5th March 2018), the animals were submitted to a startle test in their home pens. For the purposes of the test, a box with an

automated latch had been crafted and placed above and at the centre of each pen, in both rooms, prior to the arrival of the animals in November. The box contained a ball attached with an elastic cable. Small bells were attached to the cable, to produce both a visual and sound startling effect. The ball used was a different colour compared to the one used as means of enrichment in the NT room.

On the morning of the test, the animals were equipped with the ECG equipment, which they kept until the following morning at 8h00. For the purposes of this test, only four animals per room and 8 animals in total were monitored (from pens 1, 3, 5, 7) on the first day due to equipment limitations. The test was repeated a second day in order to equip eight more animals (from pens 2, 4, 6, 8) and increase the number of animals tested for statistical purposes.

ECG was recorded for pre-set times and a separate file was created for each pre-set time. ECG recording commenced 2h 50 min prior to the ball drop and continued for 2h 50 min after the ball was dropped. Four-hour monitoring period files were created for the night period (22h00 - 02h00) and (02h00 - 06h00). "Observer" was also used to record the animals' behaviour from 5 minutes before the ball drop to 30 minutes after. The ball drop was launched at 13h30, a time when most animals were not engaged in eating behaviours (concentrate and hay had been distributed in the morning). This was also a timeframe during which the MCS animals had not previously been submitted to stressors.

Unfortunately, due to equipment failure, the ball did not fall during the second day the test was repeated in two pens (6 and 8) in the MCS stress room. Therefore, behaviour observations were not explored further for this day. Heart rate, as the animals were startled in all pens due to the ball falling in their own or adjacent pens, was used from both days.

4.4.3.4.3.2 Novel environment ball test

The following days (7th -11th March 2018) the animals were tested in a novel pen (dimensions 4m x 4m) with high panels around it (3m height) in a separate area of the same building in which the animals were housed. The animals were moved into this area 30 min before the ball drop and left to explore their surroundings. The pen was devoid of stimuli. Bedding, as in the home pens, was woodchips, and no food or water sources were available. A box (similar to the one mentioned previously) contained a ball attached to it by a cord with bells. This box, fitted with an automated system, would open upon releasing the latch. The ball would then drop, startling the animals.

The sheep were equipped with the ECG monitors at 08h00. ECG was recorded for 2 h 50 min prior to moving them and 25 min after. Each recording was saved as an individual file of predetermined duration (2 h 50 min to 25 min before ball drop, 25 min before ball drop and 25 min post ball drop, 50 min post ball drop, and 2 h 50 min post ball drop).

The test was repeated twice for each pen. On the first day we tested pens 1, 5, 3 and 7 respectively. One group of animals was tested at any given time. Testing started at 13h00 (animals from pen 1 were moved into the novel pen), the first ball drop was scheduled at 13h30 and animals were returned to their home pen at 13h55.

Pen 5 was moved to the novel pen at 14h00, the ball drop was scheduled at 14h30 and return to the home pen was scheduled at 14h55. Pen 3 was moved at 15h00, the ball dropped at 15h30 and the animals returned to their pen at 15h55. Finally, pen 7 was moved at 16h00, the ball was dropped at 16h30 and the animals were moved back at 16h55.

On the second day, we repeated the process for pens 8, 4, 6 and 2, respectively. The schedule followed was as previously described. On the third day, we replicated the procedures of the first day and tested pens 1, 5, 3, 7. On the fourth day, we repeated the procedures of the second day and retested pens 8, 4, 6, 2. The order in which the pens were tested facilitated having comparable test times for NT and MCS animals.

4.4.3.4.3.3 Continuous behavioural observations during the ball test (home-pen test & novel pen ball test)

On the days of the suddenness test in the home-pens, recordings were made from 5 min prior to the ball drop to 30 min post ball drop. Continuous focal animal observations were made for all animals using Observer, using the ethogram in**Table 4.2** and we investigated the frequency of these behaviours (occurrence), the average duration of these behaviours and the latency of different activities, such as approaching the ball and resuming a different activity, such as eating hay for example, indicating a loss of interest in the stimulus.

When the suddenness test was performed in the novel pen, “Observer” was launched 25min prior to the ball drop and stopped 25min post ball drop. The files were analysed continuously for each animal. Behaviours “drinking water”, “eating concentrate” and “resting” with under 10 observations or grouped as “other” (“interaction with congener”, “resting head not visible” and “not visible”) were not included in subsequent analyses.

Table 4.2 Ethogram used during the various tests performed (4h Time budgets on days when no stressor was used, individual restraint test, home-pen ball test and novel pen ball test), alongside descriptions of what consisted a certain behaviour during observations.

24h Time Budgets		
Behaviour	**Description**	**Comments**
Eating hay	Animal standing, pulling hay, chewing hay at a max distance of 5 cm from feeder, or with head/nose in relevant feeder	Start: Pulling hay End: Moving move than 5 cm away from feeder
Eating concentrates	Animal standing, with legs on the ground or forelegs in the feeder, head in relevant feeder, or head in upright position chewing and looking around	Start: Nose in feeder End: Moving more than 5 cm away from feeder
Resting	Animal lying down, upright head posture	
Sleeping	Animal lying down, head on the ground, side of the body or resting on animal's front legs	
Lying, head not visible	Animal lying down, head not visible	
Lying, other	Animal lying down performing activity other than described, i.e., interacting with its environment or another individual, grooming	Low occurrence, not included in results
Moving	Animal pacing, running or jumping in the pen	
Immobile	Animal standing immobile not performing any activity at a distance of more than 5 cm from feeders/ water bowl	
Drinking	Animal interacting with water bowl, nose/ head in water trough	Low occurrence not included in results. Start: Nose in bowl End: Animal moves more than 10 cm away from bowl

Mineral Lick	Animal with head in the bucket where mineral lick was placed, or visibly nibbling/licking mineral lick	Low occurrence not included in results. Start: Nose in bucket End: Head/nose out of bucket
Other	Animal standing, performing any other non-described behaviour such as grooming, interacting with other individuals, interacting with environment.	Not included in results
Individual restraint		
Behaviour	**Description**	**Comments**
Posture 0	Animal immobile, head immobile	No effort to escape
Posture 1	Animal immobile, head moving	Minimal effort to escape
Posture 2	Animal standing on rear legs, immobile, forelegs restrained	Moderate effort to escape
Posture 3	Animal thrashing (rolling on ground/ arching back or jumping on rear legs)	Strenuous effort to escape
High bleats	Animal bleating with open mouth, emitting a high-pitched sound	
Low bleats	Animal bleating with mouth closed, emitting low pitched sound	
Ball Test- Home Pen		
Behaviour	**Description**	**Comments**
Contact with ball	Physical contact with ball: exclusively with the face, muzzle or the side of the face	Start: muzzle, cheek or forehead touches ball End: Animal moves more than 5 cm away or licks, bites, pushes, headbutts the ball
Interaction with ball	Animal licking, chewing, pushing, kicking or using its legs as a means of interacting with the ball	

Eating hay	As described above	
Contact with environment	Animal licking, chewing, pushing, kicking or using its legs as a means of interacting with the bars, feeders, brushes or any other aspect of their pen	
Ball Test- Novel Environment		
Behaviour	**Description**	**Comments**
Contact with environment	Smelling or sniffing to explore the novel pen	
Interaction with environment	Licking, biting, attempting to climb, digging or pushing any surface or aspect of the novel pen	
Contact with ball	As described above	
Interaction with ball	As described above	
Vigilance	Animal immobile, head in upright position, fixed gaze, ears in upright- side position, with open auricle orientation	

4.4.3.5 Heart rate monitoring

Heart rate activity was recorded using a telemetry ECG monitoring system by EMKA Technologies. Three disposable skin-adhesive electrodes (Philips, Ag/AgCl electrodes with foam, pre-gelled) were placed on each animal. Ultrasound gel (Alcyon, Lyon) was added to improve conductivity. The animals were shorn locally before every monitoring session on two locations: 1) On the right shoulder of the animal, slightly upwards, behind the point of the elbow and 2) on the thorax and behind the left leg, at the intersection of the fore-flank and the point of the elbow.

The LA (black) electrode was placed near the caudal angle of the scapula; the LL (red) electrode on the left abdominal area within the rib cage frame and the RA (white) electrode was placed slightly to the right of the sternum. The placement of the electrodes followed the main principles for ECG recording, including being as far as possible from the heart to reduce ruminal interference, and forming a triangle, the gravity centre of which corresponded to the

electric centre of the heart (right ventricle). Optimisation tests had been previously performed by the INRAE group and EMKA had been consulted.

A 450 g transmitter was secured on the back of the animal using lumbar belts (Thuasne, France), adaptable to the size and weight of the lambs as they grew. Data was acquired via an EMKA pack 4G receptor, converted, and transferred onto a laptop for live monitoring using the EMKA iOx software (2.9.5.73 version). Due to cost restrictions and limited availability of monitoring systems, eight (twin or sibling) animals were monitored in each session Recording sessions were:

Three recording sessions of 20h (10h00 - 06h00) during the 6-week stress period on eight animals in each room, on Days 10-11, Days 28-29 and Days 40-41. On the first day of each session, four animals from each room were monitored, and on the second day, four different animals were recorded in each room. The animals and the monitoring order were always the same. These sessions allowed investigation of any changes in heart rate due to treatment over time.

Three monitoring sessions from 16h00 prior to the individual restraint test to 08h00 the next morning (40h). The eight animals from the MCS group were monitored during this period, while the eight animals from the NT group wore lumbar belts but were not equipped with electrodes or monitored. The animals wearing the heart rate equipment were consistently sampled in the first group of 3 animals restrained. These sessions allowed assessment of the effect of this acute stressor on heart rate levels by comparing with the closest timeframes available from the non-stress days, and the respective night “Steps”. In addition, these measurements allowed investigation of differences over time, on the three occasions this test was performed.

Heart rate was monitored for four animals per room per day, for 24h on the days the animals were subjected to the suddenness and novelty test, where a ball automatically fell into the pen, allowing investigation of any differences between the two treatment groups at a heart rate level. On these occasions, the equipment was placed on the animals on the morning of the tests, and the equipment was removed the following morning. The timeframes of interest for this test are presented below (**Table 4.3**).

Similarly, for the ball test conducted in the novel environment, the equipment was placed on the animals on the morning of the test (8h00), and the equipment was removed 24h later on the day after the test, to re-equip the second group. The timeframes of interest for this test are presented below. Heart rate data was also acquired for the night periods once for each pen (the

first night after exposure to the test). Four NT and MCS animals were monitored each day, and pens were tested in the order mentioned above.

Table 4.3 Description of timeframes (Steps) used to segregate heart rate monitoring sessions by Test (no stress, acute stress i.e., individual restraint, suddenness test i.e. ball test)

Steps for Heart Rate Measurements No-Stress days		
Steps	**Time**	**Comments**
Step1	10h00 – 14h00	
Step2	14h00 – 18h00	
Step3	18h00 – 22h00	
Step4	22h00 - 02h00	
Step5	02h00 – 06h00	
Steps for Heart Rate Measurements- individual restraint		
Steps	**Time**	**Comments**
Step1	Gambrel Restrainer + 20min	Gambrel Restrainer: time point 0 (S1)
Step2	S1+20 min	(S2)
Step3	S2 +20 min	(S3)
Step4	S3 +20 min	(S4)
Step5	S4 +20 min	
Step6	22h00 – 02h00	
Step7	02h00 – 06h00	
Steps for Heart Rate Measurements- Ball Test (Home Pen & Novel Pen)		
Steps	**Time**	**Comments [Ball drop (B) =Time point 0]**
Step1	Ball drop – 2h50	(B -25 min) – 2h25
Step2	Ball drop -25 min	B- 25 min
Step3	Ball drop +25 min	B+ 25 min (Ball drop: time point 0)
Step4	Ball drop +2h50	(B +25 min) +2h25
Step5	22h00 – 02h00	
Step6	02h00 – 06h00	

4.4.3.6 Rumen and faecal Sample Collection

For the collection of rumen and faecal samples pre- and post-trial, the animals were exposed to the handling process and insertion of the wood bit in their mouth twice before the actual sampling, as a means of habituation. Rumen sampling was performed via rumino-oesophageal intubation using a small pump.

A handler sat the sheep on their hindquarters while keeping the neck straight and the bit in place. A second person inserted the tube down the oesophagus, while a third person pumped as soon as the tube reached the rumen. Approximately 40ml was sampled from each animal. The rumen content was directly aliquoted into 15ml Falcon tubes and dipped in liquid nitrogen for DNA extraction. Fresh rumen fluid (0.8 ml) was added to 0.5 ml of 0.5 N HCl solution containing 0.2% (wt /vol) metaphosphoric acid and 0.4% (wt /vol) crotonic acid, for protozoan fixation and counting. After collection of rumen contents from each animal, the tube and DURAN bottle were rinsed out with 70% alcohol.

The animals were monitored for 30 min post-sampling to assess lack of appetite or signs of pain and discomfort. They did not present any of these symptoms in any of the sampling sessions.

Faecal samples were collected directly from the rectum. In order to avoid inducing pain or discomfort as much as possible, gloves were moistened before sampling. Two to ten grams were collected from each animal and placed in dry ice containers. The rumen and faecal samples for DNA extraction were dipped in liquid nitrogen as soon as they had been aliquoted and then placed for long-term storage at -80°C. Samples obtained for protozoa counts were stored in a cool dark place, whereas the samples for VFA analysis were kept at -20° C until further analysis.

4.4.4 Sample & data processing

4.4.4.1 Sample processing- blood plasma: NEFA, glucose and BHB

Blood plasma was photometrically analysed by a different group within INRAE (UMRH1213) (Isabelle Constant, PERAQ) and sample analysis was conducted on an Arena 20XT Automated Analyser (ThermoFisher Scientific, France). Kits used were a Sobiod NEFA kit (Lot 11G17 & Lot 10G17), a ThermoFisher Scientific kit for BHB (Lot N616) and a GOD-POD ThermoFisher kit for Glucose (Lot MA83). The controls used were Randox CT1 (Lot 201SL) by RANDOX and Nortrol (Lot N249) by ThermoFisher Scientific (France). All samples were analysed in duplicate, and an average was recorded. CVs were under the manufacturer's suggested threshold and if not, sample analysis was repeated.

4.4.4.1.1 Cortisol

Cortisol samples were analysed via ELISA as described in Chapter 2, (**Section 2.4.3.6**). Coefficient of Variance (CV) was ensured as <15%. This threshold was based on validation assays performed by Andanson *et al*. (2018). Analysis was repeated, as necessary.

4.4.4.1.2 Serotonin

A serotonin ELISA kit (ADI-900-175) by Enzo Life Sciences (ELS) AG (Villeurbanne, France) was used for measuring serotonin levels in the blood plasma samples according to the manufacturer's instructions and previous literature available. A dilution of 1:100 was selected as the optimum concentration, to avoid background signalling. All samples were analysed in duplicate, and an average was recorded. CV was ensured as <15% (according to manufacturer's advice) and analysis was repeated, as necessary.

4.4.4.2 Rumen and faecal samples

4.4.4.2.1 DNA extraction and library preparation from rumen and faecal samples at INRAE

The methods used for sample crushing and DNA extraction from the faecal and rumen samples was as described in **Chapter 2** (**Section 2.4.3.1**) and DNA extraction was conducted randomly, regardless of experimental stage (pre- post or Experimental group) over ten days.

4.4.4.2.2 Protozoa counts

Protozoa fixing and readings were conducted as described in **Chapter 2** (**Section 2.4.3.7).**

4.4.4.2.3 Volatile Fatty Acid Content Determination in the rumen Liquid

Analysis of VFA was performed as described in (Lettat *et al.*, 2010; Jeyanathan *et al.*, 2019a) on centrifuged samples (CP 9002 Gas Chromatography, Chrompack, Middelburg, Germany), using crotonic acid as an internal standard (Morgavi *et al.*, 2003). The equipment used was a PerkinElmer Clarus 580 Gas Chromatograph (GC) and the data acquisition software was PerkinElmer TotalChrom TCNAV (Paris, France). The VFAs quantified were Acetic acid, Propionic acid, Isobutyric acid, Butyric acid, Isovaleric, Valeric acid and finally Caproic acid. Coefficient of Variation (CV) was ensured as <7.00%, otherwise the analysis was repeated.

4.4.4.3 Heart monitoring data processing

After acquiring the relevant data via an EMKA pack 4G receptor, converting and transferring it onto a laptop for live monitoring using the EMKA iOx software (2.9.5.73 version), preliminary treatment and transformation was performed using the EMKA ECG-Auto software (version 3.3.3.10, Sept 8th, 2015).

Heart rate variability analysis was then realised using the CHART software (Version 5.4.2, INRAE de Theix, group ACS/URH, Herve Chandeze). Analysis was performed for 20 min, 25min, 1h25 or 4h time periods, depending on the test, with the help of Excel Macros. The software used the interval between successive Rs (where R is a point corresponding to the peak of the QRS complex of the ECG wave) as a reference point for calculating the duration of the intervals. Previous tests and studies had been conducted by the team, defining the parameters best adapted for sheep heart monitoring.

All calculations were based on the interval duration, making it possible to separate temporal (average heartbeat and heartbeat variability) and spectral analyses (percentage of frequencies of activation of the heart rate originating from the sympathetic and parasympathetic system).

The variables calculated using CHART were:

RR: The intervals between to R waves of the electrocardiogram

Total Power: The Power of our spectrum selected for analysis

CVRR: Coefficient of variation of R intervals

LF: Low Frequency; This parameter reflects the sympathetic tone

HF: High Frequency; This parameter reflects the vagal tone

LF/HF: The ratio based on Low and High Frequencies. This parameter evaluates the balance between the sympathetic and the vagal tone

Max RR: The longest RR interval

Min RR: The shortest RR interval

Mean RR: The mean of R intervals

Average Heart Rate (HR): Number of R beats divided by the duration of the monitoring period, expressed in beats per minute (bpm)

SD of NN: Standard deviation of R intervals (overall HR variance)

SDΔRR or RMSSD (Root of the Mean Square of Successive Differences): Standard deviation of the change between successive beat intervals, indicator for vagal modulation of HR

The outputs were then saved in Excel spreadsheets for further analyses. The parameters selected for further analysis were the average RR (RR_aver), average HR (HR_aver), SDNN, RMSSD and the ratio of Low to High Frequencies (LF/HF), which taken together, allow the assessment of the sympathovagal balance (A. Destrez *et al.*, 2013a; Kim *et al.*, 2018).

4.4.4.4 Experimental Stages

Multiple samples were taken on different days to assess baseline levels, investigate effectiveness of acute stress and explore the potential presence of chronic stress. As different tests were conducted on different days during the trial, the term "experimental stage" has been included in the statistical analyses. For example, for heart rate, this is expressed as "Day", whereas for behavioural observations this is expressed as "Pretrial, Start of trial, Middle of trial and End of trial" measurements. Additionally, since rumen and faecal samples were sampled Pretrial (before the repeated stressors were applied) and Post trial (at the end of the stressor implementation), these were described as "experimental stages". The timeline related to these measurements has been presented in **Figure 4.3.**

4.5 Statistical analyses

Statistical analyses were conducted in R (R Core Team, 2019) and figures were produced using the package "ggplot2" (Wickham, 2009). Other packages used in R included: "lme4" (Bates *et al*., 2015), to perform generalised linear models (GLMs), generalised linear mixed models (GLMMs) or linear models (LMs) using the "glmer()", "glmer.nb, "lmer()","glm()" or "lm()"

functions. Other packages used frequently were: “car”, “phyloseq” (McMurdie and Holmes, 2013a); “MASS” (Riplley *et al.*, 2018); “survival”, using the “coxph()” package to analyse latency data.

Test statistics, degree of freedom, P-value, means or predicted means and standard errors of means (SEM) or Standard Deviation (SD) were reported as appropriate. Means obtained from transformed data were backtransformed and reported with 95% confidence intervals [95% CIs]. Individual statistical models were built for each of the data analyses, and the effect of Treatment groups, Experimental Stage and other variables (such as Interactions and repeated measure influences) were explored. Significant differences in tables are reported with the compact letter display system (CLD) and capitalised when $p < 0.01$. In boxplots figures, individual values, mean and interquartile range (IQR) are reported. Tables of all the models are available in the Appendix (**Tables 7.23-7.27)**. Results are considered as significant and discussed when $p \leq 0.05$.

4.5.1 Body weight and average daily gain

GLMMs were used for the investigation of Treatment group and Experimental Stage (Arrival, Pre1, Start, Week3, Week5, Week7 and End- post suddenness tests) and interaction between the two, with the inclusion of animal ID as a random factor on animal weight.

The ADG was calculated for each animal as the average gain from one weighing session to the next (i.e., $ADG = \frac{Weight(g)Week5 - Weight(g)Week3}{N\ Days}$). The period of interest was limited to the Trial (Experimental Stage: Start, Week3, Week5, Week7 and End- post suddenness tests). ADG was also calculated for each lamb for the entire period of (Start-End).

$$Weight: f(y) = Treatment\ group + Experimental\ Stage + Treatment\ group * Experimental\ Stage + (1|ID)$$

$$ADG: f(y) = Treatment\ group + Experimental\ Stage + (1|Experimental\ Stage)$$

Overall ADG for the duration of the period (Start of Trial to End) was compared between Treatment groups using a t-test after assessing normality.

4.5.2 Behaviour observations- 24h +ime budgets

To investigate the effect of Treatment group (MCS, NT), Experimental Stage (Pre, Start, Mid-trial and End of trial) and potential interactions on the various parameters measured for each behaviour, GLM Poisson models (for Occurrence, Number of bouts and Synchronisation which represent count data), GLMMs or GLMs (when no random variables were appropriate) were used. Data was not transformed.

Experimental Stage (ExpStage) was included as a continuous variable, and after testing, the Individual (ID) and/or ExpStage were included in the model where appropriate to account for repeated measurements. Normality of residuals was assessed and confirmed. Comparison of models to assess the best fit was conducted via the "Anova" function from the "car" package in R. Whilst non-significant terms were dropped from models, the starting model was:

*Treatment group + ExpStage +Treatment group * ExpStage + (ExpStage | ID)*

4.5.3 Behaviour Observations- individual restraint

In order to analyse the animals' effort to escape restraint from the Gambrel Restrainer, scan samples made every 30 sec were grouped into four 5 min time frames (time1, time2, time3 and time4), attempting to capture the variability in behaviour throughout the test. Chi-squared test were used, as variables were categorical with expected frequency over 5. Observed and estimated values for the occurrence of each posture (posture0, posture1, posture2 and posture3) were calculated to compare each time point between repetitions (i.e., time1a, time1b, time1c) and for the sum of observations for each posture in the entire testing session (20min) repeated 3 times (test1, test2 and test3).

4.5.4 Behaviour observations- ball test home-pen (latency, frequency and duration)

Treatment group effect was explored on the latencies of the animals to "Interact with the ball" and latency to "eat hay" (considered to be an indicator of loss of interest in the novel stimulus) after the ball had dropped, using Cox regression survival analysis, and coding the absence or presence of the events as "0" or "1". The "cox.me ()" function was used from the "survival" package on R.

For the duration and frequency of "contact with the ball/environment" and "interaction with the ball/environment" and "eating hay". Treatment group effect was explored using Wilcox Rank Sum tests or ANOVA, depending on whether data was normalised (i.e., SRT

transformation of “eating hay” duration) and met normality assumptions (i.e., not met for frequency data).

Treatment group, Day and Interaction effects were explored on the latencies (sec) of the animals to “Interact with the ball”, and “contact/interact with the environment” (considered to be an indicator of loss of interest in the stimulus) after the ball had dropped, using Cox regression survival analysis and coding the absence or presence of the events as “0” or “1”. The “cox.ph ()” function was used from the “survival” package on R to incorporate Day and Animal ID as repeated measurements. GLMMs with Poisson distribution were used for the frequency data, and GLMMs for the duration measured in seconds. Pre- and Post-ball drop were analysed separately.

The models used were:

Latency and Frequency: $f(y) = Treatment\ group * Day + (Day|\ ID)$, in accordance with R syntax

Duration: $f(y) = Treatment\ group * Day + (1|ID)$

Vigilance: $f(y) = Treatment\ group * Day$

4.5.5 Blood biomarkers (Cortisol, glucose, BHB, NEFA, serotonin)

4.5.6 Heart rate no stress days

In order to explore differences for each of the heart rate parameters on a Day basis, the average value of each step was used to compute the area under the curve for each of the four days of ECG monitoring. This allowed comparison between Days and Treatment groups over the course of the experiment, since the number of observations per animal/step/day did not allow the use of a model. Excel was used to compute the best fitting curve, and the area under the curve was calculated by imputing the lowest value (1) and the highest (5) (Available in Appendix **(Figures 7.25-7.44).**

In addition to this, as an average of the 5 steps did not capture the variability present within each day for each animal, each step was explored individually. The effect of Treatment, Day (repetition of the monitoring test) and potential interactions were explored using GLMMs, which allowed the integration of animal ID as a random factor and permitted a different baseline for each animal. Day was included as a continuous factor, to allow fitting of different intercepts per day, to reflect the nonlinear evolution of each step within days (Repeated Measures). Comparison of models to assess the best fit was conducted via the “Anova”

function from the “car” package in R. The general model upon which adaptations were made is presented below, and full details of the models chosen are available in the Appendix (Tables 7.23-7.27)*:*

Model:

*f(y) =Treatment group+Experimental Stage + Treatment group*Experimental Stage + (1|ID)*

4.5.7 Heart rate individual restraint test

To assess the effect of the stressor on the MCS group, we compared the three closest (by Date) NS Step1 files with the three S1 individual restraint files for each heart rate variable via repeated measures ANOVA in R. Similarly, we compared night Step5 and Step6 files.

Model: *f(Step/Heart rate variable) =Day +Error(ID| Test)*

4.5.8 Heart rate; Home-pen, ball test

In order to assess the effect of the ball drop on heart rate variability parameters, step 3 from the ball test files (ball drop+25min) was compared with step 2 (14h00- 18h00) from the closest NS day file (D4).

Model*: f(y) =Treatment group+ Day + Treatment group*Day+(1|ID)*

The influence of day on the variance was investigated and not included. Animal ID was included as a random factor. Analyses were performed on a step level. Night steps were compared with the closest respective Steps from a NS day for an effect of Treatment, Day and Interactions for the heart rate variables.

The effect of Treatment (MCS, NT) was explored on the different heart rate variables on the two days the test was repeated in the home pen. Data was not transformed as normality assumptions were met and ANOVAs were conducted. When not met, non-parametric Kruskal-Wallis tests were applied.

4.5.9 Heart rate; Novel pen, ball test

Analyses were performed on a Step level. The effect of Treatment (MCS, NT), Day and Interactions were explored on the different heart rate variables using GLMMs, Poisson GLMMs (for RR_aver count data), or GLMs. Data was not transformed. Day was included as a continuous variable and after testing, when contributing to the variance, included as a

repeated measure. Individuals (ID) were included in the models where appropriate to account for repeated measurements on the same animal. Normality of residuals was assessed and confirmed. Comparison of models to assess the best fit was conducted via the "Anova" function from the "car" package in R. For step4 (ball drop +2h50) on D4, only 2 observations out of 4 were available due to equipment failure.

Furthermore, a comparison between step 2 (14h00- 18h00) of the last no-stress test day was conducted with step 2 (ball drop -25min) and step 3 (ball drop +25min) of the Ball Test to explore the effect that moving the animals in a novel environment and the suddenness test had on heart rate. Step 2 of non-stressed days was considered the best time frame as it was closest to the time the ball test was performed (in terms of diurnal rhythm). Night step 5 (22h00 – 02h00) and 6 (02h00 – 06h00) was recorded on the 1st night the test was conducted the [MCS (n = 4) and NT (n = 4)]. These steps were respectively compared with step 5 and 6 from the last no-stress monitoring session.

Model: $f(y) = Treatment\ group + Day + Treatment\ group * Day + (Day|ID)$

4.5.10 Rumen samples

Treatment and Experimental Stage, as well as potential interaction effects were explored on the number of rumen protozoa counted (Large Entodiniomorphs, Small Entodiniomorphs, Isotricha, Dasytricha and total counts per sample). GLMM Poisson models were used (after assessing best fit):

Models:

$$f(y) = Treatment + Experimental\ Stage + Treatment * Experimental\ Stage + (1|ID)$$

$f(y) = Treatment + Experimental\ Stage + (1|ID)$ (for Small Entodiniomorphs)

Treatment group, Experimental Stage, and potential Interactions were explored on the concentration of the rumen VFAs [acetic acid (C2), propionic acid (C3), butyric acid a (C4) and the ratio C2:C3 (acetate: propionate)], pre- and post-trial using GLMMs:

$$f(y) = Treatment + Experimental\ Stage + Treatment * Experimental\ Stage + (1|ID)$$

4.5.11 Microbiota rumen and faecal samples

Rumen and faecal 16S rRNA gene amplicon sequencing files were imported in Galaxy and processed as described in **Chapter 2** (**Section 2.4.3**). All further analyses were carried out in R (Version 6.3.1) using different packages and functions cited in the appropriate sections.

Calculation of Relative Abundances (RA) and percentages of the most abundant phyla, classes etc. were carried out using QIIME. The RA tables produced for archaea and bacteria were used for further statistical analysis at each taxonomic level. rumen and faecal samples were explored separately by adopting squared root transformation (SRT). This analysis was performed for all phyla, for orders with RA over 0.5% (due to low abundance levels observed), and genera with RA over 1.00%.

Normality was assessed via Shapiro-Wilk tests. Normality assumptions were not met for most of the phyla, orders and genera explored, therefore non-parametric tests (Kruskal-Wallis) were used on grouped variables (Treatment*Experimental Stage), resulting in 4 subgroups: PreNT, PreMCS, PostNT and PostMCS. Pairwise comparisons were conducted via Wilcoxon Rank Sum tests with Bonferroni correction.

Faecal and rumen OTU tables were acquired from QIIME, Galaxy. These were imported into "phyloseq" R, along with a taxonomic ranking related to each OTU and a "metadata" file with all information related to each lamb (Treatment Experimental Stage, Pen, ID, etc.).

Unidentified reads at a kingdom level, labelled as "None" or "Other", were removed. Archaeal and bacterial diversity were investigated separately. For the rumen samples, 19810 bacterial OTUs (max reads: 18834) and 60 archaeal OTUs (max reads: 314) were acquired, while for the faecal samples, 22270 bacterial OTUs (max reads: 15292) and 58 archaeal OTUs (max reads: 260) were acquired. Alpha diversity metrics were explored as described in Chapter 2 (Shannon, Simson, Inverse Simpson, Chao1, ACE and Observed diversity).

Tests were conducted on rarefied data, a method used for normalisation. This was done by randomly selecting reads from each sample to match the lowest count of reads present in the dataset. For rumen archaea this was 10 sequences, for rumen bacteria 4832, for faecal archaea 32 and faecal bacteria 5846. GLLMS were used to investigate Treatment group, Experimental Stage, and Interaction effects on the diversity indices. The models used (Appendix, **Table 7.27**) accounted for individual variability by adding ID as a random factor, as well as Pen effect, where the model allowed. Experimental Stage did not contribute to the variability and was not included as a Random factor (to account for repeated measures) and was only added

as a fixed factor, according to the function and syntax of the "lme4" package in R. The model, used as a basis, was:

*Treatment+ Stage +Stage*Treatment + (1|Pen/ID)*

Multiple comparisons of means were performed using the "lsmeans" package for Post-Hoc analysis, with Bonferroni adjustment.

Beta diversity was calculated on Hellinger transformed data for Treatment, Experimental Stage, and combined variables using PERMANOVA on R, as described in Chapter 2, after exploring dispersion using the "betadisper()" function in "vegan". Similarly, PCoA plots were created for visual exploration of the data. Canonical Analysis Principal coordinates (CAP) plots, computed on "phyloseq", indicating the potential influence of cortisol and serotonin on rumen and faecal bacteria.

4.5.12 Partial least squares modelling

PLS modelling was used to investigate the relationship between "log(x+1)" transformed values of cortisol or "log" transformed values of serotonin and different taxonomic levels (phylum, order and genus) of the rumen and faecal microbiota sequences. The "mix0mics" package in R (Lê Cao, González and Déjean, 2009; Rohart *et al.*, 2017) was used for this purpose.

PLS models explored the relationship between cortisol/serotonin and groups: PostNT and PostMCS, as comparisons between Treatment groups post-trial allowed identification of the influence of the repeated stressors.

A further step in exploring the relationship of the different Taxonomic levels with cortisol and serotonin data was conducted by correlating the phyla with VIP >1 and orders and genera with VIP values >1.5, from the PLS analysis, with each of the variables. Reasoning for these thresholds is presented in **Chapter 2** (**Section 2.4.3.10)**

Normality was tested and assumptions not met. A correlation matrix was visualised in the form of a heatplot to explore potential covariations between phyla, orders and genera selected and the variables of interest, using Pearson correlation in R [library("Hmisc"), ("Hmisc package | R Documentation" 2019); library ("corrplot"), (Taiyun Wei *et al.*, 2017)]. The Correlations between hormones and RA are presented in the main Results section, whereas the heatplots can be found in the Appendix (**Sections 7.5.4, 7.5.5, 7.5.6, 7.5.7**).

4.6 Results

4.6.1 Weight and ADG

4.6.1.1 Weight

No significant effect of Treatment [NT (n = 168), mean = 38.7 ± 9.21 kg; MCS (n = 168), mean = 37.7 ± 8.49 kg] was observed. There was a Day and Interaction effect [F (1, 48) = 5.05; p <0.01 and F (1, 48) = 5.05; p = 0.02] which was due to the animals gaining weight over time, as there was no Treatment effect at any Experimental Stage (**Figure 4.4**).

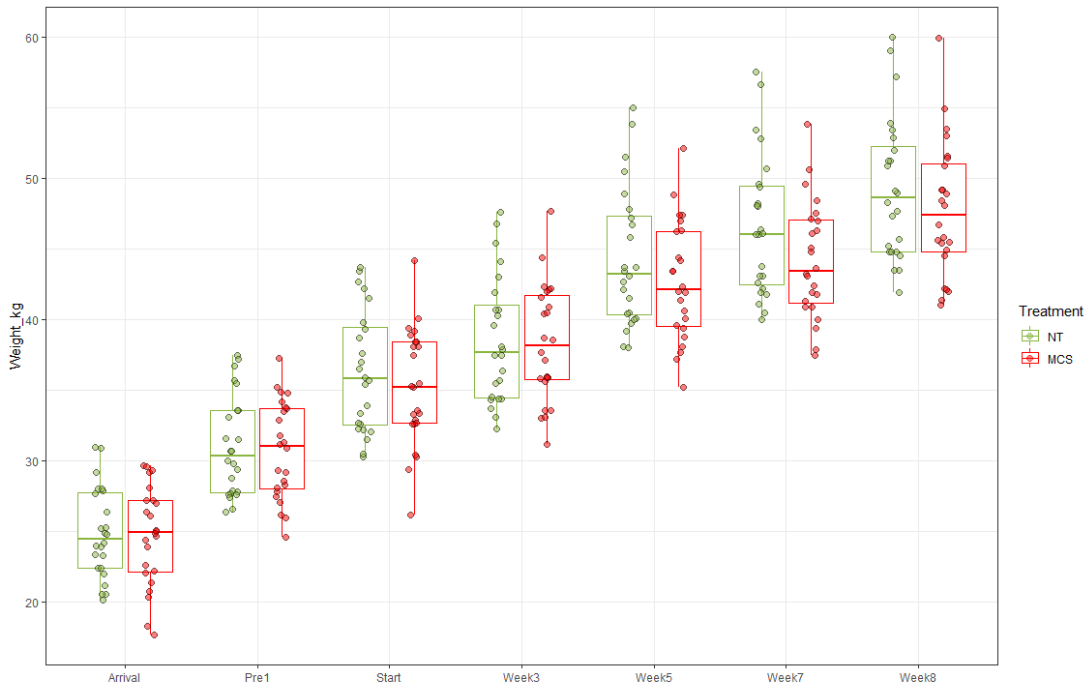

Figure 4.4 Weight (kg) of animals from their arrival to the experimental farm until the end of the trial. No Treatment effect was observed over time (p = 0.38). The points represent individual values by Treatment, and the boxplots include the median and interquartile range (IQR).

4.6.1.2 ADG

ADG was calculated for the 5 times the animals were weighed during the Trial [A: Week3-Start; B: Week5 –Week3; C: Week7 (after the end of the MCS trial) –Week5 and D: Week 8 (Post ball) –Week 7 (end of MCS trial)] and analysed via GLMM. ADG was significantly affected by Treatment [NT (n = 96), mean = 0.21 ±0.09 kg; MCS (n = 96), mean = 0.18 ± 0.08 kg; SE: 0.01 p = 0.01]. Specifically, NT animals had a significantly higher ADG at Stages B and C, but as described by Error! Reference source not found.. ADG was the same for the last m easurement period. There was no significant Treatment [NT (n =24, mean = 0.20 ±0.03); MCS (n = 24, mean = 0.19 ± 0.03) effect on the average daily gain for the overall experimental period (p = 0.48).

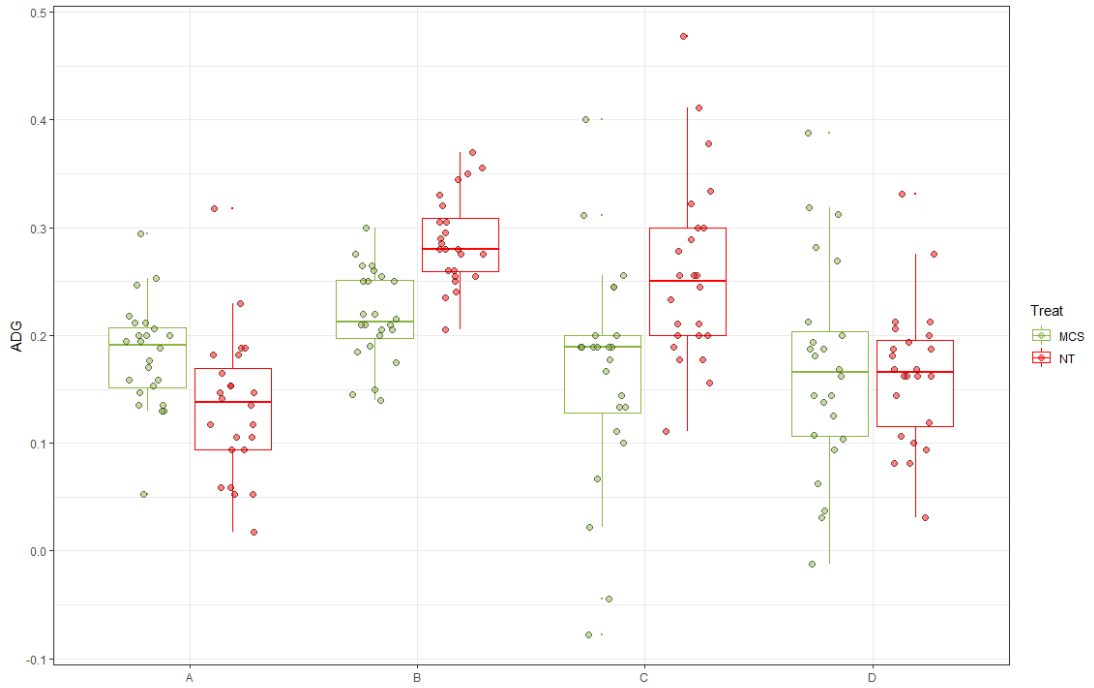

Figure 4.5 ADG (kg/day) of animals from the Start to the End of the Trial. ADG was calculated after each weighing session and presented in Experimental Stages (A: Start- Week3; B: Week3-Week5; C: Week5-Week7 and D: Week7 to Week8 (End)). There was a treatment effect (p <0.01) as ADG NT >ADG MCS. The points represent individual values by Treatment and the boxplots include the median and IQR.

4.6.2 Blood plasma biomarkers

4.6.2.1 Acute stress effects

Treatment, Experimental stage, and interaction effects were observed for cortisol, NEFA, BHB and glucose concentrations in blood plasma. Specifically, there was an increase in hormone levels of the MCS animals post individual restraint, compared to the NT group and their pre-trial values, as presented in **Table 4.4** below.

Table 4.4 GLMM results for blood plasma biomarkers measures for NT animals (n = 24) and MCS (n = 24) animals Pre-trial (n = 48) and after the third repetition of the individual restraint test (n = 48). Treatment, Experimental Stage and Interaction effects were observed for all. Mean ± SD values, p- and F-value) are presented. Differences are indicated with the CLD system and capitalised when p <0.01.

Cortisol				
		mean ±SD	**F-value**	**p-value**
Treatment group	NT	0.27 ± 0.38^{A}	24.71	<0.01
	MCS	0.77 ± 0.64^{B}		
Experimental stage	Pre	0.31 ± 0.38^{A}	38.14	<0.01
	Stress	0.73 ± 0.67^{B}		
Interactions	PreNT	0.29 ± 0.38^{A}	47.01	<0.01
	Pre MCS	0.32 ± 0.39^{A}		
	Stress NT	0.25 ± 0.40^{A}		
	Stress MCS	1.22 ± 0.51^{B}		
Glucose				
		mean ±SD	**F-value**	**p-value**
Treatment Group	NT	0.63 ± 0.10^{A}	10.52	<0.01
	MCS	0.73 ± 0.17^{B}		
Experimental Stage	Pre	0.64 ± 0.14^{A}	15.50	<0.01
	Stress	0.72 ± 0.14^{B}		
Interactions	PreNT	0.63 ± 0.12^{A}	10.83	<0.01

	Pre MCS	0.65 ±0.16^{A}		
	Stress NT	0.64 ±0.08^{A}		
	Stress MCS	0.80 ±0.14^{B}		
NEFA				
		mean ±SD	**F-value**	**p-value**
Treatment Group	NT	0.17 ±0.08A	33.91	<0.01
	MCS	0.37 ±0.32B		
Experimental Stage	Pr	0.16 ±0.09^{A}	35.51	<0.01
	Stress	0.36 ±0.33^{B}		
Interactions	PreNT	0.17 ±0.09^{A}	79.95	<0.01
	Pre MCS	0.12 ±0.06^{A}		
	Stress NT	0.15 ±0.08^{A}		
	Stress MCS	0.58 ±0.30^{B}		
BHB				
Factor	**Mean ±SD**	**mean ±SD**	**F-value**	**p-value**
Treatment Group	NT	0.22 ±0.38^{A}	8.41	<0.01
	MCS	0.26 ±0.64^{B}		
Experimental Stage	Pre	0.21 ±0.38^{A}	30.29	<0.01
	Stress	0.26 ±0.57^{B}		
Interactions	Pre NT	0.22 ±0.37^{A}	24.45	<0.01
	Pre MCS	0.21 ±0.39^{A}		
	Stress NT	0.22 ±0.39^{A}		
	Stress MCS	0.30 ±0.51^{B}		

4.6.2.2 Chronic stress effects

There was no effect of Treatment group or Experimental Stage on any of the biomarkers measured (cortisol, glucose, NEFA, serotonin; p>0.05), apart from BHB. For BHB, there was a significant Day [Pre: 0.21 ± 0.38; Post: 0.26 ± 0.67; F (1, 48) = 15.75, p <0.01] and Interaction [F (1, 24) = 5.98] effect [PreNT (0.22 ± 0.04) <PostNT: (0.26 ± 0.05), p <0.01; PostNT: (0.26 ± 0.05) > PostMCS (0.22 ± 0.04), p = 0.03] (**Figure 4.6**).

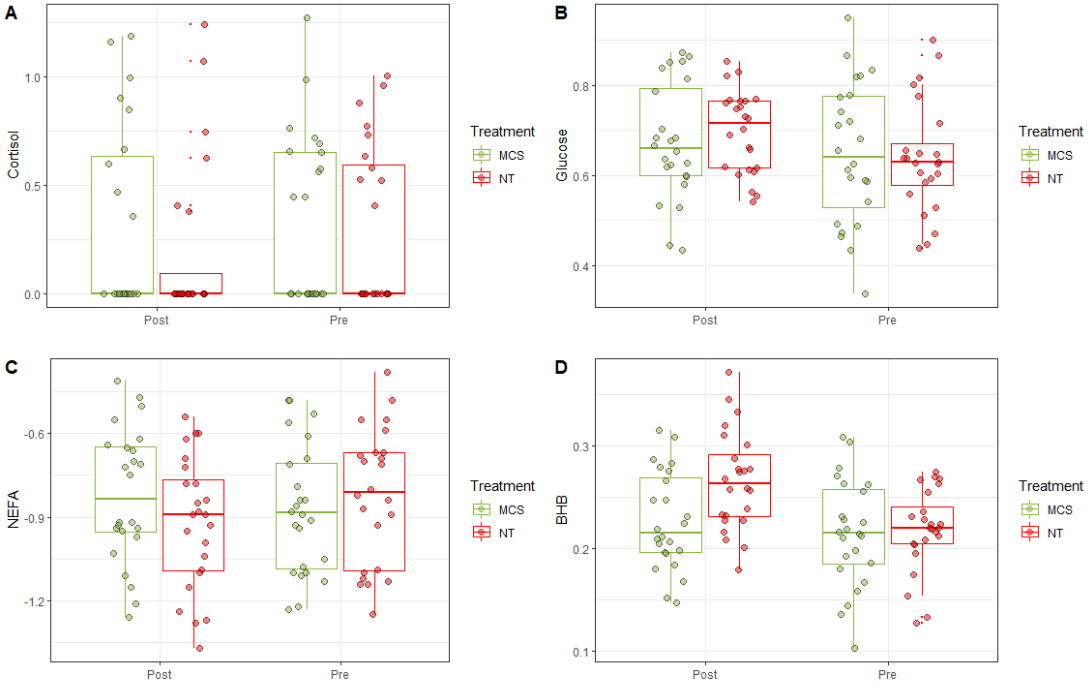

Figure 4.6 Panel A) Cortisol (ng/µl) B) glucose (g/l) C) NEFA (mmol/l) and D) BHB (mmol/l) by Experimental Stage and Treatment group. The points represent individual values by Treatment group and the boxplots include the median and IQR. Day and Interaction had a significant effect on BHB concentration (p <0.01; p = 0.02).

4.6.3 Behaviour observations- effort to escape, individual restraint

4.6.3.1 Chi-squared tests

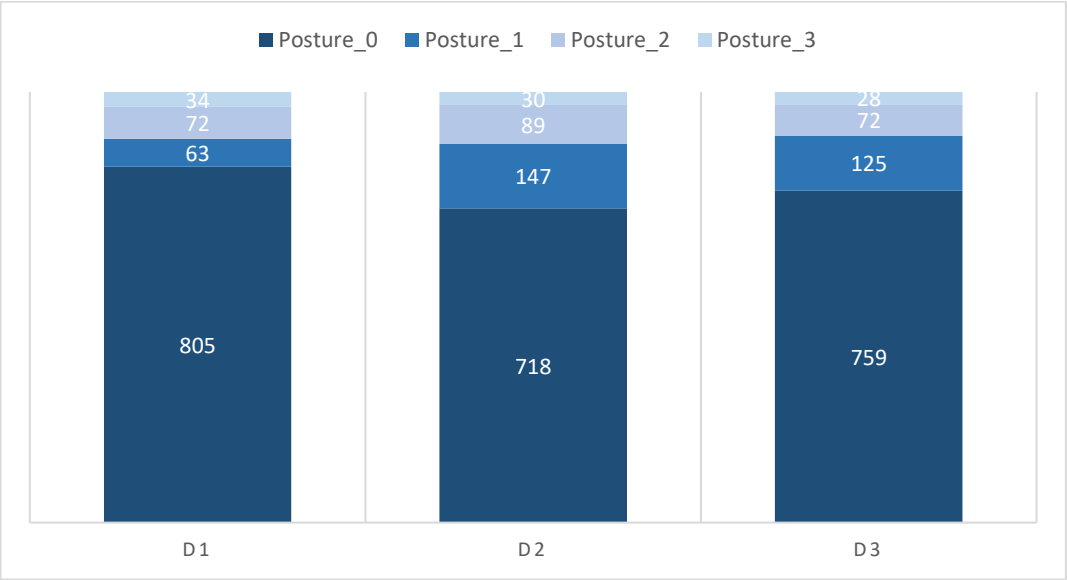

Figure 4.7 Figure representing the Sum of Postures (0, 1, 2 and 3), where posture 0: immobile, posture 1: moving head, posture 2: standing on hind legs, posture 3: vigorous effort to escape, for all animals on the three Days the Individual restraint test was performed (D1: first repetition, D2: second repetition and D3 third repetition). Observations were made every 30 seconds for each animal tested (in groups of 3).

Chi-square analysis was conducted to explore the sum of postures between days (**Figure 4.7**) and indicated a significant difference between days (chi-square = 42.10, $p < 0.01$). Post-Hoc analysis indicated significant differences between D1 and D2 ($p < 0.01$), D2 and D3 ($p < 0.01$), as well as D1 and D3 ($p < 0.01$). More specifically on D2 there is an increase again. Postures "2" and "3" remain relatively stable.

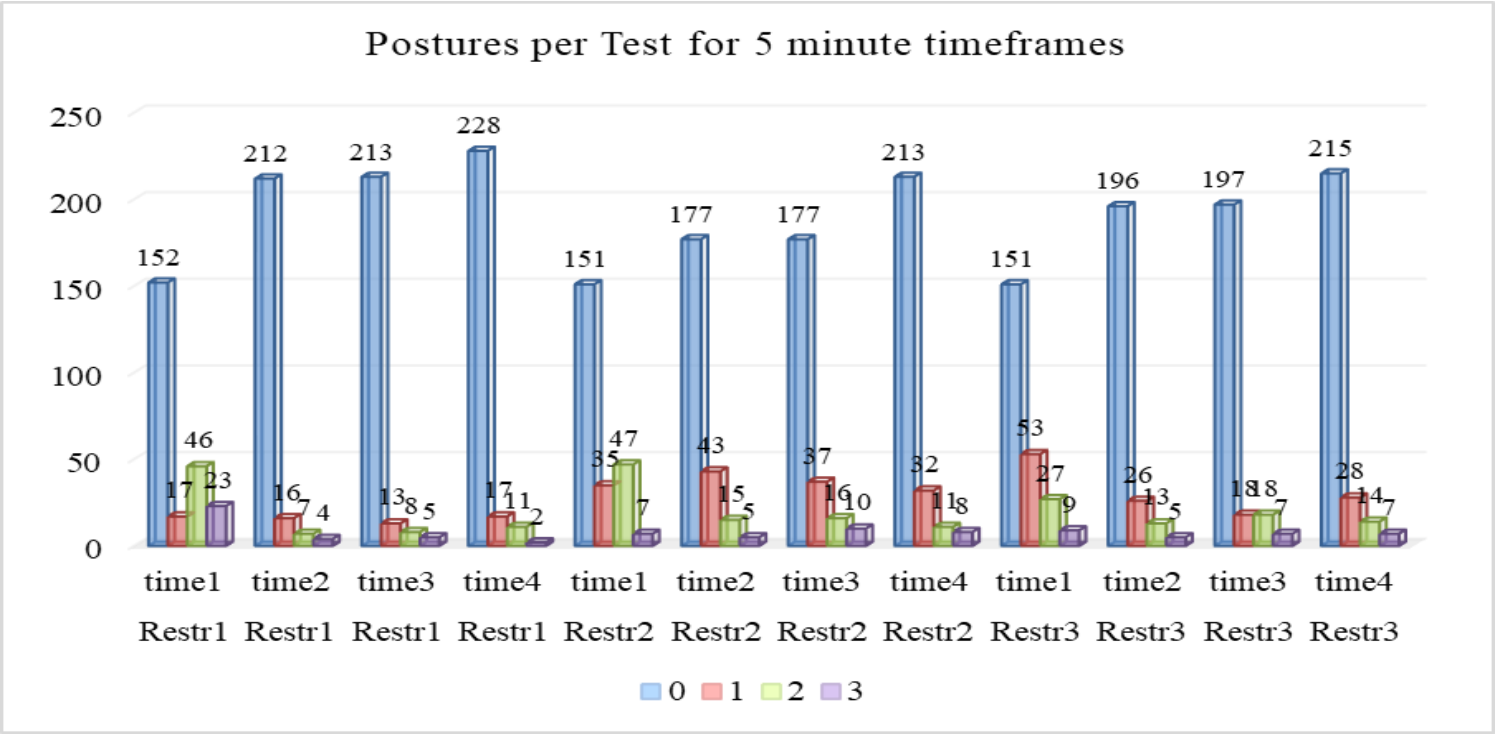

Figure 4.8 Change in animal posture over time during Gambrel restraint. Postures ("0", "1", "2", and "3") summed per 5-minute timeframes (time1, time2, time3, time4) and presented by test repetition (Restr1-Restr3).

Chi-square analysis on a timeframe level (time1: first 5 min, time2: next 5 min, time3: following 5 min and time4: last 5 min of individual restraint), indicated that for time1, there was a significant Day effect (chi-square 36.59; $p < 0.01$ between all days) (**Figure 4.8**). posture "0" was consistent, but during the first five minutes of the test an increase in posture "1" was observed for each repetition of the test followed by a decrease in posture "3".

For time 2, chi-square analysis indicated a significant difference between D1 and D2 (chi-square = 19.42, $p < 0.01$), which translates to a decrease in posture "1" and an increase in posture "2" on D2. For time3, a significant difference was observed between D1 and D2 with an increase in posture "0" and an increase in efforts to escape expressed by postures "1", "2" and "3". There was no difference observed for posture "4".

4.6.4 Behaviour observations- 24h time budgets

4.6.4.1 Eating hay

Treatment group, Experimental stage and Interaction effects were explored for several variables of "eating hay": frequency (i.e., number of observations), number of sequences performed (a sequence was considered more than 2 continuous observations), average sequence length, and average synchronisation of animals performing a specific behaviour .

Treatment group and Interaction effect had a significant effect on all parameters except frequency, where only Experimental Stage and Interaction effects were observed. More specifically, an increase of consumption of hay over time can be seen, with a drop in the Middle stage which is significantly lower than the End stage. Particularly for the NT animals at the Middle and End of the trial, a significant difference compared to earlier stages is observed. NT animals also consume hay at a lower frequency compared to the MCS animals at the Middle stage of the trial (MCS: 60.50±10.50 > NT: 54.50 ±10.30). At the End measurement of the trial, a significant drop of frequency is observed for both groups. Means and SD values are presented in **Table 4.5.**

Bouts of eating hay are reduced over time consistently for both treatment groups, whereas bout length for the NT animals is significantly longer (NT: 3.04±0.66> MCS: 2.74±0.57). Finally, NT animals are more synchronised (2.93±0.28 vs 2.70±0.36). Synchronisation increases over time but drops significantly at the End stage of the trial and this is more pronounced for the NT group (Middle NT: 2.97±0.25> End NT: 2.58±0.32).

Table 4.5 GLMM results "Eating Hay". Day, Treatment group and Interaction Effect on frequency "Eating Hay", number and average length of sequences "Eating Hay", synchronisation and average length of synchronisation bouts are reported for this behaviour. Mean and SD values are presented, as well as the p- and chi-square or F-value. N = 48 for all Experimental Stages, n = 96 for Treatment groups and n = 24 for Interaction groups. Differences are indicated with the CLD system and capitalised when p <0.01.

Frequency Eating Hay =number of scans				
		mean ±SD	**chi-square**	**p-value**
Treatment Group	NT	55.00 ±11.60^{a}	0.67	0.41
	MCS	56.10 ±10.60^{a}		
Experimental Stage	Pre	49.90 ±9.63^{B}	24.78	<0.01
	Start	57.90 ±10.80^{Abc}		
	Middle	55.50 ±11.70^{Ab}		
	End	59.20 ±10.00^{C}		
Interactions	Pre NT	56.80 ±13.60^{ac}	8.95	0.03
	Start NT	59.50 ±10.90^{ac}		
	Middle NT	54.50 ±10.30^{abc}		
	End NT	49.20 ±9.13^{Bc}		
	Pre MCS	54.20 ±9.61^{abc}		
	Start MCS	58.90 ±9.32^{a}		
	Middle MCS	60.50 ±10.50^{a}		
	End MCS	50.50 ±10.30^{BC}		
Bouts Eating Hay				
Factor	**Mean ±SD**	**mean ±SD**	**chi-square**	**p-value**
Treatment Group	NT	20.11 ±3.98^{A}	15.90	<0.01
	MCS	18.90 ±3.93^{B}		
Experimental Stage	Pre	19.8 ±4.36^{a}	11.93	<0.01
	Start	20.4 ±4.26^{b}		

	Middle	20.0 ±3.32[ab]		
	End	18.4 ± 3.89[c]		
Interactions	Pre NT	22.40 ±3.70[a]	10.95	<0.01
	Start NT	21.70 ±4.10[a]		
	Middle NT	19.30 ±3.42[ac]		
	End NT	17.30 ±3.14[bc]		
	Pre MCS	18.40 ±3.49[c]		
	Start MCS	19.20 ±4.14[ac]		
	Middle MCS	20.80 ±3.11[ac]		
	End MCS	18.50 ±4.32[bc]		
Bout length Eating Hay				
		mean ±SD	**F-value**	**p-value**
Treatment Group	NT	3.04 ±0.66[a]	6.15	0.01
	MCS	2.74 ±0.57[b]		
Interactions	Pre NT	2.55 ±0.56[a]	5.52	0.02
	Start NT	2.80 ±0.58[a]		
	Middle NT	2.87 ±0.61[a]		
	End NT	2.72 ±0.52[a]		
	Pre MCS	3.19 ±0.62[b]		
	Start MCS	3.15 ±0.56[b]		
	Middle MCS	2.98 ±0.66[a]		
	End MCS	2.84 ±0.75[a]		
Average Synchronisation				
Factor		**mean ±SD**	**F-value**	**p-value**
Treatment Group	NT	2.93 ±0.28[A]	16.01	<0.01
	MCS	2.70 ±0.36[B]		
Experimental Stage	Pre	2.78 ±0.39[a]	5.47	<0.01
	Start	2.86 ±0.35[b]		

	Middle	2.91 ±0.25^{c}		
	End	2.72 ±0.33^{d}		
Interactions	Pre NT	2.55 ±0.33^{a}	12.91	<0.01
	Start NT	2.70 ±0.38^{ac}		
	Middle N	2.97 ±0.23^{bcd}		
	End NT	2.58 ±0.32^{a}		
	Pre MCS	3.02 ±0.30^{bd}		
	Start MCS	3.01 ±0.24^{bd}		
	Middle MCS	2.84 ±0.26^{cd}		
	End MCSb	2.86 ±0.28^{cd}		

4.6.4.2 Eating concentrates

Frequency of "eating concentrates" behaviour was significantly influenced by Interaction effects between Treatment and Experimental Stage (Type II Wald Test, chi-square = 8.71; p <0.01) (**Figures 4.9-4.10**). Interaction effects were also observed for average synchronisation (chi-square = 8.43; p <0.01). Treatment differences were observed with MCS animals demonstrating lower frequency (13.50±5.26<15.50±5.93 NT). There is a drop of frequency from the Pre-trial measurement (14.90±5.15) compared to the Start (12.80±4.13) and End measurement (18.20±6.08). NT animal values at the Pre-trial stage are higher compared to the Start and Middle of the trial, and do not differ compared to the End of the trial. For the MCS animals, a consistent and sharp increase can be observed at the End of the trial. Additionally, regarding synchronisation at the Middle measurement, animals appear to be more synchronised. Means and SD are reported for all observations in **Table 4.6**.

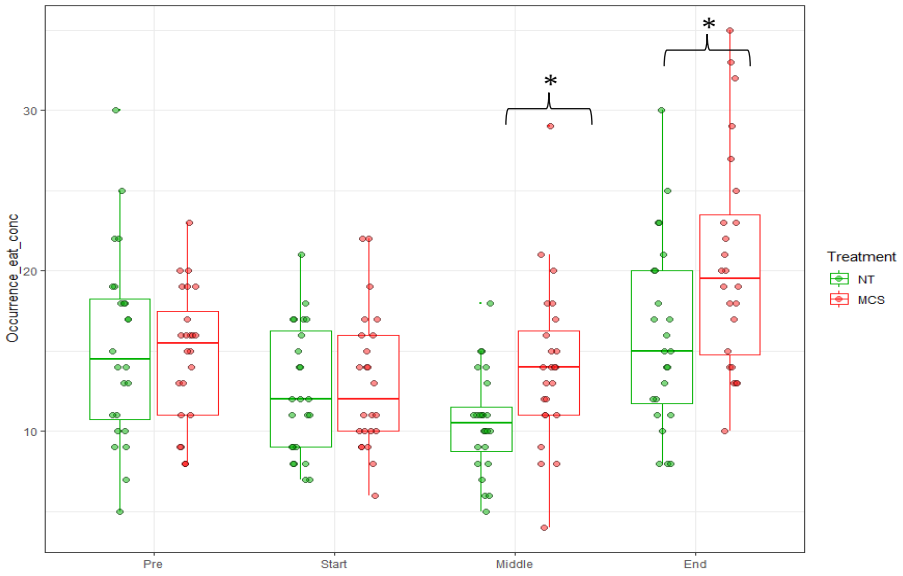

Figure 4.9 Frequency or Occurrence of "eating concentrates" behaviour by Experimental Stage (Pre-trial, Start, Middle and End of Trial), measured in 24h Time budgets with 5min intervals. There was a significant Treatment Group, Experimental Stage and Interaction effect ($p < 0.01$). The points represent individual values by Treatment and the boxplots include the median and IQR.

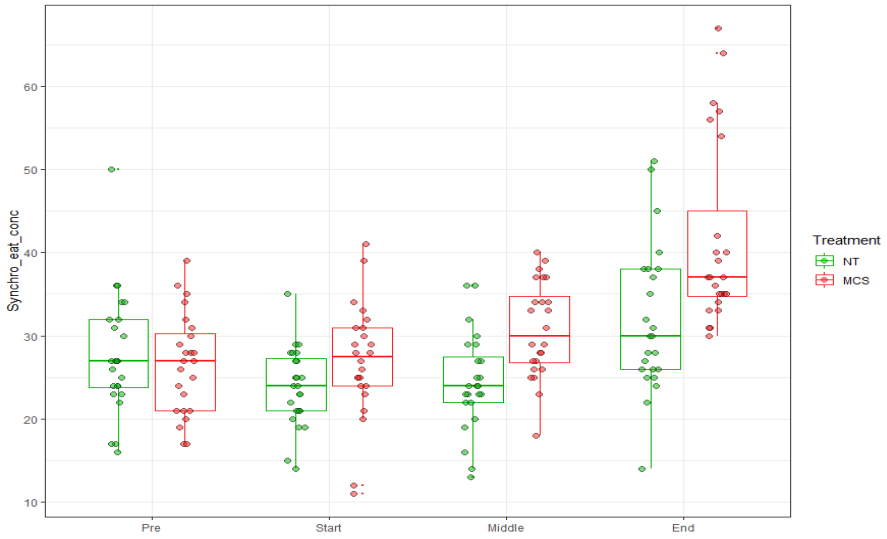

Figure 4.10 Number of times animals were synchronised in "eating concentrates" bouts by Experimental Stage (Pre-trial, Start, Middle and End of Trial), measured in 24h time budgets with 5min intervals. There was a significant Experimental Stage effect ($p < 0.01$). The points represent individual value by Treatment and the boxplots include the median and IQR.

Table 4.6 Model results for Frequency and Synchronisation of "Eating Concentrates". P-values, chi-square or F-values are reported according to the model used, as well as mean ±SD. N = 48 for all Experimental Stages, n = 96 for Treatment groups and n = 24 for Interaction groups. Differences are indicated with the CLD system and capitalised when p <0.01.

Frequency of Eating Concentrates				
		mean ±SD	**chi- square**	**p-value**
Treatment Group	NT	15.60 ±5.93[a]	8.17	<0.01
	MCS	13.50 ±5.26[b]		
Experimental Stage	Pre	14.90 ±5.15[a]	52.48	<0.01
	Start	12.80 ±4.13[b]		
	Middle	12.30 ±4.54[b]		
	End	18.20 ±6.68[c]		
Interactions	Pre NT	15.20 ±5.96[ad]	13.53	<0.01
	Start NT	12.50 ±4.02[de]		
	Middle NT	10.50 ±3.16[e]		
	End NT	15.90 ±5.77[ac]		
	Pre MCS	14.60 ±4.31[acd]		
	Start MCS	13.00 ±4.30[ade]		
	Middle MCS	14.00 ±5.07[acd]		
	End MCS	20.50 ±6.83[b]		
Average Synchronisation Eating Concentrates				
	Factor	**mean ±SD**	**F-value**	**p-value**
Treatment Group	NT	0.95 ±0.0005[a]	0.0002	0.99
	MCS	0.97 ±0.03[a]		
Experimental Stage	Pre	1.93 ±0.48[a]	9.34	<0.01
	Start	2.09 ±0.51[a]		
	Middle	2.40 ±0.61[b]		
	End	2.12 ±0.49[a]		

4.6.4.3 Resting

GLMM analysis indicated that for "resting" behaviour, the number of sequences performed was significantly influenced by Interactions between Treatment group and Experimental Stage effects (Type II Wald Test, chi-square = 5.16; p = 0.02).No differences were observed after post-Hoc analysis using Least Square Means for Multiple Comparisons performed with Tukey correction.

The average length of these sequences was influenced by Treatment group (NT: n = 96; mean = 4.10 ±0.73 and MCS: n = 96, mean = 4.37 ±0.76; chi-square = 6.21; p = 0.01). Treatment group had an effect animals' average synchronisation (NT: n = 96; mean = 3.37 ±0.19 and MCS: n = 96, mean = 3.52 ±0.17; chi-square = 16.06; p <0.01, respectively).

4.6.4.4 Sleeping

Experimental Stage influenced average synchronisation of "sleeping" behaviour (Type II Wald Test, chi-square = 17.25; p <0.01) (**Figure 4.11**). Similarly, the average length of synchronisation for this behaviour was influenced by Experimental Stage (Type II Wald Test, chi-square = 8.14; p <0.01). At the Middle observations, synchronisation significantly increases and from figure 4.20, this appears to be driven by the NT group's behaviour. P-values and mean ± SD are presented in **Table 4.7**.

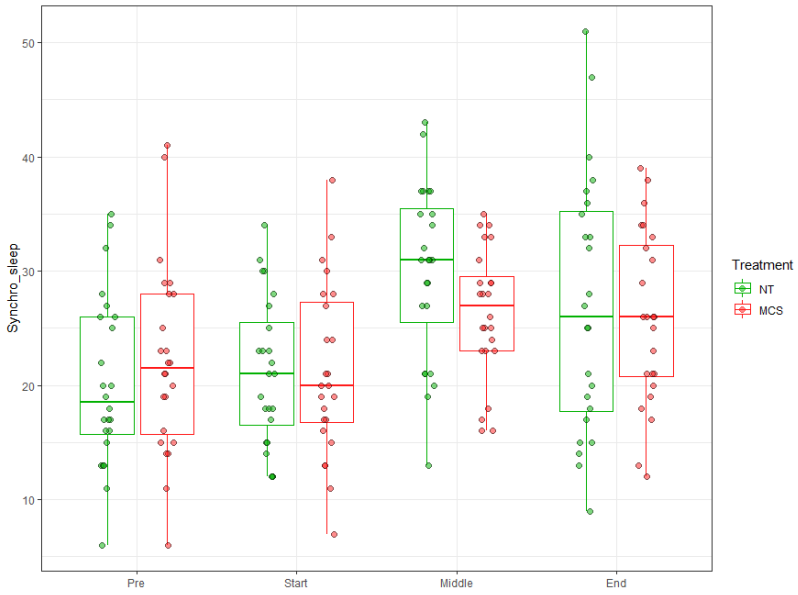

Figure 4.11 Average synchronisation for "sleeping" by Experimental Stage (Pre-trial, Start, Middle and End of trial), measured in 24h Time budgets with 5min intervals. There was a significant Experimental stage effect (p <0.01 between all Stages). The points represent individual values by Treatment and the boxplots include the median and IQR.

Table 4.7 Model results for Synchronisation of "Sleeping". P-values, F-values and mean ±SD are reported. N = 48 for all Experimental Stages, n = 96 for Treatment groups and n = 24 for Interaction groups. Differences are indicated with the CLD system and capitalised when p <0.01.

Average length of synchronisation bouts "Sleeping"				
		mean ±SD	**F-value**	**p-value**
Treatment Group	NT[a]	1.04 ±0.28	1.72	0.19
	MCS[a]	0.99 ±0.25		
Experimental Stage	Pre	0.91 ±0.25[a]	8.34	<0.01
	Start	0.91 ±0.27[a]		
	Middle	1.13 ±0.24[b]		
	End	1.08 ±0.21[a]		

4.6.4.5 Moving

GLMM analysis indicated that for "Moving", average synchronisation was influenced by Treatment and Interaction with Experimental Stage (Type II Wald Test, chi-square = 5.14; p = 0.02; chi-square = 5.15; p <0.01, respectively) (**Figure 4.12**). For this behaviour, there were significant effects of Treatment Group before treatments were actually imposed MCS > NT (0.94±0.48> 0.47±0.35, p <0.01), which evens out progressively. At the Middle stage, NT movement synchronisation is higher compared to MCS animals (0.61 ±0.32>0.53 ±0.53, p<0.01) but this does not persist until the End stage of the trial (NT: 0.44 ±0.36; MCS: 0.41 ±0.33).

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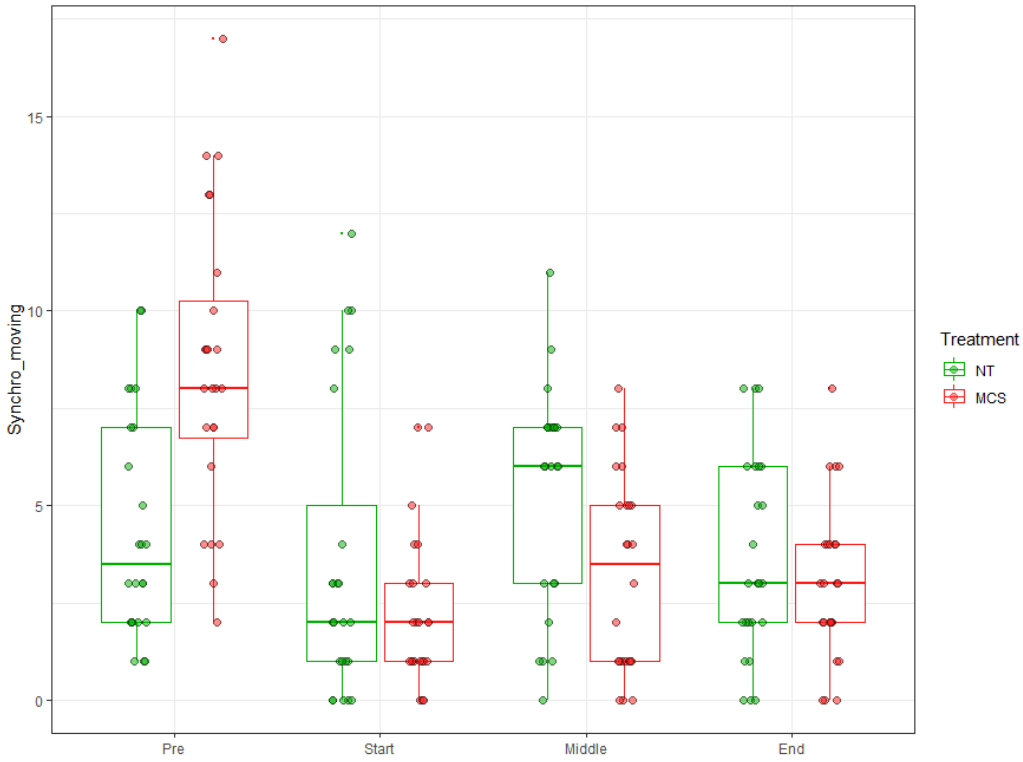

Figure 4.12 Synchronisation of "Moving" by Experimental Stage (Pre-trial, Start, Middle and End of Trial), measured in 24h Time budgets with 5min intervals. There was a significant Experimental Stage effect (p <0.01 between all Stages). The points represent individual values by Treatment group and the boxplots include the median and IQR.

4.6.4.6 Immobile

GLMM analysis indicated that the frequency of "Immobile" behaviour was significantly influenced by Experimental Stage (Type II Wald Test, chi-square = 5.73; p = 0.02) **(Figures 4.15 -4.16**). There was also an Experimental Stage and Interaction effect on the average synchronisation for this behaviour (chi-square = 5.05; p <0.01; chi-square = 5.15; p <0.01, respectively). With regards to this behaviour, animals remained immobile with a significantly higher frequency at the End of the trial compared to the previous stages (p<0.01). While NT animals' synchronisation increases over time, for MCS the opposite is true, as particularly in the Middle stages and the End measurements, synchronisation is significantly lower compared to NT, as well as the Pre-trial measurements recorded for the MCS animals. In **Table 4.8** below, post-hoc pairwise analysis p-values and mean ± SD are reported.

Table 4.8 Model results for Frequency and Synchronisation of "Immobile" behaviour. P-values, chi/F-values and mean ±SD are reported. N = 48 for all Experimental Stages, n = 96 for Treatment groups and n = 24 for Interaction groups. Differences are indicated with the CLD system and capitalised when p <0.01.

Frequency Immobile				
Factor		**mean ±SD**	**chi-square**	**p-value**
Treatment Group	NT	25.00 ±8.15[a]	1.47	0.15
	MCS	24.40 ±7.69[a]		
Experimental Stage	Pre	23.90 ±7.07[a]	3.08	<0.01
	Start	22.80 ±6.92[a]		
	Middle	24.30 ±9.92[a]		
	End	27.80 ±9.40[b]		
Synchronisation bouts immobile				
Factor		**mean ±SD**	**F-value**	**p-value**
Treatment Group	NT	1.79 ±0.41[a]	5.23	0.02
	MCS	1.62 ±0.37[b]		
Experimental Stage	Pre	1.59 ±0.35[a]	3.25	0.02
	Start	1.75 ±0.35[b]		

	Middle	1.75 ±0.45[b]		
	End	1.74 ±0.45[ab]		
Interaction	Pre NT	1.53 ±0.33[a]	10.76	<0.01
	Start NT	1.77 ±0.40[a]		
	Middle NT	2.03 ±0.34[b]		
	End NT	1.85 ±0.45[b]		
	Pre MCS	2.03 ±0.34[c]		
	Start MCS	1.85 ±0.45[b]		
	Middle MCS	1.46 ±0.37[d]		
	End MCS	1.64 ±0.42[a]		

4.6.5 Behaviour observations- ball best

4.6.5.1 Ball Test-Home Pen

4.6.5.1.1 Latencies

4.6.5.1.1.1 Latency to interact with the ball

A significant Treatment effect [NT (n = 24): 117 ± 279 sec, MCS (n = 24): 603 ± 666 sec) was observed on latency of lambs to "Interact with the ball" (chi-squared =14.48; p <0.01), indicated in **Figure 4.13** below.

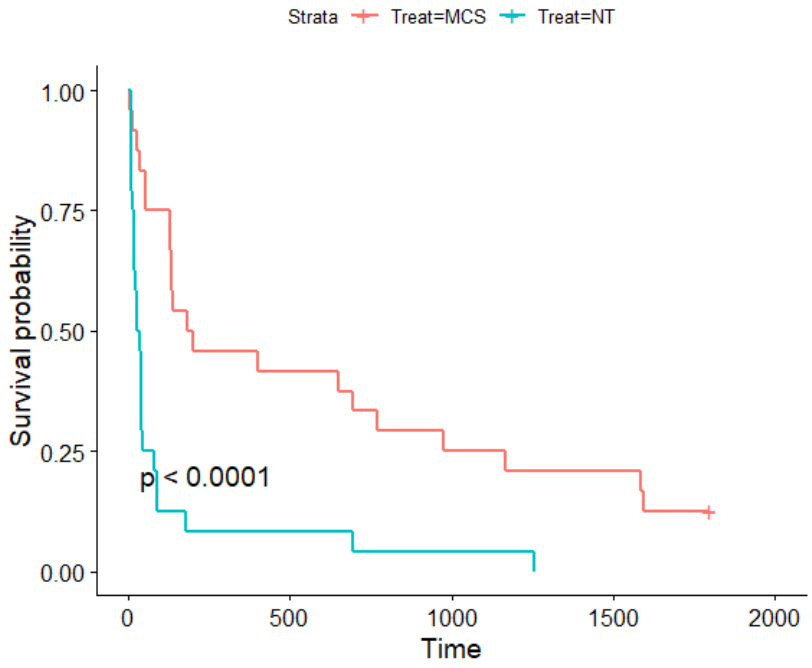

Figure 4.13 Survival Probability plot presenting the latency to approach the ball (sec). Survival Probability describes the probability of the animal approaching the ball faster or slower. The Red line represents the Probability over time for the MCS animals and the blue line the probability for the NT animals. MCS animals were significantly slower to interact with the ball p<0.01).

4.6.5.1.1.2 Latency to eat hay

Cox Survival Analysis indicated a significant Treatment effect [NT (n =24): 552 ± 350 sec, MCS (n =24): 964 ± 527sec) on the latency to "Eat Hay" (chi-squared = 5.75; p = 0.01), indicated in **Figure 4.14** below.

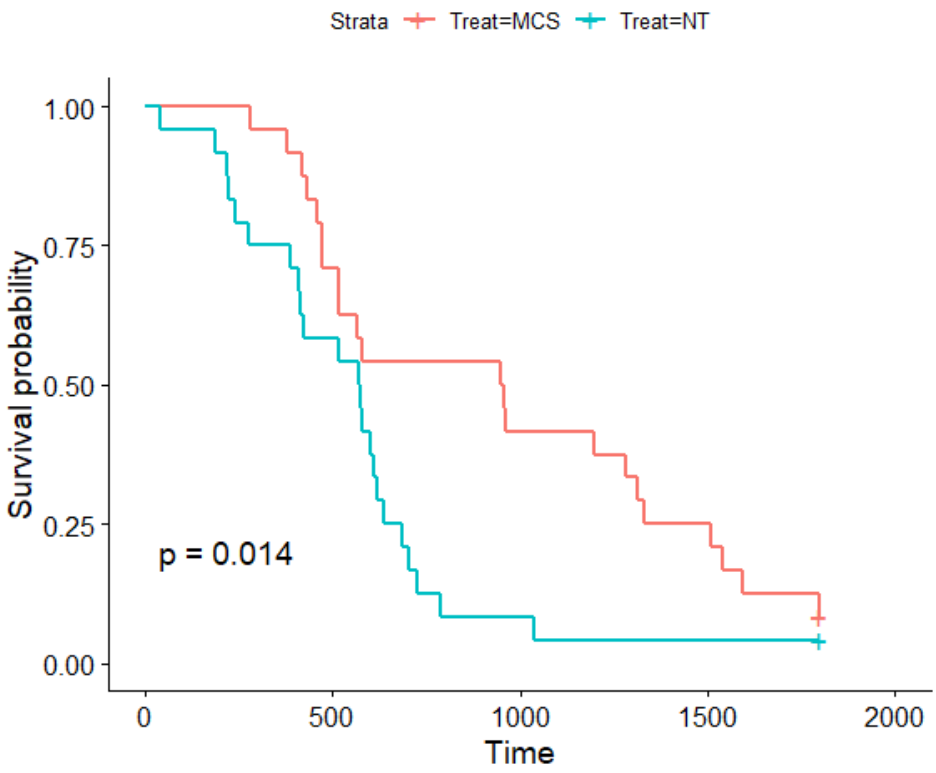

Figure 4.14 Survival Probability plot presenting the latency to eat hay (sec). Survival Probability describes the probability of the animal performing the behaviour "Eating Hay" faster or slower. The red line represents the Probability over time for the MCS animals and the blue line the probability for the NT animals. MCS animals were significantly slower to perform this activity (p = 0.01).

4.6.5.1.2 Duration and frequency

Wilcoxon Rank Sum tests for the duration of "Contact/Interaction with Ball", and "Interaction with the environment". ANOVA analysis for the duration of "Eating Hay" indicated a significant Treatment effect on all behaviours, apart from "Interaction with the Environment". Details are presented in **Table 4.9**.

Table 4.9 Results from the Wilcoxon Rank Sum and ANOVA tests conducted on the effect of Treatment (NT, n =24; MCS, n =24) on the Frequency and Duration of "Eating Hay", "Contact with Ball", "Interaction with Ball" and "Interaction with the Environment" behaviours. Significant results are presented by reporting mean ± SD by Treatment and the F/W and p-value.

DURATION			
Eating Hay			
Treatment	**mean ± SD**	**F-value**	**p-value**
NT	83.40 ±83.10	11.07	<0.01
MCS	231 ±213		NT <MCS
Contact Ball			
Treatment	**mean ± SD**	**W**	**p-value**
NT	722 ±91.50	143	<0.01
MCS	218 ±360		NT >MCS
Interaction with Ball			
Treatment	**mean ±SD**	**W**	**p-value**
NT	361 ±260	160	0.01
MCS	199 ±254		NT >MCS
FREQUENCY			
Contact Ball			
Treatment	**mean ±SD**	**W**	**p-value**
NT	10.80 ±8.96	425	<0.01
MCS	6.17 ±9.59		NT >MCS
Interaction with Ball			
Treatment	**means ±SD**	**W**	**p-value**
NT	23.30 ±13.00	402.5	0.02
MCS	15.90 ±15.50		NT >MCS

4.6.5.2 Ball Test-Novel Pen

Cox Survival Regression Analysis was conducted on the effect of Treatment, repetition of test (Day: D1, D2, D3, D4, where D1 - D3 and D2 - D4 the same pens were tested) and potential interactions. Significant results for Latency to Interact with the ball, and "Contact/Interaction with the environment" post ball drop, as well as Duration and Frequency of "Vigilance", "Contact/Interaction with ball" and "Contact/Interaction with the Environment" pre- (where relevant) and post- ball drop are presented in **Tables 4.10 -4.14**.

MCS animals took longer to interact with the ball (358±513> 151±335), on D2 animals took longer to interact with the ball, whereas on D4 they took the longest.

Regarding their contact and interaction with the environment it appears to be day dependent (which also relates to the groups tested on a given day).

Duration of vigilance was higher pre-ball drop for the NT animals (478.00± 330.00 ± 149.00, $p < 0.01$).

Pre ball drop interaction and contact with the environment was higher for the MCS animals and this was also day dependant, as contact with the environment increased significantly until D3 but dropped on D4 again. Frequency of vigilance was day dependent (higher on the two compared to D3 and D4) and particularly the group of MCS animals tested on D2 had higher vigilance frequency compared to D4 (same group of animals, 112.00± 29.90 > 67.80± 24.80, $p< 0.01$) and yet again there was a day effect. this is more pronounced comparing NT animals and MCS animals on D1 (8.67± 7.50< 18.80± 8.90) and D4 (11.60± 8.00< 25.80± 10.50).

Post ball drop vigilance duration is day dependent and increases significantly overtime for the NT animals (544.00± 192.00> 250.00± 100.00, $p< 0.01$). Frequency of vigilance was higher for MCS animals on D1 (59.30± 22.10> 48.10± 19.30) and for NT animals on D4 (82.00± 19.00> 59.50± 19.70). NT animals came into contact with the ball more than the MCS group (52. 60± 56.90> 19.70± 33.80) and all animals came into contact with the ball on D1 (60.80± 52.10) but this significantly decreases over D2, D3 and D4. On the contrary MCS animals come into contact with the environment more (104.00± 4.80> 70.10± 38.40, $p< 0.01$) and this behaviour is significantly higher for all animals on D2 ($p< 0.01$). frequency of contacting the environment is higher for NT animals overall, but frequency of interaction is higher for MCS animals.

NT animals come into contact with the ball more frequently (16.60± 12.00 vs 9.42± 14.30 call, $p< 0.01$) in day dependant manner for all animals. interaction with the ball is also day dependent and D1> D3 and D2>D4.

Table 4.10 Cox Survival Regression Analysis results for Treatment, Day and Interaction Effects on Latencies (sec) to Interact with the ball and come into Contact or Interact with the Environment post ball drop. Mean and SD values are presented for each Day (D1, D2, D3, D4, n = 24 for each) Treatment group (NT, n = 24; MCS, n = 24) and Interaction (n = 12 for each subgroup) where significant, as well as the p/chi squared values (from the type III Wall-test for the Poisson model) reported from the GLMM Model. Differences are indicated with the CLD system and capitalised when p <0.01.

Latency				
Interaction with ball				
Factor		**mean ± SD**	**F-value**	**p-value**
Treatment Group	NT	151 ±335[a]	4.44	0.04
	MCS	358 ±513[b]		
Day	D1	158 ±414[a]	4.60	0.03
	D2	221 ±340[b]		
	D3	194 ±307[ab]		
	D4	441 ±617[c]		
Interaction with environment				
Factor		**mean ± SD**	**F-value**	**p-value**
Treatment Group	NT	410 ±448	2.29	0.13
	MCS	300 ±281		
Interaction	NT D1	232 ±129[ab]	4.90	<0.01
	NT D2	466 ±456[c]		
	NT D3	142 ±108[b]		
	NT D4a	362 ±190[c]		
	MCS D1	626 ±496[d]		
	MCS D2	366 ±270[c]		
	MCS D3	519 ±616[d]		
	MCS D4	130 ±90.30[b]		
Contact with Environment				
Factor		**mean ± SD**	**F-value**	**p-value**

Treatment group	NT	164 ±171	0.42	0.51
	MCS	144 ±121		
Day	D1	187 ±183[a]	5.58	0.02
	D2	181 ±156[a]		
	D3	151 ±144[b]		
	D4	95.50 ±78.50[c]		

Table 4.11 GLMM results for duration of vigilance and contact/interaction with environment pre ball drop in the novel pen. Treatment group (NT, n = 24; MCS, n = 24), Day (D1, D2, D3, D4, n = 24 for all) and interaction effects (n = 12 for each subgroup) were explored. Mean ± SD values, p- and chi-or F-values are reported. Differences are indicated with the CLD system and capitalised when p <0.01.

Duration pre–Ball drop				
Vigilance				
Factor		**mean ± SD**	**chi-square**	**p-value**
Treatment Group	NT	478 ±147[A]	24.42	<0.01
	MCS	330 ±149[B]		
Interaction with environment				
Factor		**mean ± SD**	**chi-square**	**p-value**
Treatment Group	NT	104 ±48.80[A]	15.50	<0.01
	MCS	147 ±56.80[B]		
Day	D1	161 ±74.60[a]	72.18	<0.01
	D2	148 ±38.50[a]		
	D3	97.30 ±42.50[b]		
	D4	94.20 ±28.20[b]		
Contact with environment				

Factor		mean ± SD	F-value	p-value
Treatment Group	NT	39.50 ±40.90[A]	7.90	<0.01
	MCS	70.10 ±48.30[B]		
Day	D1	37.30 ±33.00[a]		<0.01
	D2	49.60 ±41.00[bd]		
	D3	75.50 ±56.70[c]		
	D4	57.00 ±48.60[d]		

Table 4.12 GLMM with Poisson regression results for (NT, n = 48; MCS, n = 48), Day (D1, D2, D3, D4, n = 24 for all) and Interaction Effects (n = 12 for each subgroup) on frequency to contact or interact with the environment and express "vigilance" behaviour pre ball drop. Mean and SD values are presented for each treatment, day and interaction when differences were significant, as well as the P and chi- squared values reported from the GLMM. Differences are indicated with the CLD system and capitalised when p <0.01.

Frequency pre ball drop				
Vigilance				
Factor		**mean ± SD**	**chi- square**	**p-value**
Treatment Group	NT	106.00 ±27.20	0.14	0.70
	MCS	91.50 ±30.70		
Days	D1	98.10 ±23.30[ac]	9.84	0.02
	D2	115.00 ±27.90[a]		
	D3	88.70 ±29.00[bc]		
	D4	93.00 ±32.80[c]		
Interaction	D1 NT	99.50 ±21.40[a]	31.25	<0.01
	D1 MCS	96.70 ±26.00[a]		

	D2 NT	117 ±26.80[a]		
	D2 MCS	112 ±29.90[a]		
	D3 NT	87.60 ±31.90[a]		
	D3 MCS	89.80 ±27.20[a]		
	D4 NT	118 ±15.80[a]		
	D4 MCS	67.80 ±24.80[b]		
Contact with Environment				
Factor		**mean ± SD**	**chi- square**	**p-value**
Treatment Group	NT	48.80 ±19.10[A]	26.18	<0.01
	MCS	73.50 ±20.10[B]		
Day	D1	68.90 ±30.70[ac]	35.00	<0.01
	D2	70.60 ±18.70[a]		
	D3	49.50 ±19.70[bc]		
	D4	55.50 ±14.20[c]		
Interaction with Environment				
Factor		**mean ± SD**	**chi-square**	**p-value**
Treatment Group	NT	13.10 ±9.60[A]	11.31	<0.01
	MCS	21.70 ±10.50[B]		
Day	D1	13.70 ±9.50[ac]	35.73	<0.01
	D2	16.50 ±10.20[a]		
	D3	20.70 ±11.40[b]		
	D4	18.70 ±11.70[bc]		
Interaction	D1 NT	8.67 ±7.50[a]	26.06	<0.01
	D2 NT	14.50 ±9.40[abc]		
	D3 NT	17.80 ±11.50[bc]		
	D4 NT	11.60 ±8.00[ab]		
	D1 MCS	18.80 ±8.90[bc]		
	D2 MCS	18.60 ±10.90[b]		

	D3 MCS	23.60 ±11.00^{bc}		
	D4 MCS	25.80 ±10.50^{c}		

Table 4.13 GLMM results for reatment, Day and interaction effects on duration (sec) to contact or interact with the ball and the environment and express "vigilance" behaviour post ball drop. Mean and SD values are presented for each are presented for each Treatment (NT, n = 48; MCS, n = 48), Day (D1, D2, D2, D4, n = 24 for all) and Interaction for significant differences (n = 12 for subgroups), as well as the p and F-values, reported from the GLMM. Differences are indicated with the CLD system and capitalised when p <0.01.

Duration post ball drop				
Vigilance				
Factor		**mean ± SD**	**chi-square**	**p-value**
Treatment Group	NT	354 ±182	0.90	0.30
	MCS	224 ±108		
Day	D1	251 ±123^{a}	48.18	<0.01
	D2	258 ±128^{a}		
	D3	251 ±128^{a}		
	D4	397 ±212^{b}		
Interaction	D1 NT	227 ±135^{a}	25.96	<0.01
	D2 NT	327 ±124^{ab}		
	D3 NT	318 ±110^{ab}		
	D4 NT	544 ±192^{b}		
	D1 MCS	276 ±109^{ab}		
	D2 MCS	189 ±918^{a}		
	D3 MCS	183 ±111^{ab}		
	D4 MCS	250 ±100^{a}		
Contact with ball				
Factor		**mean ± SD**	**chi-square**	**p-value**
Treatment Group	NT	52.60 ±56.90^{A}	8.28	<0.01

	MCS	19.70 ±33.80[B]		
Day	D1	60.80 ±52.10[a]	9.32	<0.01
	D2	26.60 ±39.30[b]		
	D	44.20 ±61.80[c]		
	D4	13.20 ±25.70[d]		
Interaction with ball				
Factor		**mean ± SD**	**chi-square**	**p-value**
Treatment Group	NT	109.00 ±91.20	1.09	0.30
	MCS	81.90 ±98.80		
Day	D1	152.00 ±112[a]	6.94	<0.01
	D2	67.00 ±48.20[b]		
	D3	115.00 ±115[c]		
	D4	47.70 ±51.10[b]		
Contact with environment				
Factor		**mean ± SD**	**F-value**	**p-value**
Treatment Group	NT	70.10 ±38.40	14.33	<0.01
	MCS	104.00 ±4.80		
Day	D1	90.30 ±48.00[ab]	15.55	<0.01
	D2	113.00 ±8.20[b]		
	D3	75.10 ±32.00[a]		
	D4	69.50 ±33.40[a]		

Table 4.14 GLMM with Poisson regression results for (NT, n = 48; MCS, n = 48), Day (D1, D2, D3, D4, n = 24 for all) and Interaction Effects (n = 12 for each subgroup) on frequency to contact or interact with the ball/environment and express "Vigilance" behaviour post ball drop. Mean and SD values are presented for each Treatment, Day and Interaction when differences were significant, as well as the p- and chi- squared values, reported from the GLMM. Differences are indicated with the CLD system, significance forp <0.05.

Frequency Post Ball Drop				
Vigilance				
Factor	**mean ±SD**	**p-value**	**chi- square**	**p-value**
Treatment Group	NT	61.90 ±21.70	1.76	0.19
	MCS	56.80 ±22.20		
Day	D1	53.70 ±21.10[a]	42.02	<0.01
	D2	60.80 ±20.40[b]		
	D3	51.90 ±20.30[a]		
	D4	71.00 ±22.00[c]		
Interaction	D1 NT	48.10 ±19.30[a]	2705	<0.01
	D1 MCS	59.30 ±22.10[b]		
	D2 NT	63.20 ±16.20[b]		
	D2 MCS	58.20 ±24.30[b]		
	D3 NT	54.30 ±17.10[ab]		
	D3 MCS	49.50 ±23.50[ab]		
	D4 NT	82.00 ±19.00[c]		
	D4 MCS	59.50 ±19.70[b]		
Contact with Environment				
Factor		**mean ±SD**	**chi- square**	**p-value**
Treatment Group	NT	23.30 ±13.00[A]	36.42	<0.01
	MCS	15.90 ±15.50[B]		
Interaction with Environment				
Factor		**mean ±SD**	**chi- square**	**p-value**
Treatment Group	NT	10.80 ±9.63[A]	9.73	<0.01
	MCS	16.60 ±8.78[B]		
Contact with Ball				
Factor		**mean ±SD**	**chi- square**	**p-value**
Treatment Group	NT	16.60 ±12.00[A]	3.14	<0.01

	MCS	9.42 ±14.30[B]		
Day	D1	21.90 ±15.60[a]	27.83	<0.01
	D2	9.62 ±10.00[bc]		
	D3	15.80 ±14.30[c]		
	D4	4.79 ±6.67[b]		
Interaction with Ball				
Factor		**mean ±SD**	**chi- square**	**p-value**
Treatment Group	NT	18.10 ±10.90	2.57	0.11
	MCS	16.00 ±17.10		
Day	D1	25.60 ±13.50[a]	29.11	<0.01
	D2	13.90 ±8.25[bc]		
	D3	19.10 ±16.10[b]		
	D4	9.58 ±8.04[c]		

4.6.6 Heart Rate individual restraint test

Investigation of Day effects on Step1 Gambrel restraint period for the three repetitions of the test, and non- stress (NS) day Step1 of the three closest (by date) days on which heart rate was monitored. Similarly, night step (22h00 -02h00 and 02h00 -06h00) differences were explored on individual restraint stress days and NS days. No significant differences were observed for any of the heart rate variables in these time frames.

4.6.7 Heart Rate; No stress days

4.6.7.1 Areas under curve

The area under the curve was computed in Excel by fitting the best polynomial equation for each heart rate variable within each day, as a crude way to explore overall daily heart rate variables by Treatment. These values are presented below in **Error! Reference source not found.**. Overall, average RR (milliseconds) fluctuated more for the NT group and the area under the curve was larger on D1, D3 compared to MCS values. Average HR (bpm) surface was generally bigger for the NT group compared to the MCS group, but for D4, where MCS HR was larger than NT HR. Additionally, values fluctuated greatly from day to day for the MCS.

Regarding RMSSD, MCS areas were larger compared to the NT group on D1 and D2, whereas on D3, NT was greater than MCS, whilst there was a decline over time for the MCS group. SDNN areas under the curve were higher for the MCS group except on D3, and for the MCS there was a decline, whereas for the NT the surface fluctuated. The ratio LF/HF was larger for the MCS animals on all days except D4. For the MCS, there was an increase on D2, D3 and a decrease on D4, whereas for the NT group there was a gradual decline up to D4, where an increase was observed.

Table 4.15 Area under the curve, computed in Excel as a result of fitting the best polynomial equation, by individual variable (RR_aver, HR_aver, RMSSD, SDNN and LF/HF) and day (D1-D4) of heart rate monitoring sessions.

AREA UNDER CURVE					
		D1	**D2**	**D3**	**D4**
RR_Aver	**MCS**	239.63	234.72	60.62	213.86
	NT	255.56	-57.64	235.82	-4.12
HR_aver	**MCS**	45.37	4.36	-8.82	12.80
	NT	8.76	5.74	1.54	3.73
RMSSD	**MCS**	45.37	-14.86	-31.46	-23.10
	NT	8.76	0.20	17.83	-16.36
SDNN	**MCS**	32.22	4.29	-22.74	-5.75
	NT	5.92	-8.96	15.90	-8.78
LF/HF	**MCS**	-0.27	0.32	0.31	-0.63
	NT	-1.13	-1.16	-1.05	0.37

4.6.8 Significant results by "step" (timeframe within each day)

4.6.8.1 Step 1 (10h00 -14h00)

Treatment group had a significant effect on the average number of RR intervals (distance between each heartbeat) (NT: 461 ± 46.3; MCS: 501 ± 53.3; n = 62; chi-squared = 6.09, model SE = 0.05, p = 0.01) (**Figure 4.15**).

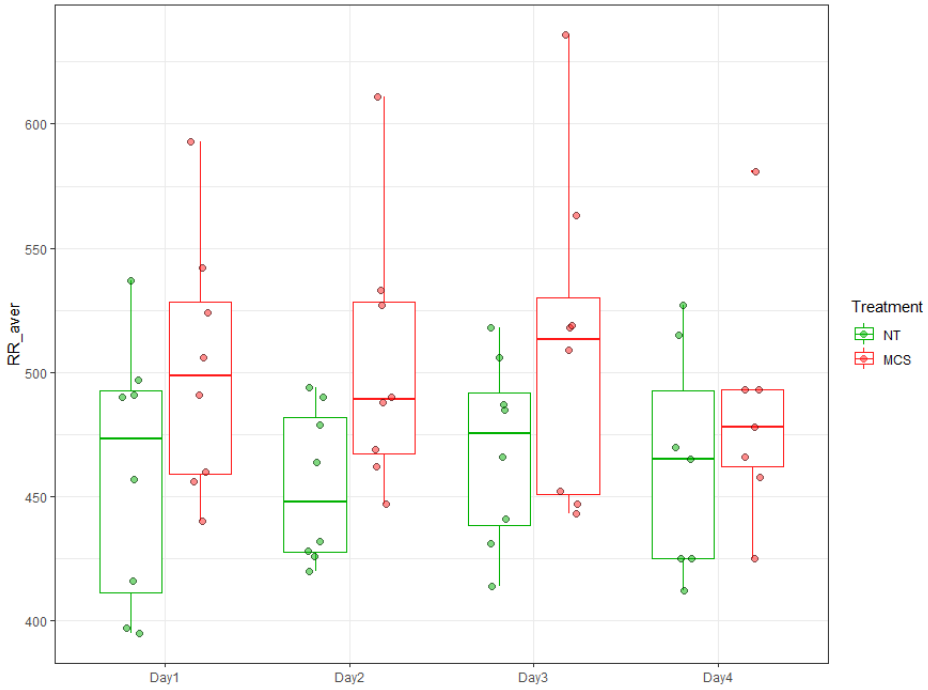

Figure 4.15 RR_average counts by Day of monitoring [Day1 (Start of trial, Day 2 (2 weeks into the trial), Day 3(4 weeks into the trial) and Day 4 (End of trial), before the Suddenness test] and Treatment group. The points represent individual values by Treatment and the boxplots include the median and IQR. Treatment had a significant effect ($p = 0.01$).

4.6.8.2 Step 2 (14h00 – 18h00)

No differences were observed for any of the heart rate variables explored (RR_aver, HR_aver, RMSSD, SDNN and LF/HF).

4.6.8.3 Step 3 (18h00 -22h00)

Treatment (NT: 555 ± 53.5; MCS: 591 ± 50.5) had a significant effect on the average number of RR intervals (n = 62; chi-squared = 6.92, model SE = 0.03, p = 0.05) (**Figures 4.16-4.17**).

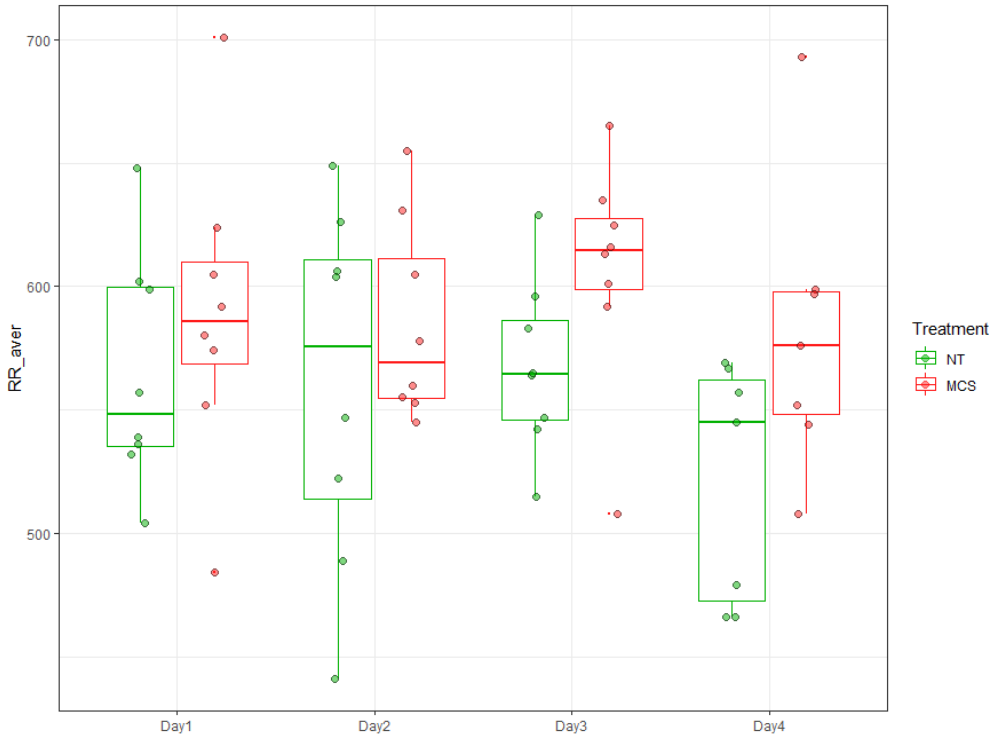

Figure 4.16 RR_average counts (milliseconds) by Day of monitoring [Day1 (Start of trial, Day 2 (2 weeks into the trial), Day 3(4 weeks into the trial) and Day 4 (End of trial), before the Suddenness test] and Treatment group. The points represent individual values by Treatment and the boxplots include the median and IQR. Treatment had a significant effect (p = 0.05).

Day (D1: mean = 81.20 ± 17.30; D2: mean = 73.10 ± 15.40; D3: mean = 78.00 ± 16.00; D4: mean = 69.8 ± 17.20) had a significant effect on SDDN (n = 62; chi-squared = 4.78, model SE = 1.20, p = 0.03).

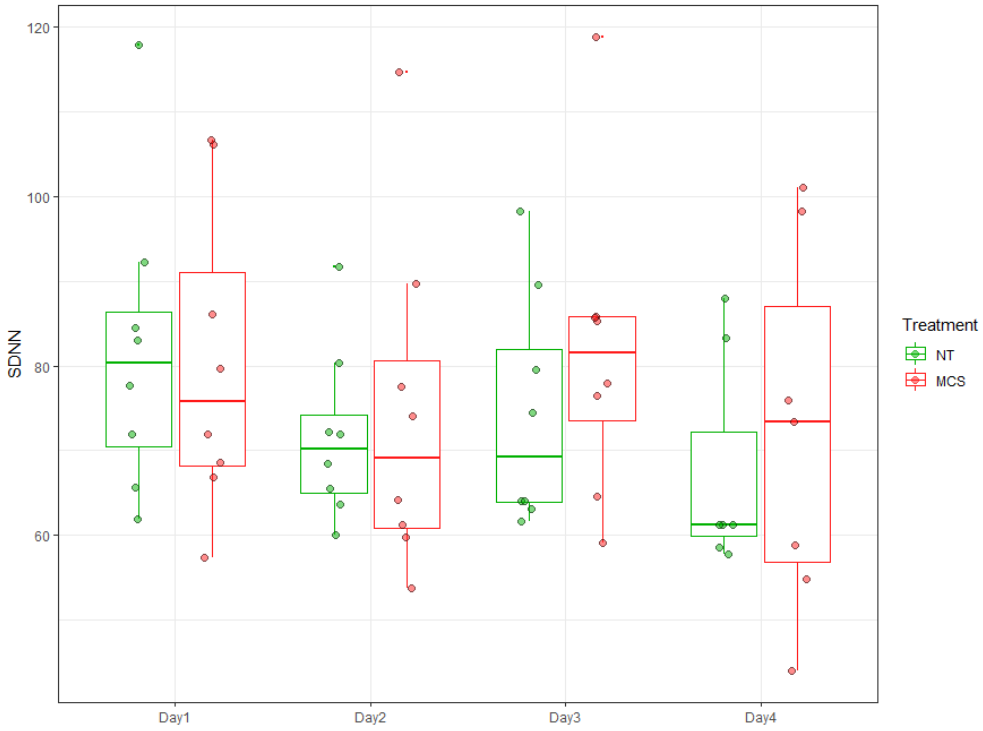

Figure 4.17 SDNN by Day of monitoring [Day1 (Start of trial, Day 2 (2 weeks into the trial), Day 3(4 weeks into the trial) and Day 4 (End of trial), before the Suddenness test] and Treatment group. The points represent individual values by Treatment and the boxplots include the median and IQR. Day had a significant effect (p = 0.03).

4.6.8.4 Step 4 (22h00 – 02h00)

Treatment (NT: 633 ± 54.4; MCS: 676 ± 52.5), and Day (Day1: mean = 672 ± 52.9; Day2: mean = 660 ± 55.70; Day3: mean = 664 ± 53.70; Day4: mean = 620 ± 58.40) had a significant effect on the average number of RR intervals (n = 62: Treatment: chi-squared = 5.34, model SE = 0.02, p = 0.03; Day: chi-squared = 8.88, model SE = 0.003, p <0.01) (**Figure 4.18 -4.20**).

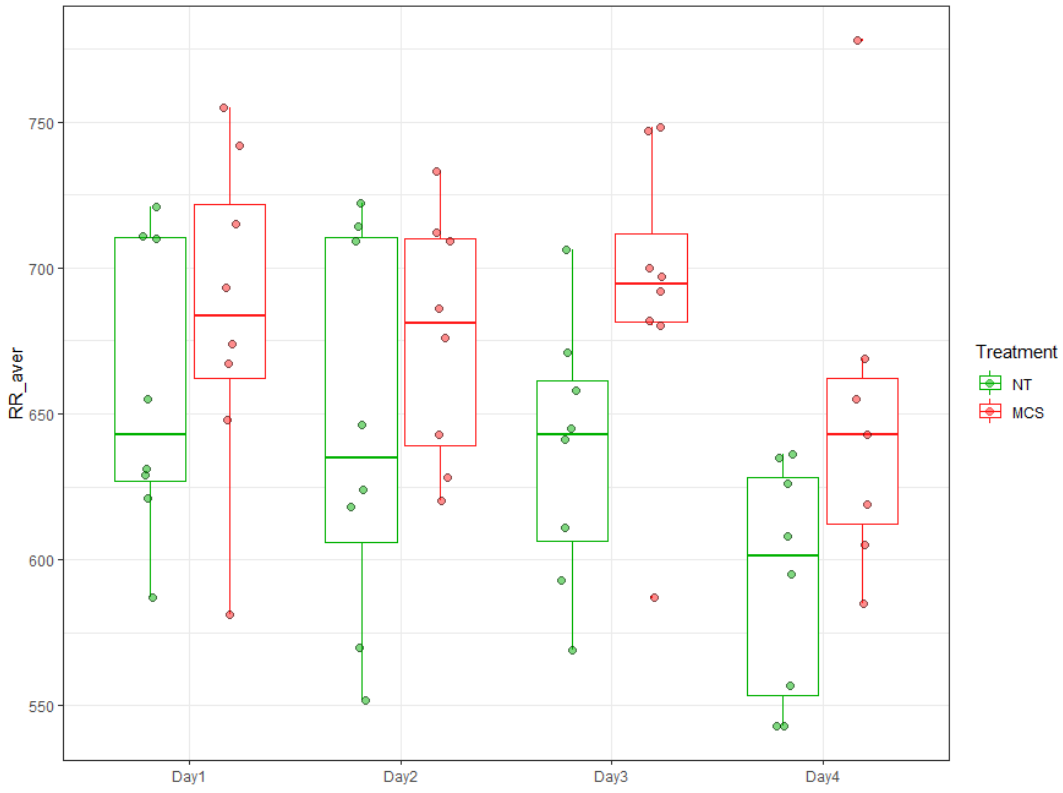

Figure 4.18 RR_average counts by Day of monitoring [Day1 (Start of trial, Day 2 (2 weeks into the trial), Day 3(4 weeks into the trial) and Day 4 (End of trial), before the Suddenness test] and Treatment group. The points represent individual values by Treatment and the boxplots include the median and IQR. Day had a significant effect (p <0.01), as did Treatment (p = 0.02).

Treatment (NT: 95.50 ± 8.23; MCS: 89.20 ± 7.06), and Day (Day1: mean = 89.90 ± 7.25; Day2: mean = 91.50 ± 8.08; Day3: mean = 90.0 ± 7.57; Day4: mean = 97.50 ± 8.60) had a significant effect on the average HR (n = 62: Treatment: chi-squared = 11.43, model SE = 2.86, p = 0.03; Day: chi-squared = 4.59, model SE= 2.56, p < 0.01).

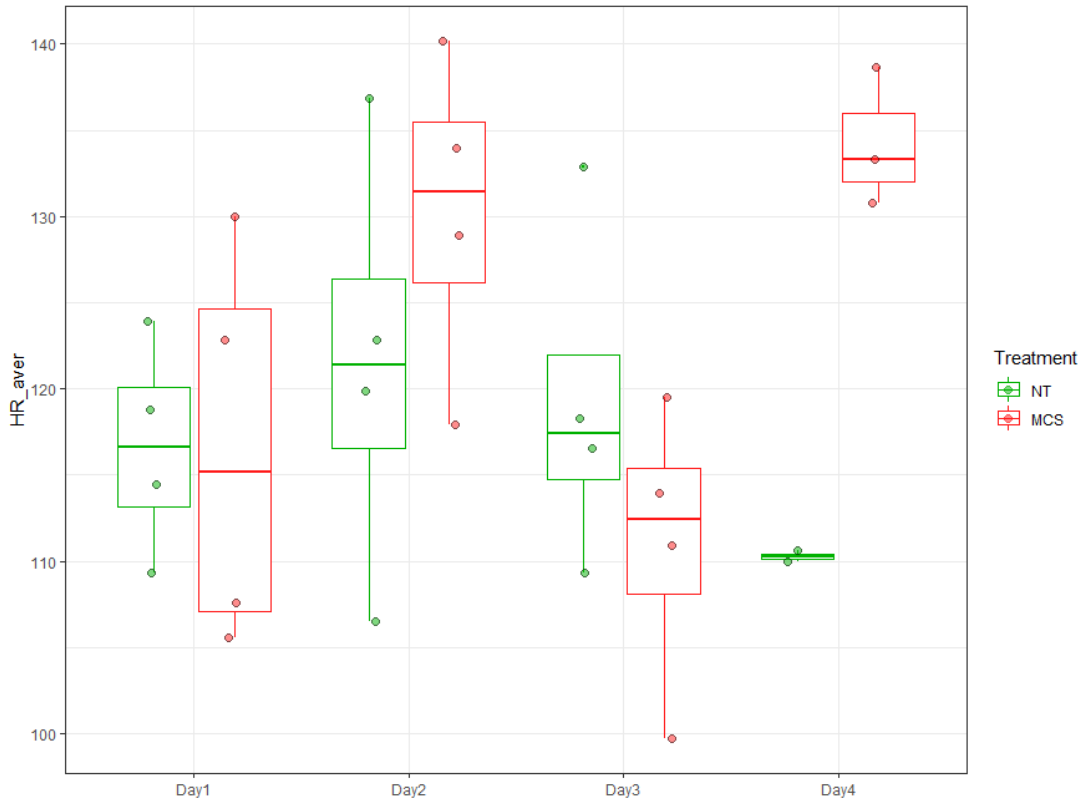

Figure 4.19 HR_average by Day of monitoring [Day1 (Start of trial, Day 2 (2 weeks into the trial), Day 3(4 weeks into the trial) and Day 4 (End of trial), before the Suddenness test] and Treatment group. The points represent individual values by Treatment and the boxplots include the median and IQR. Day had a significant effect ($p < 0.01$), as did Treatment ($p = 0.03$).

RMSSD was significantly different by Day (Day1: mean = 88.20 ± 37.70; Day2: mean = 70.90 ± 37.90; Day3: mean = 81.80 ± 38.80; Day4: mean = 64.50 ± 34.60; n = 62; chi-squared = 9.48, model SE = 2.40, $p < 0.01$).

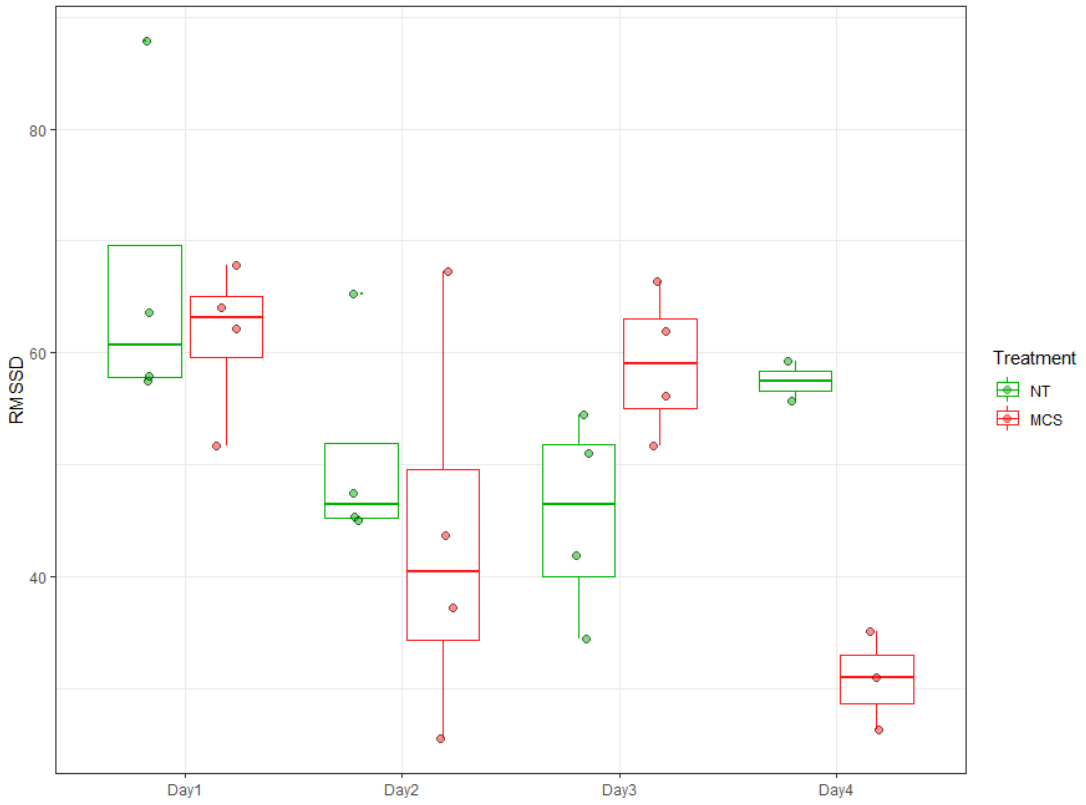

Figure 4.20 RMSSD by Day of monitoring [Day1: the Start of the trial, Day 2 (2 weeks into the trial), Day 3(4 weeks into the trial) and Day 4: (End of trial), before the Suddenness test] and Treatment group. The points represent individual values by Treatment and the boxplots include the median and IQR. Day had a significant effect ($p < 0.01$).

4.6.8.5 Step 5 (02h00 – 06h00)

Treatment (NT: 686 ± 54.60 ; MCS: 731 ± 59.60), and Day (Day1: mean = 728 ± 56.40; Day2: mean = 725 ± 55.50; Day3: mean = 714 ± 6.60; Day4: mean = 672 ± 62.20) had a significant effect on the average number of RR intervals, as indicated by the GLMM Poisson model used and the subsequent deviance test used to acquire the p-values in R (n = 62: Treatment: chi-squared = 4.41, model SE = 0.03, p = 0.04; Day: chi-squared = 9.88, model SE = 0.007, p <0.01) (**Figures 4.21-4.22**).

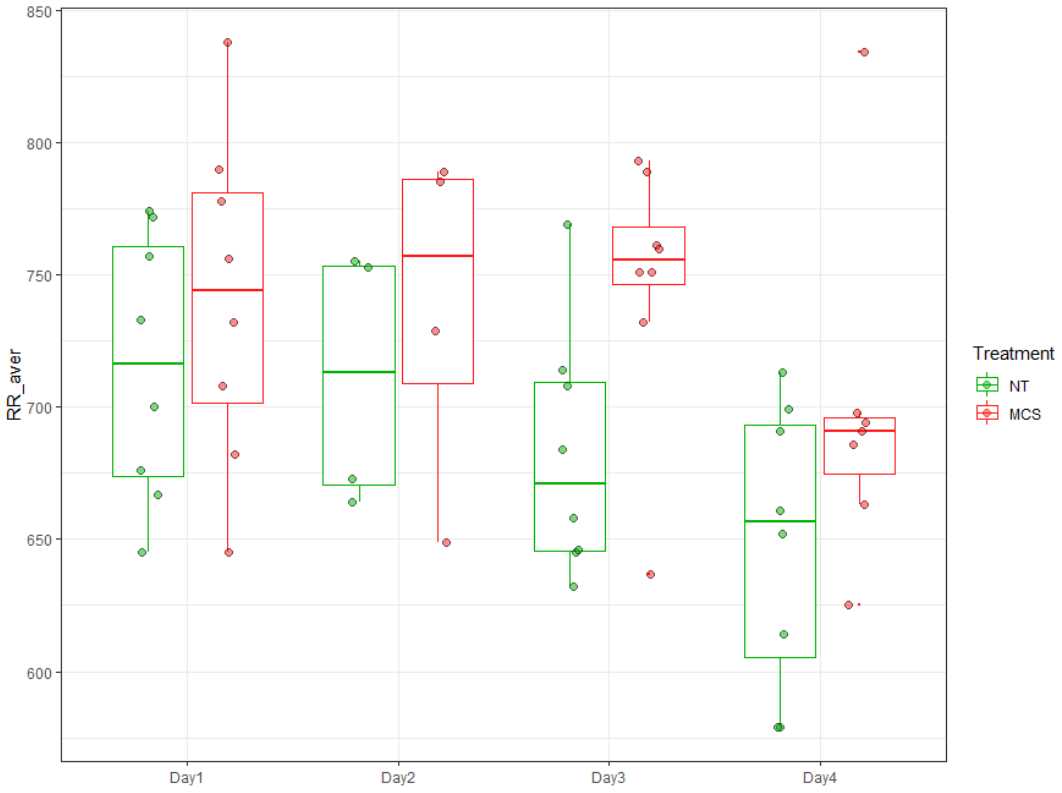

Figure 4.21 RR_average by Day of monitoring (Day1: the Start of the trial, Day 2: 2 weeks into the trial, Day 3: 4 weeks into the trial and Day 4: End of the trial, before the Suddenness test) and Treatment group. The points represent individual values by Treatment and the boxplots include the median and IQR. Day had a significant effect (p <0.01), as did Treatment (p = 0.05).

Treatment (NT: 88.00 ± 7.12; MCS: 82.60 ± 6.85), and Day (D1: mean = 82.80 ± 60.42; D2: mean = 83.20 ± 6.52; D3: mean = 84.50 ± 6.85; D4: mean = 90.00 ± 8.02) had a significant effect on the average HR, as indicated by the GLMM Poisson model used and the subsequent deviance test used to acquire the P-values in R (n = 62: Treatment: chi-squared = 3.71, model SE = 2.57, p = 0.05; Day: chi-squared = 15.40, model SE = 0.14, p <0.01).

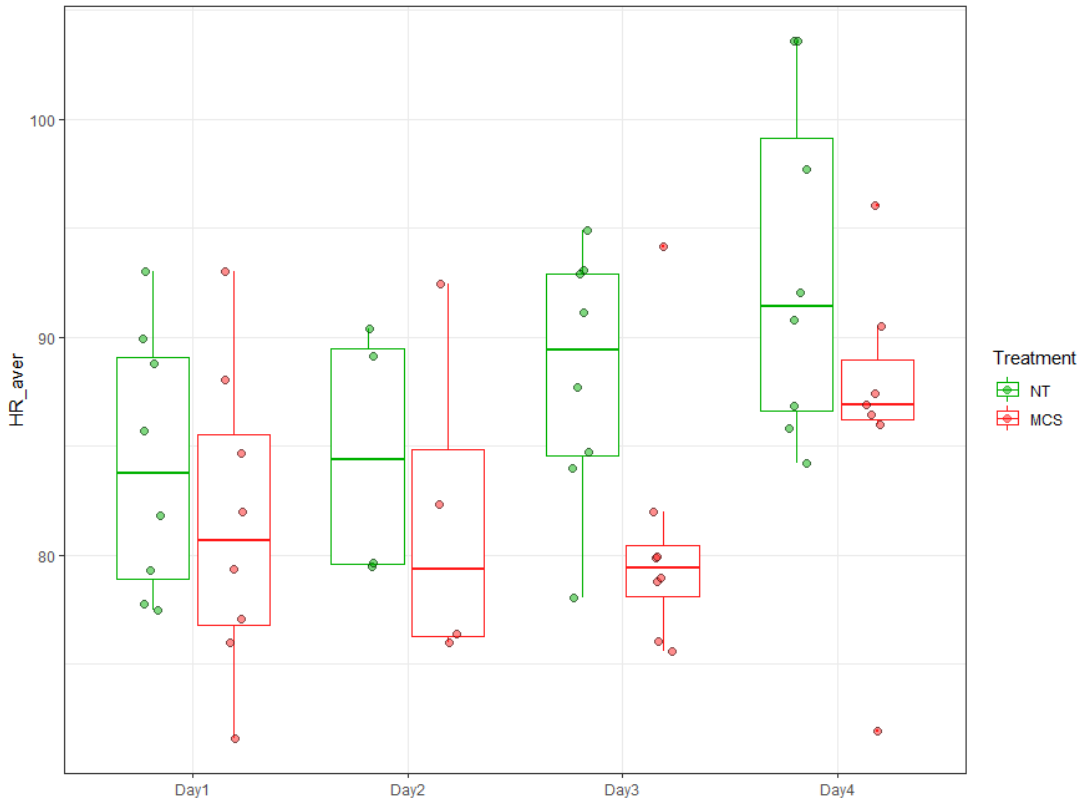

Figure 4.22 HR_average by Day of monitoring [Day1: Start of trial, Day 2: two weeks into the trial, Day 3: four weeks into the trial, and Day 4: end of trial)] and Treatment group. The points represent individual values by Treatment and the boxplots include the median and IQR. Day had a significant effect ($p < 0.01$).

4.6.9 Heart rate; Home-pen, ball test

No effect of Treatment or Day was observed for Steps 1-4. When adding Step 3 from the Non-stressed day (closest to the suddenness test), as a means of assessing the effect the suddenness test had (Ball Test Step3), an effect of Day was found on the average RR (D0: mean = 450 ± 41.20 msec; D1: mean = 491 ± 65.00; chi-squared = 16.40, $p < 0.01$) and for average HR [D0: mean = 450 ± 41.20 msec; D1: mean = 491 ± 65.00, $F(1, 16) = 8.26$, $p < 0.01$].

For the night steps, Step 5 and Step 6 from No-stress day 4 (the recording session performed closest to the date of the suddenness test, referred to as D0) were compared to Step5 and Step 6 of the Suddenness test available for one night (D1). For the average RR (msec) and the average HR (bpm) there was a Treatment, Day and Interaction effect at a Step 5 (22h00 - 02h00). At Step 6 (02h00 - 06h00), there was a Day, Treatment and interaction effect for the

average RR (msec) and a Treatment group and Day effect for the average HR. For RMSSD and SDNN there was a Day effect and for LF/HF a Treatment Group effect.

More specifically, average RR for Step5 on the no stress day compared to the Step 5 of the homepen test day, was higher for the NT animals compared to the MCS (487.00±166>473.00±128), while average RR on the test day is significantly lower compared to the NS day (348.00±30.10 <620.00±58.40) for both groups. Average heart rate is higher for the MCS group (97.30±8.47>92.10±7.10) but is lower the NS day (D0 NT 102.00±6.97 >D0 MCS 92.90±8.27). Step 6 average RR is higher for the MCS animals (477.00±181.00> 472.00±134.00) and significantly higher on D0 for both groups, compared to the test day (D1). Average heart rate is higher for the NT group (94.00±10.60>87.70±8.35, $p<0.01$), but overall lower on the test day compared to the control day (D1: 84.40±6.75< D0:97.50±8.60, $p<0.01$). Consistently, RMSSD and SDNN are higher on the test day ($p<0.01$; $p=0.02$, respectively), while LF/HF is higher for the NT animals compared to the MCS (1.30 ±0.34>0.86 ±0.48, $p=0.03$). Further information on means, SD and p-values are reported in **Table 4.16** below.

Table 4.16 Results for Day, Treatment and interaction effect on RR (msec) and HR (bpm) for Step5 No Stress Day (D0: 22h00 – 02h00) and Step 6 (D0: 02h00 – 04h00) with Step 5 (D1: 22h00 – 02h00) and Step 6 (D1: 02h00 – 04h00) respectively, from the Suddenness Test day conducted in the home pen. Mean and SD values are presented, as well as the p- and chi square value from the type II or III Wall-test. For D0 (the No- stress day), n = 16. For the ball test days n = 8. Differences are indicated with the CLD system and capitalised when p <0.01.

Step5NS – Step5Ball				
RR_aver				
Factor		**mean ±SD**	**chi- square**	**p-value**
Treatment Group	NT	487 ±166	11.97	<0.01
	MCS	473 ± 128		
Day	D0	620 ±58.40	652.316	<0.01
	D1	348 ±30.10		
Interaction	D0 NT	593 ±40.00[a]	10.29	<0.01
	D0 MCS	651 ±63.30[b]		
	D1 NT	352 ±18.70[c]		

	D1 MCS	344 ±39.40[c]		
HR_Aver				
Factor		**mean ±SD**	**chi- square**	**p-value**
Treatment Group	NT[A]	92.10 ±7.10	8.87	<0.01
	MCS[B]	97.3 ±8.47		
Day	D0	97.50 ±8.60	0.56	0.45
	D1	92.30 ±6.90		
Interaction	D0 NT	102.00 ±6.97[a]	7.28	<0.01
	D0 MCS	92.90 ±8.27[ab]		
	D1 NT	93.00 ±7.93[b]		
	D1 MCS	91.50 ±6.36[ab]		
Step6NS – Step6Ball				
RR_aver				
Factor		**mean ± SD**	**chi-square**	**p-value**
Treatment Group	NT	472 ±134[A]	21.16	<0.01
	MCS	477 ±181[B]		
Day	D0	620 ±58.40[A]	480.48	<0.01
	D1	338.00 ±67.00[B]		
Interaction	D0 NT	593 ±40.00[a]	38.22	<0.01
	D0 MCS	651 ±63.30[a]		
	D1 NT	352 ±63.00[b]		
	D1 MCS	324 ±72.20[b]		
HR_aver				
Factor		**mean ± SD**	**chi- square**	**p-value**
Treatment Group	NT	94.00 ±10.60[A]	54.50	<0.01
	MCS	87.70 ±8.35[B]		
Day	D0	97.50 ±8.60[A]	64.43	<0.01
	D1	84.40 ±6.75[B]		

RMSSD				
Factor		**mean ±SD**	**chi- square**	**p-value**
Treatment Group	NT	63.30 ±27.70	0.81	0.37
	MCS	78.90 ±38.90		
Day	D0	64.50 ±34.60^{A}	6.95	<0.01
	D1	76.80 ±33.30^{B}		
SDNN				
Factor		**mean ±SD**	**chi- square**	**p-value**
Treatment Group	NT	78.70 ±17.40	0.0009	0.98
	MCS	79.40 ±16.90		
Day	D0	75.90 ±17.60^{A}	5.44	0.02
	D1	82.00 ±16.20^{B}		
LF/HF				
Factor		**mean ± SD**	**chi- square**	**p-value**
Treatment Group	NT	1.30 ±0.34^{A}	4.95	0.03
	MCS	0.86 ±0.48^{B}		

4.6.10 Heart Rate; Novel pen, ball test

No effect of Treatment or Day was observed for the Steps 1-4. When adding Step 2 from the Non-Stressed day (closest to the Suddenness test), as a means of assessing the effect of moving the animals to the novel Pen (Ball Test Step2) and the effect of the Suddenness test (Ball Test Step3), an effect of Day was found on the average HR, RMSSD and LF/HF. Details are reported below (**Tables 4.17- 4.18**).

Regarding Step 2 (14h00 – 16h00) of NS day compared to the timeframe prior to the ball drop (Step2Ball), there was a Day effect on average heart rate. On the NS day, average heart rate was lower compared to D1, D2, D3 and D4 test days ($p<0.01$). Similarly, RMSSD was higher on D0 and the ratio of low frequencies to high frequencies LF/HF was higher on D1, D2 and D3 compared to D0. On D4 the ratio is the highest (4.61±2.19).

During the ball drop (Step3 ball), no Treatment group difference was observed. There is however a drop in average RR compared to the NS day (D0) and all the test days (D1, D2, D3,

D4). Again, the inverse is true for LF/HF, particularly for the animals (same pens) tested on D2 and D4.

Step 5 in the evenings indicated that average HR was higher for the MCS animals compared to the NT ones (96.40±9.60 > 93.40±7.02, p<0.01), but lower compared to the NS day for the NT animals (D0 NT: 101.20±6.97> 92.90±8.27, p<0.01). For Step 6, average heart rate is higher for the MCS animals (p<0.01). Average heart rate is also significantly lower on the test day compared to the control day (D0) (83.90±8.40<93.10±7.73, p<0.01).

Table 4.17 Results for Day Effect on average HR (bpm), RMSSD and LF/HF, for Step2 No Stress Day (14h00 – 16h00) with Step 2 (Ball-25min) and Step 3(Ball+25 min) for the Suddenness Test conducted in a novel environment. Mean and SD values are presented for each Day, as well as the p - and chi-square -value reported from the GLMM. For D0 (the No- Stress day), n = 16. For the ball test days n = 8 and NT, MCS n = 24. Differences are indicated with the CLD system and capitalised when p <0.01.

Step2NS - Step2Ball				
HR_aver				
Factor		**mean ± SD**	**chi- square**	**p-value**
Treatment Group	NT	136 ±14.50	0.07	0.78
	MCS	133 ±13.20		
Day	D0	124 ±15.70[a]	6.69	<0.01
	D1	137 ±7.53[b]		
	D2	145 ±11.20[b]		
	D3	132 ±8.50[b]		
	D4	143 ±9.64[b]		
RMSSD				
Factor		**mean ±SD**	**chi- square**	**p-value**
Treatment Group	NT	47.90 ±12.30	0.30	0.59
	MCS	45.20 ±11.70		
Day	D0	54.80 ±15.40[a]	25.44	<0.01
	D1	41.40 ±4.10[b]		

	D2	42.3 ±6.25[b]		
	D3	45.1 ±11.1[b]		
	D4	41.8 ±9.39[b]		
LF/HF				
Factor		**mean ±SD**	**chi- square**	**p-value**
Treatment Group	NT	3.62 ±1.89	0.23	0.63
	MCS	3.06 ±1.56		
Day	D0	1.74 ±0.87[a]	38.71	<0.01
	D1	3.46 ±1.31[b]		
	D2	4.43 ±1.33[bc]		
	D3	3.66 ±1.12[b]		
	D4	4.61 ±2.19[c]		
Step2NS – Step3Ball				
RR_aver				
Factor		**mean ±SD**	**chi-square**	**p-value**
Treatment Group	NT	465 ± 53.10	0.04	0.84
	MCS	457 ±48.10		
Day	D0	491 ±65.00[a]	5.31	0.02
	D1	462 ±19.30[b]		
	D2	422 ±37.90[c]		
	D3	478 ±27.40[b]		
	D4	429 ±29.20[c]		
HR_aver				
Factor		**mean ±SD**	**chi-square**	**p-value**
Treatment Group	NT	131 ±13.60	0.14	0.70
	MCS	133± 13.90		
Day	D0	124 ±15.70[a]	7.61	<0.01
	D1	130 ±5.47[a]		
	D2	143 ±12.30[b]		
	D3	126 ±7.01[a]		

	D4	140 ±10.80[b]		
LF/HF				
Factor		**mean ±SD**	**chi-square**	**p-value**
Treatment Group	NT	2.62 ±1.59	1.09	0.30
	MCS	3.10 ±1.56		
Day	D0	1.74 ±0.87[a]	15.14	<0.01
	D1	2.75 ±1.13[a]		
	D2	3.79 ±1.20[b]		
	D3	2.53 ±1.09[a]		
	D4	4.32 ±2.16[c]		

Similarly for the night steps, Step 5 and Step 6 from No-Stress day 4 (the recording session performed closest to the date of the Suddenness test, referred to as D0) were compared to Step5 and Step 6 of the Suddenness test available for one night (D1). For the average HR (bpm), there was a Day and interaction effect (D0 >D1; D0 NT >D1 NT). Further information on means, SD and p-values are reported in **Table 4.18** below.

Table 4.18 Results for Day effect on HR (bpm) for Step5 No stress day (D0: 22h00 – 02h00) and Step 6 (D0: 02h00 – 04h00) with Step 5 (D1: 22h00 – 02h00) and Step 6 (D1: 02h00 – 04h00) respectively from the suddenness test conducted in a novel environment. Mean and SD values are presented for each day, as well as the p- and chi square result from the type II/III Wall-test reported from the GLMM. For D0 (the no-stress day), n =16. For the ball test days n =8. Differences are indicated with the CLD system and capitalised when p <0.01.

Step5NS – Step5Ball				
HR_aver				
Factor		**Mean ±SD**	**chi-square**	**p-value**
Treatment Group	NT	93.40 ±7.62^{A}	9.11	<0.01
	MCS	96.40 ±9.60^{B}		
Day	D0	97.50 ±8.60	0.02	0.87
	D1	92.50 ±8.26		
Interaction	D0 NT	101.20 ±6.97^{a}	10.57	<0.01
	D0 MCS	91.10 ±9.25^{b}		
	D1 NT	92.90 ±8.27^{b}		
	D1 MCS	93.30 ±7.54^{b}		
Step6 NS – Step6Ball				
HR_aver				
Factor		**Mean ±SD**	**chi-square**	**p-value**
Treatment Group	NT	85.70 ±6.68	5.02	0.02
	MCS	88.50 ±9.12		
Day	D0	90.00 ±8.02	14.40	0.43
	D1	84.40 ±7.32		
Interaction	D0 NT	93.10 ±7.73^{a}	5.36	0.02
	D0 MCS	86.50 ±7.31^{ab}		
	D1 NT	83.90 ±8.40^{b}		
	D1 MCS	85.00 ±6.49^{ab}		

4.6.11 Rumen samples

4.6.11.1 Protozoa

Experimental Stage and Treatment group effects were observed for total protozoa counts. Specifically, an increase in total protozoa was observed for NT and MCS lambs post-trial. Experimental Stage had a significant effect on the concentration of small *Entodiniomorphs*. Treatment Group and Interaction effects were observed on the concentration of large *Entodiniomorphs*. Results are presented in **Table 4.19**below.

Table 4.19 GLMM results for rumen protozoa NT animals (n =24) and MCS (n = 24) animals Pre (N=48) and Post-trial (n = 48). Mean ±SD values, p- and chi-square values are reported. Differences are indicated with the CLD system and capitalised when p <0.01.

Total count				
Factor		**mean ± SD**	**chi-square**	**p-value**
Treatment Group	NT	89.90 ±48.00	0.38	0.54
	MCS	96.90 ±48.00		
Experimental Stage	Pre	80.50 ±44.60^{A}	13.90	<0.01
	Post	106.00 ±48.00^{B}		
Large *Entodiniomorphs*				
Factor		**mean ± SD**	**chi-square**	**p-value**
Treatment Group	NT	21.30 ±48.00^{A}	7.65	<0.01
	MCS	13.90 ±48.00^{B}		
Experimental Stage	Pre	16.20 ±14.10	0.28	0.59
	Post	18.90 ±18.90		
Interaction	PreNT	11.50 ±12.10^{a}	9.64	<0.01
	PreMCS	21.00 ±18.60^{b}		
	PostNT	16.20 ±16.20^{c}		
	PostMCS	21.70 ±21.20^{b}		
Small *Entodiniomorphs*				
Factor		**mean ± SD**	**chi-square**	**p-value**

Treatment Group	NT	78.60 ±30.50	1.48	0.22
	MCS	60.80 ±30.50		
Experimental Stage	Pre	58.10±33.30^{A}	70.00	<0.01
	Post	81.40 ±37.20^{B}		

4.6.11.2 VFAs

Experimental Stage effects were observed on Total VFAs, propionate, acetate, butyrate and the ratio of propionate: acetate (C2:C3) concentrations.

Specifically, an increase in propionate and subsequently of C2:C3 was observed post-trial, whereas butyrate levels were higher pre-trial. No effect was observed for acetate (p =1.21). Details are presented in **Table 4.01** below.

Table 4.20 GLMM results for rumen VFA concentrations (mmol/l) pre- (N =48) and post-trial (n = 48). Experimental Stage effects were observed for propionate, butyrate, the propionate/acetate ratio and total VFAs. Mean ± SD values, p- and chi-square values reported from the GLMM are presented.

Total VFAs				
Factor		**mean ± SD**	**chi-square**	**p- value**
Treatment Group	NT	0.958 ±0.009	2.23	0.45
	MCS	0.955 ±0.009		
Experimental Stage	Pre	0.954 ±0.009	113.90	0.05
	Post	0.959±0.008		
Propionate				
Factor		**mean ± SD**	**chi-square**	**p-value**
Treatment Group	NT	0.2000 ±0.0193	0.22	0.63
	MCS	0.197 ±0.0233		
Experimental Stage	Pre	0.1920 ±0.0191	5.47	0.02
	Post	0.2050 ±0.0216		
Butyrate				
Factor		**mean ± SD**	**chi-square**	**p-value**

Treatment Group	NT	0.0988 ±0.0145	0.77	0.38
	MCS	0.0956 ±0.0145		
Experimental Stage	Pre	0.0992 ±0.0153[a]	4.48	0.04
	Post	0.0952 ±0.0137[b]		
C2:C3				
		mean ± SD	**chi-square**	**p-value**
Treatment Group	NT	3.280 ±0.369	1.07	0.30
	MCS	3.370 ±0.495		
Experimental Stage	Pre	3.440 ±0.442[A]	9.16	<0.01
	Post	3.200 ±0.402[B]		

4.6.11.3 Taxonomy analyses: phyla, orders and genera

The overall rumen microbial community was comprised 98.90% of bacteria (range: 96.50% to 99.50%), 0.40% of archaea (range: 0.10% to 2.70%) and 0.70% was Unclassified.

At the phylum, order and genus levels, the most abundant bacteria are presented in **Figures 4.23-4.25** below. The only archaeal phylum was Euryarcheota, and the most abundant orders were E2 aned Methanobacteriales, whilst the most abundant genus was Methanobrevibacter. No significant differences were observed for Treatment and Experimtal stage groups. Bacterial phyla representing less than 1.00% of the overall population included *Spirochaetes, Lentisphaerae, Tenericutes, Cyanobacteria, Plantomycetes, Actinobacteria* and *WPS-2*. Other orders < 1.00 % included: *Gammaproteobacteria, Spirochaetes, Unidentified_WPS-2, Mollicutes, Alphaproteobacteria, Plantomycetes, Lentispheaeria, Anaerolineae* and *Coriobacteria*. genera <1.00% included: *Treponema, Sharpea, Anaeroplasma, Succinivibrio, Shuttleworthia, Moryella* and *Clostridium*. More details are reported in **Table 7.28** in the Appendix.

The most abundant bacteria and archaea at a phylum, order and genus level by grouped Factors: Experimental Stage and Treatment (PreNT, PreMCS, PostNT and PostMCS) are presented **Table 7.28** in the Appendix.

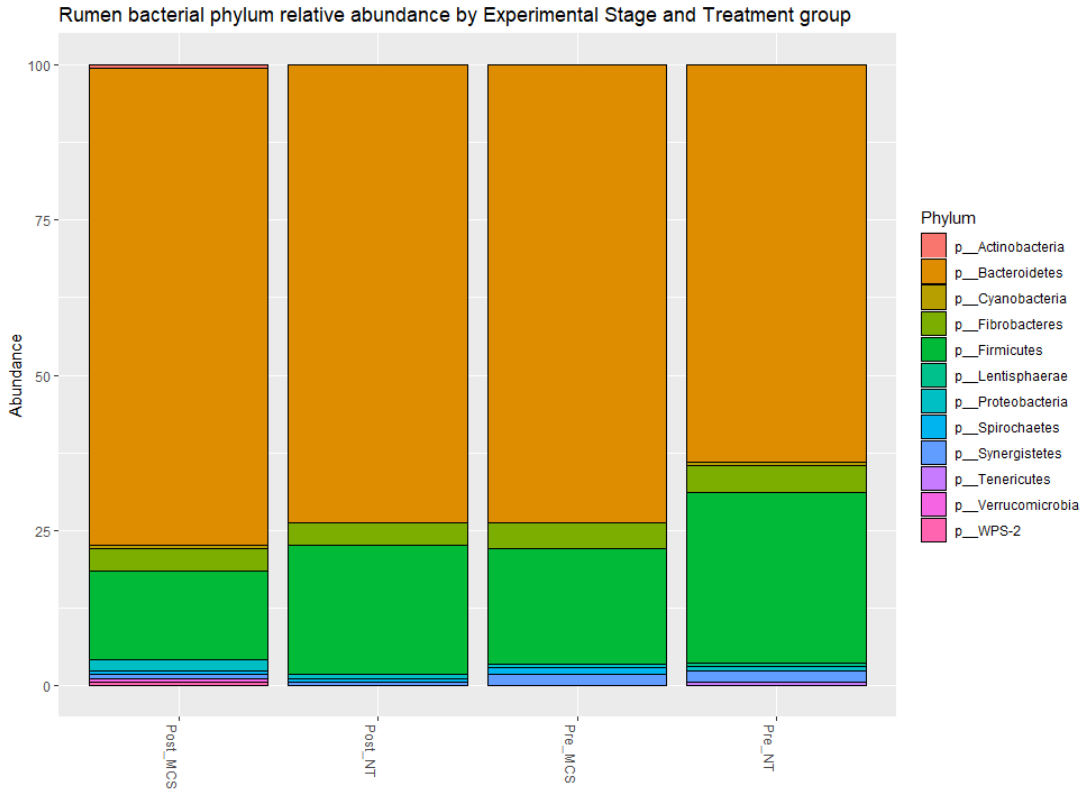

Figure 4.23 Most abundant rumen bacterial phyla by Experimental Stage and Treatment groups (Pre-MCS, Pre-NT, Post-MCS and Post-NT).

Statistical tests at the phylum level were conducted using all phyla present (n = 21) after square root transformation of the RA. Non-parametric Kruskal-Wallis followed by Wilcoxon test with Bonferroni correction indicated significant differences for the phyla*: Actinobacteria*, *Cyanobacteria*, *Firmicutes*, *Proteobacteria* and *Spirochaetes*. More details are available in **Table 4.21** and **Table 4.22** below.

Statistical tests at the order level (n = 19) were conducted between Groups (PreNT, PreMCS, PostNT and PostMCS), taking into account RA over 0.05, a lower threshold compared to the previous studies, due to lower abundance levels observed. *Spirochaetale*s, *YS2* (*Cyanobacteria*), *Coriobacteriales* and *RF32* (*Alphaproteobacteria*) were significantly different between groups. Further details are presented in Table 4.4.11.3.3 below. Statistical tests at the genus level were conducted for RA over 0.1 (n =14). No significant differences were observed.

Table 4.21 phyla with significantly different rumen RA between groups: PreNT, PostNT, PreMCS, PostMCS (n = 24 for each). The p- and chi-square values acquired from the Kruskal Wallis non-parametric testing, are reported with mean and SD values for each group. Differences are indicated with the CLD system.

Significantly different bacterial rumen phyla						
Phyla	**Kruskal p-value**	**chi-square**	**mean ±SD**			
			PreNT	**PreMCS**	**PostNT**	**PostMCS**
Actinobacteria	0.02	10.35	0.04 ±0.01[a]	0.04 ±0.02[a]	0.05 ±0.01[b]	0.05 ±0.02[b]
Firmicutes	0.03	9.05	0.44 ±0.06[a]	0.46 ±0.08[ab]	0.48 ±0.04[b]	0.46 ±0.05[ab]
Proteobacteria	0.02	10.43	0.08 ±0.04[ab]	0.09 ±0.03[a]	0.1 ±0.03[ab]	0.08 ±0.01[b]
Spirochaetes	0.02	9.65	0.07 ±0.04[a]	0.08 ±0.03[ab]	0.07 ±0.02[a]	0.09 ±0.02[b]

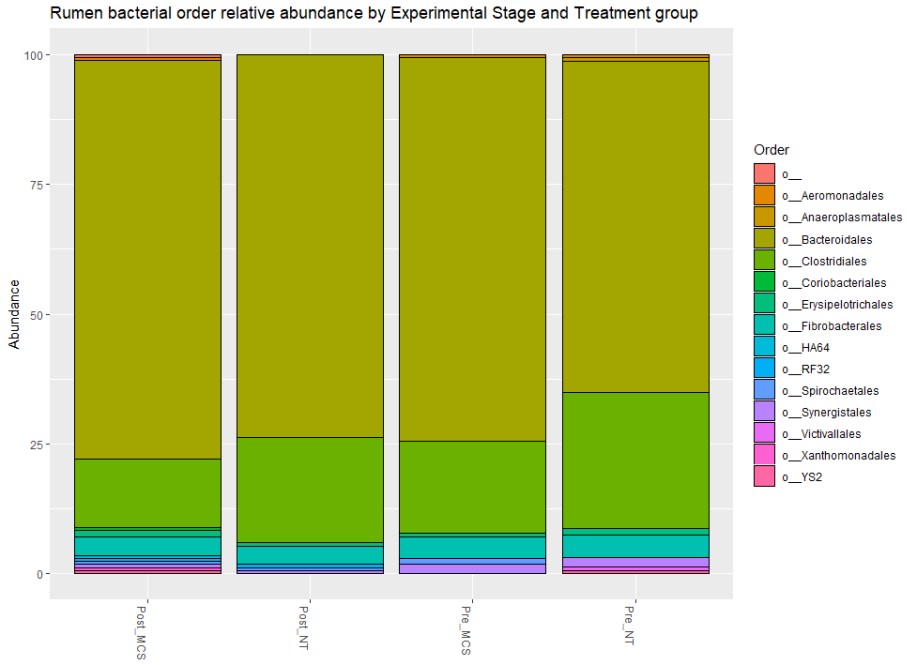

Figure 4.24 Most abundant rumen bacterial orders by Experimental Stage and Treatment groups (Pre-MCS, Pre-NT, Post-MCS and Post-NT).

Table 4.22 Orders present in RA over 0.5 per cent with significantly different rumen RA between groups: PreNT, PostNT, PreMCS, PostMCS (n = 24 for each). The p-/ chi-square values acquired from the Kruskal Wallis nonparametric testing, are reported with average and SD values for each group. Differences are indicated with the CLD system.

Orders over 0.5 percent rumen						
Orders	**Kruskal p-value**	**chi-square**	**mean ±SD**			
			PreNT	**PreMCS**	**PostNT**	**PostMCS**
Spirochaetales	0.02	9.99	0.08 ±0.04^{ab}	0.09 ±0.03^{ab}	0.09 ±0.03^{a}	0.07 ±0.01^{b}
YS2 Cyanobacteria	0.03	8.96	0.04 ±0.02^{a}	0.05 ±0.04^{ab}	0.05 ±0.02^{ab}	0.06 ±0.03^{b}
Coriobacteriales	0.01	10.58	0.0004 ±0.002^{a}	0.008 ±0.02^{b}	0.0004 ±0.002^{a}	0.004 ±0.008^{b}
RF32	<0.01	20.11	0.01 ±0.01^{a}	0.01 ±0.01^{a}	0.03 ±0.02^{b}	0.03 ±0.02^{b}

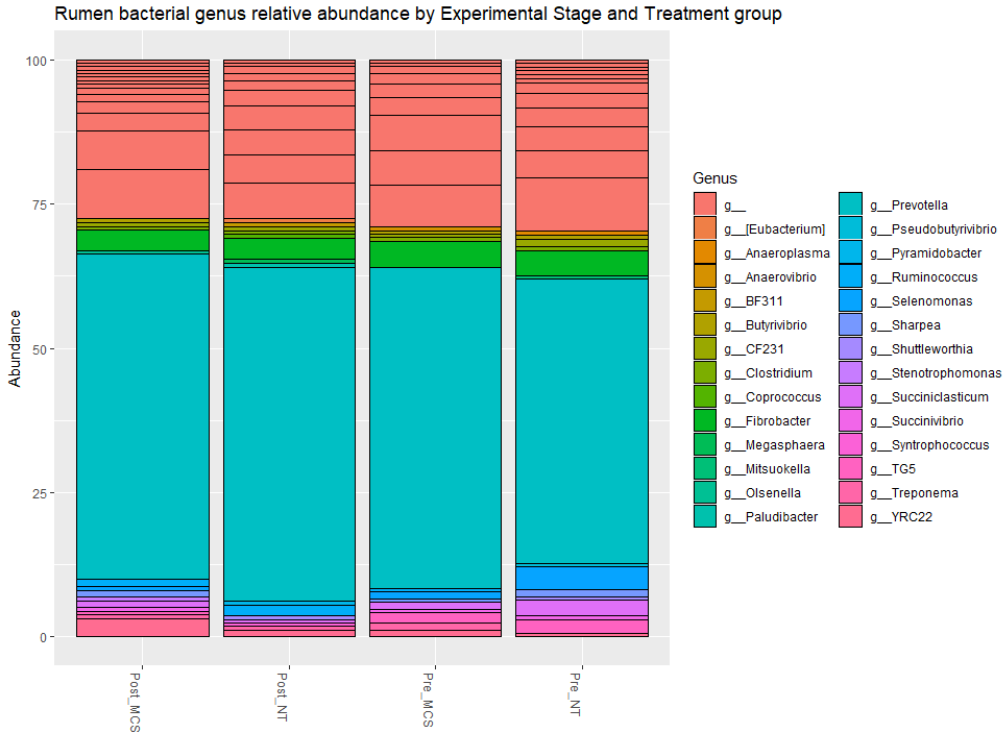

Figure 4.25 Most abundant rumen bacterial genera by Experimental Stage and Treatment groups (Pre-MCS, Pre-NT, Post-MCS and Post-NT).

4.6.11.4 Rumen bacterial and archaeal diversity Exploration

4.6.11.4.1 Rumen bacterial and archaeal alpha diversity

Experimental Stage and Interaction effects were observed for the rumen bacterial and archaeal Shannon, Simpson and Inverse Simpson indices for the NT groups pre- and post-trial. Simpson diversity also differed pre-trial between NT and MCS groups, whilst an Experimental Stage effect was observed for all indices except ACE, suggesting overall increased diversity post-trial. Boxplots were created in "phyloseq" using rarefied reads, to visualise differences in diversity indices calculated for the bacterial and archaeal rumen OTUs (**Figures 4.26-4.27**) Statistically significant differences are reported in **Tables 4.23-4.24**.

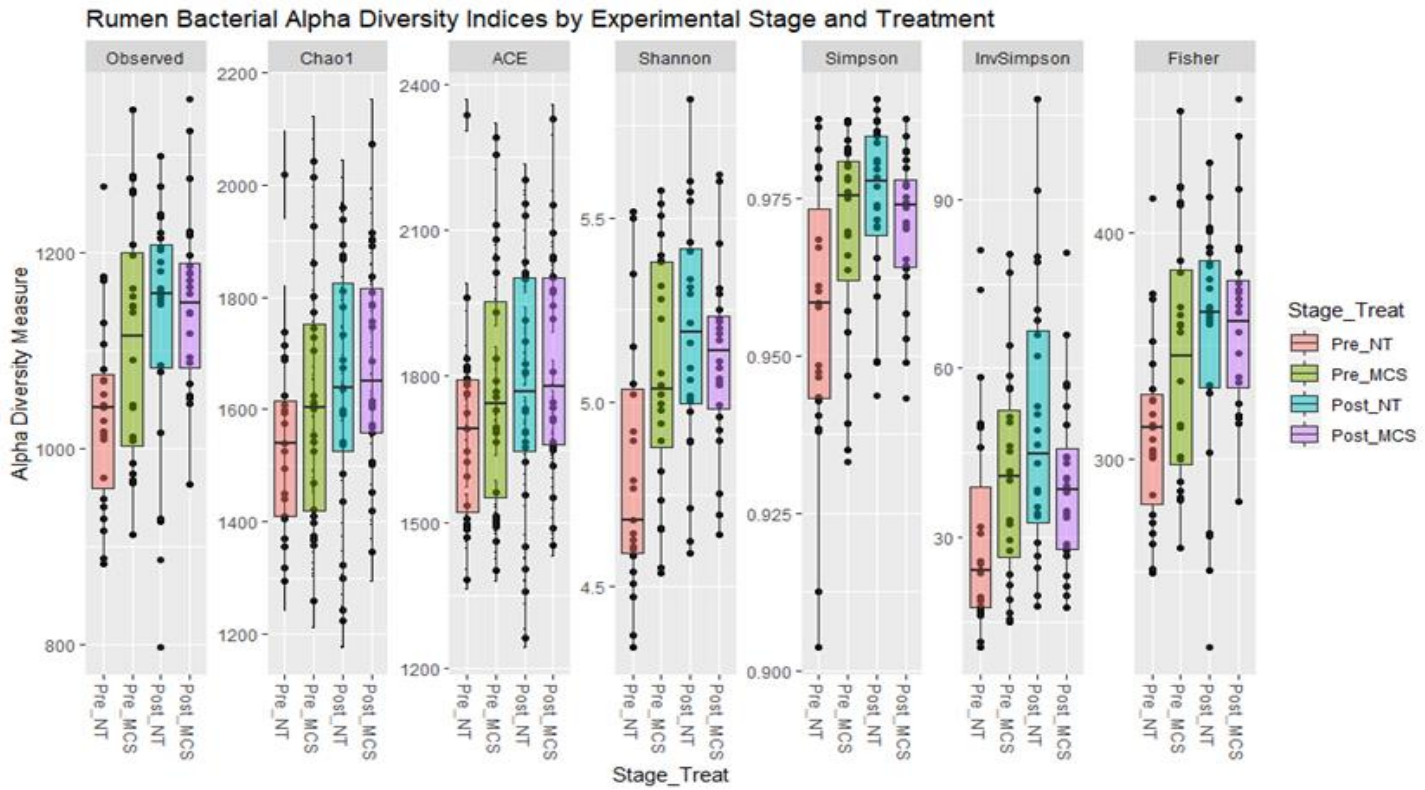

Figure 4.26 Boxplot of the Diversity Indices (Observed, Chao1, Ace, Shannon, Simpson and Inverse Simpson) calculated using "phyloseq" on rarefied counts, to examine differences in Rumen Bacterial diversity between Experimental Stages and Treatment groups (PreNT: pink; PreMCS: green; PostNT: blue; PostMCS: purple). Significant differences were observed at an Experimental Stage and mainly between PreNT and PostNT ($p < 0.01$) for the Shannon, Simpson and Inverse Simpson Indices. Means, IQR are presented.

Table 4.23 GLM results on the effect of Treatment (Treat: NT, MCS) and Experimental Stage (Pre, Post-trial) on rumen bacterial alpha diversity indices. Means and SD of each group (PreNT, PreMCS, PostNT and PostMCS, n = 24 for each), the p-values acquired from the model are reported. Differences are indicated with the CLD system and capitalised when p <0.01.

Alpha diversity index results for Rumen bacteria						
Diversity Index	**Variable**		**mean ±SD**	**P(>Chisq)**		
				Stage	**Treatment**	**Interaction**
Observed	**Experimental Stage**	Pre^{A}	1144 ±322	<0.01	0.13	0.26
		Post^{B}	1168 ±228			
	Interaction	PreNT^{a}	1139 ±201			
		PreMCS^{a}	1149 ±243			
		PostNT^{a}	1135 ±228			
		PostMCS^{a}	1202 ±211			
Shannon	**Experimental Stage**	Pre^{A}	5.10 ±0.34	<0.01	0.23	<0.01
		Post^{B}	5.30 ±0.29			
	Interaction	PreNT^{A}	4.90 ±0.34			
		PreMCS^{B}	5.20 ±0.33			
		PostNT^{B}	5.30 ±0.33			
		PostMCS^{B}	5.24 ±0.25			
Simpson	**Experimental Stage**	Pre^{A}	0.98 ±0.02	<0.01	0.2	0.01
		Post^{B}	0.98 ±0.01			
	Interaction	PreNT^{a}	0.96 ±0.02			
		PreMCS^{b}	0.99 ±0.02			
		PostNT^{b}	0.98 ±0.01			
		PostMCS^{b}	0.98 ±0.01			
Inverse Simpson	**Experimental Stage**	Pre^{A}	32.05 ±19.50	0.01	0.95	<0.01
		Post^{B}	44.39 ±20.12			
	Interaction	PreNT^{A}	24.15 ±20.13			

		PreMCS[B]	40.94 ±18.85			
		PostNT[B]	49.47 ±23.94			
		PostMCS[B]	39.31 ±16.3			
Fisher's	**Experimental Stage**	Pre[A]	343 ±82	<0.01	0.13	0.24
		Post[B]	375 ±85			
	Interaction	PreNT[a]	317 ±70			
		PreMCS[a]	339 ±94			
		PostNT[a]	361 ±88			
		PostMCS[a]	348 ±81			
Chao1	**Experimental Stage**	Pre[a]	1566 ±344	0.03	0.21	0.8
		Post[b]	1633 ±320			
	Interaction	PreNT[a]	1530 ±246			
		PreMCS[a]	1602 ±342			
		PostNT[a]	1629 ±316			
		PostMCS[a]	1638 ±323			
ACE	**Interaction**	PreNT[a]	1692 ±282	0.13	0.19	0.75
		PreMCS[a]	1738±414			
		PostNT[a]	1928 ±364			
		PostMCS[a]	1949 ±330			

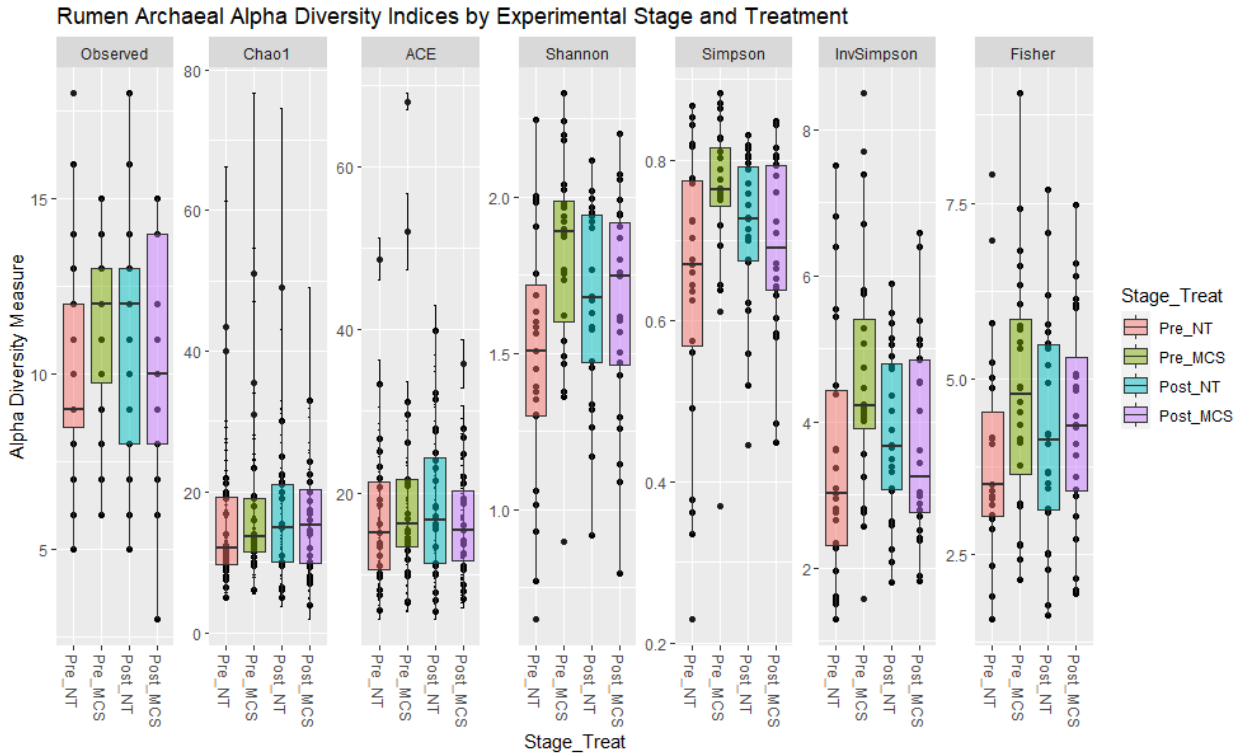

Figure 4.27 Boxplot of the Diversity Indices (Observed, Chao1, Ace, Shannon, Simpson and Inverse Simpson) calculated using "phyloseq" on rarefied counts, to examine differences in Rumen Archaeal diversity between Experimental Stages and Treatment groups (PreNT: pink; PreMCS: green; PostNT: blue; PostMCS: purple). Significant differences were observed at an Experimental Stage and mainly between PreNT and PostNT ($p < 0.01$) for the Shannon, Simpson and Inverse Simpson Indices. Means, IQR and individual values are presented.

Table 4.24 GLM results on the effect of Treatment (NT, MCS) and Experimental Stage (Pre, Post-trial) on rumen archaeal alpha diversity indices. The mean and SD of each group (PreNT, PreMCS, PostNT and PostMCS, n = 24 for each), the p-values acquired from the model are reported. Differences are indicated with the CLD system and capitalised when p <0.01.

Alpha diversity Index results for Rumen archaea						
Diversity Index	**Variable**		**Mean ±SD**	**P(>Chisq)**		
				Stage	**Treatment**	**Interaction**
Observed	**Interaction**	PreNT[a]	10.05 ±3.27	0.38	0.13	0.89
		PreMCS[a]	11.00 ±2.60			
		PostNT[a]	10.96 ±3.57			
		PostMCS[a]	10.56 ±3.45			
Shannon	**Experimental Stage**	Pre[A]	1.65 ±0.37	<0.01	0.19	<0.01
		Post[B]	1.67 ±0.33			
	Interaction	PreNT[A]	1.48 ±0.41			
		PreMCS[B]	1.81 ±0.33			
		PostNT[B]	1.67 ±0.31			
		PostMCS[B]	1.67 ±0.34			
Simpson	**Experimental Stage**	Pre[A]	0.70 ±0.15	<0.01	0.18	<0.01
		Post[B]	0.71 ±0.11			
	Interaction	PreNT[A]	0.64 ±0.18			
		PreMCS[B]	0.75 ±0.11			
		PostNT[B]	0.71 ±0.10			
		PostMCS[B]	0.70 ±0.11			
Inverse Simpson	**Experimental Stage**	Pre[a]	4.19 ±1.72	0.02	0.96	<0.01
		Post[b]	3.82 ±1.26			
	Interaction	PreNT[A]	3.52 ±1.77			
		PreMCS[B]	4.67 ±1.68			

		PostNT^{B}	3.83 ±1.15			
		PostMCS^{B}	3.80 ±1.37			
Fisher's	**Experimental Stage**	Pre^{A}	4.40 ±1.62	<0.01	0.08	0.48
		Post^{B}	4.40 ±1.55			
	Interaction	PreNT^{a}	3.95 ±1.51			
		PreMCS^{a}	4.85 ±1.72			
		PostNT^{a}	4.28 ±1.63			
		PostMCS^{a}	4.52 ±1.47			
Chao1	**Interaction**	PreNT^{a}	15.59 ±9.67	0.33	0.15	0.80
		PreMCS^{a}	17.16 ±10.01			
		PostNT^{a}	16.44 ±9.51			
		PostMCS^{a}	15.33 ±7.02			
ACE	**Interaction**	PreNT^{a}	17.38 ±9.67	0.38	0.13	0.89
		PreMCS^{a}	20.52 ±13.93			
		PostNT^{a}	18.38 ±8.99			
		PostMCS^{a}	16.95 ±7.13			

4.6.11.4.2 Rumen bacterial and archaeal beta diversity

A permutation test for homogeneity of multivariate dispersions was applied on Bray-Curtis distances, to investigate the dispersion of archaeal and bacterial samples between groups: PreNT, PreMCS, PostNT and PostMCS. Dispersion was significantly different between groups ($p < 0.01$). No differences were observed for the archaea.

A Multivariate Analysis of Variance test (Adonis) indicated significant differences between NT and MCS lambs pre- and post-trial for the rumen bacteria. No differences were observed for the archaea. Results are presented in **Table 4.25** below.

Table 4.25 Results from the beta-dispersion and Adonis tests are presented below for PreNT, PreMCS, PostNT and PostMCS rumen bacteria and archaea groups. p- values, F-values, squared estimate of errors (SSE) and mean squared errors (MSE) have been reported accordingly. Significance has been noted with the presence of a "*" symbol.

Bacterial β-Dispersion results				
	PreNT-PreMCS	**PreNT-PostNT**	**PreMCS-PostMCS**	**PostNT- PostMCS**
P-value	0.42	0.02*	<0.01	0.005*
F-value	1.05	1.27	1.36	1.24
SSE	0.21	0.50	0.53	0.47
Bacterial Adonis results				
	PreNT-PreMCS	**PreNT-PostNT**	**PreMCS-PostMCS**	**PostNT- PostMCS**
P-value	0.02*	<0.01*	0.03*	0.03*
F-value	1.052	1.29	1.47	1.24
MSE	0.41	0.50	0.58	0.47

4.6.11.4.3 Principal coordinate analysis (PCoA) of the rumen bacterial community

PCoA plots for Treatment, Experimental Stage and grouped variables (PreNT, PreMCS, PostNT and PostMCS) were computed (**Figures 4.28- 4.29**). For the bacteria, the percentage explained by each axis (Axis 1: 4.5%, Axis 2: 2.9%) was particularly low. This is likely due to the high number of bacteria present, but in particularly low abundances. For the archaea, description levels are higher, with Axis 1 explaining: 10.80% and Axis 2: 8.30%, which is moderate. Distinct clusters due to Treatment group* Experimental Stage are not clear, apart from potentially PreNT, PostNT and PostMCS groups for rumen bacteria, a result supported by the statistical tests for alpha diversity presented above.

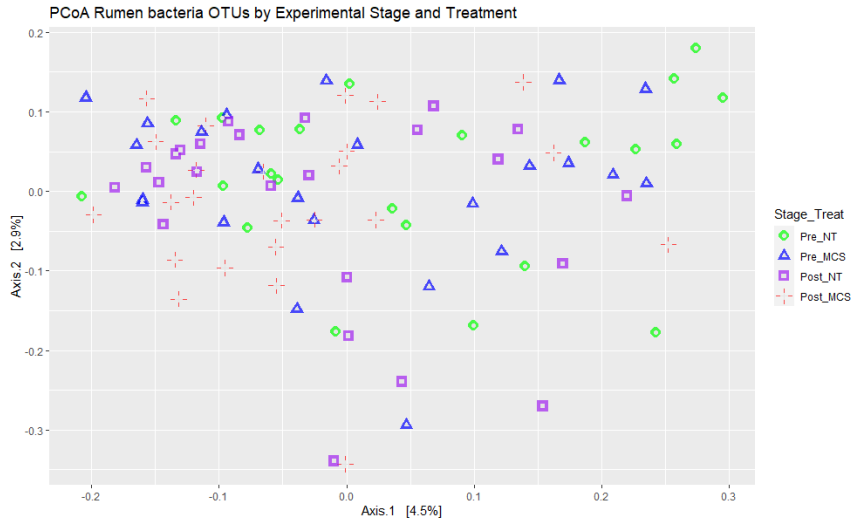

Figure 4.28 Principal coordinate analysis (PCoA) of the rumen bacterial community based on the SRT RA data of OTUs. The relative distances of all points represent the relative dissimilarities of the samples according to the Bray-Curtis index. Treatment groups are colour coded and distinguished by shape (green circle = PreNT, blue triangle = PreMCS, purple square = PostNT, red cross = PostMCS). The percentage of total variation explained by each PCoA axis is shown in brackets.

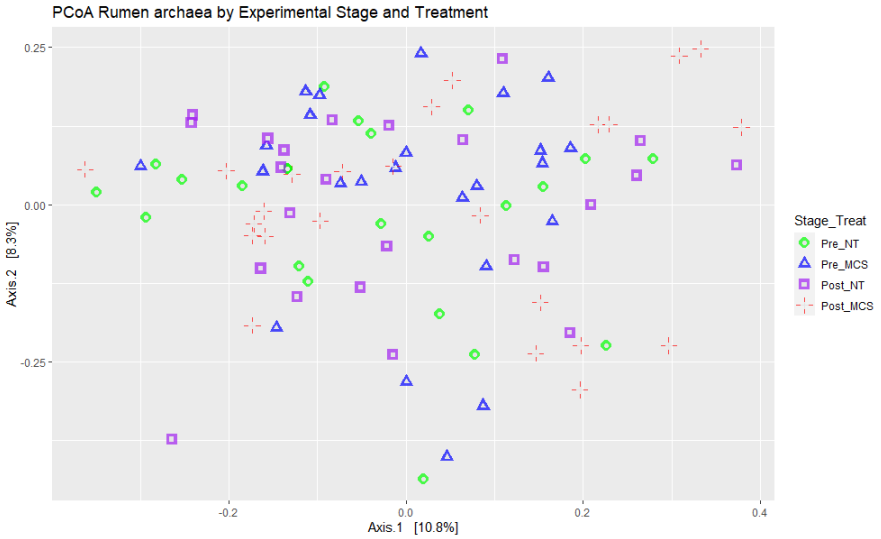

Figure 4.29 Principal coordinate analysis (PCoA) of the rumen archaeal community based on the SRT RA data of OTUs. The relative distances of all points represent the relative dissimilarities of the samples according to the Bray-Curtis index. Treatment groups are colour coded and distinguished by shape (green circle = PreNT, blue triangle = PreMCS, purple square = PostNT, red cross = PostMCS). The percentage of total variation explained by each PCoA axis is shown in brackets.

4.6.11.5 Correlation analyses

4.6.11.5.1 Correlation between rumen protozoa and plasma cortisol/ serotonin

Cortisol and serotonin both pre- and post-trial were not found to be correlated, as assessed via Spearman Correlation of untransformed data (Pre: Rho = -0.01, p = 0.94; Post: Rho =-0.15, p = 0.30).

The Spearman Rho Correlation Coefficient between cortisol post-trial and the Total protozoa counts was Rho = -0.01 (p = 0.94) indicating a very poor correlation. Spearman Rho between cortisol and Dasytricha resulted in Rho = 0.23 (p = 0.12), indicating the relationship between the two was poor. Similarly, cortisol and small Entodiniomorphs showed no significant relationship (Rho = -0.01, p = 0.92). Spearman-rho ranked correlation for cortisol- Isotricha and cortisol- Large-Entodiniomorphs did not indicate the presence of any statistically significant relationship between variables (Rho = -0.16, p = 0.27 and R = -0.07, p = 0.03 respectively).

Correlations of all Protozoa with serotonin were also non-significant (Small Entodiniomorphs: Rho = -0.001, p = 0.99; Large-Entodiniomorphs: Rho = -0.07, p = 0.63; Dasytricha: Rho = -0.17, p = 0.23, Isotricha: Rho = 0.19, p = 0.19 and Total count: Rho = 0.190, p = 0.50).

4.6.11.5.2 Canonical analysis of principal coordinates ordination plots

Canonical analysis of principal coordinates (CAP) plot produced using Bray Curtis distances calculated on the Hellinger transformed OTU data for rumen bacteria and archaea are depicted in **Figures 4.30**, **4.31**. The first two principal component axes (containing the most variability) were used for this plot, but the amount of variability explained is particularly low. A CAP plot aids in the visualisation of differences in the location or relative dispersion *between a priori* set groups. Here the length of the vectors and the lack of evident clustering suggest a weak link between OTUs, cortisol and serotonin.

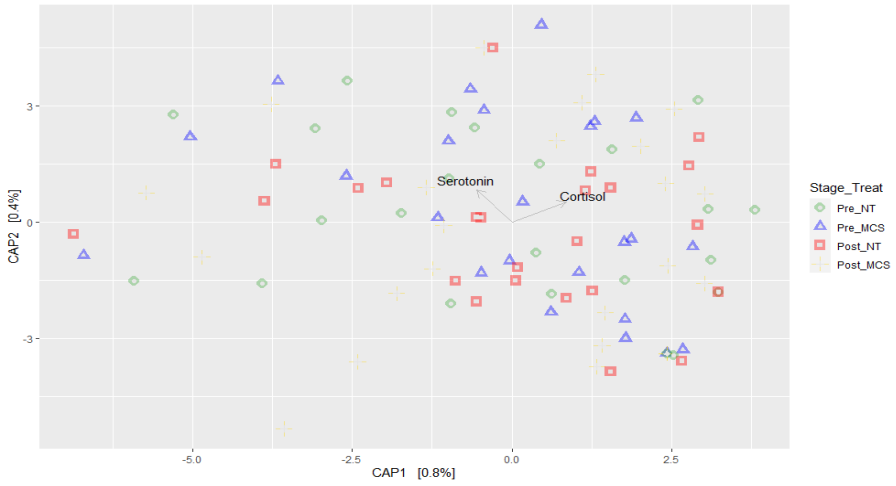

Figure 4.30 Canonical analysis of principal coordinates (CAP) ordination plot (based on Bray-Curtis of SRT rumen bacterial OTU abundances) showing canonical axes that best discriminate the bacterial community assemblages across grouped factors: Experimental Stage and Treatment (PreNT, PreMCS, PostNT, PostMCS) cortisol and serotonin values have been overlaid on the plot as vectors. Vector length corresponds to correlation strength.

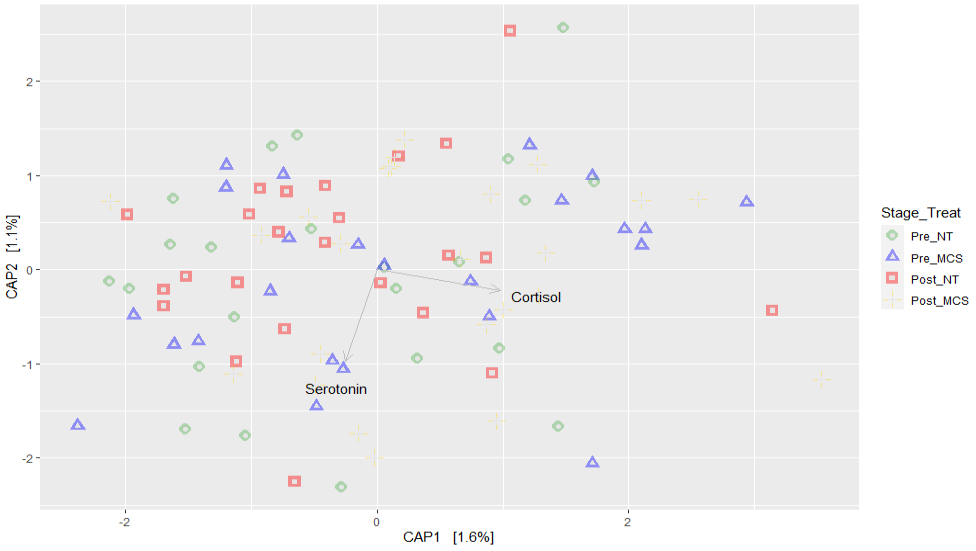

Figure 4.31 Canonical analysis of principal coordinates (CAP) ordination plot (based on Bray-Curtis of SRT rumen archaeal OTU abundances) showing canonical axes that best discriminate the bacterial community assemblages across grouped factors: Experimental Stage and Treatment (PreNT, PreMCS, PostNT, PostMCS). Cortisol and serotonin values have been overlaid on the plot as vectors. Vector length corresponds to correlation strength.

4.6.11.5.3 PLS Rumen relative abundances and cortisol/serotonin

4.6.11.5.3.1 PLS phylum level, order and genus level

A PLS regression between SRT RA of all rumen phyla with "log(x+1)" transformed cortisol values and "log" transformed serotonin values was carried out for the PostNT and PostMCS rumen samples.

The percentage of variability in cortisol and serotonin explaining variability present in the phyla, orders and genera RA is presented in **Table 4.26**. Following the PLS regression, Pearson correlation was conducted between phyla that had a VIP score >1.00, orders with VIP >1.50 and genera with VIP >1.50 and cortisol/serotonin. The correlation coefficient (R) was mainly used as an indication of the direction of the relationship.

Table 4.26 Percentage of cortisol and serotonin variability, and percentage of explained variability of RA for each Variable. The higher the percentage of variability explained in the RA, the higher our confidence in the relationship and VIP scores reported.

Group	% Cortisol variability	% Variability explained	% Serotonin variability	% Variability explained
Phyla				
PostNT	29.99	43.50	30.58	42.49
PostMCS	25.80	61.89	23.97	48.35
Orders				
PostNT	14.85	91.98	13.69	82.55
PostMCS	17.69	82.59	15.15	91.21
Genera				
PostNT	16.00	95.92	10.73	97.18
PostMCS	14.61	91.73	13.25	96.48

The correlation coefficient (R) was not higher than 0.8, or lower than -0.8 indicating that the phyla, orders and genera in question do not have a particularly strong relationship with cortisol or serotonin. phyla with PLS VIP scores >1.00, as well as orders and genera with VIP scores >1.50 for each group and their relationship with cortisol and serotonin have been reported in the Appendix **Tables 7.29-7.36.** VIP scores, R values and RA for the phyla with VIP >1.00

for each group can be found in Appendix (**Tables 7.37- 7.48).** Heatplots created on R to explore the relationship between cortisol and phyla by variable are also available in the Appendix (**Figures 7.45 -7.56).**

4.6.12 Faecal samples

4.6.12.1 Taxonomy analyses

The overall faecal community was comprised 98.90% of bacteria (range: 96.50% to 99.50%), 1.10% of archaea (range: 0.00% to 3.50%) and 1.90% was unclassified.

At the phylum, order and genus level, the most abundant bacteria and archaea are presented in **Figures 4.32- 4.36** below. Phyla representing less than 1.00% of the overall population included *Cyanobacteria*, *Actinobacteria*, Synergistetes, *Verrucomicrobia*, *Plantomycetes* and *Proteobacteria*. Other orders <1.00% included: *YS2_Cyanobacteria*, *ML515J-28_RF3_Tenericutes*, *RF39_Moliccutes*, *Erysipelotrichales*, *Fibrobacterales* and *Verrucomicrobiales*. genera <1.00% included: *Clostridium, Fibrobacter, YS2_Cyanobacteria, Roseburia, S24-7_Bacteroidales and Unidentified_Paraprevotellaceae.* More details can be found in Appendix **Table 7.28**.

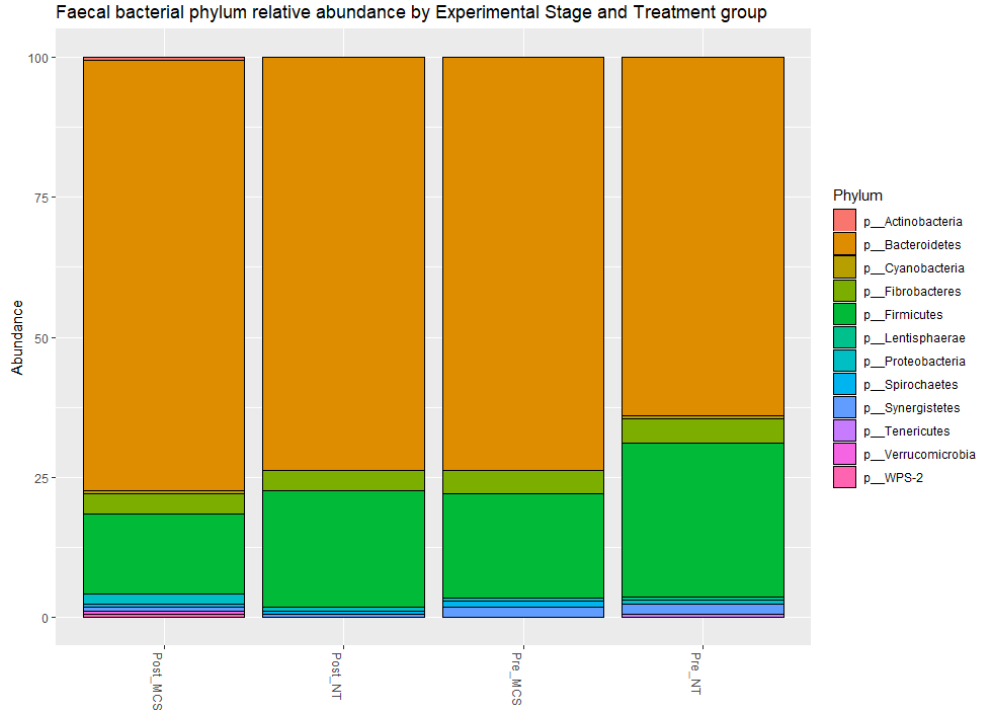

Figure 4.32 Faecal bacterial phyla by Experimental Stage and Treatment groups (Pre-MCS, Pre-NT, Post-MCS and Post-NT).

Table 4.27 Phyla with significantly different faecal RA between groups: PreNT, PostNT, PreMCS, PostMCS. The p-values acquired from the Kruskal Wallis nonparametric testing, as well as the F-value are reported alongside mean and SD values for each group.

Faecal Phyla						
Phyla	**Kruskal p-value**	**chi-square**	**mean ±SD**			
			PreNT	**PreMCS**	**PostNT**	**PostMCS**
Euryarcheota	<0.01	16.46	0.10 ±0.02[a]	0.09 ±0.02[a]	0.11 ±0.01[ab]	0.10 ±0.01[b]
Actinobacteria	<0.01	16.80	0.04 ±0.01[a]	0.03 ±0.01[a]	0.04 ±0.02[ab]	0.05 ±0.02[b]
Cyanobacteria	<0.01	11,58	0.06 ±0.01[a]	0.06 ±0.01[ab]	0.07 ±0.01[b]	0.07 ±0.01[b]
Lentisphaerae	0.03	9.13	0.04 ±0.02[a]	0.05 ±0.01[ab]	0.05 ±0.02[ab]	0.05 ±0.01[b]
Spirochaetes	<0.01	18.44	0.11 ±0.03[a]	0.15 ±0.03[b]	0.13 ±0.02[a]	0.13 ±0.03[ab]

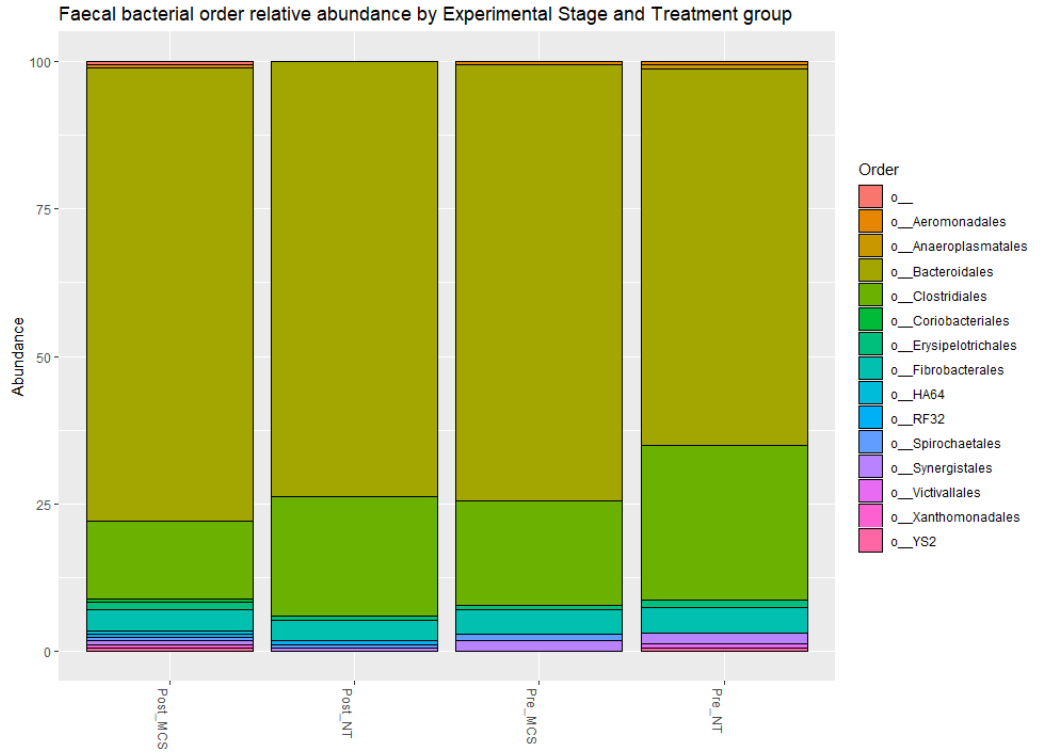

Figure 4.33 Faecal bacterial orders by Experimental Stage and Treatment groups (Pre-MCS, Pre-NT, Post-MCS and Post-NT).

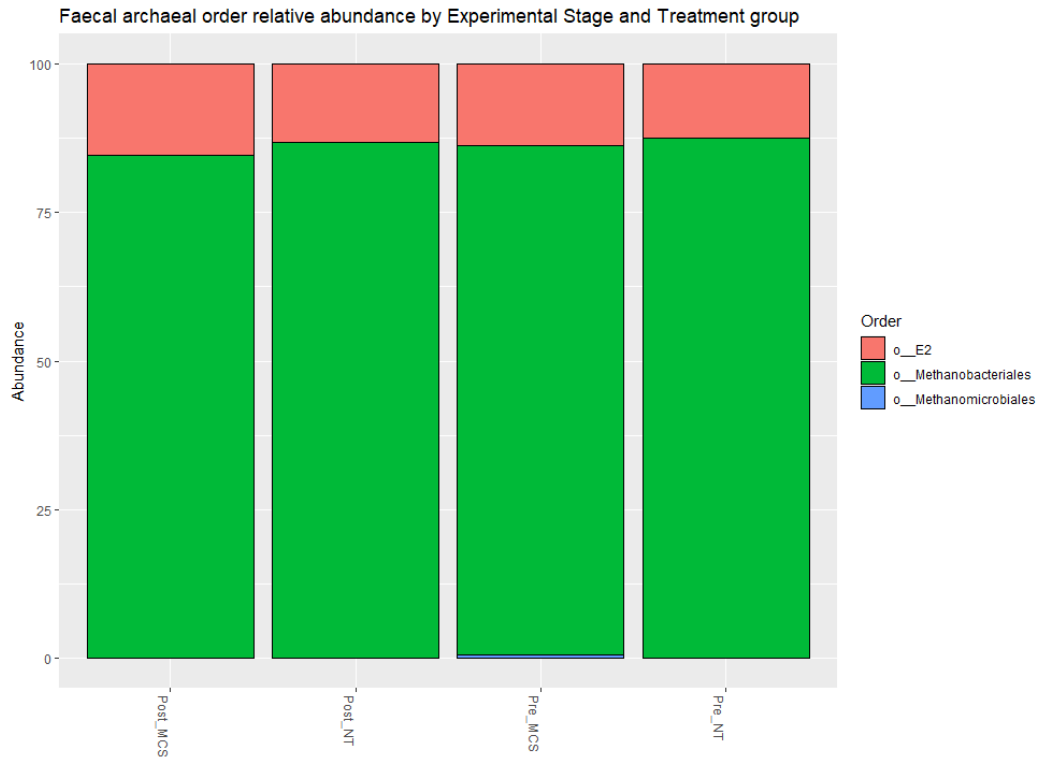

Figure 4.34 Faecal archaeal orders by Experimental Stage and Treatment groups (Pre-MCS, Pre-NT, Post-MCS and Post-NT).

Table 4.28 Orders over 0.5 % with significantly different faecal RA between groups: PreNT, PostNT, PreMCS, PostMCS. The p-values and F-values acquired from the Kruskal Wallis tests and mean ±SD values are reported for each group.

Orders over 0.5 percent faecal						
Orders	**Kruskal p-value**	**chi-square**	**mean ±SD**			
			PreNT	**PreMCS**	**PostNT**	**PostMCS**
Methanomicrobiales	<0.01	11.85	0.009 ±0.004[a]	0.009 ±0.003[a]	0.01 ±0.004[b]	0.01 ±0.003[ab]
YS2	0.02	10.17	0.004 ±0.002[a]	0.005 ±0.002[ab]	0.006 ±0.002[b]	0.005 ±0.002[ab]
Spirochaetales	<0.01	13.06	0.01 ±0.008[a]	0.02 ±0.007[b]	0.02 ±0.009[a]	0.02 ±0.008[a]

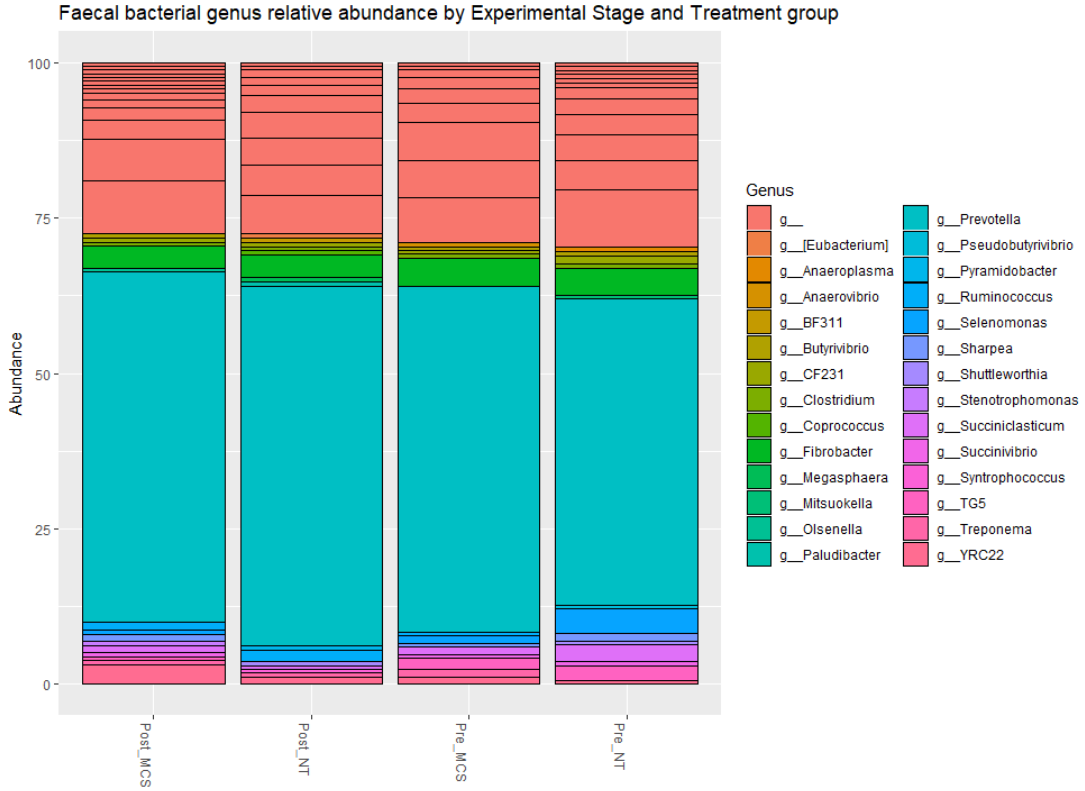

Figure 4.35 Faecal bacterial genera by Experimental Stage and Treatment groups (Pre-MCS, Pre-NT, Post-MCS and Post-NT).

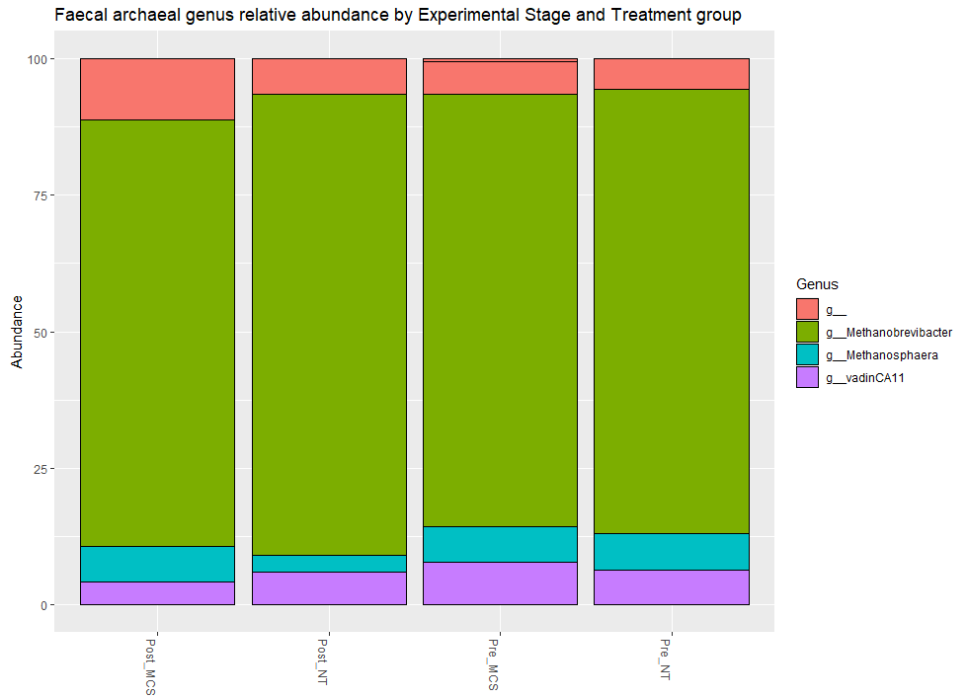

Figure 4.36 Faecal archaeal genera by Experimental Stage and Treatment groups (Pre-MCS, Pre-NT, Post-MCS and Post-NT).

Table 4.29 Genera over 1% with significantly different faecal RA between groups: PreNT, PostNT, PreMCS, PostMCS. The P-values acquired from the Kruskal Wallis non-parametric testing, as well as the Wilcoxon rank test P-value (used for post-Hoc analysis) are reported alongside mean and SD values for each group.

Genera over 1 percent faecal						
Genera	**Kruskal p-value**	**chi-square**	**mean ±SD**			
			PreNT	**PreMCS**	**PostNT**	**PostMCS**
Unidentified_ Methanocorpusculaceae	<0.01	11.75	0.09 ±0.02^{a}	0.09 ±0.02^{a}	0.1 ±0.02^{b}	0.09 ±0.02^{a}
Bacteroides	<0.01	23.63	0.1 ±0.03^{a}	0.1 ±0.02^{b}	0.1 ±0.03^{c}	0.1 ±0.05^{a}
Unidentified_ Bacteroidaceae	0.02	9.30	0.2 ±0.06^{a}	0.3 ±0.06^{a}	0.3 ±0.04^{a}	0.2 ±0.05^{a}
Unidentified_ Rikenellaceae	<0.01	13.61	0.25 ±0.03^{a}	0.25 ±0.03^{a}	0.2 4±0.03^{ab}	0.22 ±0.03^{b}
Treponema	<0.01	19.66	0.12 ±0.03^{a}	0.10 ±0.03^{b}	0.1 1±0.02^{ab}	0.12 ±0.03^{a}

4.6.12.2 Faecal alpha diversity bacteria and archaea

An effect of Experimental Stage was observed for the faecal bacterial alpha diversity indices overall, which were higher post-trial compared to pre-trial, except Chao1 and ACE, reflecting an overall increase in diversity. Inverse Simpson was also significantly higher for the MCS group pre-trial, but this difference was not observed post-trial, while NT Simpson increased post-trial compared to pre-trial. No differences were observed for the faecal archaea indices. Boxplots were created in "phyloseq" using rarefied reads, to visualise differences in diversity indices calculated for the bacterial and archaeal faecal OTUs. The boxplots presented here (**Figures 4.37-4.38**) are by grouped variables: PreNT, PreMCS, PostNT, PostMCS and for all variables.

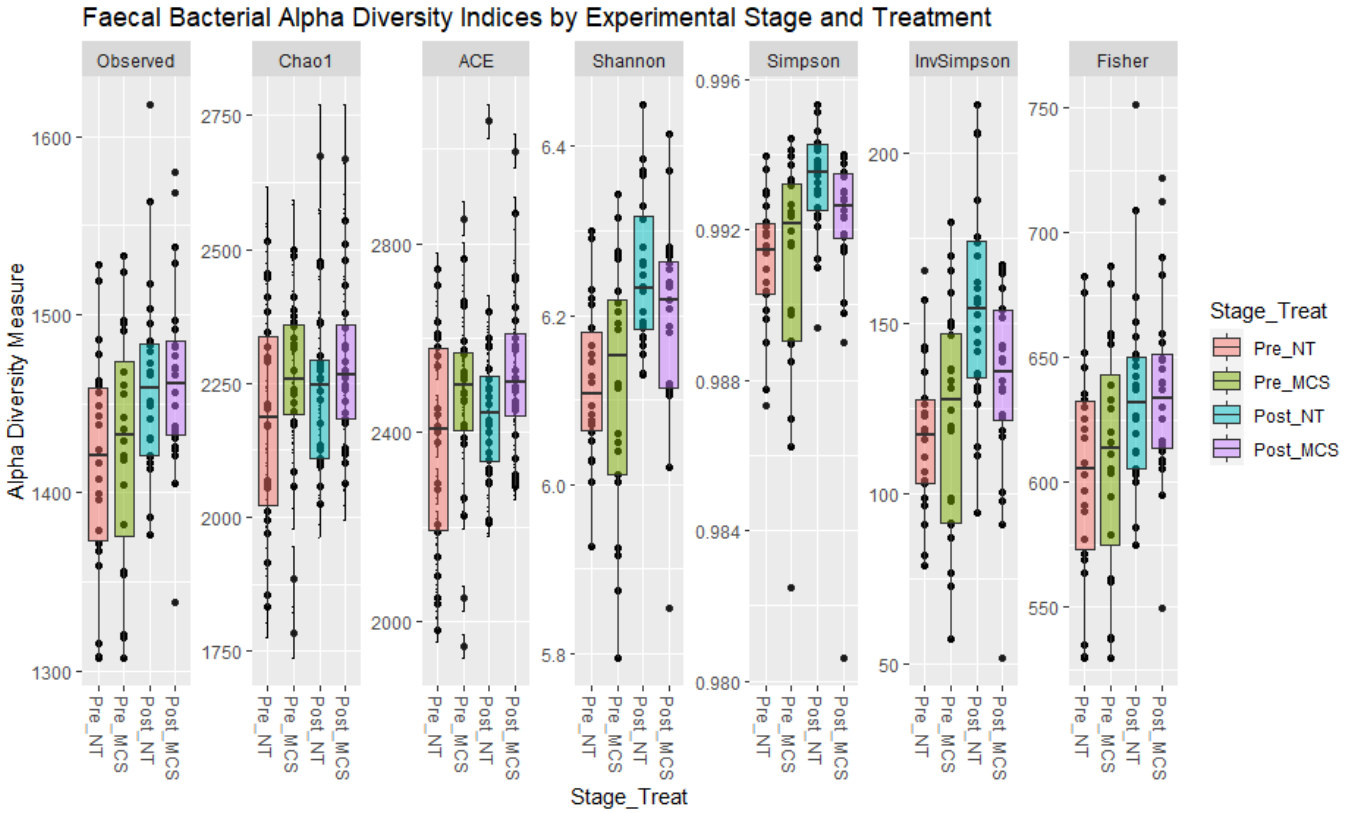

Figure 4.37 Boxplot of the Diversity Indices (Observed, Chao1, Ace, Shannon, Simpson and Inverse Simpson) calculated via "phyloseq" on rarefied counts, to examine differences in Faecal Bacterial diversity between Experimental Stages and Treatment groups (PreNT: pink; PreMCS: green; PostNT: blue; PostMCS: purple). Significant differences were observed at an Experimental Stage and further Kruskal-Wallis tests indicated differences mainly between PreNT and PostNT ($p < 0.01$) for the Shannon, Simpson and Inverse Simpson Indices. Means, SD and IQR are presented.

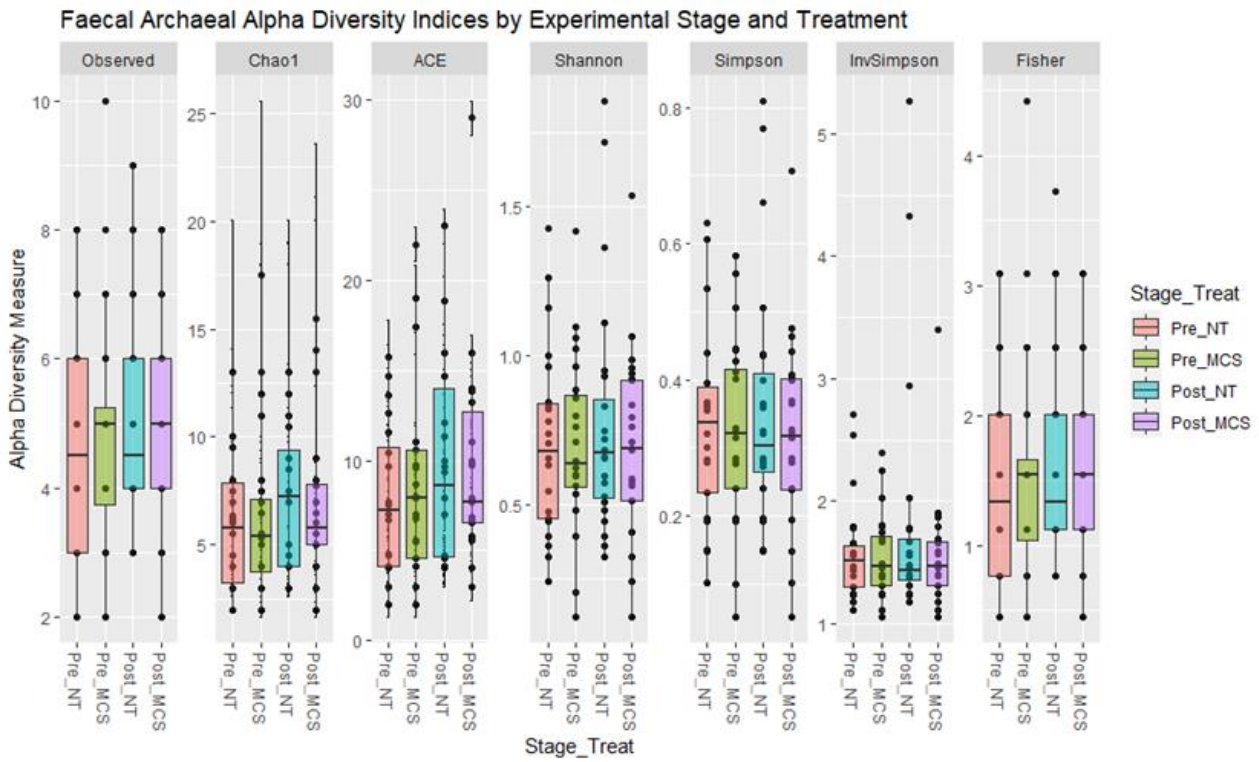

Figure 4.38 Boxplot of the Diversity Indices (Observed, Chao1, Ace, Shannon, Simpson and Inverse Simpson) calculated via "phyloseq" on rarefied counts, to examine differences in Faecal Archaeal diversity between Experimental Stages and Treatment groups (PreNT: pink; PreMCS: green; PostNT: blue; PostMCS: purple). No differences were observed. Means, SD and IQR are presented.

4.6.12.3 Faecal beta diversity

Dispersion of bacterial samples between groups: PreNT, PreMCS, PostNT and Post MCS, were significantly different but did not differ for the archaea.

A Multivariate Analysis of Variance test (Adonis) indicated significant differences between Groups (PreNT, PreMCS, PostNT, Post MCS) for the archaea and bacteria. Further details are reported in **Table 4.30** below

Table 4.30 Results from the beta-dispersion and Adonis tests are presented below for PreNT, PreMCS, PostNT and PostMCS faecal bacteria groups. P-values, F-values, squared estimate of errors (SSE) and mean squared errors (MSE) have been reported accordingly. Significance has been noted with the presence of a "*" symbol.

Bacterial β-Dispersion results				
	PreNT-PreMCS	**PreNT-PostNT**	**PreMCS-PostMCS**	**PostNT- PostMCS**
p-value	<0.01*	<0.01*	<0.01*	<0.01*
F-value	1.26	1.31	1.41	1.47
SSE	0.45	0.46	0.49	0.50
Bacterial Adonis results				
	PreNT-PreMCS	**PreNT-PostNT**	**PreMCS-PostMCS**	**PostNT- PostMCS**
P-value	0.03*	<0.01*	<0.01*	0.04*
F-value	1.26	0.03	1.41	1.62
MSE	0.44	0.46	0.30	0.60
Archaeal Adonis results				
	PreNT-PreMCS	**PreNT-PostNT**	**PreMCS-PostMCS**	**PostNT- PostMCS**
P-value	0.02*	0.14	0.42	0.02*
F-value	0.75	1.12	0.90	0.76
MSE	0.42	0.32	0.25	0.21

4.6.12.4 PCoA plots

PCoA plots for Treatment, Experimental Stage and grouped variables (PreNT, PreMCS, PostNT and PostMCS) are presented below. For the bacteria, the percentage explained by each axis (Axis 1: 2.2%, Axis 2: 1.1%) is very low, indicating we are not capturing the variability present in this way. This may be once again since there were many bacterial OTUs present at very low abundance. For the archaea, description levels are slightly higher, with Axis 1 explaining: 9.00% and Axis 2: 7.40%. Distinct clusters due to Treatment* Experimental Stage are not clear, although overall the NT and MCS treatments both appear to evolve over time (**Figures 4.39-4.40**).

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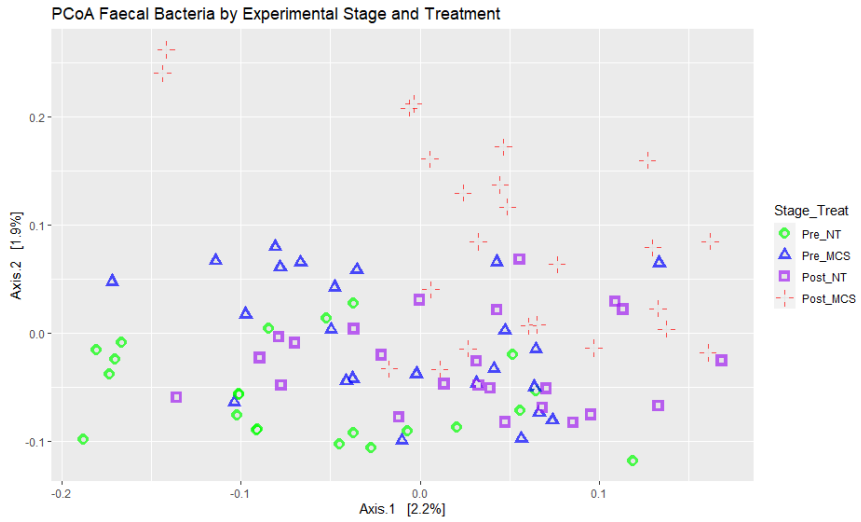

Figure 4.39 Principal coordinate analysis (PCoA) of the rumen bacterial community based on the SRT RA data of OTUs. The relative distances of all points represent the relative dissimilarities of the samples according to the Bray-Curtis index. Treatment groups are colour coded and distinguished by shape (green circle =PreNT, blue triangle =PreMCS, purple square =PostNT, red cross =PostMCS). The percentage of total variation explained by each PCoA axis is shown in brackets.

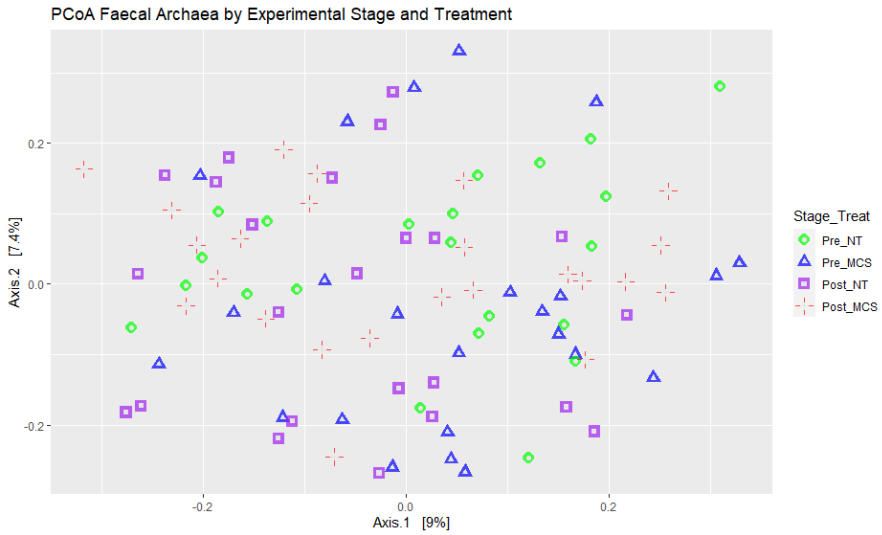

Figure 4.40 Principal coordinate analysis (PCoA) of the rumen archaeal community based on the SRT RA data of OTUs. The relative distances of all points represent the relative dissimilarities of the samples according to the Bray-Curtis index. Treatment groups are colour coded and distinguished by shape (green circle = PreNT, blue triangle = PreMCS, purple square = PostNT, red cross = PostMCS). The percentage of total variation explained by each PCoA axis is shown in brackets.

4.6.12.5 Correlation analyses

4.6.12.5.1 CAP Plots

The CAP plots exploring cortisol and serotonin relationships with OTU data for faecal bacteria and archaea are depicted in **Figures 4.11-4.42**. The first two principal component axes (containing the most variability) were used for this plot, but the amount of variability explained is particularly low. Here, the length of the vector for serotonin in the opposite direction to PreMCS and PostNT faecal bacterial clusters and in the direction of a PostMCS cluster, suggesting a correlation between the OTUs of this subgroup and serotonin. For the archaea, clustering is not as evident.

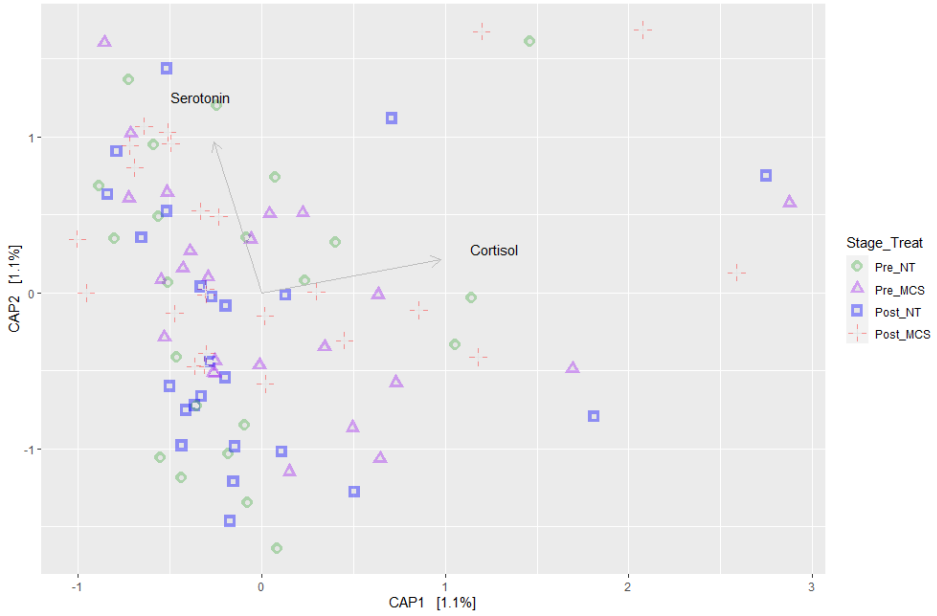

Figure 4.41 Canonical analysis of principal coordinates (CAP) ordination plot (based on Bray-Curtis distances of SRT faecal bacterial OTU abundances) showing canonical axes that best discriminate the bacterial community assemblages across grouped factors: Experimental Stage and Treatment (PreNT, PreMCS, PostNT, PostMCS). Cortisol and serotonin values have been overlaid on the plot as vectors. Vector length corresponds to the strength of the correlation.

Figure 4.42 Canonical analysis of principal coordinates (CAP) ordination plot (based on Bray-Curtis distances of SRT faecal archaea OTU abundances) showing canonical axes that best discriminate the bacterial community assemblages across grouped factors: Experimental Stage and Treatment (PreNT. PreMCS, PostNT, PostMCS) Cortisol and serotonin values have been overlaid on the plot as vectors. Vector length corresponds to the strength of the correlation.

4.6.12.5.2 PLS Faecal relative abundances and cortisol/serotonin

4.6.12.5.2.1 PLS phylum, order and genus level

A PLS regression between SRT RA of all faecal phyla with "log(x+1)" transformed cortisol and "log" serotonin values was carried out for PostNT and PostMCS groups. The percentage of Variability in cortisol explaining Variability present in the RA is presented in **Table 4.39**below. Following the PLS regression, Pearson correlation was conducted between phyla that had a VIP score >1.00, orders with VIP >1.50 and genera with VIP >1.50, in each group and cortisol/serotonin concentration. The correlation coefficient (R) was mainly used as an indication of the direction of the relationship.

Table 4.31 Percentage of cortisol and serotonin variability and percentage of explained variability of RA for each variable. The higher the percentage of variability explained in the RA, the higher our confidence in the relationship and VIP scores reported.

Group	% Cortisol variability	% Variability explained	% Serotonin variability	% Variability explained
Phyla				
PostNT	25.94	41.97	24.46	46.06
PostMCS	24.14	61.59	19.92	48.98
Orders				
PostNT	13.73	90.94	13.53	83.42
PostMCS	11.93	88.26	14.03	95.36
Genera				
PostNT	12.31	98.57	12.31	98.57
PostMCS	12.39	96.82	12.50	96.72

The correlation coefficient (R) was not higher than 0.8, or lower than -0.8 indicating that the phyla, orders and genera in question do not have a particularly strong relationship with cortisol or serotonin. Phyla with PLS VIP scores >1.00, as well as orders and genera with VIP scores >1.50 for each group and their relationship with cortisol and serotonin are reported in the Appendix **Tables 7.50-7.57**.

Heatplots created on R to explore the relationship between cortisol/ serotonin and phyla, orders and genera are also available in the Appendix (**Figures 7.57- 7.68** and corresponding data is available in Tables **7.58-7.69).**

4.7 Discussion

The MCS treatment had a significant effect on animal behaviour in terms of altered response to novelty (such as increased interaction with the novel environment and decreased contact/interaction with the ball). Heart rate parameters did not act as clear indictors of chronic stress, although average RR fluctuations for the MCS group during the night periods assessed, compared between days, may act as an indication of sympathetic hyperactivation. Apart from BHB concentrations, the biomarkers used did not indicate differences between Pre and Post trial estages and Treatment groups.

Bacterial alpha diversity increased in rumen and faecal samples for both groups over time, particularly for the NT group. Interesting findings in terms of changes in relative abundances in the rumen were a significant increase of *Firmicutes* post trial for the NT group, and of *Proteobacteria* and *Spirochaetes* (indicators of disease and stress) for the MCS group.

A significant decrease in *Euryarcheota* was observed for NT group Post trial and a significant increase in *Actinobacteria* abundance increase for the MCS lambs Post-Trial. *Euryarcheota* are linked to methanogenesis, so potentially a drop in relative abundance could be considered beneficial, whereas increased *Actinobacteria* have been reported in animals and humans expressing depressive states.

Finally, PLS and correlation analyses between cortisol and serotonin and PostNT, PostMCS RA at a phylum, order and genus level did not result in significant correlations (R >0.8 or <-0.8) despite some high VIP scores. This is likely due to the power of these analyses, as generally large quantities of data are required for efficient and accurate correlation analyses, or simply the hormone levels observed did not, in this case, reflect accurately the dynamics within the rumen and faecal microbial community.

4.7.1 Weight and ADG

There was no significant Treatment effect on animal weight, taking into consideration their weight prior to the trial -although Treatment did appear to influence ADG. Starting the trial, the MCS animals had higher ADG compared to the NT group, but this was reversed during the 6 weeks as NT animals had significantly higher ADG. Despite this, the lambs at the final weighing after all tests had been conducted did not differ in terms of ADG.

This suggests that the stressors applied to the MCS group could have an effect on their motivation to eat, as seen in frequency of Eating Hay for the MCS, which was significantly higher in the middle compared to the end of the trial, and from the number of Eating Hay

bouts, which was consistently higher for the NT group. Finally, the decline in growth rate for both groups towards the end of the trial is explained by the fact that these were lambs, still growing when the trial started, but at the end of the trial were almost 8 months of age, when their weight may be reaching a plateau as they were closer to achieving mature weight.

4.7.2 Hormones and Stress metabolites

As observed after the individual restraint test, this stressor did significantly affect cortisol, glucose, NEFA and BHB plasma levels as there was an increase in all biomarker concentrations compared to Pre-trial levels, and the NT animals sampled on the same day. This means that the HPA axis was mobilised leading to an increase in cortisol levels and, an increase in glucose, BHB and NEFAs, which may reflect the mobilisation from fat and lipid stores in response to negative energy balance but are regarded (mainly glucose) as indicators of stress in ruminants. The fact that these results are from the 3rd repetition of the stressor demonstrate that the stressor was still effective and that the animals exhibited a stress response, 5 weeks into the trial.

In terms of chronic stress, there was no Treatment effect on plasma cortisol, glucose and NEFA concentrations indicating any differences in fat mobilisation or an effect of the stressors on animal metabolism. Of note, cortisol levels were particularly low in these animals both pre- and post-trial, with concentrations frequently under the detection threshold of the ELISA. However, as these samples were analysed at the same time as the samples from chapter 2, there was no reason to consider these findings erroneous. Similarly, there was no significant effect of Treatment or experimental stage on plasma serotonin levels.

However, there was an effect of experimental stage and interaction with Treatment on BHB, as for both groups BHB was higher post-trial and NT levels were higher compared to the MCS group at the end of the trial. These levels were normal (<0.08 mmol/l for sheep) (MacNeill, 2009) and this finding could yet again be related to the fact that throughout the trial NT animals had higher ADG and had a higher number of hay eating bouts, reflecting that potentially the MCS animals' eating motivation and metabolism were affected by the stress treatment.

4.7.3 Effect on behaviour

4.7.3.1 Individual restraint

Regarding the reactions the animals displayed to the individual restraint test using the Gambrel Restrainer, on D1 there was an observed higher number of Posture "0" meaning the animal was immobile, and Posture "3": strenuous effort to escape. On D2, Posture "1" was significantly lower, and overall using chi-squared tests, the interpretation of the results is not clear, apart from the fact that there was an obvious aversion towards being restrained and that from the metabolites tested (cortisol, BHB, NEFA and glucose), there was a response to the stressor as indicated by higher blood plasma concentrations of these markers.

4.7.3.2 Time budgets, non-stress days

As mentioned above, one of the most significant findings was that the frequency of Eating Hay declined over time, but that the NT group performed longer bouts and were more synchronised (both in bouts and duration of bouts). Disruption of eating habits due to the implementation of stressors throughout the day, as well as placing a grid once a week over the MCS concentrate feeders, may have influenced the MCS lambs' motivation to eat and their eating habits. Alternatively, the constant physiological activation via cumulative stress responses may have started to impact metabolism reflected in the behaviour. Eating behaviour and metabolism are interlinked, and the direction of cause-effect relationships is not clear.

Also, in terms of concentrate consumption, there was a significant drop in frequency between the first and third 24h Time Budget (i.e. in the middle of the trial), but an increase during the last monitoring session. MCS animals displayed more synchronised bouts compared to the NT group, which may also be an effect that the limitation of access to the concentrate had on the MCS lambs, by motivating them to eat faster and grouped together due to the potential event of being disrupted or not being allowed to have access. These differences could potentially be linked to metabolism factors such as the lower BHB levels in MCS animals compared to the NT group at the end of the trial (although the relationship is not clear), and also the difference between Pre and Post experimental stages in terms of microbial diversity in the NT lambs.

As a gregarious species, sheep tend to synchronise their behaviours and activities with their flock mates as a defence mechanism against predators, whilst increasing social cohesion which allows physical and emotional security (also observed in other species) (Duranton and Gaunet, 2016). Therefore, the investigation of disruption or differences between Treatment groups could act as a good indicator of the effect of stress (Caroprese *et al.*, 2016; Richmond *et al.*, 2017; Mattiello *et al.*, 2019). Interestingly, the lambs in the MCS group showed a decline in

resting bouts over time (and an increase in NT animals although post-Hoc analysis did not render significant results), and more/ longer synchronised bouts of this behaviour compared to the NT group. This may be an influence the repeated stress had on the animals, motivating them to increase their social cohesion mentioned above as a response.

In terms of the "Sleeping" behaviour, there was an overall increase in synchronisation over time and the average length of synchronisation bouts, particularly in the middle of the trial for both groups. Both groups became less synchronised while "Moving" towards the end of the trial, while MCS animals had longer synchronised bouts of moving during the middle part of the trial compared to the NT group, again indicating that the MCS animals sought an increased cohesion. In contrast, the frequency of standing "immobile" increased over time for both groups, as did the synchronisation and length of synchronisation bouts for this activity. In this case, at the middle of the study, more synchronisation bouts were observed for the NT group, potentially suggesting a calmer state. These findings could also be related to age, as ruminants, as other animals appear to maintain "personality" traits, but activity and general behaviours change with age (Hedlund and Løvlie, 2015; Foris *et al.*, 2018; Neave *et al.*, 2018; Zablocki-Thomas *et al.*, 2018).

4.7.3.3 Ball Test

4.7.3.3.1 Home-pen

The NT animals were faster to interact with the ball and faster to start eating hay again after the ball drop, indicating a loss in interest in the stimulus or that the event did not have a lasting impact on their motivation to eat. This is in contrast with literature findings where sheep demonstrated lower motivation to feed after being stressed (Doyle *et al.*, 2015), although seeking food may be compensatory mechanism to induce a positive state (Keen-Rhinehart, Dailey and Bartness, 2010; Volkow, Wang and Baler, 2011).

In both cases, latency was higher for the MCS meaning that the animals were more hesitant to interact with the novel object (increased fearfulness) and that their motivation to eat hay was affected. Despite this, in the 30 minutes of continuous observation, the MCS group spent more time eating hay overall. Furthermore, the frequency with which the animals contacted and interacted with the ball was lower in the MCS group, once again pointing towards increased fearfulness toward the novel object. These results are in agreement with findings by (Mendl *et al.*, 2009; Doyle *et al.*, 2015) , which indicated that stressed animals may exhibit differences in bias, as well as higher activity levels.

4.7.3.3.2 ***Novel pen***

Latency to contact and interact with the ball was once again higher for MCS animals meaning they were more hesitant towards this object. There was also a Day effect on latency, but considering that D1, D3 and D2, D4 were the days that the same pens were tested, a significant increase in time lapsed to approach the ball on D4 compared to D2, which may be a result of loss of interest in the ball after repetitions of the test, as was expected. Latency to contact and interact with the environment was assessed post ball drop as an indicator of motivation to explore, versus motivation to interact with the novel object.

There was a Day effect with D1 latency to interact with the environment being higher compared to D3 and D2 latency being higher compared to D4, which is logical since potentially the first time the ball dropped, it presented more of a stimulus and interested the animals more than exploring the environment. Additionally, on D1 and D3, MCS animals were slower to interact with the environment compared to NT lambs, which in combination with MCS animals being slower overall to interact with the ball, indicates that this subgroup of MCS animals was more fearful.

Similarly, latency to contact the environment was reduced over time for both groups, meaning that potentially they lost interest in the ball and/or became less fearful and explored the pen more. MCS animals were observed to contact and interact more frequently and for a longer overall time (with) the environment, which can be interpreted either as lack of fearfulness or as frustration. As “Interaction with the Environment” also included behaviours such as climbing the panels making up the pen and the door with their front legs, and head-butting or licking/chewing surfaces. It would warrant further investigation to assess the motivation and emotion behind these behaviours (Reefmann *et al.*, 2009a, 2009b).

Both pre and post ball drop, vigilance duration was higher for NT lambs compared to MCS overall, and particularly frequency of vigilance was higher for the NT group compared to the MCS on D4, a potential explanation for which may be that the NT animals of this group were negatively pre-disposed and expecting the ball to drop (faster learning skills). This is purely speculative; therefore, an alternative could be that the MCS lambs were more anxious and took longer to habituate, describing a greater startle response and tendency towards negative bias. In general, and not surprisingly, frequency of vigilance declined over the repetitions of the test. This was not in agreement with Reefmann *et al.*'s (2009b) findings, where ear postures used to describe valence, indicated that calmer animals had less valence occurrences. However, increased activity as mentioned before and lower vigilance pre-ball drop may be

indicators of negative effects on cognitive bias and increased arousal (Mendl *et al.*, 2009; Doyle *et al.*, 2015).

Post ball drop, NT animals spent more time and approached/ interacted with the ball more frequently compared to the MCS animals. There was a decline in both duration and frequency over the repetitions of the test particularly for the animals tested on D1 and D3. In terms of contact and interaction with the environment, duration was higher for MCS lambs. Frequency of contact was higher for NT animals although frequency of interaction was higher for MCS, suggesting once again that MCS may be less fearful or more agitated. A decline in interest in the environment over the repetitions of the test is considered normal as the animals become used to the procedures and the pen.

Once again these findings are consistent with literature where motivation and biases towards novelty are affected by stress (Doyle *et al.*, 2011, 2015; Alexandra Destrez *et al.*, 2013; Destrez *et al.*, 2014; Lee *et al.*, 2016; Monk *et al.*, 2018)

4.7.4 Heart rate

4.7.4.1 Individual restraint

Surprisingly, when comparing Step1 from non-stressed monitoring days to the 20-minute periods the animals were individually restrained, no significant effect of Treatment was observed. This may be due to the animals' posture, as they were not able to move, or potentially the 4h time frames were more variable compared to the 20 minutes, which did not allow efficient assessment of the effect of the stressor. A different explanation may be that the HR increased when placing the restrainer, but as the animals were mainly in Posture 0 (immobile), HR dropped and there was a potential delayed effect after freeing the animal from the restrainer. This potential effect was not investigated further.

4.7.4.2 No stress days

Using the area under the curve to investigate overall differences in heart rate variables by day of monitoring, average RR was higher in MCS compared to the NT animals. RR is a measure of the distance between consecutive peaks in the electrocardiogram, and a higher number reflects potentially shorter distances between peaks, as a result of a higher heartrate. Subsequently, HR areas under the curve were overall higher in the MCS animals, except on D3 in the middle of the trial.

RMSSD was lower overall for MCS animals. RMSSD reflects the communication between the vagus nerve and autonomic control of the heart. Low levels have been linked to several pathologies, and in humans with depression and unexplained death in epilepsy (Francis *et al.*, 2009; DeGiorgio *et al.*, 2010). The ratio between Low Frequency and High Frequency, considered to be a potential indicator of PNS and SNS balance, was higher overall for MCS animals, an indication of sympathetic dominance.

In order to capture the variability due to circadian rhythms and animal management practices, the analysis conducted by Step (4h time frames during the day) demonstrated that for most Steps (1,3,4,5), average RR counts were higher for MCS animals. Unfortunately, this cannot be evaluated further since it was a consistent finding from the pre-experimental stage day (D0). However, although there was a decline in average RR over time for the NT animals, RR increased at each monitoring session for the MCS animals and particularly on D3 (at the middle of the trial) before a significant drop occurred on the last monitoring session. This finding was also observed for Step 4 on these two days. Since these were the periods when lights went out and theoretically the animals would be resting, it poses an interesting timeframe to inspect the influences of stress on the SNS and heart rate. These irregularities over time for the MCS animals may indicate hyperactivation of the SNS system since irregularities in RR intervals have also been linked to increased mental stress (Dimitriev and Saperova, 2015).

Regarding Step4 (night period: 22h00 -02h00), average heart rate did not differ pre-trial for the two Treatment groups, but on D2 it was significantly higher for MCS animals, followed by a big drop on D3 and an increase again on D4. This fluctuation could be a further indication of disturbance for the MCS group. Surprisingly, during Step 5 (night period: 02h00 – 06h00), average HR (bpm) is significantly lower on all days in MCS animals and particularly on D3. HR tended to increase over time for the NT animals, while for the MCS animals the tendency was the opposite. On D4, the last monitoring session at the end of the trial, heart rate was higher than normal levels (65-85 bpm) for both groups and particularly for the NT group. Chronic stress is known to have an impact on circadian rhythm and most studies on chronically stressed animals describe a blunted circadian rhythm (e.g. less difference over time) than non-stressed animals (Veissier, Le Neindre and Trillat, 1989; Gorka, Moryl and Papp, 1996; Dwyer and Bornett, 2004; Lightman, 2008; Koch *et al.*, 2017; Veissier, Mialon and Sloth, 2017). For example, in the study conducted by Veissier, Mialon and Sloth (2017), the circadian rhythm of activity in dairy cows in commercial settings was investigated, using a real-time positioning system. Three hundred and fifty cows' individual positions were recorder over a priod of 5 months in a Danish dairy farm to assess activity (i.e., resting, feeding, in alley). The research group found that the average level of activity of a cow on a given day and its variations during

that day depended on physiological states such as estrus, lameness and mastitis, all of which can cause distress to an animal and lead to physical stress. Interestingly, circadian variations in activity appeared to vary in a more pronounced way 1 to 2 days before the farmer detected a disorder (Veissier, Mialon and Sloth, 2017).

In the present study it is also of note that all animals had relatively high average HR, even during calm situations that may be due to age and breed effects.

Due to the fluctuations seen for average RR and higher HR for the MCS animals, we hypothesise that the repeated stressors did have an impact on the lambs, albeit short lived, as for the 4th heart rate monitoring session, conducted 5 days after no stressors, the night period HR and average RR results did not reflect the results of the previous days.

4.7.4.3 Ball test

4.7.4.3.1 Home pen

Comparison of Step 2 of NS D4 (the last day of monitoring heart rate after the stress trial and before the ball test) with the 25-minute step post-ball drop in the home pens, did not indicate any significant changes in any of the HR variables. As above, the variability in the longer time frames may have masked the variability present in the 25 minutes and resulted in non-significant findings. Treatment groups did not respond differently in terms of heart rate variables.

During the first night step (22h00- 02h00), average RR was significantly lower for both Treatment groups compared to the NS night, meaning that the stressor impacted this variable by decreasing the distance between peaks. HR was also lower compared to the NS day particularly for the NT group. The fact that there were lower RR and HR at this step for the ball test night may be an effect of the SNS still being activated, as HR recovery involves PNS reactivation while SNS activity remains elevated.

During the second night step (02h00 -06h00), the same effects as above were observed with RR and HR significantly lower for both Treatment groups compared to the NS night. RMSSD and SDNN were higher for the ball drop night step, which is in accordance with the findings above, since RMSSD reflects the beat-to-beat variance in HR and is used to estimate the vagally mediated changes reflected in HRV. Furthermore, there was a significant difference in the LF/HF ratio, which was lower on the ball test night, indicating parasympathetic dominance.

4.7.4.3.2 Novel environment

Comparison of Step 2 from D4 with the 25min step from moving the animals to the test pen to the ball drop, demonstrated higher HR, which increased with every repetition of the test. Higher LF/HF and reduced RMSSD were also observed, all indicating sympathetic dominance and a stress response. Similarly, comparing the same time frame from the NS day with the 25 minutes post ball drop to the end of the test in the novel environment, there was a significant decrease in average RR and a subsequent increase in HR and LF/HF, again indicating an initiation of flight or fight response. Significant differences observed during these steps compared to NS days (considering no effect was found on the heart rate variables in the home pen ball test,) may indicate that the suddenness aspect and novel object may have a greater impact on the animals when in an unfamiliar environment, or that the change of environment itself stimulated the animals more. Comparisons of night Steps had, as above, confounding results as average HR was higher on the NS nights compared to the ball test nights for both time frames, particularly for the NT animals. This effect may be random, as on the night of D4 there may have been disturbances, which we are not aware of influencing the results such as age, feed availability at night and the presence of rodents disturbing the animals etc. However, as described above changes in diurnal rhythms are likely to have contributed to this finding.

4.7.5 Rumen samples

4.7.5.1 Protozoan counts

Total protozoan count was significantly increased post-trial particularly for the NT group, mainly due to the higher presence of Large Entodiniomorphs in the NT group and higher Presence of Small Entodiniomorphs in the MCS group at this stage. This increase could be an effect of diet or may be linked to the increased alpha diversity indices for bacteria in the rumen, as certain protozoa such as Large Entodiniomorphs have been attributed to have bacterial breakdown capacity, therefore growth in bacterial abundance may lead to greater availability for protozoa and an opportunity to proliferate. Significant correlation was not observed between cortisol and any of the protozoa, or the total count, which agrees with findings from Chapter 2.

4.7.5.2 VFAs

An increase in propionate and butyrate was observed post trial for both groups, while the ratio C2:C3 decreased, an observation usually driven by changes in forage/concentrate ratio in the diet, which was not the case here. As propionate is linked to appetite mediation, this could be linked with the progressive decrease in frequency of eating hay. Propionate availability is considered to mediate glucose availability (Leng, Steel and Luick, 1967), but since no significant differences were observed in terms of glucose levels in blood plasma, these observations could be an effect of age, as these animals were at a growing stage which may be decreasing. An increase in butyrate, frequently considered as a positive indicator of microbiome health and positive emotionality in humans (Bourassa *et al.*, 2016), may be related to the increase observed in rumen alpha diversity Post-trial, and *Firmicutes* RA, particularly for the NT group.

4.7.5.3 Rumen microbiome

Rumen samples mainly comprised of *Bacteroidetes* (68.7% of the total abundance) and *Firmicutes* (22.00%), with other less abundant phyla present (i.e. *Fibrobacteres, Synergistetes and Spirochaetes* etc.), in agreement with literature (Deusch et al., 2017) and the community structure described in Chapter 2. Main orders were *Bacteroidales, Clostridiales Fibrobacterales* and *Synergistales* and main genera: *Prevotella* (50.60%), an Unidentified member of the *Veillonellaceae* family (5.60%) and an Unidentified member of the *Bacteroidales* order (5.5%), which again is consistent with the community structure of the Lafage Romane animals.

In terms of differences in RA, very few were significant. At a phylum level, Firmicutes abundance was increased for the NT group post trial, which may be related to an increase in VFAs, particularly in relation to *Clostridium* clusters which play significant inhibitory or promoting roles in VFA production (Salsali, Parker and Sattar, 2008; Andersen *et al.*, 2017; Islam *et al.*, 2017). Importantly, *Proteobacteria* abundance was higher in MCS animals post-trial. *Proteobacteria* are considered as possible indicators of disease and stress, as many studies in humans have correlated these bacteria with a number of pathologies (Rizzatti et al., 2017) (Yang and Jobin, 2014; Bradley and Pollard, 2017; Sun *et al.*, 2019).

Of further importance, Langgartner *et al*., (2017) observed an increase in the abundance of *Proteobacteria* in a mouse model for chronic psychosocial stress, while also in mice, Jang *et al.* (2018) reported that immobilisation stress led to an increase in *Proteobacteria* abundance and more specifically, *E.coli* abundance. *Spirochaetes* abundance was also significantly higher in MCS animals compared to NT animals post-trial, and bacteria of this phylum have also been linked to pathogenesis (Gay and Dick, 1986; Haake, 2000). At an order level, *Spirochaetales* abundance was higher for the NT animals compared to the MCS ones post-trial, and *RF32_Aphaproteobacteria* abundance was higher post trial for both groups. No significant information is available for these orders linking them to stress responsiveness or specific pathologies. For the rumen samples, genera with a RA of over 1 percent were not significantly affected by Treatment or Experimental Stage.

With regards to alpha diversity for the rumen bacteria, there was an overall increase for all indices explored (except Chao1) post-trial. Particularly Simpson, measuring the probability that randomly selected OTUs will belong to the same species acting as a measure of both richness and evenness, was significantly higher for the NT group post-trial compared to pre-trial. The observation that Simpson was significantly different pre-trial between NT and MCS animals did not persist Post trial. Inverse Simpson, used as a measure of species present, was also significantly higher for the NT group post-trial. Beta diversity, explaining the rate of change in species composition from one stage to another, indicated that Treatment groups differed pre-trial and that this persisted post-trial, but also that pre-trial beta diversity was significantly lower for both NT and MCS groups compared to their post-trial diversity levels. In agreement with this, beta dispersion showed the same pattern, increasing significantly for both groups post-trial.

The low variability explained in the PCoA plots and the lack of clustering or evident visual separation of the samples on the two main axes may be explained by the high number of OTUs present with particularly low abundances. CAP plots also indicated that cortisol and serotonin

explained low levels of variability and correlated poorly with the OTUs grouped by Treatment group and Experimental stage, as indicated by vector length on the plot. Further exploration of relationships between cortisol and serotonin concentrations post-trial with phyla, orders and genera presenting high VIP scores in the PLS analyses, indicated that although VIP scores were high, the correlation scores remained under 0.8 or higher than -0.8, which means the relationship was not particularly strong.

Phyla from the MCS samples post-trial that had the highest VIP (1.67 and 1.66) scores and highest positive correlation with cortisol were *LD1* (R = 0.43/ RA: 0.0001) and *Spirochaetes* (R = 0.43/ RA: 0.60). The later, as mentioned previously, was in higher abundance post-trial in the MCS animals and has been linked to pathogenesis. *LD1* was found to have a low positive correlation with cortisol in the Lafage animals too. For the NT group, the highest VIP score (1.97) was observed for *Armatimonadetes*, which had an R = 0.41 (RA: 0.0008). This is a recently discovered phylum and seems to prevail in carbon/nitrogen free ecosystems (Tamez-Guerra *et al.*, 2017).

The MCS phyla with the highest VIP scores and R with serotonin were *Fibrobacteres* (VIP: 2.07, R = -0.50, RA: 4.67) and *Firmicutes* (VIP: 1.79, R = 0.45, RA: 21.00). The negative relationship observed between *Fibrobacteres*, the order level *Fibrobacterales* and the genus *Fibrobacter* with serotonin is surprising, as these bacteria are linked to butyrate production which in turn is related to colonic serotonin production (Reigstad *et al.*, 2015). However, there have been reports that butyrate in the colon can act as an inhibitor of the serotonin transporter (Gill *et al.*, 2013; Baudry *et al.*, 2019), therefore the relationship between butyrate producers and serotonin is not clear. Many *Firmicutes* species are also butyrate producers and so a positive relationship with serotonin, as observed here, is more expected. However high abundance of this phylum in the human gut has previously been linked to depression (Huang *et al.*, 2018).

At an order level, cortisol had the higher correlation score (R = 0.61) with the order *Pseudomonadales* (VIP: 2.27 /RA: 0.003) and *PL-11B10_Spirochaetes* (R = 0.52, VIP: 2.05 and RA: 0.005) for the MCS group. For serotonin, the highest VIP and R score was observed for *Thermoplasmata_E2* but there is no known relationship between archaea and serotonin levels. NT animals' post-trial cortisol levels were more correlated with the *Unidentified_Alphaproteobacterium* (R = 0.61, VIP: 3.25, RA <0.0001) and the *Myxococcales* order (R =0.51, VIP: 2.42 and RA: 0.0001) neither of which have significant references in literature linking them to cortisol or stress response.

The order *Deltaproteobacteria GMD14H09* (R = 0.39, VIP: 2.08, RA: 0.007) presented the highest R with serotonin, once again contrasting literature findings where *Deltaproteobacteria* were significantly increased in stress susceptible mice after social defeat stress (Yang *et al.*, 2017).

At a genus level for the MCS animals, the highest R score (R = 0.61) was observed between cortisol and *Pseudomonas* (VIP: 2.54, RA: 0.03). Lyte and Ernst (Lyte *et al*., 1992) had shown that noradrenaline and adrenaline catalysed *Pseudomonas aeruginosa* growth, which *was later found to* actively disrupted the cortisol-binding activity of the corticosteroid-binding globulin (Simard *et al.*, 2014) indicating that there are links between corticosteroids and this genus. An interesting case is the order *Victivallaceae* with a correlation score -0.50 and VIP: 2.08, which was also consistently negatively correlated with cortisol in MCS faecal samples too.

Cortisol correlations at a genus level with NT animals at the post-trial stage were inconsistent, as *Succinimonas*, with an R score of <0.01 was presented with a VIP score of 3.03 and *Unidentified_Proteobacterium* with R = 0.15 had the same VIP score. *Bifidobacterium* appeared to have the most coherent relationship between VIP score and R-value (3.03 and 0.61 respectively), as did *Shuttleworthia* which presented a negative relationship with cortisol (R = -0.49, VIP: 2.69). *Shuttleworthia* in pigs has been found to be affected by sanitary stress (Kubasova *et al.*, 2018) and higher abundances were observed in Type 2 diabetes mellitus patients in a human study (F. Liu *et al.*, 2017). Serotonin in PostNT animals was found to be negatively related to *p-75-a5_Erysipelotrichaceae* and *Unidentified_Coriobacteriaceae* (VIP: 2.76, RA: 0.006, R = -0.53 and VIP: 2.60, RA: 0.003, R = 0.51 respectively). Interestingly, higher abundance for *Coriobacteriaceae* and *Erysipelotrichaceae* families has been linked to metabolic perturbations and stress in animals and observed in higher abundances after sleep deprivation in humans (Bangsgaard Bendtsen *et al.*, 2012; Benedict *et al.*, 2016).

4.7.6 Faecal microbiota

Main faecal phyla were *Firmicutes* (56.30%), *Bacteroidetes* (35.50%) and *Spirochaetes* (2.20%), in alignment with what was expected (O' Donnell *et al*., 2017) and the faecal community composition in the Lafage Romane animals (Chapter 2). The most abundant orders were *Clostridiales* (55.60%), *Bacteroidales* (35.40%) and *Spirochaetales* (1.80%). Most abundant genera included an unidentified member of the *Ruminococcaceae* family (23.60%), *Bacteroidaceae_* J_7N15 (8.40%) and an unidentified member of the order *Bacteroidales* (8.60%). *Ruminococcus* was also high at an overall presence of 5.70% in the community. High

Ruminococcus abundance has been identified in people on the autism spectrum (Wang *et al.*, 2013).

In terms of differences in RA, at a phylum level there was a significant decrease in *Euryarcheota* in the NT group post-trial and *Actinobacteria* abundance increased post-trial for the MCS lambs. *Euryarcheota* are linked to methanogenesis, therefore a drop post-trial in non-stress conditions is considered a welcome and positive result. However, increased *Actinobacteria* have been reported in "depressed" mice and humans with mental disorders (Hemmings *et al.*, 2017; Luo *et al.*, 2018; Huang *et al.*, 2019). *Cyanobacteria* abundance was also significantly higher post-trial compared to pre-trial for the NT group.

Related to the findings at a phylum level, orders that differed significantly for the NT group were *Methanomicrobiales* and *YS2_Cyanobacteria*, which were higher pre-trial. At a genus level, *Unidentified_Methanocorpusculaceae* abundance was higher post-trial for the NT group compared to pre-trial abundance, whereas the genera *Bacteroides* and *Unidentified_Rikenellaceae* were more abundant post-trial for the MCS group compared to pre-trial levels. Previous studies have demonstrated a decrease in *Bacteroides* and an increase in the *Clostridium* genus due to stress (Bailey *et al*., 2011; O'Mahony *et al*., 2009), which is not in agreement with the findings for the MCS animals.

Alpha diversity for the faecal bacteria increased for all indices explored (except Chao1 and ACE) post-trial. Inverse Simpson was significantly different for the two groups but did not differ post-trial. It was however significantly higher for the NT group post-trial compared to pre-Trial. Beta diversity for the faecal samples was exactly as described for the rumen, as differences were observed between Treatment pre- and post-trial, while diversity and dispersion also increased post-trial for both groups.

Again, the low variability explained in the PCoA plots by the two main axes may be explained by the high number of OTUs present with particularly low abundances. For the faecal samples however, segregation between Experimental stages and Treatment groups is present, a higher dispersion or scatter may be observed for the MCS OTUs post-trial.

Cortisol and serotonin explained low levels of variability and were correlated poorly with the OTUs grouped by Treatment groups and Experimental Stage according to the CAP plots. However, vector length was longer compared to the rumen CAP plots and serotonin appeared to be in "covariation" with a cluster of MCS OTUs post-trial.

Further exploration of relationships between cortisol and serotonin concentrations post-trial with faecal phyla, orders and genera presenting high VIP scores in the PLS analyses, indicated

that similarly to the observations for the rumen samples, VIP scores were high, but the correlation scores remained under 0.8 or higher than -0.8, which indicated a weak relationship between RAand cortisol/serotonin.

At the faecal MCS phylum level, cortisol had the highest negative relationship (VIP: 2.03, R = 0.58) with *Bacteroidetes*, the second most abundant phylum (RA: 35.88) and a positive relationship with *Firmicutes* (VIP: 1.64, R = 0.47), the most abundant phylum (RA: 52.90). This is interesting as many studies focus on the ratio between *Firmicutes* to *Bacteroidete*s to ascertain host health, and as observed for the RA, *Bacteroides* was also higher for the MCS post trial, indicating some relationship with the PLS findings.

At an order level, this translated to a higher relationship between cortisol with *Bacteroidales* (VIP: 2.49 R = -0.57, RA: 35.85) and *Clostridiales* (VIP: 1.98, R = 0.46, RA: 54.16). *Bacteroidales* is seen as a "beneficial" microbe and a negative relationship with cortisol would mean that any increase in stress could lead to drop in abundance and potential dysregulations. *Clostridiales* on the other hand is also thought to be associated with functional connectivity between brain regions and the GI tract in terms of sensorimotion, and several species have been associated with irritable bowel syndrome (Labus *et al.*, 2019).

In contrast, at a genus level, negative relationships were observed between cortisol and *Clostridium* and *02d06_ Clostridiaceae* (VIP: 2.79, R = -0.60, RA: 0.04; VIP: 2.18, R = -0.58, RA: 0.20). *Clostridium* is an opportunistic pathogen, associated frequently with suboptimum health status, while higher abundance of *Clostridium difficile* has been associated with depressive states in humans (Rogers *et al.*, 2016). Other relationships of interest between MCS genera and cortisol were with *Sutterella* (VIP: 2.41, R = 0.54, RA: 0.007), increased abundance of which has been found in faecal samples of children with ASD (Wang *et al.*, 2013) and *Unidentified_ Bifidobacteriaceae* (VIP: 2.25, R = 0.50, RA: 0.003) which in humans has been linked to pathological conditions such as gastritis (Mattarelli *et al.*, 2014).

In contrast, for the NT group cortisol was more correlated with phyla of lower abundances such as *Elusimicrobia* (VIP: 1.77, R = -0.37, RA: 0.04) and *Planctomycetes* (VIP: 1.72, R =0.40, RA: 0.35). At an order level though, higher VIP scores and R was observed between cortisol and *Flavobacteriales* (VIP: 2.18, R = 0.51, RA: 0.009) and *Other_Firmicutes* (VIP: 1.84, R = -0.42, RA: 0.008). As in the rumen, this shows inconsistent relationships between cortisol and the NT group. At a genus level, cortisol did have a positive relationship (VIP: 2.41, R =0.54) with *Afipia*, species of which are pathogenic and respond to increases in circulating cortisol levels.

Relevant to serotonin, important relationships based on literature findings included the negative relationship presented for the NT group with *Elusimicrobia* (VIP: 1.77, R =0.40, RA: 0.04). Ahmad *et al.* (2019) have identified that diabetic patients had reduced RA of this phylum, while previous findings have outlined the influence serotonin exerts on diabetic platelets (Malyszko *et al.*, 1994), potentially justifying this relationship. The genus *Burkholderiales* (RA: 0.13) demonstrated a positive relationship with serotonin (VIP: 2.48, R = 0.51) for the MCS group post-trial. This genus includes many pathogenic species and its relationship with tryptophan pathways is unclear.

For the NT group, the negative relationship between *Anaerostipes* and serotonin is also controversial, as butyrate produced by this genus, as mentioned above, is implicated in tryptophan production but also inhibits SERT. Finally, of mention, *Mycoplasma,* which exhibited a positive relationship with serotonin (VIP: 1.62, R = 0.28, RA: 0.007), is related to *M. pneumoniae*, previously associated with significantly greater numbers of mast cells in patients with chronic asthma. Furthermore, in rodents, this organism has been found to induce activation of mast cells with a release of serotonin (Hoek *et al.*, 2002).

4.7.7 Examination of hypotheses

- Stressed animals responded to acute stressors imposed over the MCS 6-week period, meaning cortisol, glucose increased after certain stressors compared to the NS animals. Heart rate values did not increase but there were discrepancies in the way heart rate variability changed over time during the days that stressors were tested.
- Repeated unpredictable mild stressors for a duration of 6 weeks led to behavioural changes (time spent resting, increased synchronisation for moving amongst the MCS animals, time spent eating concentrates, synchronisation of eating hay between animals and time spent eating hay (which reduced towards the end of the trial)). Physiological changes as a result of chronic stress were not as evident. However, heart rate variability measures were erratic for the MCS animals, indicating a disturbance which could be attributed to stress.
- Stressed animals responded differently to a suddenness test, when compared to NS animals tested under the same conditions. There were differences in behaviours such as vigilance and motivation to interact with the ball, as well as changes in heart rate measurements, though these were less pronounced than expected.
- Repeated unpredictable mild stressors for a duration of 6 weeks did not alter rumen and faecal profiles. There were shifts in microbial community indices with diversity

increasing over the study for untreated animals, whilst it declined in animals experiencing mild chronic stress.

- Cortisol and serotonin levels can act as predictors of the presence/absence or abundance of certain bacterial phyla, orders or genera. Despite extensive correlation analyses and PLS regression, there were no statistically significant relationships between relative abundances of microbial taxa in the rumen or faeces and plasma serotonin and cortisol.

In conclusion, mild chronic stress affected both lamb behaviour and physiology. Taken together with other studies in this thesis, as well as other studies with ruminant livestock, it appears that effects of mild chronic stress on the intestinal microbiome is small. However, considering differences in synchronisation of resting, eating activities, vigilance and irregular heart rate variables we can assume that the animals were affected by the stressors. This means that for animal management and welfare purposes, despite not seeing immediate physiological changes, animals should not be distressed and subjected to unnecessary treatments.

Chapter 5

General Discussion

5 General Discussion

5.1 Introduction

In many mammalian species, stress has a pronounced impact on the gut microbiome, affecting host health and behavioural responses (Borre *et al.*, 2014a; Furness *et al.*, 2014; Stilling, Dinan and Cryan, 2014a; Rea, Dinan and Cryan, 2016; Davidson *et al.*, 2018; O'Mahony, McVey Neufeld, *et al.*, 2020). Current exploration of the microbiome in ruminants mainly focuses on its manipulation via diet and genetic selection, with the aim of improving productivity and reducing methane emissions. This narrow focus has resulted in fundamental gaps in knowledge regarding the relationship between stress susceptibility and management/emotional distress and the gastrointestinal microbiome of ruminants, and specifically sheep.

As such, the overall aim of this study was to perform an initial exploration of this relationship from a microbial 16S rRNA gene amplicon sequencing perspective, i.e., investigating taxonomy and diversity in correlation with biomarkers of interest. The novelty lies in the fact that this is the first study of its type to look at the stress effects on both rumen and faecal microbial community in ruminants and particularly in sheep.

5.2 Practical considerations

5.2.1 Experimental procedures

5.2.1.1 Stress Responsiveness - Measures and Methodologies

Methodologies used to assess stress response are laborious and challenging, while the responses may be difficult to define, due to variable and ambiguous responses (dependent on the stressor and the intensity of the stressor), and to high individual variability. Furthermore, the experimental environment and the procedures used to collect samples may unintentionally affect responses, while repetition of events may also lead to animal adaptation. Hence, this work used a wide range of physiological and behavioural measurements to assess the effectiveness of acute stressors and measure chronic responses. Stressors in general must have a biological significance for the species studied and the observations made are frequently quantitative, meaning that they assess behavioural changes, but do not always reflect motivation or emotional state in predictable ways.

For example, in Project 4 the MCS group interacted more with the environment and less with the ball during the novel pen ball test, indicating that the MCS procedures themselves affected

lamb responses to novelty and suddenness. However, the observations included in "interaction with the environment" were climbing and chewing panels, behaviours that cannot easily be distinguished as frustration or exploration, despite research devoted to the investigation of emotion and cognition in farm animals (Désiré *et al.*, 2004, 2006; Panksepp, 2004; Boissy *et al.*, 2007; Richmond *et al.*, 2017). Differences in latencies to interact with the ball and the environment, as well as differences in vigilance, may be indicators of differences in attention bias and evidence that MCS animals took longer to adapt to novel situations. Therefore, the inclusion of a "Universal" test at the end of the trial was particularly important in comparing responses between Treatment groups.

Additionally, stress treatments may exert effects on the rumen/gut indirectly (e.g. by affecting meal patterns and feed intake), as well as through the mechanisms of interest (stress hormones), affecting particular microbial communities. In Chapter 4 there was significant evidence of this type of impact, as expressed by fluctuations for average RR and higher HR for the MCS animals, as well as differences in the number of hay eating bouts and the synchronisation of several activities (eating hay, concentrate, moving and resting), particularly in the middle of the experiment. Consequently, this work used three different experimental models with both direct and indirect methods to alter stress responses.

The influence of these types of indirect effects in Chapter 2 and 3 studies would affect all animals in a similar way, as the animals in both of these projects were managed as a group prior to sampling, and no known stressful events (other than standard husbandry practices) contributed to abrupt changes. Furthermore, rapid changes due to management, other than diet, are unlikely, due to the large volume of digesta in the rumen and gut, and relatively slow turn-over rates. However, in Chapter 3, one significant influence on the microbiota community structure was the different diets offered for males and females in the last 10 days prior to sampling. It is evidently important to avoid known large effects such as diet changes to differentiate true sex effects in this experiment. Despite this, the presence of many significant differences between females and males of the Blue Neonatal Treatment is an important indication that Neonatal treatment may have had an effect on rumen and community structure development.

5.2.1.2 Sample processing and analyses

Both sample matrices explored (i.e. rumen and faecal) are high in material that can make DNA extraction and downstream analysis challenging. In the rumen, this material consists of mucosal proteins and undigested feedstuffs, whilst in the faecal samples of shed intestinal

epithelial cells, as well as undigested food and a vast array of endogenous secretions, all of which compromise the quality of the DNA collected for sequencing. Therefore, it is important to use techniques that circumvent these issues, such as crushing the samples prior to using extraction protocols, and including multiple purification steps. The rumen DNA yield in studies described in Chapters 2 and 4 was significantly higher compared to Chapter 3, where the samples were not crushed.

Standardisation of methodologies is frequently discussed in microbiome reviews, as it can complicate comparisons between studies. Furthermore, an observation from the studies in the project where the samples were crushed is that the faecal material contains so much microbial material that smaller quantities of crushed samples were necessary in order to achieve optimal DNA extraction.

Library preparation and sequencing methods are factors that can result in unwanted microbial variation. Next generation sequencing is limiting in the sense that only small parts of the 16S rRNA gene (here the hypervariable V4 region) can be sequenced. Yang *et al.* (2016) suggested that hypervariable regions V4 -V6 are the most reliable for determining bacterial species, whilst V2 and V8 hypervariable regions are the least reliable. Both hypervariable regions and pyrosequencing platforms have effects on relative abundances and which bacterial taxa are represented in the community (Salipante *et al.*, 2014). However, Caporaso *et al.* (2012) demonstrated an agreement between MiSeq and HiSeq platforms. In analysing microbiota sequences, the assignment of taxonomic units is performed using SILVA, RDP, Greengenes or NCBI, which can lead to discrepancies. Despite this, (Balvočiute and Huson, 2017) noted a large overlap between Greengenes (used in the present study) and SILVA, indicating that comparisons using these two annotation tools are reliable. It has to be noted that in this study only prokaryotes (mainly bacteria and some archaea) were targeted and that taxonomic analyses do not provide information on the functions bacteria carry out.

When sampling blood to assess biomarkers of stress, it is crucial that sampling is performed using standardised and consistent methodologies within and between studies. Time of sampling is important as many hormones and metabolites are influenced by interactions between the effects of sleep and the intrinsic circadian systems, as well as external pressures (temperature, light, food supply, social factors and disease). Similarly, methods of analyses such as the ELISAs conducted for the cortisol samples are best conducted in a standardised and uniform way as there is evidence of laboratory and kit effects (Haddad *et al.*, 2019). Furthermore, many of the ELISA kits used for the analyses of the other biomarkers (e.g. serotonin) had to be tested prior to use, as sheep plasma may have interfering or cross-reacting

substances and antibodies against the reagents or the analytes. In general, while researching options for processing the plasma samples for various biomarkers, it was evident that not all kits were species-specific and sensitive enough for use in sheep.

As observed across all studies, cortisol may not be the ideal biomarker to assess chronic stress. Although it is implicated in many metabolic and stress pathways, and certain bacteria respond to this hormone, high individual variability and a lack of multiple measures (particularly in projects from Chapters 2 and 3) that would allow identification of disrupted baseline levels, render interpretation of plasma or serum cortisol problematic. Furthermore, serum cortisol, due to its protein-binding capacity, becomes difficult to evaluate (Lee *et al*., 2015).

Potential alternative biomarkers that are linked with behavioural observations (i.e. differences in hay and concentrate consumption), such as BHB and glucose, which also increase after stressful events, could be used. In general, use of any single biomarker (i.e. EBVs, cortisol) can be problematic and one dimensional, so allostatic load models which utilise a suite of neuroendocrine and immune-related or even behavioural and genetic indicators have been suggested as an alternative approach, measuring stress effects across multiple physiological systems (Lupien *et al.*, 2009).

5.2.1.3 Statistical analyses

Microbial communities are complex, and many methods have been used to describe them, using a vast range of tools and statistical tests across the literature. The approaches followed in this study were in accordance with currently methodologies in human and rodent microbiome studies (Zhao, 2013; Zijlmans *et al.*, 2015; Bradley and Pollard, 2017; Foster, Rinaman and Cryan, 2017; Jašarević *et al.*, 2017a; Karl *et al.*, 2018). A first step is an initial exploration of the community structure at an OTU level and from a relative abundance perspective, exploring differences at various taxonomic levels.

A second step involves correlations with biomarkers and variables of interest as performed here, with the use of PLS and Correlation analyses which allow the identification of bacteria, acting as markers or predictors of interest. Of note, correlations and PLS can give misleading results when low sample numbers are used (Aggarwal and Ranganathan, 2016). For example, a study exploring sex and neonatal effects would ideally require 6 animals per grouping of variables, meaning the overall sample number would have to be 72 lambs (36 of each sex).

Cortisol, for reasons previously described, was used as the common and comparative link between the microbiota and stress throughout all three experiments. The PLS analysis and

Correlations allowed investigation of whether higher or lower concentrations of this hormone were associated with higher or lower relative abundance of bacterial phyla, orders and genera for all three projects. As mentioned above, since significant correlations were scarce and since cortisol can be an ambiguous biomarker for chronic stress assessment, the use of alternative biomarkers or different methods to determine links between biomarkers, such as allostatic load models or network analysis, are potential alternative approaches. Weighted correlation network analysis is more frequently used in gene-expression exploration (Auffret *et al.*, 2017; Kaliannan *et al.*, 2018; Lima *et al.*, 2019), but has potential in this type of investigation as described by Kelder *et al.*, (2014) and Ma and Li, (2019).

5.3 General findings

The overall aim of this study was to shed light on how emotional distress and genetics may influence relative abundance and structure of gut and rumen microbes in sheep, and in the case of the MCS trial, how stressors applied unpredictably and repeatedly, can affect sheep behaviour and various physiology aspects, as well as the microbial community. Three factors that are known to affect the microbiome: 1)Genetic background (expressed as EBVs) (Kostic, Howitt and Garrett, 2013; Stilling, Dinan and Cryan, 2014b; Goodrich *et al.*, 2016; Li, *et al.*, 2019b); 2)Early colonisation factors (such as prenatal and early life stress) (O'Mahony *et al.*, 2009; Jašarević *et al.*, 2015, 2018; Carlson *et al.*, 2018; Dong and Gupta, 2019; Singh and Mittal, 2019; Webster, Consuegra and Leaniz, 2020) and 3)External influences in the form of stress (i.e repeated and unpredictable stressors; Yáñez-Ruiz, Abecia and Newbold, 2015, Bharwani, M Firoz Mian, *et al.*, 2016; Marin *et al.*, 2017c; Karl *et al.*, 2018; Molina-Torres *et al.*, 2019; Siopi *et al.*, 2020), were explored as complementary approaches, targeting this multifaceted question.

These questions, in relation to the sheep gut microbiota (using faecal samples as a proxy), are of interest due to the fact that in other species, such as rodents, birds, pigs and humans, significant links have been found between the gut microbiome, host health and behavioural responses (Borre *et al.*, 2014b; Furness *et al.*, 2014; Stilling, Dinan and Cryan, 2014a; Rea, Dinan and Cryan, 2016; Davidson *et al.*, 2018; O'Mahony,McVey Neufeld, *et al.*, 2020). They are also interesting from an ethical point of view, in terms of animal welfare, considering that management procedures, viewed as standard practice, may not have direct and extreme effects on the animals' stress levels, but can progressively lead to a chronically distressed state. This in turn, has a practical aspect, as chronic stress or continuous stimulation of the HPA axis can lead to subclinical health conditions, suboptimal performance (in terms of growth, milk

production, reproduction etc.) (Glaser and Kiecolt-Glaser, 2005; Sutherland *et al.*, 2006; Rabasa and Dickson, 2016; Min *et al.*, 2019), disruption of the microbiome, ultimately initiating a vicious cycle (Bailey *et al.*, 2011b; Neuman,Debelius, *et al.*, 2015; Bharwani,Mian, *et al.*, 2016), all of which may account for financial losses in a farming system.

In terms of exploring stress effects on the rumen microbiota of sheep, this is a significant organ for ruminants, unique in its structure and functionality amongst animal species. Within this organ lies an entire ecosystem where the organisms live in a fine balance, interacting with each other, the host and feedstuff provided to/by the host. These microorganisms have the ability to convert feedstuff into microbial matter and fermentation products, which can then be utilised by the host. Main examples are volatile fatty acids (i.e. acetate, propionate and butyrate) and gasses, such as carbon dioxide and methane. Reducing animal emissions of these two infamous gases has been the target of genetic improvement and diet modifications in ruminants for decades (Morgavi *et al.*, 2015b; Yáñez-Ruiz, Abecia and Newbold, 2015; Roehe *et al.*, 2016b; Saro *et al.*, 2018a; Jeyanathan *et al.*, 2019b), as they are known contributors to climate change, and any energy diverted from the animal's part to their formation is a loss in terms of productivity. Differences were observed mainly by sex and age related to methane producing archaeal abundance across the studies and it may be of interest to investigate these aspects with larger numbers of animals.

As stress has been shown to significantly influence gut bacteria in humans mice and other species, even resulting in pathological states (colitis, IBS and cancer) (Messer and Chang, 2018; Gao, *et al.*, 2018; Rinninella *et al.*, 2019), it was considered of interest to explore whether stress or factors related to stress response (genetic predisposition to higher or lower responsiveness) could also lead to significant shifts in the rumen microbiota. Very little is known about whether hormones can reach the rumen and if they are degraded before they have an opportunity to interact with the bacteria present (Rath *et al.*, 2016). Many bacterial species have been found to proliferate in the presence of stress hormones and have hormone receptors (Lyte, 1993; Neuman, Justine W Debelius, *et al.*, 2015), but it is unknown whether the rumen bacteria respond specifically to stress signals via changes in VFA production or interaction with the host via signalling molecules other than VFAs (Rath *et al.*, 2016). Therefore, this study consisted of an initial exploration of correlations that could act as further proof of this communication.

The approaches followed in this study were in accordance with what is currently conducted in human and rodent microbiome studies (Zhao, 2013; Zijlmans *et al.*, 2015; Bradley and Pollard, 2017; Foster, Rinaman and Cryan, 2017; Jašarević *et al.*, 2017b; Karl *et al.*, 2018). A first step

is evidently an initial exploration of the community structure at an OTU level and from a relative abundance perspective, exploring differences at various taxonomic levels. A second step involves correlations with biomarkers and variables of interest as performed here with the use of PLS and Correlation analyses which allow the identification of bacteria which could act as markers or predictors of interest.

Cortisol, for reasons previously described, was used as the common and comparative link between the microbiota and stress throughout all three experiments. The PLS analysis and Correlations allowed investigation of whether higher or lower concentration of this hormone was associated with higher or lower relative abundance of bacteria Phyla, Orders and Genera for all three projects.

Main findings

Chapter 2

There was no significant influence of the two Genetic Lines (B+ more reactive to social isolation and B- less reactive) on cortisol concentrations and diversity indices. For the rumen samples, *Fibrobacteres* within Generation G1 was higher in abundance in the B- line and *Elusimicrobiales* differed significantly between generations within the B+ line (B+G1 < B+G0), indicating a potential genetic influence. At the Genus level, an unidentified member of the *Lachnospira* family within Generation G1 was more abundant in the B+ line. Regarding the faecal samples, the *Verrucomicrobia* Order was more abundant in the B- line within Generation G1. A significant difference within Generation G0 was observed for the Orders *Z20* and *Methanomicrobiales,* with higher relative abundance in the B+ line.

Generation (G0, G1) had an effect on alpha and beta diversity as well as on the relative abundance of several Phyla, Orders and Genera (e.g. *Elusimicrobiales* Order was higher in G0 within the B+ line, while unclassified *Endomicrobia* was higher in G0 animals compared to the G1 animals within the B+ generation. These differences are interpreted as age effects rather than differences linked to genetic variation between the two generations.

Variability in cortisol explained higher levels of variability in the rumen samples compared to the faecal samples and higher levels overall compared to the variation explained by the EBVs. Although several Phyla, Orders and Genera for the faecal and rumen samples had high VIP scores indicating they contributed significantly to the model used, correlations between relative abundances and cortisol or EBVs were not significant for any taxonomic level.

Chapter 3

In the second project, the effect of Three Prenatal Treatments, in combination with Two Neonatal Treatments, on the rumen microbiota were explored in 7-month-old lambs (32-33 weeks). There was a significant sex effect for all diversity indices, which unfortunately could not be separated from diet effects. Despite there not being many abundance differences at a Phylum level, sex effects were observed for many Orders and Genera. An inversion of the abundance of Firmicutes and Bacteroidetes was observed in the females compared to the males, which has three possible interpretations. 1) This could be the influence of a diet lacking in fibre leading to higher Bacteroidetes abundance (Min *et al.*, 2019), 2) this may be a sex effect reflecting different rumen maturation rates or 3) an indicator of microbial dysregulation, as frequently described in human studies (Rinninella *et al.*, 2019).

General conclusions include: 1) Neonatal Treatment had no significant effect. 2) Alpha and Beta Diversity were higher in females, as was plasma cortisol concentration. Several bacterial abundances known to be influenced by cortisol (i.e., *Lactobacilli*) and abundance of potential pathogens (*Clostridium*, *Verruco-5*, S*elenomonas*) was higher in females. In the males, the lowest diversity was observed within the social isolation treatment group which may simply be a result of having a lower number of samples in this Treatment Grouping. Many significant differences in relative abundances were observed between Isolated males and Recognition test males, as well as between Isolated males and Isolated females, indicating that potentially the accumulation of events for this group affected the microbial community in these males in a more pronounced manner, suppressing rumen microbial richness, evenness and overall diversity.

PLS analysis between cortisol plasma concentrations acquired on the day of slaughter and relative abundances at a phylum, order and genus group, mainly took Sex and Sex*Neonatal treatment into account. Many bacteria contributing to the model in an important way also demonstrated significant correlations with cortisol. This result should be interpreted cautiously, as the low number of animals within each Treatment Grouping is a factor that can significantly affect the reliability of these results (Aggarwal and Ranganathan, 2016).

Chapter 4

In the third project, a Mild Chronic Stress Trial was conducted aiming to explore the effect management and emotional stress had on sheep physiology, behaviour and rumen/ gut microbiota. The acute stressor we assessed appears to have had an effect on the animals in

terms of stress biomarkers as cortisol, glucose, BHB and cortisol were all higher post Individual Restraint.

Severe chronic stress was not induced, as from a physiology aspect there was evidently an adaptive response since the biomarkes of stress mentioned above and serotonin were not different between treatment groups post-trial. Due to the behavioural observations made though, distress, as dicussed by Moberg 1985 (a damaging form of stress that is outwardly expressed by recognisable behaviours) appears to have ensued.

Due to the fluctuations seen for average RR and higher HR for the MCS animals, we hypothesise that the repeated stressors did have an impact on the lambs, potentially by affecting their circadian rhythm, as frequently observed in chronically stressed animals (Dumbell, Matveeva and Oster, 2016). In terms of behaviour observations, the two groups differed in the number of hay eating bouts and the synchronisation of several activities (eating hay, concentrate, moving and resting), particularly towards the middle of the experiment.

The ball test, used to investigate differences between treatment groups' reactions to novelty and suddenness, after the 6 week trial, showed there were significant differences in latencies to interact with the ball and the environment as well as differences in vigilance, which may be indicators of differences in attention bias and the MCS animals taking longer to adapt to novel situations.

Alpha and beta diversity metrics were different for the NT group Pre and Post-trial, and this shift indicates a different rate of evolution in the microbiota community between NT and MCS animals. *Proteobacteria* and *Spirochaetes*, both of which have been linked to pathogenesis (Haake, 2000; Bradley and Pollard, 2017; Rizzatti, Gianenrico & Lopetuso, Loris & Gibiino, G. & Binda, Cecilia & Gasbarrini, 2017), were more abundant Post-trial in the MCS for the rumen samples. For the faecal samples, most changes in relative abundance were between Pre- and Post- stages of the NT group.

PLS regression once again identified may bacterial phyla, orders and genera that contributed in a significant way to the prediction model investigating the relationship between cortisol/serotonin and rumen/ faecal relative abundances. Despite this, once again, there were no significant correlations.

Global Findings

Across all studies, few low abundance rumen or faecal microbes appeared to respond to stress, but most bacteria and archaea and the overall community structure did not demonstrate significant shifts. A possible reason for these differences in comparison with rodent/human

studies is that the rumen is more resilient compared to the gut of monogastric mammals in terms of perturbations and external influences. The rumen may act as a buffer, "easing" external pressures and helping the animal cope against stress. This is purely speculative, based on the volume of this organ and its functional importance to ruminants, and could be important in demonstrating that sheep are particularly resilient and adaptable to stress effects.

Furthermore, post rumen maturation, after the animal has transitioned from a "pre-ruminant" to a ruminant state, intestinal disease occurrences and abundance of opportunistic pathogens (i.e., *E. coli*) decline in ruminants (Zhao *et al.*, 2003), indicating that once a stable community has been established in the rumen, it can subsequently influence the gut microbiota and contribute to a "healthier" state (e.g. by enhancing mucosal barriers) (Malmuthuge, *et al*., 2015). A second explanation could be that immunological aspects in sheep/ruminants come into play. For example, sheep have higher NK levels compared to other species (Tizard, 2017), and, as mentioned in Chapter 3, there is a sharp drop in NK cells early in ruminant life, allowing colonisation of the rumen. Therefore, the rumen may act as a regulating mechanism interacting with many physiology systems.

Many bacteria correlated with cortisol agreed with findings in previous research conducted in human and mice studies. Some were significant (Chapter 3), while most were not. For several bacteria (*Fibrobacteres*, *Firmicutes*, *Erysipelotrichales*, *Endomicrobia*, *LD1*, *Armatimonadetes*), the relationship with cortisol was consistently in the same direction (positive or negative). These findings are noteworthy as they indicate that potentially more data would allow robust correlations. This is the only study in ruminants, whereas there is vast literature in other species collating data sets and extracting significant results.

EBVs were not significantly correlated with rumen and faecal microbial abundances in this study. These findings were difficult to relate with literature as there are very few, exclusively human, studies, which explore temperament traits (comparative to the EBV) in relation to the microbiome. Additionally, serotonin was not significantly correlated with rumen and faecal microbial abundances (Chapter 4), which may be due to the type of sample collected, i.e., plasma serotonin vs. tryptophan or cerebrospinal fluid. This suggestion for alternative sampling sites/ matrices is based on the fact that the brainstem is the site of main activity of 5-HT (Visser *et al.*, 2011), whilst 5-HT concentration in the brain and cerebrospinal fluid is correlated (Matsumoto *et al.*, 1991). Finally, there may simply be a true lack of interaction between this neurotransmitter and ruminant bacteria (alternative interaction pathways).

The vast majority of bacteria with high VIP scores were low in abundance, which may mean that, in ruminants, interactions between the microbiota in the rumen/ gut and the ENS may be

expressed via complex mechanisms, and that there are many contributors rather than specific driving forces.

There was evidence of inter-animal variability in microbiota profiles, unrelated to stress, which are currently unexplained. For example, in Chapter 2, Generation (which was mainly reflective of ewe age) had a significant effect on microbiota community structure and relative abundances. Furthermore, age contributes to changes in temperament in other mammals, so it may play a role in responsiveness in sheep and may have knock-on effects on microbiota or *vice versa*. The exploration of age-related differences in combination with stress impacts may offer potential for future studies.

Additionally, breed effects on the microbiome in ruminants are known (F. Li, Hitch, *et al.*, 2019), therefore variability in bacteria present between studies in Chapters 1/3 and 2 are expected. The bacterial phyla, orders and genera between projects in Chapters 1 and 3, where the animals were of the same breed (Romane), represents further evidence that breed and genetics contribute to microbiome structure.

With reference to the MCS trial (Chapter 4), severe chronic stress was not induced, as from a physiology perspective there was evidently an adaptive response, since the biomarkers of stress (glucose, cortisol, BHB, NEFA) and serotonin were not different between NT and MCS animals post-trial. The lack of higher biomarker concentrations does not mean that the animals were not disturbed. From the behavioural observations, distress, as discussed by Moberg (1985) "a damaging form of stress that is outwardly expressed by recognisable behaviours", ensued. Several behavioural parameters (frequency of hay consumption and synchronisation of activities), as well as fluctuations of average RR and HR, altered HRV and attention bias differences between groups, are evidence of potential disruption of behavioural and heart rate circadian rhythms (Merrow *et al.*, 2005; Wyse *et al.*, 2018).

Overall, we did not observe major effects of genetic predisposition of stress, prenatal and neonatal effects and following a MCS paradigm on the microbiome in the rumen and faecal, differences were observed in terms of behaviour. If behaviour is significantly disturbed, such as synchronisation of activities and feeding patterns, this may have potential effects on the physiology, immune system and in the long run animal productivity, metabolism and the microbiome via indirect pathways. Therefore, it is still important to maintain high welfare standards and avoid distressing animals by upholding the 3R principles and allowing them to express normal species-related behaviours.

In conclusion, despite not identifying significant correlations and a pronounced impact of stress and stress responsiveness on the microbiota, this study represents a very small amount

of data in comparison to literature available for other species. Therefore, not finding a stress impact is important, indicating sheep resilience (as discussed above), however, the fact that this is the first study of its type in ruminants means that "strict" conclusions cannot be drawn. Human and rodent studies report inconsistent relationships between bacteria, genes and immune markers, but meta-analysis and review studies allow pooling of data and extraction of significant relationships with practical applications (e.g., related to depression and cancer research).

5.4 Suggestions for future work

Very little is known about whether hormones can reach the rumen, and if they are degraded before they have an opportunity to interact with the bacteria present (Rath *et al.*, 2016). Many bacterial species have been found to proliferate in the presence of stress hormones and have hormone receptors (Lyte, 1993; Neuman, Justine W. Debelius, *et al.*, 2015), but it is unknown whether the rumen bacteria respond specifically to stress signals via changes in VFA production, or interaction with the host via signalling molecules other than VFAs (Rath *et al*., 2016).

Therefore, metabolomic and proteomic analysis, in combination with metagenomics analysis to assess potential gene functions of the bacteria present in the rumen in relation to stress mechanisms, would be of interest. As this would require a large amount of data and pooling different dataset types to extract this information, this represents a long-term goal which has been described in most research fields investigating host-microbiome interactions (cancer research, IBS, depression studies, feed efficiency and many more).

Within the rumen there is an entire ecosystem living in fine balance, interacting with each other, the host and feedstuff provided to/by the host. These microorganisms have the ability to convert feedstuff into microbial matter and fermentation products, which can then be utilised by the host. Main examples are volatile fatty acids (i.e., acetate, propionate and butyrate), and gases such as carbon dioxide and methane. Reducing animal emissions of these gases has been the target of genetic improvement and diet modifications in ruminants for decades (Morgavi *et al.*, 2015a; Yáñez-Ruiz, Abecia and Newbold, 2015; Roehe *et al.*, 2016a; Saro *et al.*, 2018b; Jeyanathan *et al.*, 2019a), as they are known contributors to climate change, and any energy diverted from the animal's part to their formation is a loss in terms of productivity.

It is not currently known what impact management and stress may have on methanogens. As indicated by findings in Chapter 3, it would be interesting to investigate differences between

sexes. Many studies focus on one or the other, and for production animals both females and males are important, since they are both used for different purposes (females: milk, reproduction; males: meat, reproduction). Differences in management and early life disturbances could lead to higher or lower abundances in important bacteria such as methanogens and opportunistic pathogens.

In addition to investigating impacts of stress on the rumen, it would be interesting to look more into faecal samples. The vast majority of studies in ruminants use samples from the rumen, while studies exploring faecal samples are comparatively few. As in other animal species, the gut is where most stress-microbiome interactions have been observed, and it could be argued that focusing on this location and sample matrix would be an evident future step. This is the only study to date exploring stress effects on ruminant gut bacteria profiles; in comparison to the proliferation of data found for other species, it is evident that further exploration and more data are required to reach firm conclusions. On this note, a further point for consideration is that the presence of bacteria unique to the small intestine of ruminants (Malmuthuge *et al.*, 2014) suggests that exclusively investigating faecal samples may not capture the entirety of the microbial diversity in the gut, overlooking important regional host–microbial interactions (Malmuthuge *et al.*, 2015).

As there was little evidence of impact of genetic lines on the microbiome, an alternative approach would be to correlate genetic markers (e.g., SNPs) directly with taxonomic profiles in the rumen/faecal samples to investigate relationships from that perspective, followed up by potential associated "omics" (transcriptomics and proteomics) analyses.

What was investigated in the context of the present studies was "who" is in the rumen and faecal samples, acquired via taxonomic analyses from 16S rRNA gene amplicon sequencing. The exact role of the bacteria and archaea present is not known, other than what has been described in previous studies. However, these have not focused on stress-host (ruminant)-microbiome interactions. It is unknown which genes may be expressed under host stress signals in ruminants, triggering microbial cell expression of genes. There are evidently metabolomic studies conducted on rumen and faecal samples in cattle (fewer in sheep) but none have looked at gene expression, stress pathway signals and proteomics al together.

Therefore, as a starting point, since the studies presented here explored a vast body of interactions with very few significant results, it may be more pertinent to perform metagenomics and metabolomics investigations with a focus on specific bacteria/ archaea (i.e. *Lactobacillus, Ruminococcus*) for which functional pathways have already been explored. This would allow the use of training datasets and Machine Learning approaches. Alternatively,

bacteria that showed some responsiveness in this study (such as *Bacteroidales*, *Prevotella*, *Methanobrevibacter*, and *Clostridiales* species) could act as starting point for metabolism exploration in relation to stress.

5.5 CONCLUSIONS

In conclusion, despite extensive investigation of the microbial structure in rumen and faecal samples, no clear relationships emerged between the microbiota and the factors selected as markers for this interplay (cortisol, serotonin, EBVs). Many of the correlations observed, despite a great part of them not being significant, agreed with literature findings in the direction of relationship present. Genetic predisposition to stress did not influence the microbial structure and abundance of rumen and faecal communities. Age and early life interventions showed potential for impacting the community in more consequential ways.

Whilst able to determine behavioural differences between treatment groups in the MCS trial, the rumen and microbiota differences were not as pronounced as expected from literature findings for other species. It is likely that the rumen acts as a "buffer" and exploration of this intricate ecosystem would not only allow a better understanding of ruminants but would also help shed light on the multifaceted mechanisms at play, allowing hosts in general to achieve a homeostatic state and co-exist with their microbiome.

Chapter 6

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Chapter 7

Appendix

7 Appendix

7.1 Appendix: Chapter 2

7.1.1 Rumen phyla, orders and genera correlograms with cortisol and estimated breeding values (EBVs)

7.1.1.1 Rumen phyla- cortisol

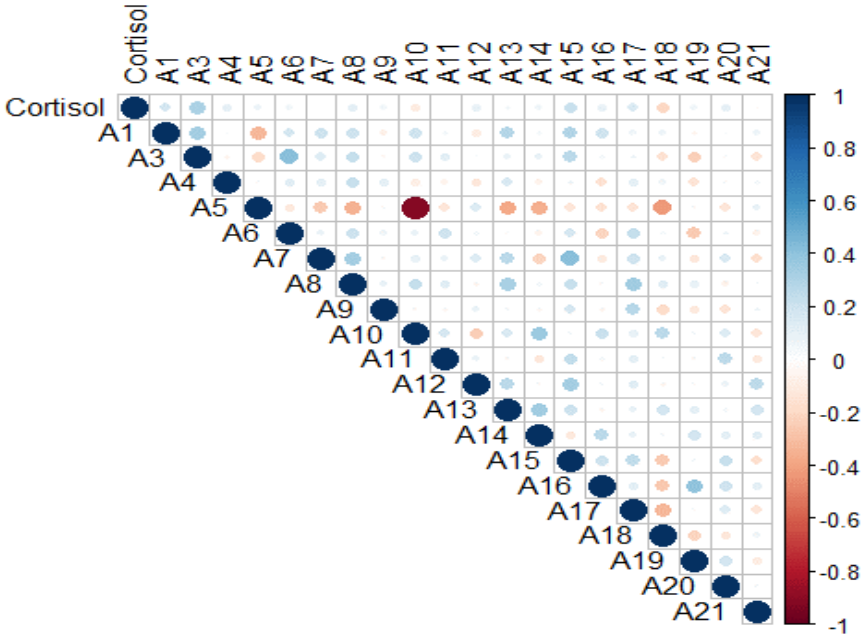


Figure 7.1 Spearman's rank correlation matrix for SRT rumen phyla abundances and SRT cortisol values. The matrix depicts relationships among phyla, and between phyla and cortisol on the top row Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two phyla. phyla and VIPs obtained from the PLS regression are also shown in **Table 7.1.**

Table 7.1 Rumen phyla, used to explore the relationship of SRT phyla relative abundances and cortisol. The VIP score from the PLS analysis, mean relative abundance of each phylum, the direction of correlation with cortisol, as well as the correlation coefficient (R) and p-values as reported from the correlation analyses. Coding is also available to relate with Correlogram **Figure 7.1.**

	Phylum	**VIP**	**Mean RA**	**Direction**	**R**	**p-value**
A1	*Euryarchaeota*	0.87	0.51	Positive	0.16	0.24
A3	*Actinobacteria*	1.17	0.08	Positive	0.31	0.02
A4	*Armatimonadetes*	0.94	0.01	Positive	0.02	0.88
A5	*Bacteroidetes*	0.92	38.94	Positive	0.07	0.61
A6	*Chloroflexi*	0.86	0.05	Positive	0.05	0.72
A7	*Cyanobacteria*	1.08	0.87	Negative	-0.06	0.67
A8	*Elusimicrobia*	1.13	0.12	Positive	0.13	0.32
A9	*Fibrobacteres*	0.95	0.71	Positive	0.02	0.89
A10	*Firmicutes*	1.02	15.18	Negative	-0.11	0.40
A11	*Fusobacteria*	0.70	0.00	Positive	0.04	0.77
A12	*LD1*	0.81	0.02	Positive	0.13	0.34
A13	*Lentisphaerae*	0.73	0.23	Positive	0.10	0.46
A14	*Planctomycetes*	0.99	0.10	Positive	0.12	0.38
A15	*Proteobacteria*	0.89	0.54	Positive	0.15	0.25
A16	*SR1*	1.07	0.01	Positive	0.15	0.26
A17	*Spirochaetes*	1.09	0.50	Positive	0.14	0.30
A18	*Synergistetes*	2.07	1.00	Negative	-0.23	0.08
A19	*Tenericutes*	0.79	0.23	Positive	0.05	0.69
A20	*Verrucomicrobia*	0.77	0.06	Positive	0.16	0.24
A21	*WPS-2*	0.77	0.01	Positive	0.10	0.45

7.1.1.2 Rumen phyla – estimated breeding values

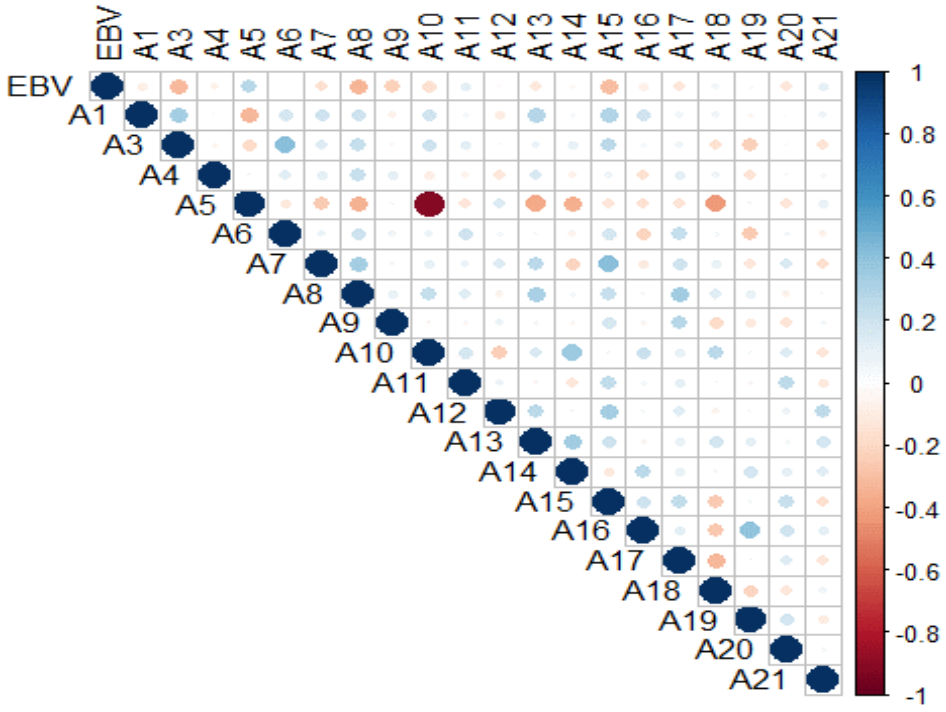


Figure 7.2 Spearman's rank correlation matrix of the SRT rumen phyla abundances and Estimated Breeding values (EBVs). The matrix depicts relationships among phyla, and between phyla and EBVs on the top row Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two phyla. Phyla and VIPs obtained from the PLS regression are also shown in **Table7.2**.

Table 7.2 Rumen phyla, used to explore the relationship of SRT phyla relative abundances and Estimated Breeding values (EBVs). The VIP score from the PLS analysis, mean relative abundance of each phylum, the direction of correlation with EBVs, as well as the correlation coefficient (R) and p-values as reported from the correlation analyses. Coding is also available to relate with Correlogram **Figure 7.2.**

	Phylum	**VIP**	**Mean RA**	**Direction**	**R**	**p-value**
A1	*Euryarchaeota*	0.57	0.51	Negative	-0.12	0.36
A3	*Actinobacteria*	0.84	0.08	Negative	-0.29	0.03
A4	*Armatimonadetes*	0.90	0.01	Negative	-0.08	0.57
A5	*Bacteroidetes*	1.00	38.94	Positive	0.19	0.15
A6	*Chloroflexi*	0.90	0.05	Negative	-0.07	0.61
A7	*Cyanobacteria*	0.73	0.87	Negative	-0.25	0.06
A8	*Elusimicrobia*	2.26	0.12	Negative	-0.31	0.02
A9	*Fibrobacteres*	0.98	0.71	Negative	-0.29	0.03
A10	*Firmicutes*	0.70	15.18	Negative	-0.11	0.43
A11	*Fusobacteria*	1.87	0.00	Positive	0.09	0.51
A12	*LD1*	0.49	0.02	Negative	-0.06	0.64
A13	*Lentisphaerae*	0.58	0.23	Negative	-0.15	0.28
A14	*Planctomycetes*	1.12	0.10	Negative	-0.04	0.79
A15	*Proteobacteria*	0.70	0.54	Negative	-0.35	0.01
A16	*SR1*	0.86	0.01	Negative	-0.09	0.48
A17	*Spirochaetes*	0.51	0.50	Negative	-0.14	0.30
A18	*Synergistetes*	0.45	1.00	Negative	0.00	0.99
A19	*Tenericutes*	1.12	0.23	Positive	0.05	0.74
A20	*Verrucomicrobia*	0.76	0.06	Negative	-0.14	0.31
A21	*WPS-2*	0.64	0.01	Positive	0.02	0.86

7.1.1.3 Rumen orders-cortisol

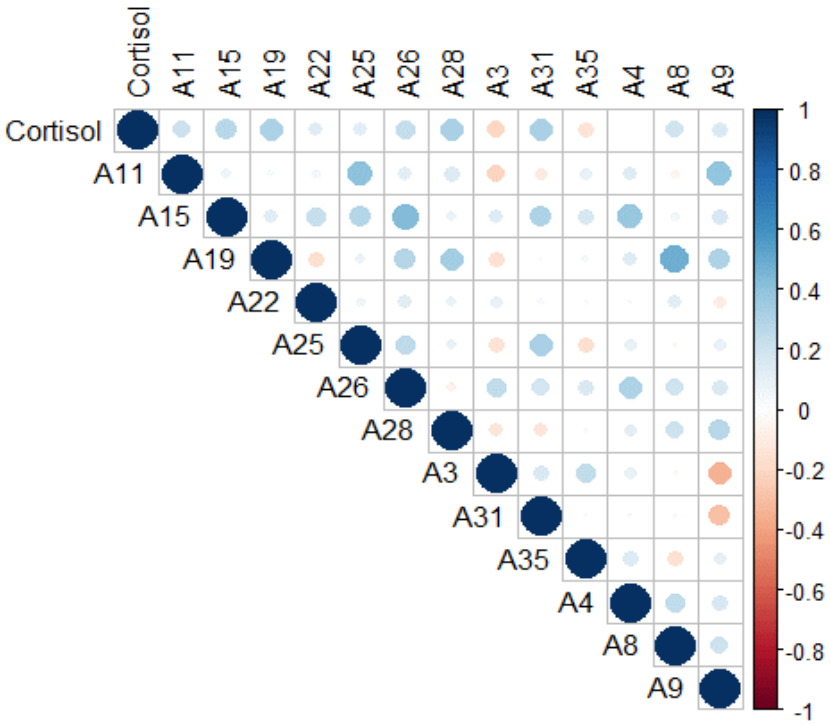


Figure 7.3 Spearman's rank correlation matrix of the SRT rumen order RA and cortisol values. The matrix depicts relationships among orders, and between orders and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two orders. Orders and VIPs obtained from the PLS regression are also shown in **Table 7.3.**

Table 7.3 Rumen orders with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT order RA and cortisol. Orders are presented along with higher taxonomic levels if necessary, for recognition purposes. The VIP score from the PLS analysis, mean RA of each order, the direction of correlation with cortisol as well as the correlation coefficient (R) and p-values as reported from the correlation analyses. Coding is also available to relate with Correlogram **Figure 7.3**.

	Orders	**VIP**	**Mean RA**	**Direction**	**R**	**p-value**
A28	*Lactobacillales*	2.03	0.02	Positive	0.35	0.01
A19	*Coriobacteriales*	1.75	0.08	Positive	0.31	0.02
A15	*p_Spirochaetes;c_MVP-15;o_PL-11B10*	1.74	0.09	Positive	0.30	0.02
A31	*Myxococcales*	1.52	0.02	Positive	0.26	0.05
A26	*c_[Lentisphaeria];o_Z20*	1.41	0.04	Positive	0.25	0.06
A3	*Synergistales*	1.41	0.98	Negative	-0.23	0.08
A22	*p_Tenericutes;c_RF3;o_ML615J-28*	1.36	0.06	Positive	0.22	0.10
A25	*c_Verruco-5;o_LD1-PB3*	1.28	0.04	Positive	0.15	0.28
A8	*Methanobacteriales*	1.15	0.23	Positive	0.20	0.13
A4	*p_Cyanobacteria;c_4C0d-2;o_YS2*	1.14	0.85	Negative	-0.06	0.63
A11	*Erysipelotrichales*	1.06	0.18	Positive	0.19	0.17
A35	*p_Firmicutes;Other;Other*	1.03	0.01	Negative	-0.17	0.20
A9	*c_Alphaproteobacteria;o_RF32*	1.00	0.22	Positive	0.15	0.28

7.1.1.4 Rumen orders-estimated breeding values

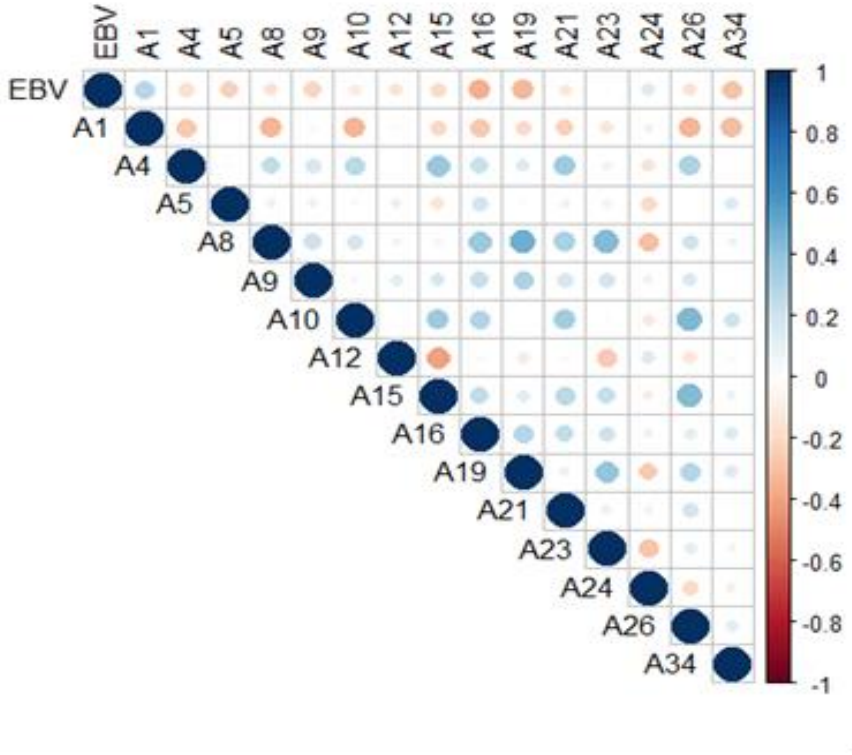


Figure 7.4 Spearman's rank correlation matrix of the SRT rumen order abundances and Estimated Breeding values (EBVs). The matrix depicts relationships among orders and between orders and EBVs on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two orders. Orders and VIPs obtained from the PLS regression are also shown in **Table 7.4.**

Table 7.4 Rumen orders with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT order relative abundances and Estimated Breeding values (EBVs). Orders are presented along with higher taxonomic levels if necessary, for recognition purposes. The VIP score from the PLS analysis, mean relative abundance of each order, the direction of correlation with EBVs as well as the correlation coefficient (R) and p-values as reported from the correlation analyses. Coding is also available to relate with Correlogram **Figure 7.4**.

	Order	**VIP**	**Mean RA**	**Direction**	**R**	**p-value**
A5	*Fibrobacterales*	1.94	0.66	Negative	-0.29	0.03
A34	*p_Planctomycetes;c_vadinHA49;Other*	1.76	0.01	Negative	-0.30	0.02
A19	*Coriobacteriales*	1.58	0.08	Negative	-0.29	0.03
A16	*Elusimicrobiales*	1.56	0.09	Negative	-0.29	0.03
A4	*p_Cyanobacteria;c_4C0d-2;o_YS2*	1.45	0.85	Negative	-0.26	0.05
A8	*Methanobacteriales*	1.43	0.23	Negative	-0.26	0.05
A9	*c_Alphaproteobacteria;o_RF32*	1.40	0.22	Negative	-0.26	0.05
A23	*c_Anaerolineae;o_Anaerolineales*	1.31	0.05	Negative	-0.06	0.65
A15	*p_Spirochaetes;c_MVP-15; o_PL-11B10*	1.31	0.09	Negative	-0.24	0.08
A12	*c_Mollicutes;o_RF39*	1.25	0.12	Negative	-0.12	0.37
A21	*c_Alphaproteobacteria;Other*	1.21	0.07	Negative	-0.22	0.10
A24	*c_Mollicutes;o_Anaeroplasmatales*	1.17	0.04	Positive	0.22	0.11
A26	*c_[Lentisphaeria];o_Z20*	1.15	0.04	Negative	-0.18	0.17
A10	*Victivallales*	1.12	0.19	Negative	-0.13	0.33
A1	*Bacteroidales*	1.05	37.54	Positive	0.19	0.15

7.1.1.5 Rumen genera- cortisol

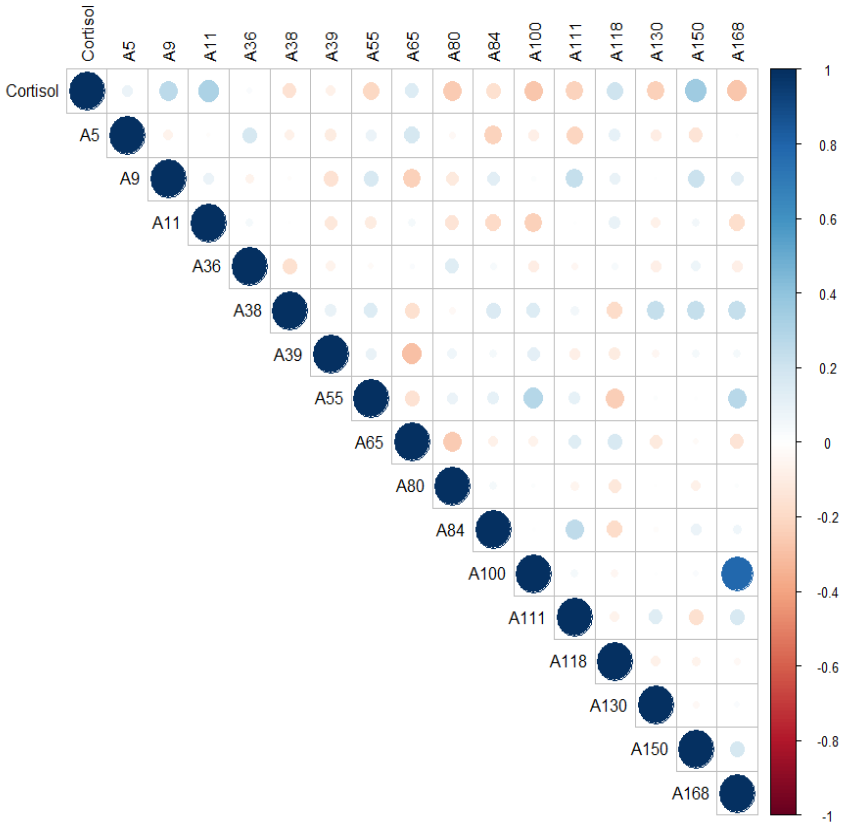


Figure 7.5 Spearman's rank correlation matrix of the SRT rumen genus abundances and cortisol. The matrix depicts relationships among genera, and between genera and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two genera. Genera and VIPs obtained from the PLS regression are also shown in **Table 7.5**.

Table 7.5 Rumen genera with VIP scores higher than 1.5, as reported by the PLS regression used to explore the relationship of SRT genera RA and cortisol. Genera are presented along with Family and higher taxonomic level if necessary, for recognition purposes. The VIP score from the PLS analysis, mean RA of each genus, the direction of correlation with cortisol, as well as the correlation coefficient (R) and p-values as reported from the correlation analyses. Coding is also available to relate with Correlogram **Figure 7.5.**

	Genera	**VIP**	**Mean RA**	**Direction**	**R**	**p- value**
A130	*Afipia*	2.30	0.200	Negative	-0.31	0.02
A168	*f_Dethiosulfovibrionaceae;g_TG5*	2.01	0.001	Negative	-0.19	0.17
A118	*f_Erysipelotrichaceae;g_p-75-a5_ Unknown*	1.86	0.045	Positive	0.10	0.45
A36	*f_[Paraprevotellaceae];Other*	1.85	0.026	Positive	0.03	0.83
A38	*f_[Paraprevotellaceae];g_CF231*	1.83	0.008	Positive	0.08	0.53
A100	*f_Veillonellaceae;Unknown*	1.83	0.008	Negative	-0.11	0.41
A80	*g_Pseudobutyrivibrio*	1.82	0.004	Negative	-0.23	0.08
A84	*f_Peptococcaceae;Unknown_*	1.81	0.020	Negative	-0.18	0.17
A9	*f_Coriobacteriaceae;Other*	1.73	0.008	Positive	0.34	0.01
A55	*p_Firmicutes;Other;Other; Other;Other*	1.72	0.007	Negative	-0.20	0.13
A39	*f_[Paraprevotellaceae];g_YRC22*	1.68	0.008	Positive	0.05	0.70
A68	*f_Lachnospiraceae;g_*	1.67	0.009	Positive	0.09	0.53
A150	*o_Myxococcales;f_0319-6G20;Unknown_*	1.66	0.015	Positive	0.31	0.02
A111	*f_Erysipelotrichaceae;Other*	1.57	0.001	Negative	-0.20	0.15
A65	*Anaerofustis*	1.56	0.001	Negative	-0.25	0.06
A11	*Adlercreutzia*	1.52	0.000	Positive	0.30	0.02
A5	*f_[Methanomassiliicoccaceae]; Other*	1.51	0.214	Negative	-0.22	0.10

7.1.1.6 Rumen genera- estimated Breeding values

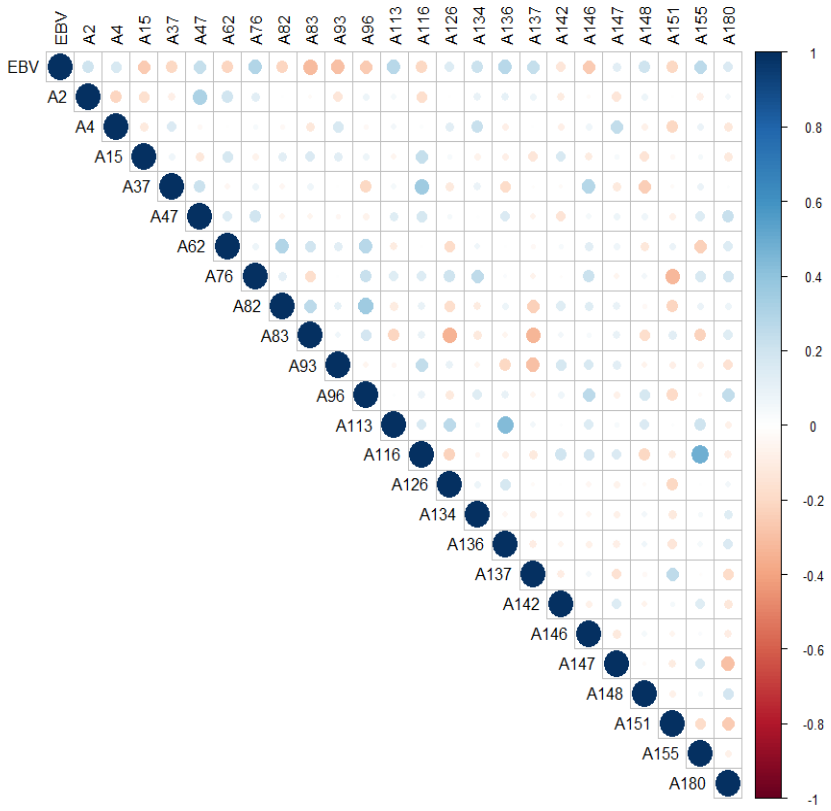


Figure 7.6 Spearman's rank correlation matrix of the SRT rumen genus abundances and Estimated Breeding values (EBVs). The matrix depicts relationships among genera, and between genera and EBVs on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two genera. Genera and VIPs obtained from the PLS regression are also shown in **Table 7.6.**

Table 7.6 Rumen Genera with VIP scores higher than 1.5, as reported by the PLS regression used to explore the relationship of SRT genera relative abundances and Estimated Breeding values. Genera are presented along with Family and higher taxonomic level if necessary, for recognition purposes. The VIP score from the PLS analysis, mean relative abundance of each genus, the direction of correlation with EBVs, as well as the correlation coefficient (R) and p-values as reported from the correlation analyses. Coding is also available to relate with Correlogram **Figure 7.6**.

	Genera	**VIP**	**Mean RA**	**Direction**	**R**	**p- value**
A113	*Anaerorhabdus*	1.96	0.004	Positive	0.24	0.07
A116	*Sharpea*	1.64	0.01	Negative	-0.20	0.13
A126	*p_Proteobacteria;Other; Other;Other;Other*	1.79	0.004	Positive	0.22	0.11
A134	*Agrobacterium*	1.57	0.0003	Positive	0.19	0.15
A136	*Novosphingobium*	1.94	0.0004	Positive	0.24	0.07
A137	*Sphingomonas*	1.78	0.001	Positive	0.22	0.10
A146	*f_Desulfovibrionaceae;g_*	1.97	0.001	Negative	-0.23	0.09
A148	*o_Desulfuromonadales; Other;Other*	1.53	0.006	Positive	0.19	0.16
A15	*Slackia*	1.85	0.001	Negative	-0.23	0.09
A151	*c_Deltaproteobacteria; o_PB19;f_;g_*	2.01	0.003	Negative	-0.25	0.06
A155	*Ruminobacter*	1.77	0.005	Positive	0.22	0.10
A180	*c_Verruco-5; o_LD1-PB3;f_;g_*	1.60	0.04	Positive	0.19	0.15
A2	*g_Methanosphaera*	1.82	0.02	Positive	0.22	0.10
A37	*f_[Paraprevotellaceae];g_*	1.98	0.95	Negative	-0.24	0.08
A4	*Methanimicrococcus*	1.62	0.01	Positive	0.20	0.14
A47	*o_Streptophyta;f_;g_*	1.71	0.01	Positive	0.21	0.12
A62	*f_Clostridiaceae;g_02d06*	1.83	0.20	Negative	-0.22	0.09
A64	*Dehalobacterium*	1.30	0.01	Positive	0.16	0.23

A76	*Lachnospira*	2.13	0.01	Positive	0.26	0.05
A82	*Shuttleworthia*	1.84	0.07	Negative	-0.22	0.10
A83	*Syntrophococcus*	2.54	0.01	Negative	-0.31	0.02
A93	*Oscillospira*	1.57	0.39	Negative	-0.18	0.18
A96	*Sporobacter*	2.14	0.01	Negative	-0.26	0.05

7.1.2 Faecal phyla, orders and genera correlograms with cortisol and estimated breeding values (EBVs)

7.1.2.1 Faecal phyla- estimated breeding values

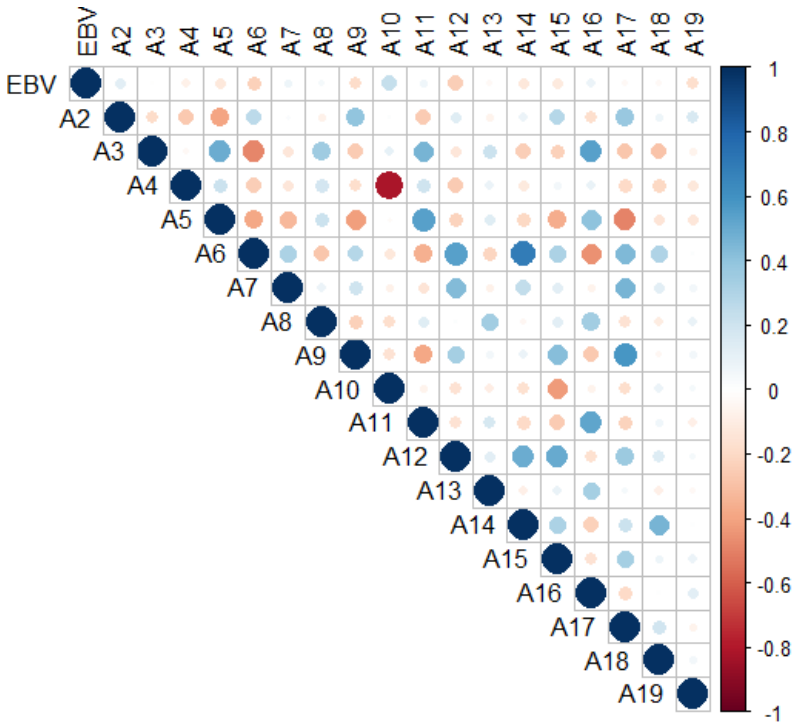


Figure 7.7 Spearman's rank correlation matrix for SRT faecal phyla abundances and SRT Estimated Breeding values (EBVs). The matrix depicts relationships among phyla, and between phyla and EBVs on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two phyla. Phyla and VIPs obtained from the PLS regression are also shown in **Table 7.7**.

Table 7.7 Faecal phyla, used to explore the relationship of SRT phyla relative abundances and Estimated Breeding values (EBVs). The VIP score from the PLS analysis, mean relative abundance of each phylum, the direction of correlation with EBVs, as well as the correlation coefficient (R) and p-values as reported from the correlation analyses. Coding is also available to relate with Correlogram **Figure 7.7**.

	Phylum	VIP	mean RA	Direction	R	p-value
A19	*WPS-2*	1.90	0.00	Positive	0.26	0.05
A13	*Planctomycetes*	1.60	0.14	Negative	-0.20	0.13
A8	*Elusimicrobia*	1.54	0.03	Positive	0.22	0.10
A17	*Tenericutes*	1.22	0.43	Negative	-0.17	0.22
A6	*Cyanobacteria*	1.00	0.53	Negative	-0.11	0.40
A1	*Euryarchaeota*	0.97	0.55	Positive	0.12	0.38
A3	*Actinobacteria*	0.93	0.13	Positive	0.07	0.63
A9	*Fibrobacteres*	0.93	0.26	Negative	-0.11	0.42
A2	*k_Bacteria;Other*	0.90	0.05	Positive	0.08	0.58
A5	*Chloroflexi*	0.90	0.01	Positive	0.08	0.54
A15	*Spirochaetes*	0.82	1.04	Negative	-0.01	0.93
A16	*Synergistetes*	0.81	0.00	Positive	0.04	0.75
A14	*Proteobacteria*	0.76	0.31	Negative	-0.08	0.57
A12	*Lentisphaerae*	0.74	0.44	Negative	-0.03	0.81
A11	*LD1*	0.68	0.00	Positive	0.05	0.72
A4	*Bacteroidetes*	0.53	24.42	Negative	-0.03	0.85
A7	*Deferribacteres*	0.49	0.01	Positive	0.01	0.93
A10	*Firmicutes*	0.36	28.05	Positive	0.05	0.70
A18	*Verrucomicrobia*	0.35	0.05	Negative	-0.04	0.77

7.1.2.2 Faecal orders-cortisol

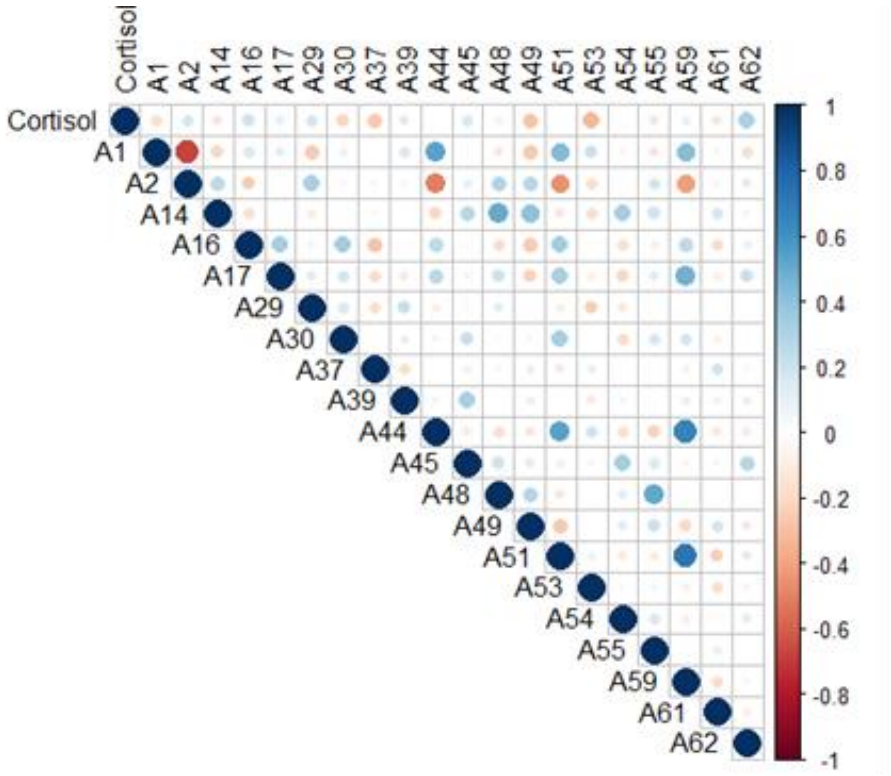


Figure 7.8 Spearman's rank correlation matrix of the SRT faecal order RA and cortisol values. The matrix depicts relationships among orders, and between orders and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two orders. Orders and VIPs obtained from the PLS regression are also shown in **Table 7.8**.

Table 7.8 Faecal orders with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT order RA and cortisol. Orders are presented along with higher taxonomic levels if necessary, for recognition purposes. The VIP score from the PLS analysis, mean RA of each order, the direction of correlation with cortisol as well as the correlation coefficient (R) and p-values as reported from the correlation analyses. Coding is also available to relate with Correlogram **Figure 7.8**.

	Order	VIP	mean RA.	Direction	R	p-value
A53	*Acholeplasmatales*	2.80	0.0006	Negative	-0.37	0.01
A62	*k_Bacteria;p__WPS-2;c_;o_*	1.94	0.0005	Positive	0.26	0.05
A37	*c_Betaproteobacteria;Other*	1.86	0.004	Negative	-0.25	0.06
A49	*Sphaerochaetales*	1.78	0.03	Negative	-0.31	0.02
A16	*Elusimicrobiales*	1.71	0.03	Positive	0.21	0.11
A30	*Pirellulales*	1.66	0.14	Negative	-0.20	0.13
A48	*c_Spirochaetes;o_M2PT2-76*	1.41	0.04	Negative	-0.18	0.18
A14	*Streptophyta*	1.37	0.04	Negative	-0.18	0.17
A17	*c_Endomicrobia;o_*	1.32	0.001	Positive	0.13	0.32
A61	*Verrucomicrobiales*	1.29	0.02	Negative	-0.18	0.17
A1	*Methanobacteriales*	1.27	0.18	Negative	-0.08	0.55
A2	*Methanomicrobiales*	1.26	0.33	Positive	0.10	0.44
A44	*Aeromonadales*	1.22	0.003	Positive	0.11	0.40
A45	*Enterobacteriales*	1.15	0.0005	Positive	0.13	0.32
A55	*c_Mollicutes;o_RF39*	1.15	0.12	Negative	-0.13	0.33
A29	*c_[Lentisphaeria];o_Z20*	1.13	0.02	Positive	0.19	0.16
A39	*Nitrosomonadales*	1.09	0.002	Positive	0.15	0.26
A54	*Anaeroplasmatales*	1.07	0.005	Negative	-0.13	0.32
A51	*Synergistales*	1.04	0.004	Positive	0.04	0.75
A59	*c_Verruco-5;o_LD1-PB3*	1.02	0.003	Positive	0.09	0.52

7.1.2.3 Faecal orders- Estimated Breeding values

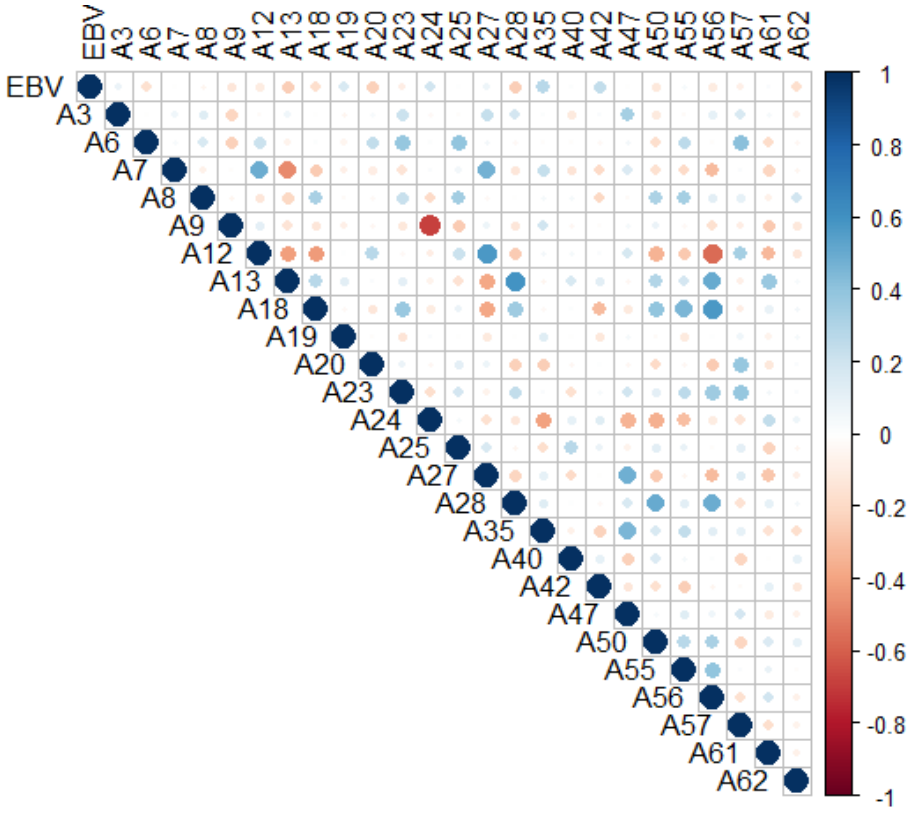


Figure 7.9 Spearman's rank correlation matrix of the SRT faecal order abundances and Estimated Breeding values (EBVs). The matrix depicts relationships among orders and between orders and EBVs on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two orders. Orders and VIPs obtained from the PLS regression are also shown in **Table 7.9.**

Table 7.9 Faecal orders with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT order relative abundances and Estimated Breeding values (EBVs). Orders are presented along with higher taxonomic levels if necessary, for recognition purposes. The VIP score from the PLS analysis, mean relative abundance of each order, the direction of correlation with EBVs as well as the correlation coefficient (R) and p-values as reported from the correlation analyses. Coding is also available to relate with Correlogram **Figure 7.9.**

	Order	VIP	mean RA	Direction	R	p-value
A20	*Bacillales*	2.10	0.02	Negative	-0.22	0.10
A12	*Anaerolineales*	1.97	0.006	Positive	0.01	0.95
A57	*c_Opitutae;o_HA64*	1.76	0.001	Negative	-0.07	0.59
A42	*Campylobacterales*	1.58	0.01	Positive	0.25	0.06
A24	*Clostridiales*	1.49	27.76	Negative	-0.16	0.23
A27	*k_Bacteria;p_LD1;c_;Unidentified*	1.47	0.003	Positive	0.20	0.14
A6	*Actinomycetales*	1.44	0.005	Positive	0.06	0.67
A23	*c_Clostridia;Other*	1.39	0.01	Positive	0.20	0.14
A8	*p_Bacteroidetes;Other;Other*	1.37	0.21	Positive	0.18	0.19
A35	*Rhizobiales*	1.36	0.005	Positive	0.27	0.04
A47	*Xanthomonadales*	1.34	0.001	Positive	0.08	0.58
A9	*Bacteroidales*	1.28	24.16	Negative	-0.24	0.08
A28	*Victivallales*	1.27	0.42	Negative	-0.30	0.02
A19	*p_Firmicutes;Other;Other*	1.26	0.01	Positive	0.17	0.20
A13	*p_Cyanobacteria;c_4C0d-2;o_YS2*	1.12	0.49	Negative	-0.26	0.05
A7	*Coriobacteriales*	1.11	0.12	Negative	-0.02	0.89
A62	*k_Bacteria;p_WPS-2;c_; Unidentified*	1.08	0.0005	Negative	-0.24	0.08
A56	*p_Tenericutes;c_RF3;o_ML615J-28*	1.07	0.30	Negative	-0.09	0.53
A18	*Fibrobacterales*	1.07	0.26	Negative	-0.24	0.07
A25	*c_Clostridia;o_SHA-98*	1.05	0.005	Positive	0.15	0.26
A55	*c_Mollicutes;o_RF39*	1.04	0.12	Positive	0.13	0.33

A61	*Verrucomicrobiales*	1.03	0.02	Positive	0.05	0.69
A50	*Spirochaetales*	1.02	0.97	Negative	-0.10	0.46
A3	*Methanosarcinales*	1.01	0.001	Positive	0.14	0.29
A40	*Desulfovibrionales*	1.01	0.01	Positive	0.10	0.46

7.1.2.4 Faecal genera- cortisol

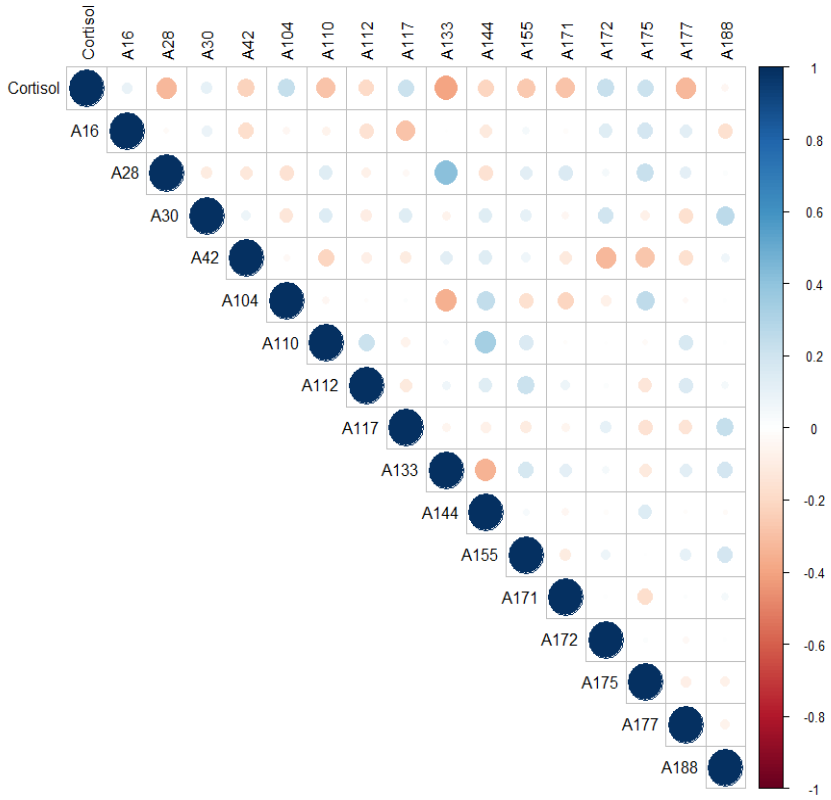


Figure 7.10 Spearman's rank correlation matrix of the SRT faecal genus abundances and cortisol. The matrix depicts relationships among genera, and between genera and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two genera. Genera and VIPs obtained from the PLS regression are also shown in **Table 7.10**.

Table 7.10 Faecal genera with VIP scores higher than 1.5, as reported by the PLS regression used to explore the relationship of SRT genera RA and cortisol. Genera are presented along with Family and higher taxonomic level if necessary, for recognition purposes. The VIP score from the PLS analysis, mean RA of each genus, the direction of correlation with cortisol, as well as the correlation coefficient (R) and p-values as reported from the correlation analyses. Coding is also available to relate with Correlogram **Figure 7.10**.

	Genera	**VIP**	**Mean RA**	**Direction**	**R**	**p-value**
A177	*o_Acholeplasmatales;Unidentified*	2.66	0.001	Negative	-0.52	0.00
A133	*Coprobacillus*	2.55	0.02	Negative	-0.40	0.002
A110	*Clostridium*	2.43	0.28	Negative	-0.52	0.000
A171	*Sphaerochaeta*	2.20	0.03	Negative	-0.16	0.24
A28	*Paludibacter*	2.12	0.67	Positive	0.02	0.88
A30	*Porphyromonas*	2.03	0.01	Negative	-0.06	0.67
A112	*Faecalibacterium*	1.87	0.01	Negative	-0.48	0.0002
A188	*p_WPS-2;c_;o_;f_;Unidentified*	1.87	0.00	Negative	-0.37	0.005
A42	*f_[Paraprevotellaceae]; Unidentified*	1.81	0.04	Negative	-0.40	0.00
A104	*f_Peptostreptococcaceae; g_[Clostridium]*	1.78	0.01	Negative	-0.16	0.25
A155	*c_Betaproteobacteria;Other; Other;Other*	1.77	0.004	Negative	-0.19	0.15
A175	*f_Dethiosulfovibrionaceae; g_TG5*	1.73	0.001	Negative	-0.19	0.16
A172	*f_Spirochaetaceae; Unidentified*	1.72	0.04	Positive	0.35	0.01
A144	*f_Pirellulaceae; Unidentified*	1.56	0.14	Positive	0.13	0.34
A117	*Subdoligranulum*	1.55	0.002	Negative	-0.31	0.02
A16	*Olsenella*	1.52	0.004	Positive	0.23	0.08

7.1.2.5 Faecal genera- Estimated Breeding values

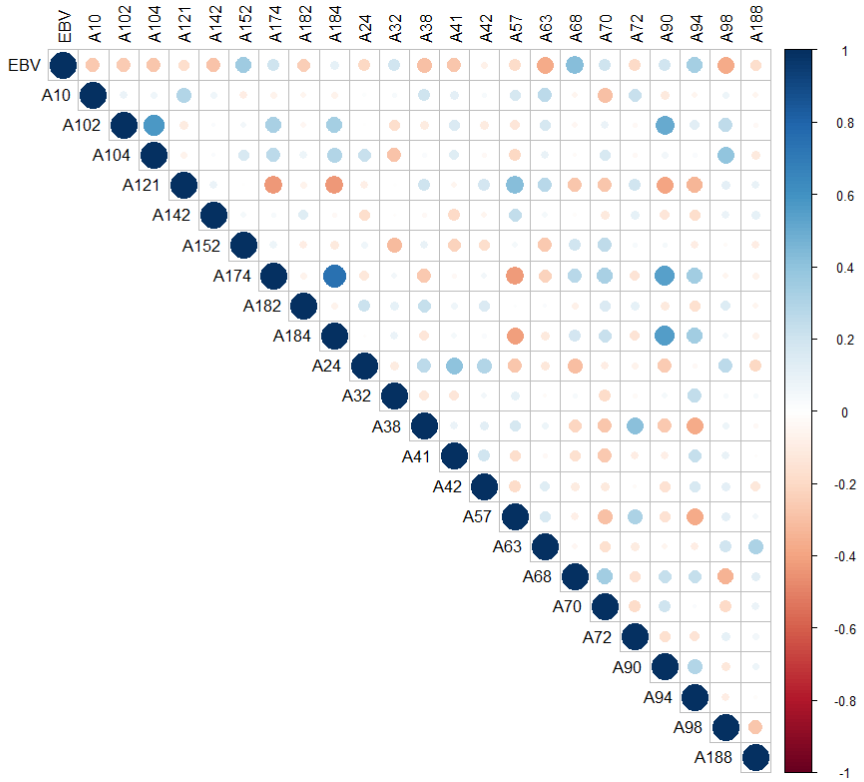


Figure 7.11 Spearman's rank correlation matrix of the SRT faecal genus abundances and Estimated Breeding values (EBVs). The matrix depicts relationships among genera, and between genera and EBVs on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two genera. Genera and VIPs obtained from the PLS regression are also shown in **Table 7.11**.

Table 7.11 Rumen genera with VIP scores higher than 1.5, as reported by the PLS regression used to explore the relationship of SRT genera relative abundances and Estimated Breeding values. Genera are presented along with Family and higher taxonomic level if necessary, for recognition purposes. The VIP score from the PLS analysis, mean relative abundance of each genus, the direction of correlation with EBVs, as well as the correlation coefficient (R) and p-values as reported from the correlation analyses. Coding is also available to relate with Correlogram **Figure 7.11**.

	Genera	**VIP**	**mean RA**	**Direction**	**R**	**p-value**
A68	*o_Clostridiales;*	2.52	3.17	Positive	0.42	0.00

	Unidentified					
A152	*Agrobacterium*	2.15	0.002	Positive	0.33	0.01
A63	*Sporosarcina*	2.14	0.004	Negative	-0.35	0.01
A41	*f_[Paraprevotellaceae];Other*	2.01	0.006	Negative	-0.31	0.02
A94	*Pseudobutyrivibrio*	2.00	0.007	Positive	0.32	0.01
A121	*Phascolarctobacterium*	1.97	0.35	Negative	-0.27	0.04
A98	*f_Lachnospiraceae;g_[Ruminococcus]*	1.93	0.01	Negative	-0.32	0.02
A174	*Pyramidobacter*	1.87	0.003	Positive	0.26	0.05
A38	*o_Bacteroidales;f_S24-7; Unidentified*	1.79	0.19	Negative	-0.29	0.03
A10	*Sanguibacter*	1.69	0.001	Negative	-0.28	0.04
A182	*c_Opitutae;o_HA64; Unidentified*	1.62	0.001	Negative	-0.26	0.05
A118	*Syntrophomonas*	1.62	0.002	Positive	0.27	0.05
A102	*f_Peptostreptococcaceae; Unidentified*	1.61	0.26	Negative	-0.25	0.06
A184	*c_Verruco-5;o_LD1-PB3; Unidentified*	1.60	0.003	Positive	0.21	0.11
A142	*Victivallis*	1.60	0.13	Negative	-0.26	0.06
A57	*Fibrobacter*	1.59	0.26	Negative	-0.25	0.06
A70	*f_Christensenellaceae; Unidentified*	1.58	0.36	Positive	0.26	0.05
A104	*f_Peptostreptococcaceae ;g_[Clostridium]*	1.58	0.01	Negative	-0.24	0.07
A188	*k_Bacteria;p__WPS-2; Unidentified*	1.55	0.0005	Negative	-0.24	0.08
A32	*Prevotella*	1.55	0.32	Positive	0.25	0.06
A24	*f_Bacteroidaceae;g_5-7N15*	1.54	3.07	Negative	-0.25	0.06
A72	*f_Clostridiaceae; Unidentified*	1.53	0.003	Negative	-0.25	0.06
A90	*Johnsonella*	1.52	0.004	Positive	0.23	0.08

7.2 Appendix Chapter 3

7.2.1 Phylum abundance correlations with cortisol

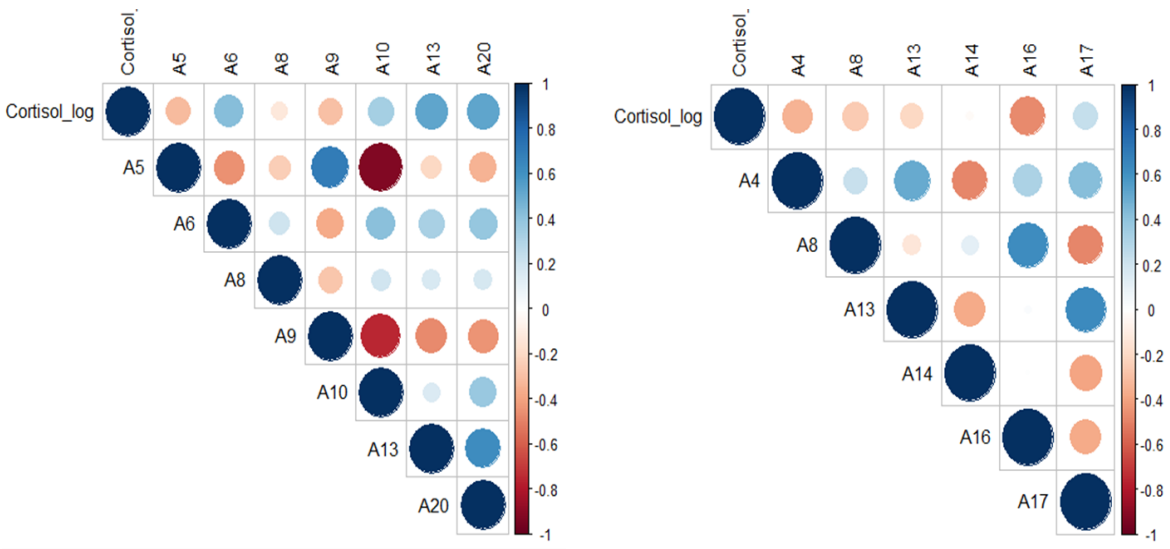
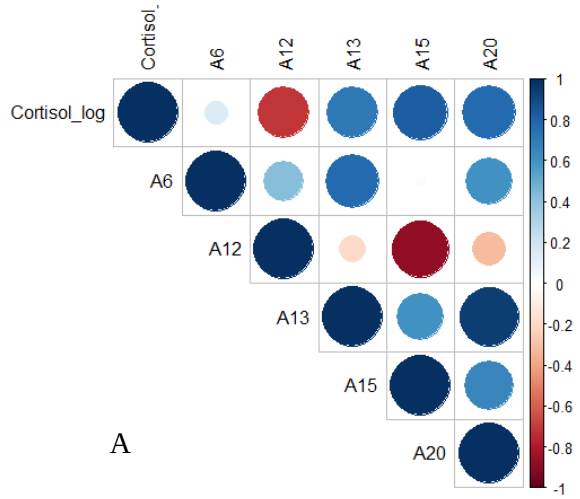


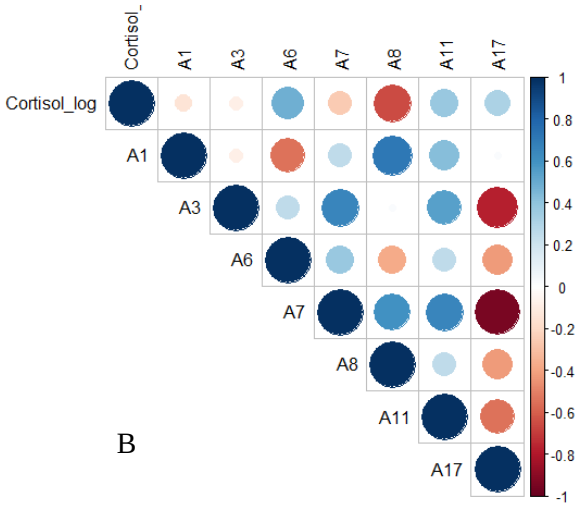
Figure 7.12 Spearman's rank correlation matrices for SRT rumen Female (A) and Male (B) phyla abundances and cortisol values. The matrix depicts relationships among phyla, and between phyla and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two phyla. Phyla and VIPs obtained from the PLS regression are also shown in **Table 7.12**.

Table 7.12 VIP scores from the PLS analyses of order RA by Sex and log transformed cortisol values. Correlation coefficients (R), as presented by the Pearson correlation analysis conducted, mean RA of each order by sex and coding numbers are also available to relate with **Figure 7.12.**

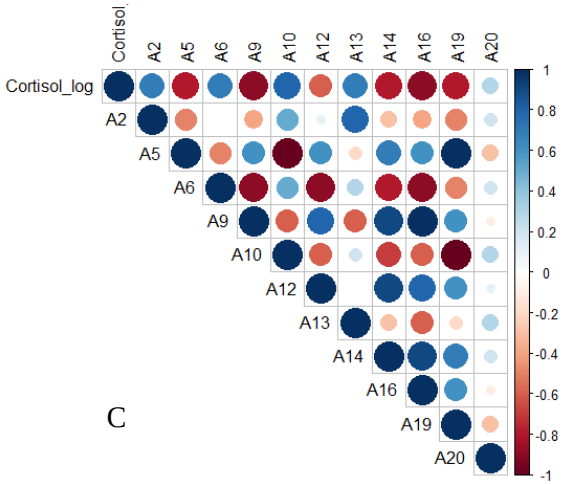
Females					**Males**				
Phyla, VIP > 1		**VIP**	**R**	**mean RA**	**Phyla, VIP > 1**		**VIP**	**R**	**mean RA**
A20	*WPS-2*	1.78	0.59	0.002	A14	*Proteobacteria*	1.8	-0.37	0.02
A6	*Chloroflexi*	1.70	0.56	0.07	A13	*Planctomycetes*	1.71	-0.32	0.007
A9	*Fibrobacteres*	1.36	-0.10	0.02	A16	*Spirochaetes*	1.48	-0.31	0.01
A13	*Planctomycetes*	1.36	0.44	0.008	A17	*Synergistetes*	1.46	0.32	0.02
A8	*Elusimicrobia*	1.31	-0.27	0.001	A8	*Elusimicrobia*	1.22	-0.16	0.001
A5	*Bacteroidetes*	1.06	-0.21	0.33	A4	*Armatimonadetes*	1.20	0.20	0.0001
A10	*Firmicutes*	1.01	0.22	0.46					


Cortisol_
A6
A12
A13
A15
A20
Cortisol_log
A6
A12
A13
A15
A20
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

A


Cortisol_
A1
A3
A6
A7
A8
A11
A17
Cortisol_log
A1
A3
A6
A7
A8
A11
A17
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

B


Cortisol_
A2
A5
A6
A9
A10
A12
A13
A14
A16
A19
A20
Cortisol_log
A2
A5
A6
A9
A10
A12
A13
A14
A16
A19
A20
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

C

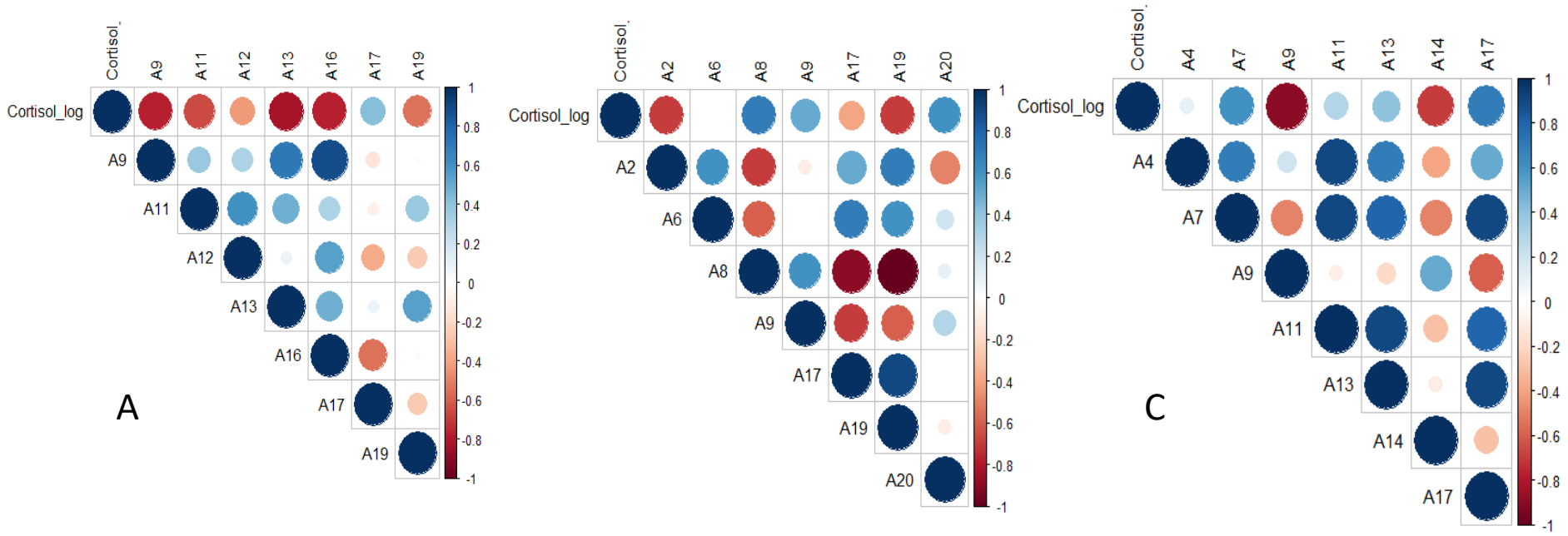


Figure 7.13 Spearman's rank correlation matrices for SRT rumen Male Control (A), Male Alternative (B) and Male Negative (C) phyla abundances and cortisol values. The matrix depicts relationships among phyla, and between phyla and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two phyla. Phyla and VIPs obtained from the PLS regression are also shown in **Table 7.13**

Table 7.13 VIP scores from the PLS analyses of phylum RA by Group (Sex * Prenatal Treatment) and log transformed cortisol values, as well as correlation coefficients (R), as presented by the Pearson correlation analysis conducted. Mean RA of each order by grouped variable and coding numbers are also reported to relate with the correlogram (**Figures 7.13, 7.14**).

Females														
Control					**Alternative**					**Negative**				
Phyla, VIP > 1		**VIP**	**R**	**mean RA**	**Phyla, VIP > 1**		**VIP**	**R**	**mean RA**	**Phyla, VIP > 1**		**VIP**	**R**	**mean RA**
A15	*SR1*	1.89	0.83*	0.0003	A6	*Chloroflexi*	2.57	0.80*	0.07	A9	*Fibrobacteres*	1.51	-0.83*	0.20
A20	*WPS-2*	1.67	0.73	0.001	A11	*LD1*	1.29	0.07	0.0003	A6	*Chloroflexi*	1.42	0.80*	0.07
A13	*Planctomycetes*	1.48	0.65	0.007	A8	*Elusimicrobia*	1.11	-0.34	0.001	A2	*Bacteria_* Unidentified	1.40	0.68	0.004
A6	*Chloroflexi*	1.07	0.47	0.08	A3	*Actinobacteria*	1.09	-0.40	0.005	A20	*WPS-2*	1.31	0.70	0.003
A12	*Lentisphaerae*	1.00	-0.43	0.002	A1	*Euryarchaeota*	1.03	-0.32	0.04	A13	*Planctomycetes*	1.28	0.54	0.01
					A7	*Cyanobacteria*	1.02	-0.30	0.006	A14	*Proteobacteria*	1.20	-0.64	0.01
					A17	*Synergistetes*	1.00	0.32	0.02	A16	*Spirochaetes*	1.19	-0.66	0.01
										A5	*Bacteroidetes*	1.19	-0.63	0.32
										A10	*Firmicutes*	1.12	0.59	0.46
										A19	*Verrucomicrobia*	1.10	-0.55	0.04
										A12	*Lentisphaerae*	1.04	-0.49	0.003
Males														

Control					Alternative					Negative				
Phyla, VIP > 1		**VIP**	**R**	**mean RA**	**Phyla, VIP > 1**		**VIP**	**R**	**mean RA**	**Phyla, VIP > 1**		**VIP**	**R**	**mean RA**
A13	*Planctomycetes*	2.04	-0.85*	0.01	A9	*Fibrobacteres*	1.70	0.91*	0.02	A14	*Proteobacteria*	1.99	-0.88*	0.02
A11	*LD1*	1.67	-0.71	0.0002	A19	*Verrucomicrobia*	1.36	-0.77	0.03	A9	*Fibrobacteres*	1.9	-0.77	0.21
A16	*Spirochaetes*	1.51	-0.64	0.01	A2	*Bacteria_ Unidentified*	1.32	-0.66	0.003	A17	*Synergistetes*	1.26	0.55	0.01
A19	*Verrucomicrobia*	1.35	-0.50	0.04	A17	*Synergistetes*	1.31	0.78	0.02	A4	*Armatimonadetes*	1.20	0.51	< 0.0001
A9	*Fibrobacteres*	1.15	-0.50	0.02	A8	*Elusimicrobia*	1.25	0.74	0.0005	A11	*LD1*	1.12	0.47	< 0.0001
A12	*Lentisphaerae*	1.19	-0.48	0.003	A20	*WPS-2*	1.22	0.72	0.001	A13	*Planctomycetes*	1.04	0.44	0.005
A17	*Synergistetes*	1.13	0.48	0.02	A6	*Chloroflexi*	1.06	-0.27	0.01	A7	*Cyanobacteria*	1.03	0.42	0.002

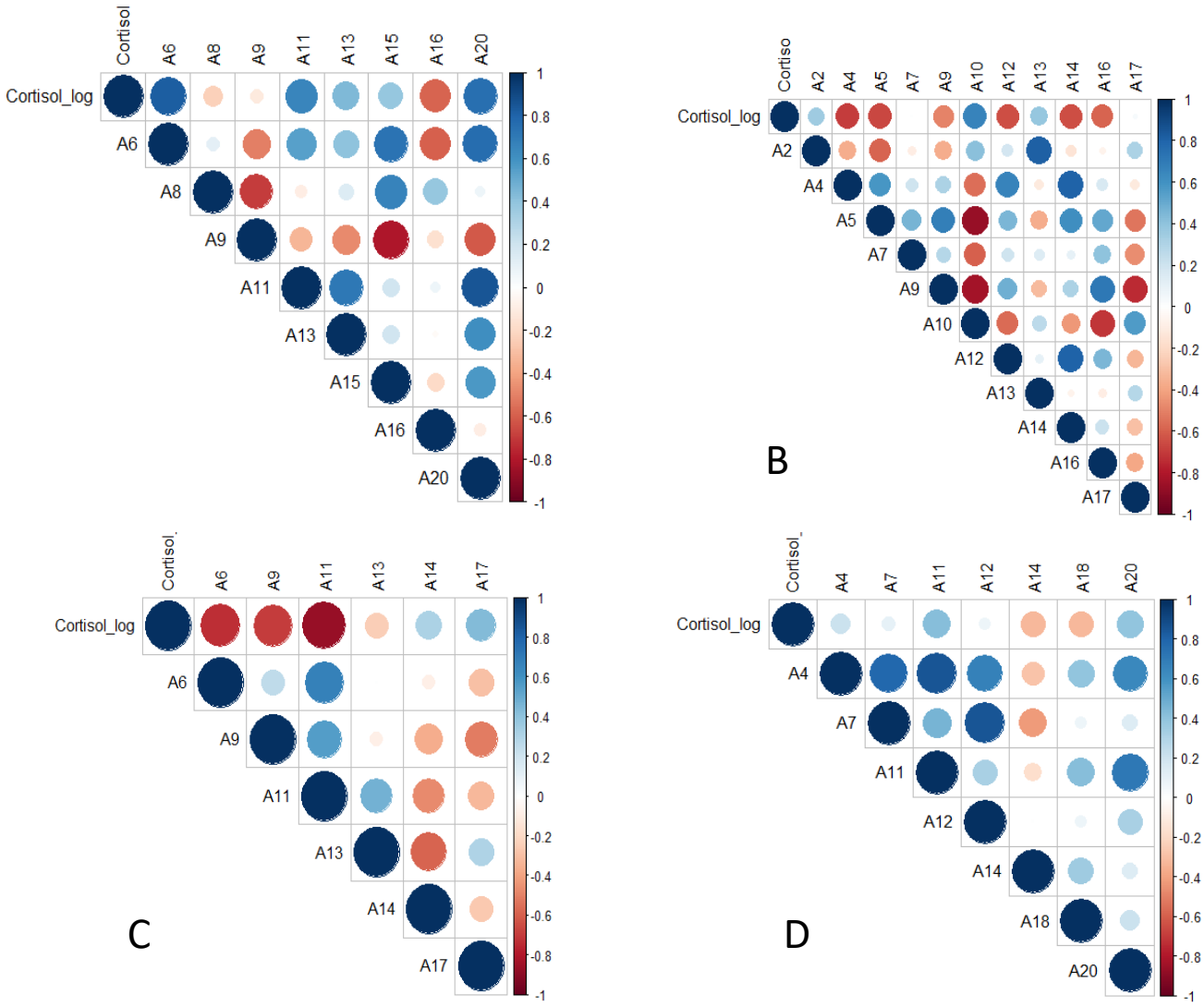


Figure 7.14 Spearman's rank correlation matrices for SRT rumen Female Red (A), Female Blue (B) and Male Red (C) and Male Blue (D) Phyla abundances and cortisol values. The matrix depicts relationships among Phyla, and between Phyla and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two Phyla. Phyla and VIPs obtained from the PLS regression are also shown in **Table 7.14**.

Table 7.14 VIP scores from the PLS analyses of phylum RA by Group1 (Sex * natal Treatment) and log transformed cortisol values, as well as correlation coefficients (R), as presented by the Pearson correlation analysis conducted. Total RA of each phylum in the microbial community and coding numbers are also reported to relate with the correlogram (**Figures 7.14, 7.15)**.

Females									
Recognition					**Isolation**				
Phyla, VIP > 1		**VIP**	**R**	**mean RA**	**Phyla, VIP > 1**		**VIP**	**R**	**mean RA**
A6	*Chloroflexi*	1.89	0.83*	0.007	A10	*Firmicutes*	1.42	0.70	0.50
A20	*WPS-2*	1.70	0.75	0.002	A17	*Synergistetes*	1.35	0.02	0.02
A8	*Elusimicrobia*	1.42	-0.35	0.001	A14	*Proteobacteria*	1.33	-0.64	0.01
A9	*Fibrobacteres*	1.05	-0.09	0.02	A4	*Armatimonadetes*	1.33	-0.65	<0.0001
A13	*Planctomycetes*	1.05	0.47	0.01	A5	*Bacteroidetes*	1.31	-0.65	0.29
A11	*LD1*	1.03	0.48	0.0002	A12	*Lentisphaerae*	1.24	-0.61	0.003
A16	*Spirochaetes*	1.02	-0.50	0.01	A13	*Planctomycetes*	1.12	0.32	0.007
A15	*SR1*	1.00	0.46	0.0002	A2	*Bacteria_* Unidentified	1.08	0.42	0.003
					A7	*Cyanobacteria*	1.06	-0.03	0.009
					A9	*Fibrobacteres*	1.05	-0.33	0.02
					A16	*Spirochaetes*	1.02	-0.47	0.01

Males									
Recognition					**Isolation**				
Phyla, VIP > 1		**VIP**	**R**	**mean RA**	**Phyla, VIP > 1**		**VIP**	**R**	**mean RA**
A6	*Chloroflexi*	1.92	-0.73	0.008	A14	*Proteobacteria*	1.74	-0.83*	0.02
A11	*LD1*	1.70	-0.73	0.0002	A4	*Armatimonadetes*	1.71	0.86*	<0.0001
A13	*Planctomycetes*	11.64	-0.61	0.009	A11	*LD1*	1.45	0.72	<0.0001
A17	*Synergistetes*	1.41	0.53	0.02	A18	*Tenericutes*	1.16	-0.47	0.006
A9	*Fibrobacteres*	1.24	-0.48	0.02	A7	*Cyanobacteria*	1.08	0.54	0.001
A14	*Proteobacteria*	1.01	0.38	0.01	A12	*Lentisphaerae*	1.03	0.40	0.001
					A20	*WPS-2*	1.01	0.51	0.001

7.2.2 Order level correlations with cortisol

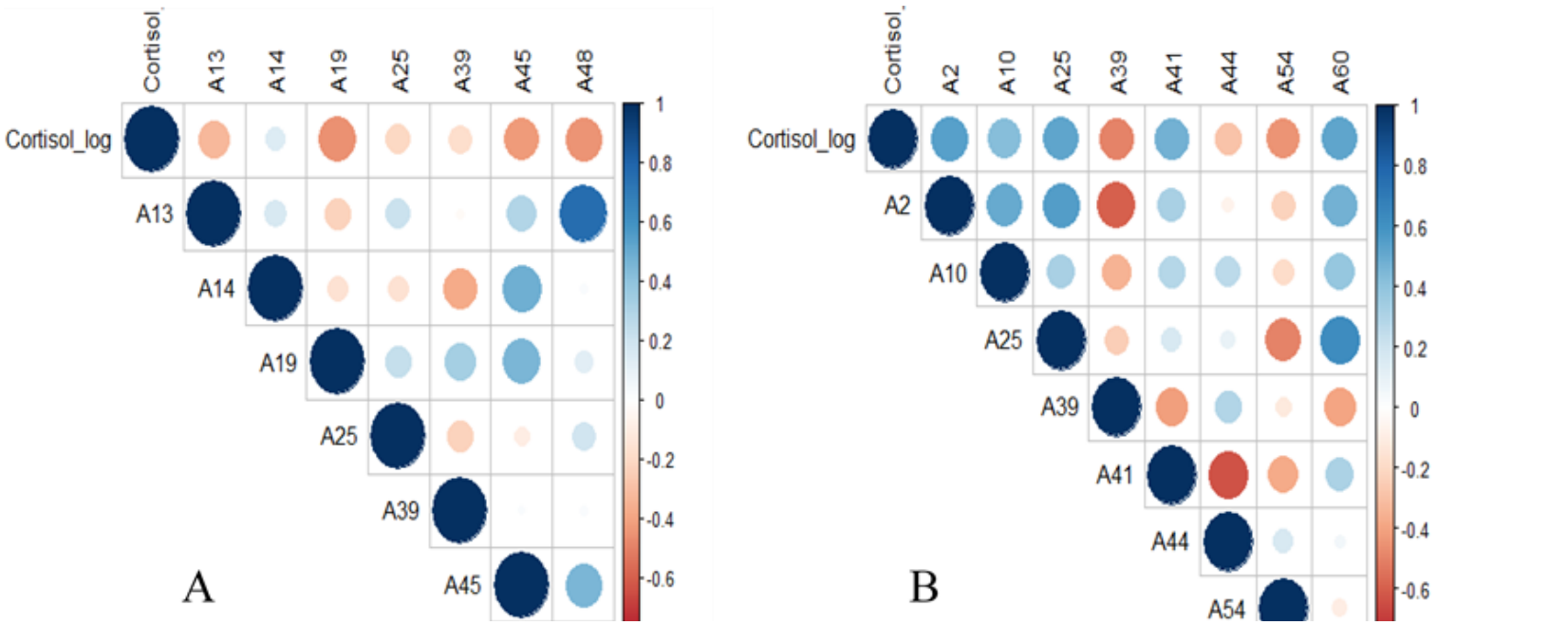


Figure 7.16 Spearman's rank correlation matrices for SRT rumen Male (A), Female (B) Order abundances with a VIP score over 1.5 and cortisol values. The matrix depicts relationships among Orders, and between Orders and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two Orders. Orders and VIPs obtained from the PLS regression are also shown in **Table 7.15.**

Table 7.15 VIP scores from the PLS analyses of order RA by Sex and log transformed cortisol values, as well as correlation coefficients (R), as presented by the Pearson correlation analysis conducted. RA for each order and coding numbers are also reported to relate with the correlogram (**Figures 7.16)**.

Females					**Males**				
Orders, VIP > 1.5		**VIP**	**R**	**mean RA**	**Orders, VIP > 1.5**		**VIP**	**R**	**mean RA**
A60	*WPS-2*	2.21	0.60	0.002	A39	*Spirochaetes; o_M2PT2-76*	2.12	-0.43	<0.001
A10	*Anaerolineales*	2.10	0.57	0.007	A19	*Clostridia;Other*	2.07	-0.43	<0.001
A41	*Enterobacteriales*	1.86	0.51	<0.0001	A48	*Mollicutes;Other*	1.72	-0.42	<0.001
A39	*Spirochaetes; o_M2PT2-76*	1.77	-0.49	<0.0001	A13	*Elusimicrobiales*	1.60	-0.37	<0.001
A54	*Tenericutes;c_RF3; o_ML615J-28*	1.75	-0.52	<0.0001	A14	*Endomicrobia_ Unidentified*	1.55	0.11	<0.001
A44	*Spirochaetes; o_M2PT2-76*	1.71	-0.40	<0.0001	A25	*Pirellulales*	1.55	-0.32	0.007
A25	*Pirellulales*	1.66	0.45	0.008	A45	*Spirochaetales*	1.53	-0.29	0.009
A2	*Thermoplasmata; o_E2*	1.54	0.40	0.001					

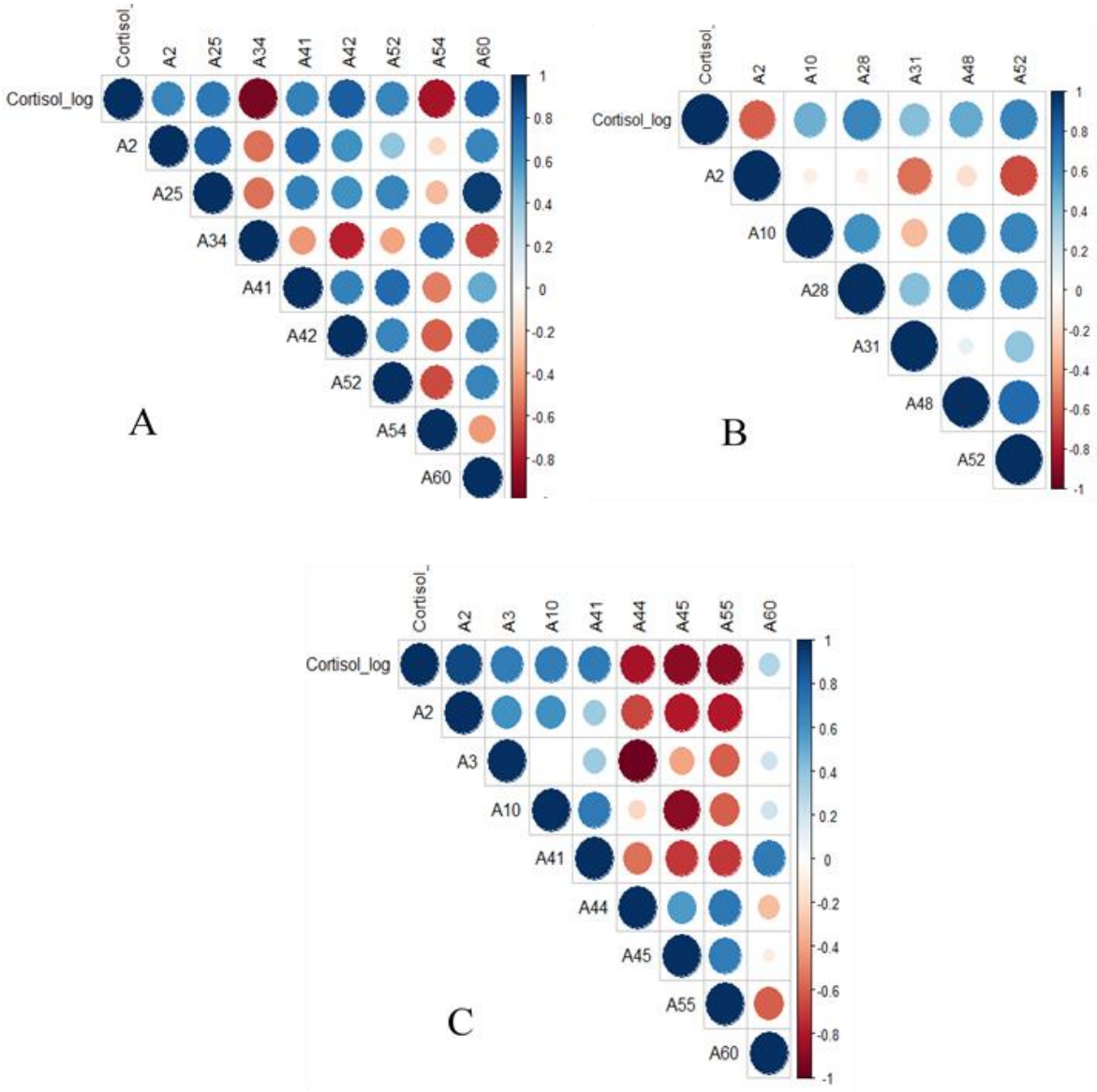


Figure 7.16 Spearman's rank correlation matrices for SRT rumen Female Control (A), Female Alternative (B) and Female Negative (C). order abundances with a VIP score over 1.5 and cortisol values. The matrix depicts relationships among orders, and between orders and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two orders. Orders and VIPs obtained from the PLS regression are also shown in **Table 7.16**.

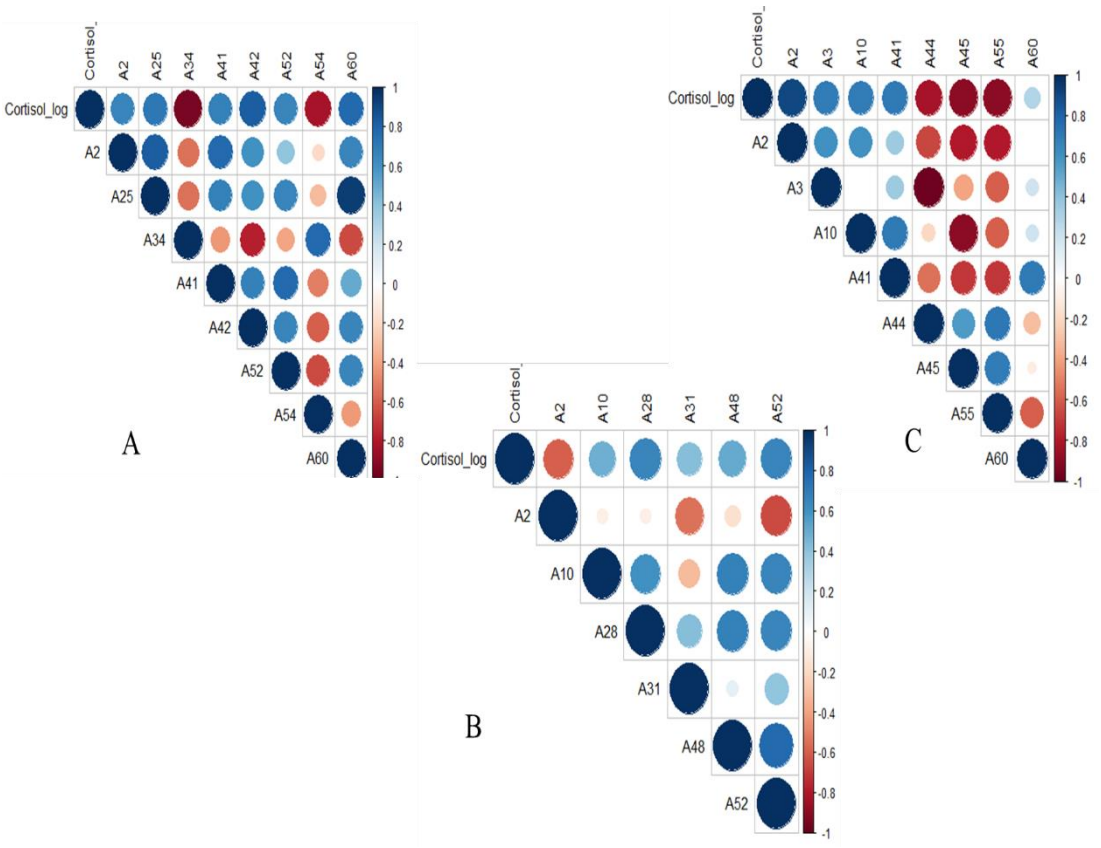


Figure 7.17 Spearman's rank correlation matrices for SRT rumen Male Control (A), Male Alternative (B) and Male Negative (C). Order abundances with a VIP score over 1.5 and cortisol values. The matrix depicts relationships among orders, and between orders and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two orders. Orders and VIPs obtained from the PLS regression are also shown in **Table 7.16**.

Table 7.16 VIP scores from the PLS analyses of order Relative Abundances by Group1 (Sex*Prenatal Treatment) and log transformed cortisol values, as well as correlation coefficients (R), as presented by the Pearson correlation analysis conducted. RA for each order is also reported, as well as coding numbers to relate with the correlogram (**Figures 7.17, 7.18)**.

Females														
Control					**Alternative**					**Negative**				
Orders, VIP > 1.5		**VIP**	**R**	**mean RA**	**Orders, VIP > 1.5**		**VIP**	**R**	**mean RA**	**Orders, VIP > 1.5**		**VIP**	**R**	**mean RA**
A41	*Enterobacteriales*	1.96	0.86	<0.001	A52	*Mycoplasmatales*	3.18	0.92	<0.001	A10	*Anaerolineales*	1.87	0.79	0.008
A52	*Mycoplasmatales*	1.93	0.85	<0.001	A10	*Anaerolineales*	2.78	0.80	0.004	A41	*Enterobacteriales*	1.83	0.90	<0.001
A42	*SR1;Unidentified*	1.90	0.83	<0.001	A2	*Thermoplasmata; o_E2*	2.48	-0.71	0.001	A55	*Verrucomicrobia; Other*	1.81	-0.94	<0.001
A54	*Tenericutes; c_RF3;o_ML615J-28*	1.71	-0.71	<0.001	A28	*Alphaproteo-bacteria; Other*	2.18	0.62	<0.001	A3	*Spirochaetes;o__M2 PT2-76*	1.63	0.69	<0.001
A60	*WPS-2_Unidentified*	1.69	0.74	0.001	A48	*Mollicutes;Other*	2.04	0.59	<0.001	A45	*Spirochaetales*	1.60	-0.71	0.009

A34	*Desulfovibrionales*	1.60	-0.70	0.020	A31	*Rickettsiales*	1.67	0.48	<0.001	A60	*WPS-2_Unidentified*	1.54	0.70	0.004
A2	*Thermoplasmata; o__E2*	1.60	0.70	0.002						A2	*Thermoplasmata;o__E2*	1.52	0.65	0.002
A25	*Pirellulales*	1.50	0.66	0.007						A44	*Spirochaetes; o_M2PT2-76*	1.52	-0.70	<0.001
Males														
Control					**Alternative**					**Negative**				
Orders, VIP > 1.5		**VIP**	**R**	**mean RA**	**Orders, VIP > 1.5**		**VIP**	**R**	**mean RA**	**Orders, VIP > 1.5**		**VIP**	**R**	**mean RA**
A26	*Planctomycetes; c__vadinHA49; Other*	2.05	-0.91	<0.001	A15	*Fibrobacterales*	2.00	0.91	0.022	A39	*Gammaproteo-bacteria;Other*	2.27	-0.94	<0.001
A25	*Pirellulales*	1.96	-0.86	0.01	A39	*Gammaproteo-bacteria;Other*	1.95	0.88	<0.001	A40	*Aeromonadales*	2.10	-0.87	0.018
A16	*Firmicutes; Other;*	1.85	0.82	0.002	A6	*Coriobacteriales*	1.92	0.89	0.003	A21	*Erysipelotrichales*	2.07	-0.86	0.046
A57	*Verruco-5; o_LD1-PB3*	1.67	-0.74	<0.001	A27	*Proteobacteria; Other;*	1.86	-0.83	0.001	A15	*Fibrobacterales*	1.98	-0.78	0.013

A19	*Clostridia;Other*	1.66	-0.72	0.001	A21	*Erysipelotrichales*	1.85	0.84	0.024	A38	*Campylobacterales*	1.95	0.78	<0.001
A55	*Verrucomicrobia; Other*	1.61	0.71	<0.001	A16	*Firmicutes; Other*	1.81	-0.81	0.001	A18	*Lactobacillales*	1.61	0.60	0.019
A22	*LD1;Unidentified*	1.60	-0.71	<0.001	A58	*Verruco-5; o_WCHB1-41*	1.75	-0.78	0.028					
A31	*Rickettsiales*	1.60	-0.66	<0.001	A46	*Synergistales*	1.67	-0.78	0.023					
A4	*Actinomycetales*	1.58	-0.71	<0.001	A3	*Bacteria; Unidentified*	1.54	-0.66	0.003					
					A14	*Endomicrobia; Unidentified*	1.52	0.66	<0.001					
					A20	*Clostridiales*	1.52	-0.68	0.364					

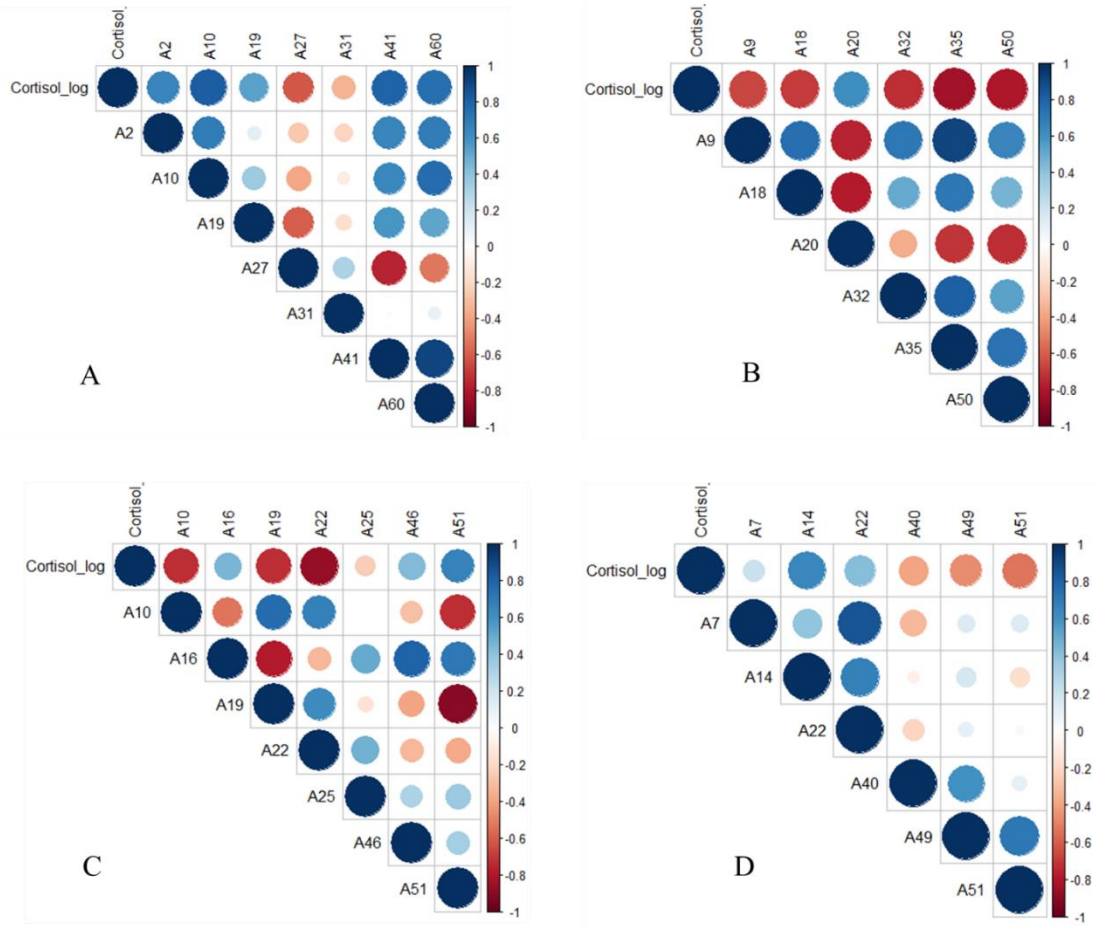


Figure 7.18 Spearman's rank correlation matrices for SRT rumen Female Recognition (A), Female Isolation (B) Male Recognition (C) and Male Isolation (D). Order abundances with a VIP score over 1.5 and cortisol values. The matrix depicts relationships among orders, and between orders and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two orders. Orders and VIPs obtained from the PLS regression are also shown **in Table 7.17**

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Table 7.17 VIP scores from the PLS analyses of order Relative Abundances by Group2 (Sex * Neonatal Treatment) and log transformed cortisol values, as well as correlation coefficients (R), as presented by the Pearson correlation analysis conducted. RA for each order are also reported as well as coding numbers to relate with the correlogram (**Figures 3.8)**.

Females									
Recognition					**Isolation**				
Orders, VIP > 1.5		**VIP**	**R**	**mean RA**	**Orders, VIP > 1.5**		**VIP**	**R**	**mean RA**
A10	*Anaerolineales*	2.11	0.84	0.007	A32	*Burkholderiales*	1.87	-0.81	<0.001
A60	*WPS-2; Unidentified*	1.94	0.75	0.002	A35	*Deltaproteobacteria; o_GMD14H09*	1.85	-0.80	0.002
A41	*Enterobacteriales*	1.81	0.72	<0.001	A50	*Acholeplasmatales*	1.73	-0.75	<0.001
A19	*Clostridia;Other*	1.73	0.67	<0.001	A20	*Clostridiales*	1.62	0.69	0.489
A27	*Proteobacteria;Other*	1.65	-0.72	<0.001	A18	*Lactobacillales*	1.61	-0.69	<0.001
A31	*Rickettsiales*	1.57	-0.57	<0.001	A9	*Bacteroidales*	1.52	-0.65	0.291
A2	*Thermoplasmata;o_E2*	1.55	0.60	0.001					
Males									
Recognition					**Isolation**				
Orders, VIP > 1.5		**VIP**	**R**	**mean RA**	**Orders, VIP > 1.5**		**VIP**	**R**	**mean RA**

A19	*Clostridiales*	2.45	-0.82	0.392	A7	*Armatimonadetes; c_SJA-176;o_RB046*	1.99	0.86	<0.001
A10	*Anaerolineales*	2.16	-0.73	0.008	A40	*Aeromonadales*	1.91	-0.83	0.018
A16	*Firmicutes;Other*	1.96	0.65	0.002	A51	*Anaeroplasmatales*	1.76	-0.72	0.002
A22	*LD1;Unidentified*	1.91	-0.73	<0.001	A22	*LD1_Unidentified*	1.68	0.72	<0.001
A25	*Pirellulales*	1.83	-0.61	0.009	A49	*Mollicutes; Unidentified*	1.52	-0.62	<0.001
A51	*Anaeroplasmatales*	1.55	0.52	0.003	A14	*Endomicrobia_ Unidentified*	1.52	0.53	<0.001
A46	*Synergistales*	1.54	0.53	0.018					

7.2.3 Genus level- correlations with cortisol

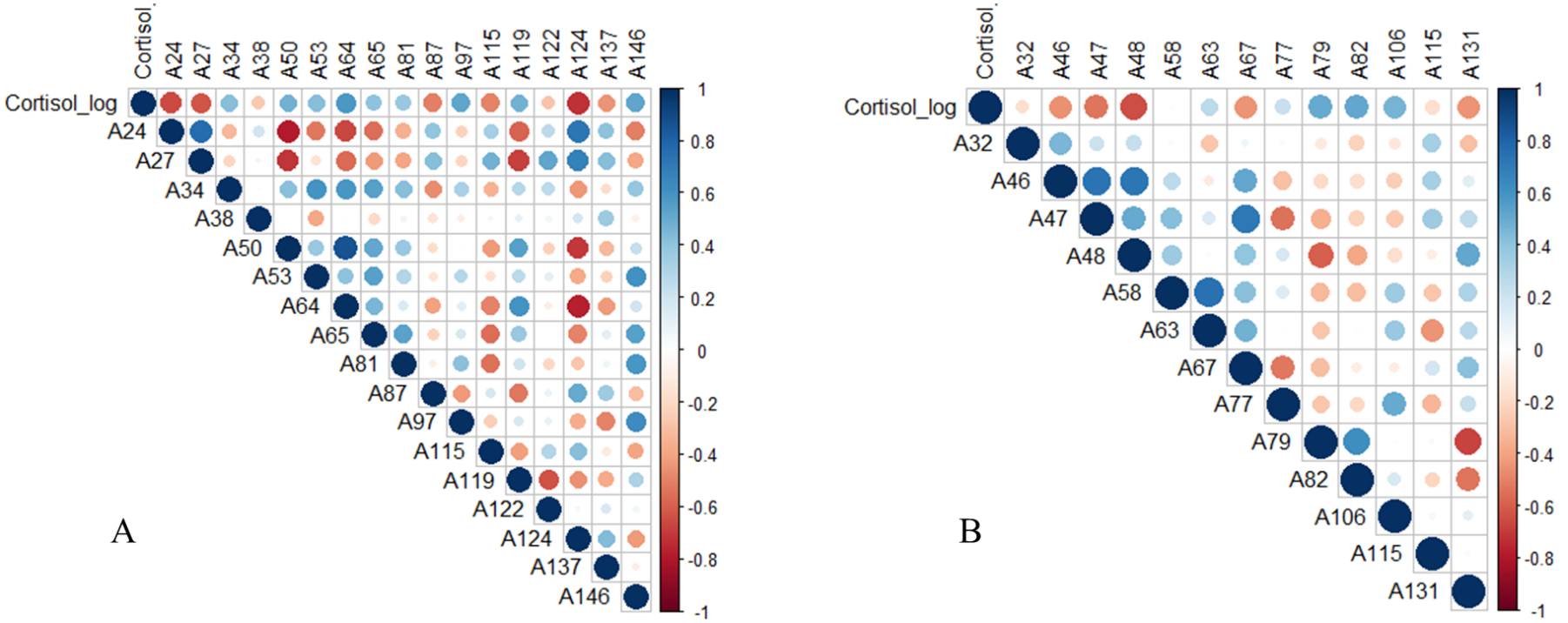

Figure 7.19 Spearman's rank correlation matrices for SRT rumen Female (A) and Male (B) genus abundances with a VIP score over 1.5 and cortisol values. The matrix depicts relationships among genera, and between genera and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two genera. Genera and VIPs obtained from the PLS regression are also shown **in Table 7.18**

Table 7.18 VIP scores from the PLS analyses of genera Relative Abundances by Sex and log transformed cortisol values, as well as correlation coefficients (R), as presented by the Pearson correlation analysis conducted. RA for each genera are also reported, as well as coding numbers to relate with the correlogram (**Figure 7.20)**.

Females					**Males**				
Genera VIP>1.5		**VIP**	**R**	**mean RA**	**Genera VIP>1.51.50**		**VIP**	**R**	**mean RA**
A24	*Paludibacter*	2.14	-0.63	0.002	A82	*Selenomonas*	2.08	0.53	0.005
A146	*WPS-2_Unidentified*	2.10	0.60	0.002	A47	*Clostridiales;Other*	1.95	-0.49	0.014
A64	*Moryella*	2.00	0.58	0.003	A63	*Defluviitalea*	1.94	0.28	<0.001
A87	*Erysipelotrichaceae; Other*	1.99	-0.52	<0.001	A106	*Brachymonas*	1.93	0.41	<0.001
A34	*Anaerolinaceae;SHD -231*	1.97	0.57	0.008	A115	*Gammaproteobacteria ;Other*	1.90	-0.43	<0.001
A50	*Clostridiaceae; 02d06*	1.85	0.52	0.014	A79	*Anaerovibrio*	1.82	0.49	0.001

A119	*Escherichia*	1.75	0.51	<0.001	A46	*Clostridia;Other*	1.76	-0.43	<0.001
A137	*Tenericutes;c_RF3;o_ML615J-28; Unidentified*	1.72	-0.52	<0.001	A48	*Clostridiales_ Unidentified*	1.72	-0.42	0.006
A27	*Bacteroidales;f_RF16_Unidentified*	1.71	-0.58	0.013	A32	*[Paraprevotellaceae]; YRC22*	1.69	-0.41	0.004
A38	*Elusimicrobium*	1.69	-0.34	<0.001	A77	*Veillonellaceae;Other*	1.68	0.35	<0.001
A65	*Pseudobutyrivibrio*	1.69	0.46	0.002	A58	*Anaerostipes*	1.65	0.13	0.001
A122	*Spirochaetes;o_M2PT2-76_Unidentified*	1.68	-0.40	<0.001	A131	*Mollicutes;Other*	1.58	-0.42	<0.001
A97	*Pirellulaceae_ Unidentified*	1.67	0.45	0.008	A67	*Syntrophococcus*	1.56	-0.35	<0.001
A115	*Gammaproteobacteria;Other*	1.64	-0.49	<0.001					

A124	*Spirochaetaceae_ Unidentified*	1.64	-0.48	0.001					
A53	*Dehalobacterium*	1.64	0.54	<0.001					
A81	*Schwartzia*	1.50	0.44	<0.001					

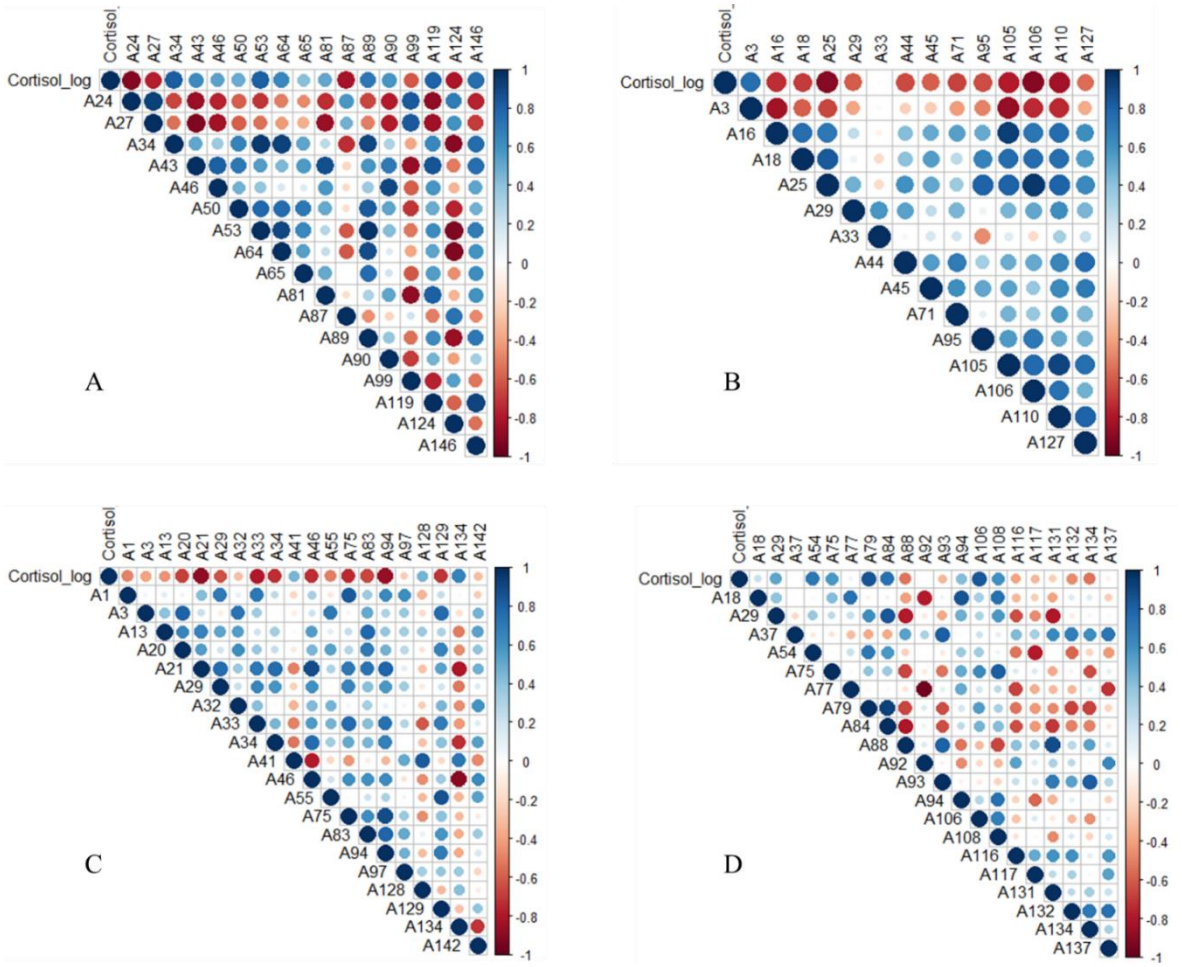

Figure 7.20 Spearman's rank correlation matrices for SRT rumen Female Recognition (A), Female Isolation (B) Male Recognition (C) and Male Isolation (D). genus abundances with a VIP score over 1.5 and cortisol values. The matrix depicts relationships among genera, and between genera and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two genera. Genera and VIPs obtained from the PLS regression are also shown in **Table 7.19**

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Table 7.19 VIP scores from the PLS analyses of genera Relative Abundances by Group2 (Sex Neonatal* Treatment) and log transformed cortisol values, as well as correlation coefficients (R), as presented by the Pearson correlation analysis conducted. RA for each genera are also reported, as well as coding numbers to relate with the correlogram (**Figure 7.21)**.

Females									
Recognition					**Isolation**				
Genera VIP>1.5		**VIP**	**R**	**mean RA**	**Genera VIP>1.5**		**VIP**	**R**	**mean RA**
A24	*Paludibacter*	2.17	-0.93	0.002	A25	*Prevotellaceae_Unidentified*	2.18	-0.92	0.012
A34	*Anaerolinaceae; SHD-231*	2.10	0.84	0.007	A106	*Brachymonas*	2.11	-0.88	<0.001
A146	*WPS-2_Unidentified*	1.91	0.75	0.002	A71	*Anaerofilum*	1.89	-0.76	<0.001
A87	*Erysipelotrichaceae;Other*	1.88	-0.69	<0.001	A110	*Deltaproteobacteria;o_GMD14H09_ Unidentified*	1.89	-0.80	<0.001
A64	*Moryella*	1.85	0.75	0.002	A105	*Sutterella*	1.88	-0.80	<0.001
A43	*Staphylococcus*	1.85	0.76	<0.001	A133	*Acholeplasmatales_Unidentified*	1.77	0.13	<0.001
A119	*Escherichia*	1.80	0.72	<0.001	A16	*Adlercreutzia*	1.65	-0.70	0.001
A50	*Clostridiaceae; 02d06*	1.78	0.70	0.013	A44	*Lactobacillus*	1.62	-0.69	<0.001
A46	*Clostridia;Other*	1.71	0.67	<0.001	A45	*Streptococcus*	1.62	-0.69	<0.001

A53	*Dehalobacterium*	1.66	0.76	<0.001	A127	*Pyramidobacter*	1.55	-0.65	0.001
A65	*Pseudobutyrivibrio*	1.66	0.65	0.002	A29	*[Paraprevotellaceae];Other*	1.54	-0.64	<0.001
A27	*Bacteroidales;f_RF16_Unidentified*	1.65	-0.84	0.015	A3	*Methanosphaera*	1.53	0.65	0.001
A99	*Proteobacteria;Other*	1.64	-0.72	<0.001	A18	*p_Armatimonadetes;c_SJA-176;o_RB046_Unidentified*	1.52	-0.65	<0.001
A89	*Bulleidia*	1.62	0.64	0.002	A95	*Victivallaceae; Unidentified*	1.51	-0.63	0.003
A124	*Spirochaetaceae_ Unidentified*	1.58	-0.72	0.001					
A90	*Erysipelotrichaceae_L7A_E11*	1.52	0.62	<0.001					
A81	*Schwartzia*	1.50	0.62	<0.001					
Males									
Recognition					**Isolation**				
Genera VIP>1.5		**VIP**	**R**	**mean RA**	**Genera VIP>1.5**		**VIP**	**R**	**mean RA**
A20	*Bacteroidales;Other*	2.76	-0.94	0.002	A79	*Anaerovibrio*	2.26	0.88	0.001
A46	*Clostridia;Other*	2.38	-0.82	0.001	A88	*Erysipelotrichaceae_Unidentified*	2.21	-0.88	<0.001

A33	*[Prevotella]*	2.23	-0.76	<0.001	A18	*p_Armatimonadetes;c_SJA-176;o_RB046_Unidentified*	2.15	0.86	<0.001
A34	*Anaerolinaceae;SHD-231*	2.11	-0.73	0.008	A116	*Succinivibrionaceae_Unidentified*	2.05	-0.82	0.017
A32	*[Paraprevotellaceae];YRC22*	2.09	-0.72	0.005	A75	*Papillibacter*	2.04	0.81	<0.001
A13	*Coriobacteriaceae;Other*	2.07	-0.72	<0.001	A134	*Anaeroplasma*	1.83	-0.72	0.002
A129	*Synergistaceae_Unidentified*	2.02	-0.73	<0.001	A106	*Brachymonas*	1.83	0.73	<0.001
A41	*Firmicutes;Other*	1.94	0.65	0.001	A93	*Erysipelotrichaceae;p-75-a5*	1.81	-0.72	0.001
A94	*LD1_ Unidentified*	1.86	-0.73	<0.001	A94	*LD1_ Unidentified*	1.80	0.72	<0.001
A97	*Pirellulaceae_ Unidentified*	1.80	-0.61	0.009	A108	*Desulfovibrionaceae_Unidentified*	1.79	0.71	<0.001
A21	*Bacteroidales_ Unidentified*	1.78	-0.76	0.031	A84	*[Mogibacteriaceae]_Unidentified*	1.77	0.69	0.007
A55	*Eubacterium*	1.74	-0.58	<0.001	A54	*Anaerofustis*	1.61	0.57	<0.001
A1	*Methanobacteriaceae_Unindentified*	1.72	-0.60	<0.001	A117	*Ruminobacter*	1.60	-0.62	0.001
A75	*Papillibacter*	1.66	-0.59	0.001	A131	*Mollicutes;Other*	1.60	-0.64	<0.001
A142	*WCHB1-41_Unidentified*	1.64	-0.57	<0.001	A137	*Tenericutes;c_RF3;o_ML615J-28; Unidentified*	1.59	-0.64	<0.001
A83	*Succiniclasticum*	1.60	-0.62	0.025	A29	*[Paraprevotellaceae];Other*	1.59	0.63	<0.001

A3	*Methanosphaera*	1.60	-0.55	0.001	A132	*Mollicutes_Unidentified*	1.58	-0.62	<0.001
A29	*[Paraprevotellaceae];Other*	1.59	-0.55	<0.001	A37	*Elusimicrobiaceae_Unidentified*	1.56	-0.62	<0.001
A128	*Dethiosulfovibrionaceae;TG5*	1.59	0.55	0.017	A77	*Veillonellaceae;Other*	1.55	0.61	<0.001
A134	*Anaeroplasma*	1.52	0.52	0.003	A92	*Sharpea*	1.51	-0.59	0.031

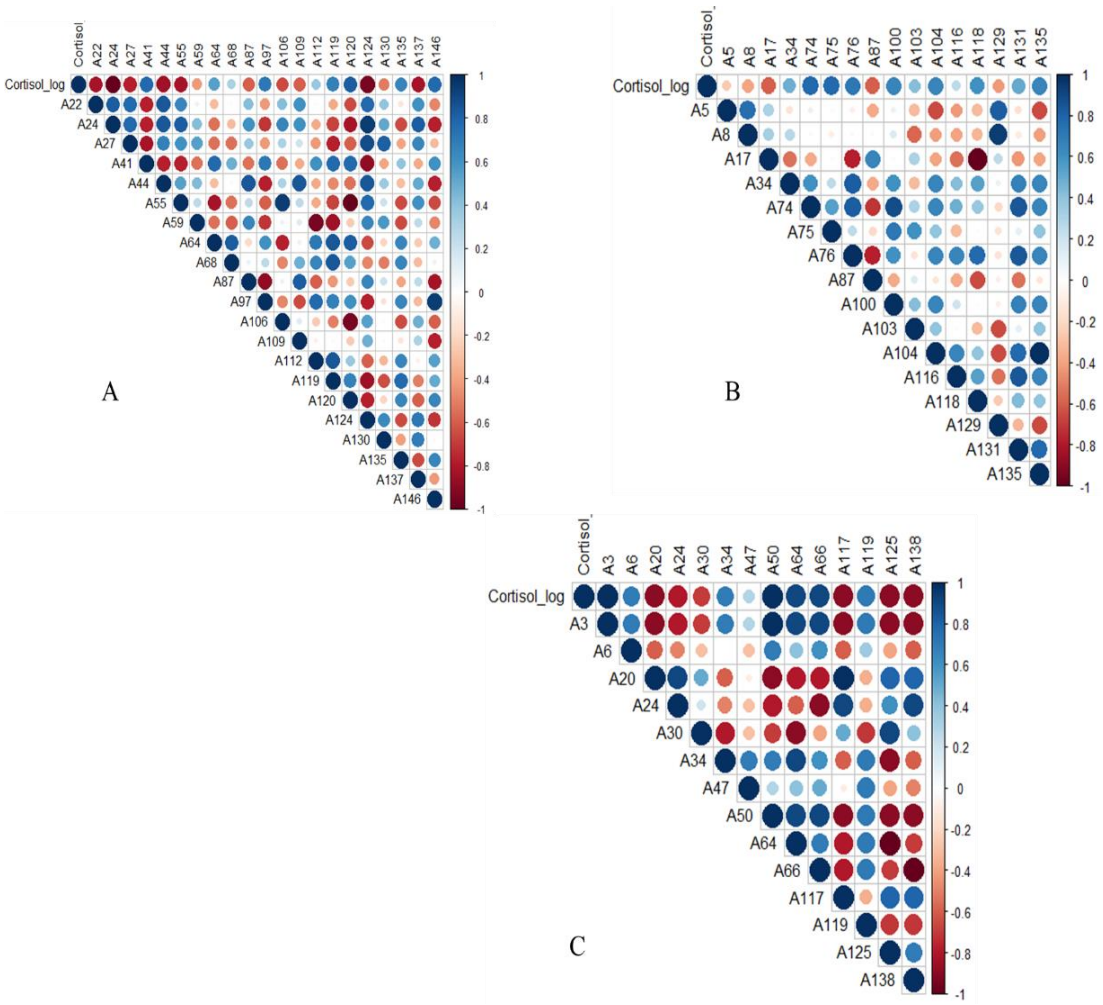

Figure 7.21 Spearman's rank correlation matrices for SRT rumen Female Control (A), Female Alternative (B) and Female Negative (C). genus abundances with a VIP score over 1.5 and cortisol values. The matrix depicts relationships among genera, and between genera and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two genera. Genera and VIPs obtained from the PLS regression are also shown in **Table 7.20.**

Table 7.20 VIP scores from the PLS analyses of genera Relative Abundances by Group1 (Females* Prenatal Treatment) and log transformed cortisol values, as well as correlation coefficients (R), as presented by the Pearson correlation analysis conducted. RA for each genera are also reported, as well as coding numbers to relate with the correlogram (**Figure 7.22)**.

Females														
Control					**Alternative**					**Negative**				
Genera VIP>1.5		**VIP**	**R**	**mean RA**	**Genera VIP>1.5**		**VIP**	**R**	**mean RA**	**Genera VIP>1.5**		**VIP**	**R**	**mean RA**
A24	*Paludibacter*	2.11	-0.90	0.002	A104	*Zea*	2.98	0.92	<0.001	A50	*Clostridiaceae ; 02d06*	1.94	0.89	0.016
A119	*Escherichia*	2.02	0.86	<0.001	A135	*Mycoplasmataceae Unidentified*	2.98	0.92	<0.001	A66	*Shuttleworthia*	1.85	0.88	<0.001
A135	*Mycoplasmataceae Unidentified*	2.01	0.85	<0.001	A34	*Anaerolinaceae; SHD-231*	2.60	0.80	0.004	A30	*[Paraprevotellaceae]_ Unidentified*	1.83	-0.84	0.003
A120	*SR1_ Unidentified*	1.96	0.83	<0.001	A75	*Papillibacter*	2.17	0.65	0.001	A34	*Anaerolinaceae; SHD-231*	1.78	0.79	0.008

A124	*Spirochaetaceae_ Unidentified*	1.90	-0.81	0.001	A100	*Alphaproteobacteria;Other*	2.14	0.62	<0.001	A119	*Escherichia*	1.78	0.90	<0.001
A27	*Bacteroidales;f_RF16_Unidentified*	1.83	-0.78	0.012	A116	*Succinivibrionaceae _Unidentified*	2.09	0.65	0.001	A47	*Clostridiales; Other*	1.77	0.78	0.016
A55	*Eubacterium*	1.81	-0.77	<0.001	A74	*Oscillospira*	2.08	0.64	0.001	A138	*Verrucomicrobia;Other*	1.73	0.75	<0.001
A81	*Schwartzia*	1.76	0.75	<0.001	A76	*Ruminococcus*	2.05	0.64	0.055	A64	*Moryella*	1.73	0.83	0.003
A5	*[Methanomassiliicoccaceae];vadinCA11*	1.74	0.75	0.001	A131	*Mollicutes; Othe*	1.89	0.59	<0.001	A3	*Methanosphaera*	1.68	0.82	0.001
A146	*WPS-2_ Unidentified*	1.74	0.74	0.001	A129	*Synergistaceae _Unidentified*	1.84	-0.58	<0.001	A117	*Ruminobacter*	1.64	-0.79	0.001
A22	*Bacteroidales; _BS11_ Unidentified*	1.72	-0.73	<0.001	A5	*[Methanomassiliicoccaceae];vadinCA11*	1.82	-0.57	0.001	A24	*Paludibacter*	1.59	-0.73	0.001

A44	*Lactobacillus*	1.69	-0.71	<0.001	A87	*Erysipelotrichaceae;Other*	1.61	-0.50	<0.001	A6	*Bacteria_Other*	1.57	0.69	0.004
A137	*Tenericutes;c_RF3;o_ML615J-28; Unidentified*	1.69	-0.71	<0.001	A118	*Succinivibrio*	1.58	0.48	<0.001	A125	*Treponema*	1.54	-0.71	0.009
A112	*o_Myxococcales; f_319-6G20_ Unidentified*	1.66	0.70	<0.001	A103	*Rickettsiales_ Unidentified*	1.57	0.47	<0.001	A20	*Bacteroidales; Other*	1.51	-0.67	0.002
A59	*Blautia*	1.65	-0.70	0.002	A8	*Microbacteriaceae;Other*	1.56	-0.48	<0.001					
A109	*Desulfovibrio*	1.64	-0.70	0.002	A17	*Enterococcus*	1.50	-0.44	<0.001					
A68	*Clostridium*	1.62	0.69	0.001										
A64	*Moryella*	1.61	0.68	0.003										
A87	*Erysipelotrichaceae;Other*	1.57	-0.67	<0.001										

A97	*Pirellulaceae_ Unidentified*	1.54	0.66	0.007										
A106	*Brachymonas*	1.54	-0.65	<0.001										
A41	*Firmicutes;Other*	1.52	0.64	0.002										
A130	*Tenericutes;Other*	1.50	-0.64	<0.001										

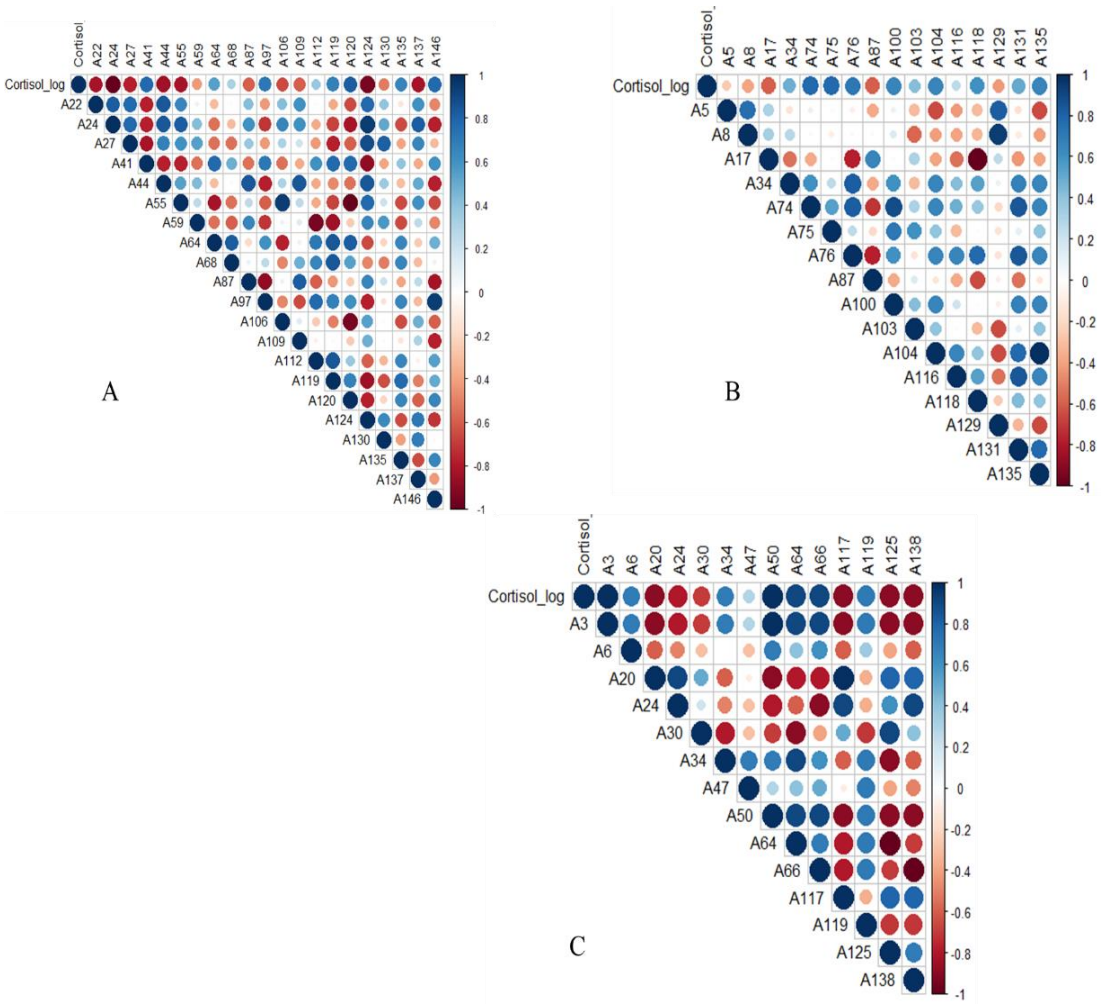

Figure 7.22 Spearman's rank correlation matrices for SRT rumen Female Control (A), Female Alternative (B) and Female Negative (C). genus abundances with a VIP score over 1.5 and cortisol values. The matrix depicts relationships among genera, and between genera and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two genera. Genera and VIPs obtained from the PLS regression are also shown in **Table 7.21**

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Table 7.21 VIP scores from the PLS analyses of genera Relative Abundances by Group1 (Females* Prenatal Treatment) and log transformed cortisol values, as well as correlation coefficients (R), as presented by the Pearson correlation analysis conducted. RA for each genera are also reported, as well as coding numbers to relate with the correlogram (**Figure 7.23)**.

Females														
Control					**Alternative**					**Negative**				
Genera VIP>1.5		**VIP**	**R**	**mean RA**	**Genera VIP>1.5**		**VIP**	**R**	**mean RA**	**Genera VIP>1.5**		**VIP**	**R**	**mean RA**
A24	*Paludibacter*	2.11	-0.90	0.002	A104	*Zea*	2.98	0.92	<0.001	A50	*Clostridiaceae ; 02d06*	1.94	0.89	0.016
A119	*Escherichia*	2.02	0.86	<0.001	A135	*Mycoplasmataceae Unidentified*	2.98	0.92	<0.001	A66	*Shuttleworthia*	1.85	0.88	<0.001
A135	*Mycoplasmataceae Unidentified*	2.01	0.85	<0.001	A34	*Anaerolinacea e; SHD-231*	2.60	0.80	0.004	A30	*[Paraprevotell aceae]_ Unidentified*	1.83	-0.84	0.003
A120	*SR1_ Unidentified*	1.96	0.83	<0.001	A75	*Papillibacter*	2.17	0.65	0.001	A34	*Anaerolinacea e; SHD-231*	1.78	0.79	0.008

A124	*Spirochaetaceae_ Unidentified*	1.90	-0.81	0.001	A100	*Alphaproteobacteria;Other*	2.14	0.62	<0.001	A119	*Escherichia*	1.78	0.90	<0.001
A27	*Bacteroidales;f_RF16_Unidentified*	1.83	-0.78	0.012	A116	*Succinivibrionaceae _Unidentified*	2.09	0.65	0.001	A47	*Clostridiales; Other*	1.77	0.78	0.016
A55	*Eubacterium*	1.81	-0.77	<0.001	A74	*Oscillospira*	2.08	0.64	0.001	A138	*Verrucomicrobia;Other*	1.73	0.75	<0.001
A81	*Schwartzia*	1.76	0.75	<0.001	A76	*Ruminococcus*	2.05	0.64	0.055	A64	*Moryella*	1.73	0.83	0.003
A5	*[Methanomassiliicoccaceae];vadinCA11*	1.74	0.75	0.001	A131	*Mollicutes; Othe*	1.89	0.59	<0.001	A3	*Methanosphaera*	1.68	0.82	0.001
A146	*WPS-2_ Unidentified*	1.74	0.74	0.001	A129	*Synergistaceae _Unidentified*	1.84	-0.58	<0.001	A117	*Ruminobacter*	1.64	-0.79	0.001
A22	*Bacteroidales; _BS11_ Unidentified*	1.72	-0.73	<0.001	A5	*[Methanomassiliicoccaceae];vadinCA11*	1.82	-0.57	0.001	A24	*Paludibacter*	1.59	-0.73	0.001

A44	*Lactobacillus*	1.69	-0.71	<0.001	A87	*Erysipelotrichaceae;Other*	1.61	-0.50	<0.001	A6	*Bacteria_Other*	1.57	0.69	0.004
A137	*Tenericutes;c_RF3;o_ML615J-28; Unidentified*	1.69	-0.71	<0.001	A118	*Succinivibrio*	1.58	0.48	<0.001	A125	*Treponema*	1.54	-0.71	0.009
A112	*o_Myxococcales; f_319-6G20_ Unidentified*	1.66	0.70	<0.001	A103	*Rickettsiales_ Unidentified*	1.57	0.47	<0.001	A20	*Bacteroidales; Other*	1.51	-0.67	0.002
A59	*Blautia*	1.65	-0.70	0.002	A8	*Microbacteriaceae;Other*	1.56	-0.48	<0.001					
A109	*Desulfovibrio*	1.64	-0.70	0.002	A17	*Enterococcus*	1.50	-0.44	<0.001					
A68	*Clostridium*	1.62	0.69	0.001										
A64	*Moryella*	1.61	0.68	0.003										
A87	*Erysipelotrichaceae;Other*	1.57	-0.67	<0.001										

A97	*Pirellulaceae_ Unidentified*	1.54	0.66	0.007										
A106	*Brachymonas*	1.54	-0.65	<0.001										
A41	*Firmicutes;Other*	1.52	0.64	0.002										
A130	*Tenericutes;Other*	1.50	-0.64	<0.001										

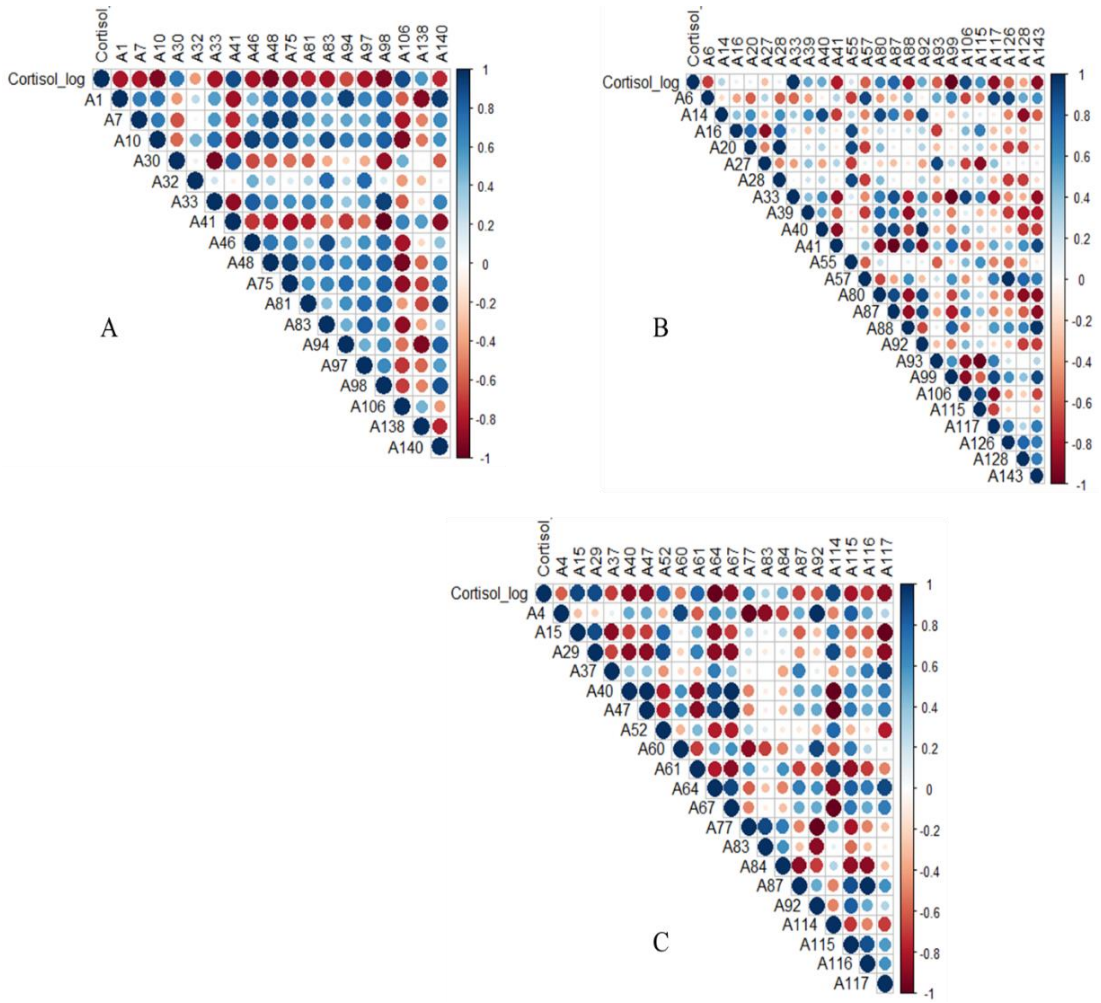

Figure 7.23 Spearman's rank correlation matrices for SRT rumen Male Control (A), Male Alternative (B) and Male Negative (C). genus abundances with a VIP score over 1.5 and cortisol values. The matrix depicts relationships among genera, and between genera and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two genera. Genera and VIPs obtained from the PLS regression are also shown in **Table 7.22.**

Table 7.22 VIP scores from the PLS analyses of genera Relative Abundances by Group1 (Males *Prenatal Treatment) and log transformed cortisol values, as well as correlation coefficients (R), as presented by the Pearson correlation analysis conducted. RA for each genus are also reported, as well as coding numbers to relate with the correlogram (**Figure 7.24**).

Males														
Control					**Alternative**					**Negative**				
Genera VIP>1.5		**VIP**	**R**	**mean RA**	**Genera VIP>1.5**		**VIP**	**R**	**mean RA**	**Genera VIP>1.5**		**VIP**	**R**	**mean RA**
A98	*c_vadinHA49; Other*	2.05	-0.91	<0.001	A55	*Eubacterium*	2.14	0.89	<0.001	A64	*Moryella*	2.27	-0.97	0.002
A81	*Schwartzia*	2.00	-0.89	<0.001	A40	*Fibrobacter*	2.14	0.91	0.022	A115	*Gammaproteobacteria;Other*	2.19	-0.94	<0.001
A97	*Pirellulaceae_ Unidentified*	1.94	-0.86	0.010	A93	*Erysipelotrichaceae;p-75-a5*	2.09	-0.86	0.001	A117	*Ruminobacter*	2.06	-0.88	0.001
A33	*[Prevotella]*	1.93	-0.86	<0.001	A28	*Bacteroidales _S24-7_ Unidentified*	2.08	0.88	0.006	A116	*Succinivibrionaceae_ Unidentified*	2.03	-0.86	0.017
A41	*Firmicutes;Other*	1.85	0.82	0.002	A14	*Coriobacteriaceae_ Unidentified*	2.07	0.91	0.001	A87	*Erysipelotrichaceae;Other*	1.89	-0.80	<0.001
A32	*[Paraprevotellaceae];*	1.85	-0.81	0.005	A115	*Gammaproteobacteria;Other*	2.05	0.88	<0.001	A47	*Clostridiales; Other*	1.88	-0.80	0.012

	YRC22													
A48	*Clostridiales_ Unidentified*	1.73	-0.77	0.070	A143	*Verruco-5;o__WCHB1-41;f__RFP12_ Unidentified*	2.05	-0.85	0.021	A92	*Sharpea*	1.88	-0.80	0.036
A10	*Nocardiopsaceae _ Unidentified*	1.72	-0.77	<0.001	A99	*Proteobacteria;Other*	1.96	-0.83	0.001	A83	*Succiniclasticum*	1.84	0.78	0.027
A1	*Methanobacteriaceae_Unindentified*	1.72	-0.76	0.001	A41	*Firmicutes; Other*	1.96	-0.81	0.001	A114	*Campylobacter*	1.84	0.78	<0.001
A30	*[Paraprevotellaceae]_ Unidentified*	1.68	0.75	0.005	A33	*[Prevotella]*	1.96	0.83	<0.001	A40	*Fibrobacter*	1.82	-0.78	0.013
A140	*Verruco-5;o__LD1-PB3_ Unidentified*	1.66	-0.74	<0.001	A92	*Sharpea*	1.87	0.83	0.016	A67	*Syntrophococcus*	1.81	-0.77	<0.001
A75	*Papillibacter*	1.65	-0.73	0.001	A128	*Dethiosulfovibrionaceae;TG 5*	1.82	-0.79	0.021	A37	*Elusimicrobiaceae_Unidentified*	1.80	-0.76	<0.001
A7	*Actinopolysporaceae _ Unidentified*	1.65	-0.73	<0.001	A20	*Bacteroidales; Other*	1.79	0.75	0.001	A4	*[Methanomassiliicoccaceae] _Unidentified;*	1.79	-0.76	<0.001

A83	*Succiniclasticum*	1.63	-0.72	0.025	A80	*Mitsuokella*	1.76	0.77	<0.001	A29	*[Paraprevotellaceae];Other*	1.79	0.76	<0.001
A46	*Clostridia;Other*	1.63	-0.72	0.001	A126	*Dethiosulfovibrionaceae;Other*	1.76	-0.73	0.001	A60	*Butyrivibrio*	1.76	-0.75	0.021
A106	*Brachymonas*	1.63	0.73	<0.001	A87	*Erysipelotrichaceae;Other*	1.75	0.73	<0.001	A15	*Adlercreutzia*	1.76	0.75	<0.001
A94	*LD1_ Unidentified*	1.61	-0.71	<0.001	A57	*Lachnospiraceae_ Unidentified*	1.72	-0.72	0.026	A61	*Clostridium*	1.67	0.71	0.008
A138	*Verrucomicrobia; Other*	1.60	0.71	<0.001	A106	*Brachymonas*	1.67	0.77	<0.001	A52	*Dehalobacteriaceae_Unidentified*	1.59	0.68	<0.001
					A88	*Erysipelotrichaceae_ Unidentified*	1.65	-0.76	<0.001	A84	*[Mogibacteriaceae]_Unidentified*	1.52	0.65	0.009
					A16	*Adlercreutzia*	1.64	0.68	<0.001	A77	*Veillonellaceae; Other*	1.52	0.65	<0.001
					A39	*Endomicrobia _Unidentified*	1.62	0.66	<0.001					
					A6	*Bacteria_Other*	1.59	-0.66	0.003					

					A117	*Ruminobacter*	1.56	-0.64	0.001					
					A27	*Bacteroidales; f_RF16_Unidentified*	1.51	-0.63	0.016					

7.3 Appendix Chapter 4

7.3.1 Stressor descriptions

7.3.1.1 Wet and soiled bedding

Wet bedding (or wet litter) has been used as a stressor in most chronic unpredictable mild stress trials as an easy means of creating an uncomfortable environment for the tested animals (Fregonesi *et al.*, 2007; Frisbee *et al.*, 2015; DeVallance *et al.*, 2017; O'Connor *et al.*, 2019). Additionally, soiled bedding represents an uncomfortable lying surface due to humidity and ammonia release (Misselbrook and Powell, 2005). It is an easy stressor to replicate but also represents an event that would potentially occur in farming conditions.

Animals were exposed to the wet bedding pens (1.50 m x 4.50 m) once a week/ pen, on different days each week and for variable durations ranging from 1.5 h to 3.5 h. Pens 5 and 6 were placed on the same wet bedding test-pen on the right side of Room 2, whereas Pens 7 and 8 were always placed in the pen in the far left of the room. The bedding consisted of matter that had previously been cleaned out of the home pens (wood chips and hay) and was moistened to a point where water was visible, making it an uncomfortable surface for the animals to lie on. This was cleared out the day after each test was performed by personnel in green. No food or water was available in the test pens.

7.3.1.2 Restricted access to concentrate

Habituating the animals to a feeding schedule meant they became accustomed to the auditory and visual cues proceeding the distribution of concentrate and hay. Limiting access to the feeder whilst performing all the cues and distributing the concentrate, which they could see but not access, was hypothesised to disrupt their routine and create frustration, increasing movement and their efforts to access the feed.

On several occasions during the trial, a grid was placed over the feeder in order to limit the animals' access to concentrates after it had been distributed (repeated 5 times). At 9h00 a person in white entered the pens and placed the grid over the feeder. The grid was secured with cables and allowed the animal to see the concentrates but not access it. A person wearing green removed the grid 20 min after the distribution of the concentrates and proceeded to also distribute hay. NT animals followed the normal protocol.

7.3.1.3 Social mixing

Sheep are known to display a gregarious social instinct. This allows them to bond closely to other sheep and display preferences to flock members. Flock mentality movements protect individuals from predators. Many studies have demonstrated that disruption of the social coherence of the group can lead to aggressiveness and an increase in stress hormone levels until social structure is re-established (Ruiz-De-La-Torre and Manteca, 1999; Blanchard, McKittrick and Blanchard, 2001; Miranda-de la Lama, Villarroel and María, 2012). Using this information, we decided to mix the animals on multiple occasions, for short periods of time to avoid habituation and using random sheep in a random order each time.

The MCS treated animals were mixed between pens 6 times during 6 weeks by switching the animals in groups of two or three between neighbouring pens. On two days they were switched in the morning period (from 10h30 to 12h30); on one occasion they were mixed twice in one day (10h30 to 12h30 and from 14h00 to 16h00) and on two occasions they were mixed in the afternoon (from 15h00 to 17h00). The combination of animals and order of mixing was random. The duration that the animals stayed in their neighbouring pen varied from 30 min to 1h30 min.

7.3.1.4 Noisy human

A study conducted by Waynert *et al*. (1999), on the effect of noise on cattle, found that movement and heart rate increased with the exposure to audios of clanging metal on metal. Interestingly, heart rate and movement increased and were significantly higher when the animals were exposed to audio recordings of humans shouting compared to the metallic sounds.

On 7 occasions, a person in white entered the pens holding items that could create a noise (metal bars, plastic bags) or illicit fear (large multi-coloured feather dusters, brooms, tinsel). The “noisy human” would then perform exaggerated movements, shout and make unnatural noises, disturbing the animals and forcing them to move out of his/her course which consisted of walking around the pen multiple times clock-wise, anti-clockwise or randomly.

The “noisy human” remained in the pens for between 3-7 minutes and on two occasions entered the pens once in the morning, and once in the afternoon. On another occasion, the test

was performed by two people in white simultaneously, who entered the pens from opposite directions of the room and worked through the pens.

7.3.1.5 Presence of dog/ guidance by dog

As sheep consider dogs as predators, they tend to flock together for protection and move away from the danger, perceiving the dog as a predator (*How has the risk of predation shaped the behavioural responses of sheep to fear and distress?*, no date; Beausoleil, Stafford and Mellor, 2005). Since these animals had not been previously exposed to a dog, we decided to include it as a stressor.

On 4 occasions the sheep were exposed to sheep dogs and dog handlers were dressed in white. The first time the animals were exposed, the dog was guided up and down in front and behind the pens for 3 min.

The second time this stressor was used, a different sheep dog, trained for guiding sheep that entered the pens and moved the animals in the corridor of Room 2, one pen at a time, returning them to their home pens after 2 min.

On the third occasion, the animals were exposed to the two previous dogs: once in the morning where the dog stayed in the room with the handler for 10 min, exited and re-entered for 2 min and once more in the afternoon when the animals were guided pen by pen in the corridor by the trained dog. For the purposes of this stressor they were guided by the dog and its handler up and down the length of the corridor for 5 min and subsequently returned to their home pens.

7.3.1.6 Lights during the night

Sheep have binocular vision, moderate acuity, are dichromats and avoid shadows or harsh contrasts between light and dark due to increased retinal sensitivity to movement (Dwyer, 2008).

The effects of stroboscope lights on a group of sheep was piloted before the trial and verified that the sweeping motion or flashing lights startled them and caused them to flock together and remain alert for at least 5 minutes.

Stroboscopic lights in the MCS Room were remotely controlled via PC using SweetLight's.dmx free downloadable software (https://sweetlight-controller.com/). We programmed the lights to perform a variety of functions (sweeping motion of a headlight,

interchanging colours, fast and slow flashing lights, and rapid rotation of light beams) for different durations, at different times in the night. This event was also sometimes combined with a wind turbine, which was programmed to launch at the same time as the lights went on.

Overall, the "lights stressor" was included in the MCS trial 12 times. The duration varied from 1 to 5 min. On 4 nights, the lights were programmed to go on twice, on two nights the lights were set off three times and the combination of lights and wind turbine was operated 5 times. The time of night the lights came on was never the same and could be from 30 min after main lights went off at 19h00, to 30 minutes prior to them coming on in the morning at 7h00.

7.3.1.7 Rough handling while conducting standard management

Rough handling of livestock, by enhancing the animals' fear of humans, can markedly affect the stress physiology and reactivity levels of animals (Hemsworth *et al.*, 2010).

Weighing and any handling for standard management in the MCS group was conducted by animal technicians in the white uniform (described above) performing exaggerated movements who were also intentionally louder. No pain was inflicted, and the animals were not misstreated. In comparison, NT animals were weighed and handled in a calmer fashion and animal technicians wore the green uniform.

7.3.1.8 Shearing

Shearing represents a management practice commonly used which requires the animal to be restricted, isolated from the flock on many occasions and exposed to noise (humans, clippers), all of which are aversive to sheep, as the restriction and noise of clippers for this procedure are known to illicit a stress response (Destrez *et al.*, 2013; Doyle *et al.*, 2011b; Rushen and Congdon, 1986).

On the sixth week of the trial, the animals in the MCS group were shorn by a trained stockworker. The animals' identification numbers were retraced on their backs with marking crayons and animals were returned to their home-pens.

7.3.1.9 Individual restraint

Sheep show a particular aversion to being restrained and as a gregarious animal, they get particularly distressed when separated from their flock/group (Destrez *et al.*, 2013b).

7.3.2 Tables with model equations

Table 7.23 GLM and LM models used to explore Treatment and Day effect on Heart Rate parameters (HR_aver, RR_aver, RMSSD, SDNN and LF/HF) for each Step within the day, when no stressor was applied.

Models		
Step	**Heart Rate Parameter**	**Model**
Step1	RR_aver	*Treatment+ Day + Treatment *Day + (Day | ID)*
	HR_aver	*Treatment+ Day + (Day | ID)*
	RMSSD	*Treatment+ Day + (1 | ID)*
	SDNN	*Treatment+ Day + (Day | ID)*
	LF/HF	as above
Step 2	RR_aver	as above
	HR_aver	as above
	RMSSD	as above
	SDNN	as above
	LF/HF	*Treatment+ Day + (1 | ID)*
Step 3	RR_aver	*Treatment+ Day + (Day | ID)*
	HR_aver	as above
	RMSSD	as above
	SDNN	*Treatment+ Day + (1 | ID)*
	LF/HF	as above
Step 4	RR_aver	*Treatment+ Day + (Day | ID)*
	HR_aver	*Treatment+ Day + (1 | ID)*
	RMSSD	as above
	SDNN	as above
	LF/HF	as above
	RR_aver	*Treatment+ Day + (Day | ID)*
	HR_aver	*Treatment+ Day + (1 | ID)*

Step 5	RMSSD	*Treatment+ Day + (Day | ID)*
	SDNN	*Treatment+ Day + (1 | ID)*
	LF/HF	as above

Table 7.24 Statistical test/model used to explore Treatment effect on Heart Rate parameters (HR_aver, RR_aver, RMSSD, SDNN and LF/HF) for each Step for the Suddenness Test in the home pen. Treatment*day, expressed the exploration of Treatment Day (NS or Suddenness Test and their Interaction.

Step	**Heart Rate Parameter**	**Statistical Test**
Step1	RR_aver	ANOVA
	HR_aver	ANOVA
	RMSSD	ANOVA
	SDNN	Kruskal-Wallis
	LF/HF	ANOVA
Step 2	RR_aver	ANOVA
	HR_aver	ANOVA
	RMSSD	Kruskal-Wallis
	SDNN	ANOVA
	LF/HF	Kruskal-Wallis
Step 3	RR_aver	ANOVA
	HR_aver	ANOVA
	RMSSD	ANOVA
	SDNN	ANOVA
	LF/HF	ANOVA
Step 4	RR_aver	ANOVA
	HR_aver	ANOVA
	RMSSD	Kruskal-Wallis
	SDNN	Kruskal-Wallis

	LF/HF	ANOVA
Step 5 With NS Step5	RR_aver	*Treatment*day+ (1|ID) (Poisson)*
	HR_aver	*Treatment*day+ (1|ID)*
	RMSSD	*as above*
	SDNN	*as above*
	LF/HF	*as above*
Step 6 With NS Step6	RR_aver	*Treatment*day+ (1|ID) (Poisson)*
	HR_aver	*Treatment*day+ (1|ID)*
	RMSSD	*as above*
	SDNN	*as above*
	LF/HF	*as above*

Table 7.25 Models appled for Analysis of Heart Rate variables by Step for the Suddenness test conducted in a novel environment. Treatment * Day describes the investigation of Treatment, Day and their Interaction.

Models		
Step	**Heart Rate Parameter**	**Model**
Step1	RR_aver	*Treatment*Day +(1|Day) (Poisson)*
	HR_aver	*Treatment *Day + (1 | ID)*
	RMSSD	as above
	SDNN	as above
	LF/HF	as above
Step 2	RR_aver	*Treatment +Day + (1 | ID) (Poisson)*
	HR_aver	*Treatment +Day + (1 | ID)*
	RMSSD	as above
	SDNN	as above
	LF/HF	as above*)*

Step 2 no stress – Step 2 Ball	RR_aver	*Treatment +Day + (1 | ID) (Poisson)*
	HR_aver	*Treatment +Day + (1 | ID)*
	RMSSD	as above
	SDNN	as above
	LF/HF	as above
Step 3	RR_aver	*Treatment +Day + (1 | ID) (Poisson)*
	HR_aver	*Treatment +Day + (1 | ID)*
	RMSSD	as above
	SDNN	as above
	LF/HF	as above
Step 2 no Stress- Step 3 Ball	RR_aver	*Treatment +Day + (1 | ID) (Poisson)*
	HR_aver	*Treatment +Day + (1 | ID)*
	RMSSD	as above
	SDNN	as above
	LF/HF	as above
Step 4	RR_aver	*Treatment +Day + (1 | ID)*
	HR_aver	*Treatment *Day + (1 | ID)*
	RMSSD	as above
	SDNN	*Treatment+ Day + (1 | ID)*
	LF/HF	as above
Step 5 No stress - Step 5 Ball	RR_aver	Treatment *Day+ *(1 | ID) (Poisson)*
	HR_aver	Treatment *Day+ *(1 | ID)*
	RMSSD	as above
	SDNN	as above
	LF/HF	as above
Step 6 No stress -	RR_aver	Treatment *Day+ *(1 | ID) (Poisson)*
	HR_aver	Treatment *Day+ *(1 | ID)*
	RMSSD	as above

Step 6 Ball	SDNN	as above
	LF/HF	as above

Table 7.26 Statistical models were used to explore the effect of Treatment (MCS, NT), Experimental Stage (ExpStage: Pre, Start, Mid-trial and End of trial) on the various parameters measured for each behaviour on days when no stress was applied. Treatment * ExpStage expresses the investigation of Treatment, Experimental Stage and their Interaction.

Models		
Behaviour	**Parameter**	**Model**
Eating Hay	Occurrence	*Treatment * ExpStage + (1 | ExpStage) (Poisson)*
	Number of bouts	*Treatment+ ExpStage (Poisson)*
	Length of bouts	*Treatment+ ExpStage+ (ExpStage |ID)*
	Synchronisation	*as above*
	Average Synchronisation	*Treatment * ExpStage + (1 | ID)*
Eating Concentrate	Occurrence	*Treatment * ExpStage + (ExpStage | ID) (Poisson)*
	Number of bouts	*as above*
	Length of bouts	*Treatment+ ExpStage+ (1 |ID)*
	Synchronisation	*Treatment * ExpStage + (1 | ExpStage)*
	Average Synchronisation	*Treatment+ ExpStage+ (1 |ID)*
Resting	Occurrence	*Treatment+ ExpStage+ (1 |ID) (Poisson)*
	Number of bouts	*as above*
	Length of bouts	*Treatment+ ExpStage+ (ExpStage |ID)*
	Synchronisation	*as above*
	Average Synchronisation	*Treatment+ ExpStage+ (1 |ID)*
	Occurrence	*Treatment+ ExpStage+ (ExpStage |ID) (Poisson)*
	Number of bouts	*Treatment+ ExpStage (Poisson)*

Sleeping	Length of bouts	*Treatment+ ExpStage+ (ExpStage |ID)*
	Synchronisation	*Treatment+ ExpStage+ (1 |ID)*
	Average Synchronisation	*Treatment * ExpStage*
Moving	Occurrence	*Treatment+ ExpStage+ (1 |ID) (Poisson)*
	Number of bouts	*as above*
	Length of bouts	*Treatment+ ExpStage+ (ExpStage |ID)*
	Synchronisation	*NA*
	Average Synchronisation	*Treatment+ ExpStage*
Immobile	Occurrence	*Treatment+ ExpStage+ (ExpStage |ID) (Poisson)*
	Number of bouts	*as above*
	Length of bouts	*Treatment+ ExpStage+ (1 |ID)*
	Synchronisation	*Treatment * ExpStage + (ExpStage | ID)*
	Average Synchronisation	

Table 7.27 Models (lmer or glm) used to explore Treatment and Experimental Stage (referred to as "Stage" in the table) effect on alpha diversity indices (Shannon, Simpson, Inverse Simpson, Observed, Chao1, ACE and Fisher) for each sample matrix i.e. rumen bacteria, rumen archaea, faecal bacteria and faecal archaea.

Models		
Sample Matrix	**Alpha diversity Index**	**Model**
Rumen Bacteria	Shannon	*Treatment+ Stage +Stage*Treatment + (1|Pen/ID)*
	Simpson	*Treatment+ Stage +Stage*Treatment + (1|ID)*
	Inverse Simpson	as above
	Observed	*Treatment+ Stage +Stage*Treatment + (1|Pen/ID))*
	Chao1	as above

	ACE	as above
	Fisher	as above
Rumen Archaea	Shannon	*Treatment+ Stage +Stage*Treatment + (1|Pen/ID)*
	Simpson	*Treatment+ Stage +Stage*Treatment + (1|ID)*
	Inverse Simpson	as above
	Observed	as above
	Chao1	*Treatment+ Stage +Stage*Treatment + (1|Pen/ID)*
	ACE	as above
	Fisher	as above
Faecal Bacteria	Shannon	*Treatment+ Stage +Stage*Treatment + (1|Pen/ID)*
	Simpson	as above
	Inverse Simpson	as above
	Observed	as above
	Chao1	*Treatment+ Stage +Stage*Treatment + (1|ID)*
	ACE	*Treatment+ Stage +Stage*Treatment*
	Fisher	*Treatment+ Stage +Stage*Treatment + (1|ID)*
Faecal Archaea	Shannon	*Treatment+ Stage+ (1|ID)*
	Simpson	as above
	Inverse Simpson	as above
	Observed	as above
	Chao1	*Treatment+ Stage*
	ACE	as above
	Fisher	*Treatment+ Stage+ (1|ID)*

7.3.3 Area -Under -the -curve figures and equations for heart rate variables by day

7.3.3.1 Day 1

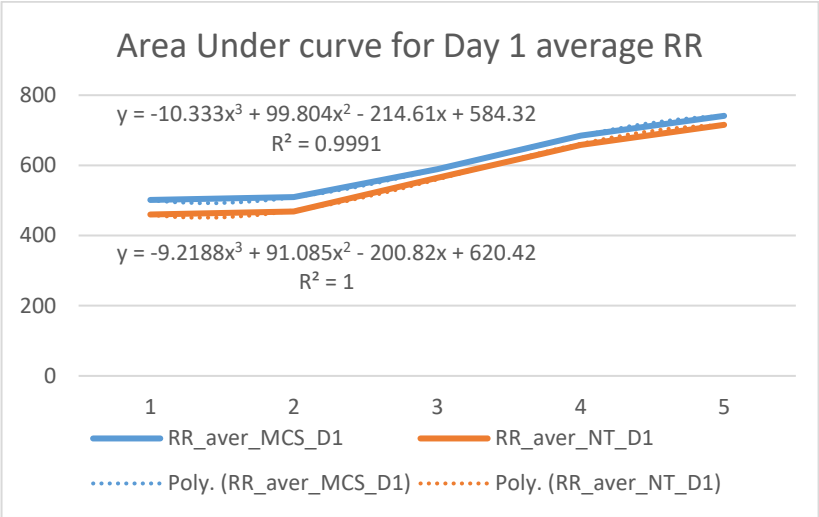

Figure 7.24 Area under the curve for the first Day (D1, Pre-trial) of heart monitoring on a No-stress day. Average RR by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported

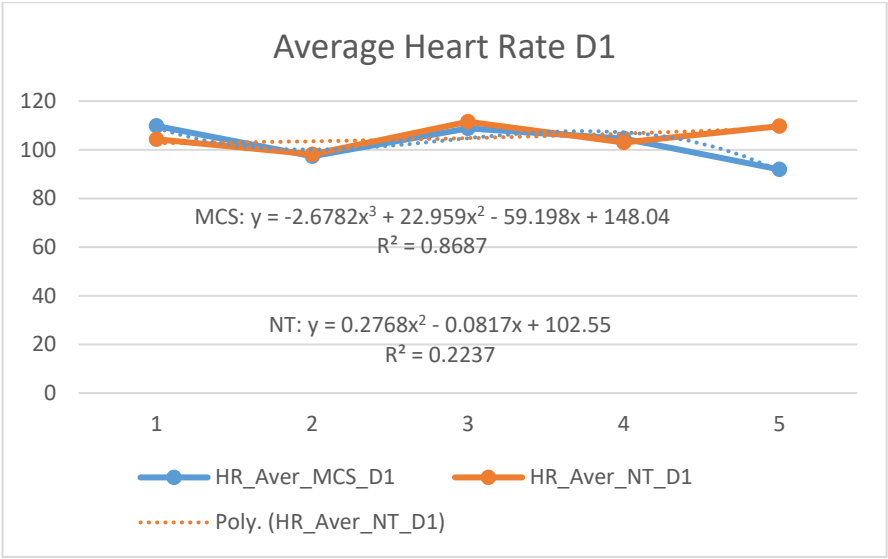

Figure 7.25 Area under the curve for the first Day (D1, Pre-trial) of heart monitoring on a No-stress day. Average HR by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

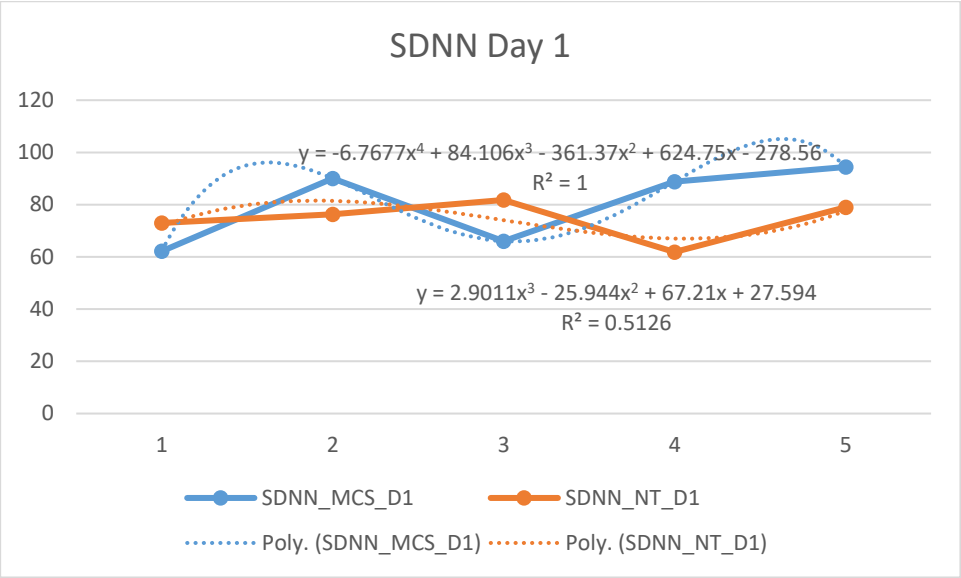

Figure 7.26 Area under the curve for the first Day (D1, Pre-trial) of heart monitoring on a No-stress day. SDNN by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

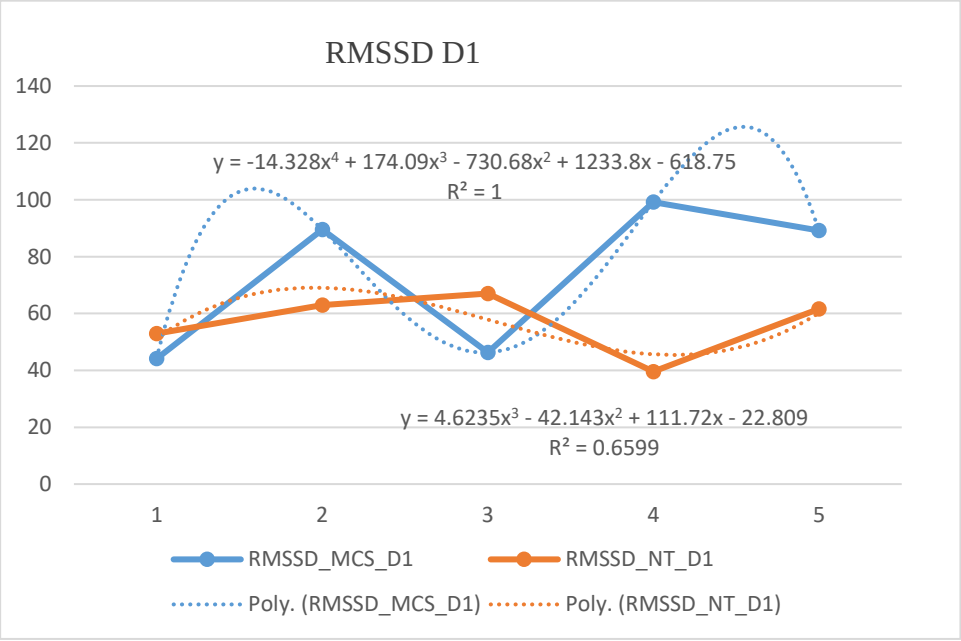

Figure 7.27 Area under the curve for the first Day (D1, Pre-trial) of heart monitoring on a No-stress day. RMSSD by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

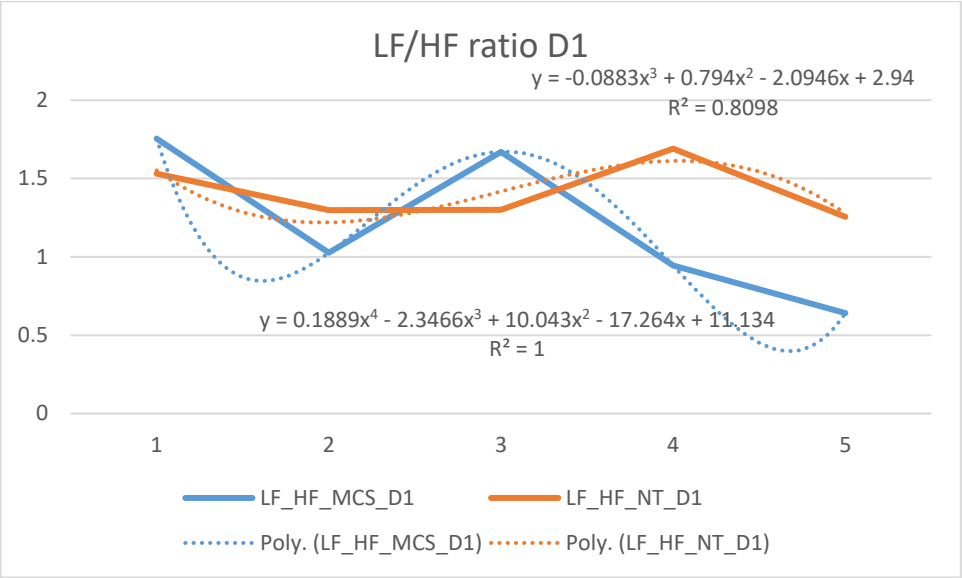

Figure 7.28 Area under the curve for the first Day (D1, Pre-trial) of heart monitoring on a No-stress day. LF/HF by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

7.3.3.2 Day 2

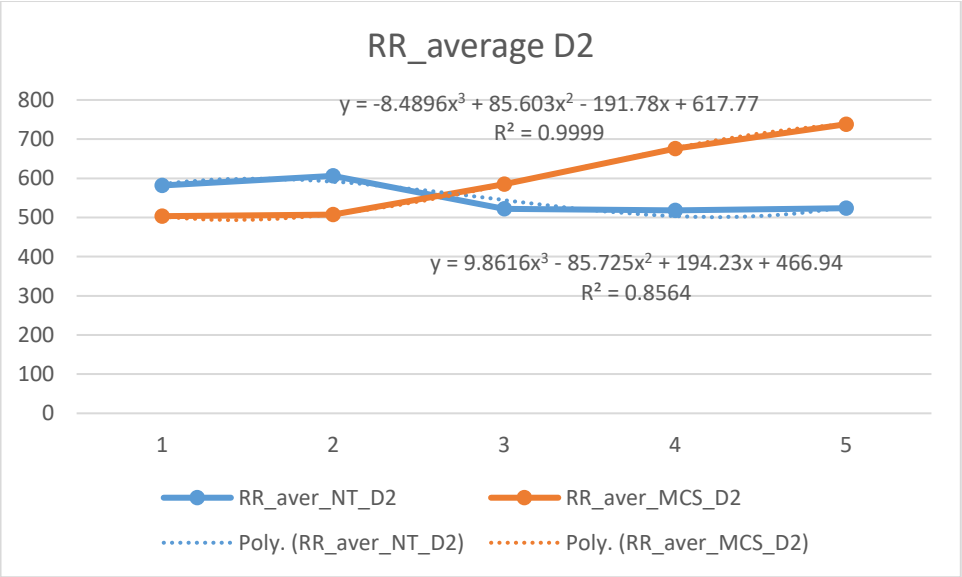

Figure 7.29 Area under the curve for the second Day (D2, Start of Trial) of heart monitoring on a No-stress day. Average RR by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

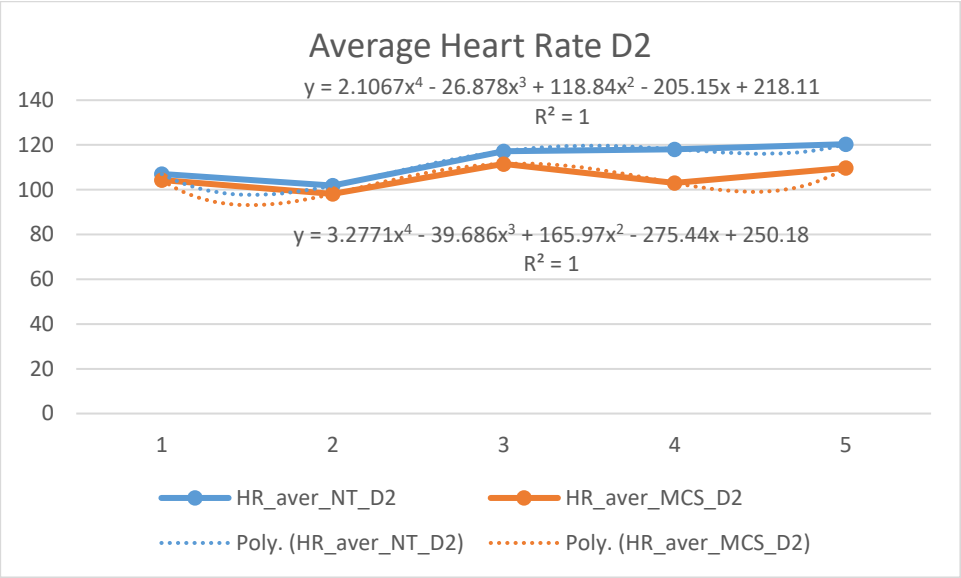

Figure 7.30 Area under the curve for the second Day (D2, Start of Trial) of heart monitoring on a No-stress day. Average HR by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

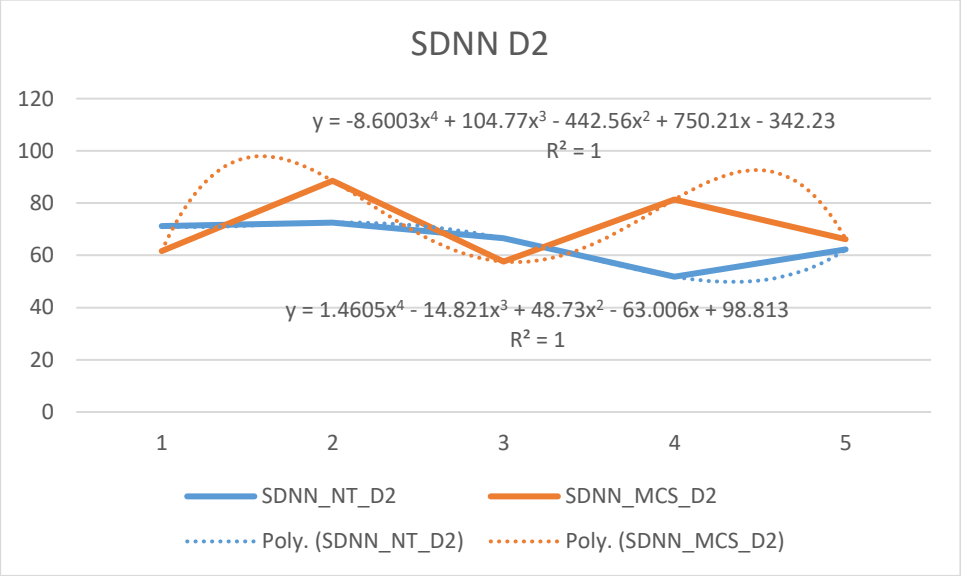

Figure 7.31 Area under the curve for the second Day (D2, Start of Trial) of heart monitoring on a No-stress day. SDNN by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

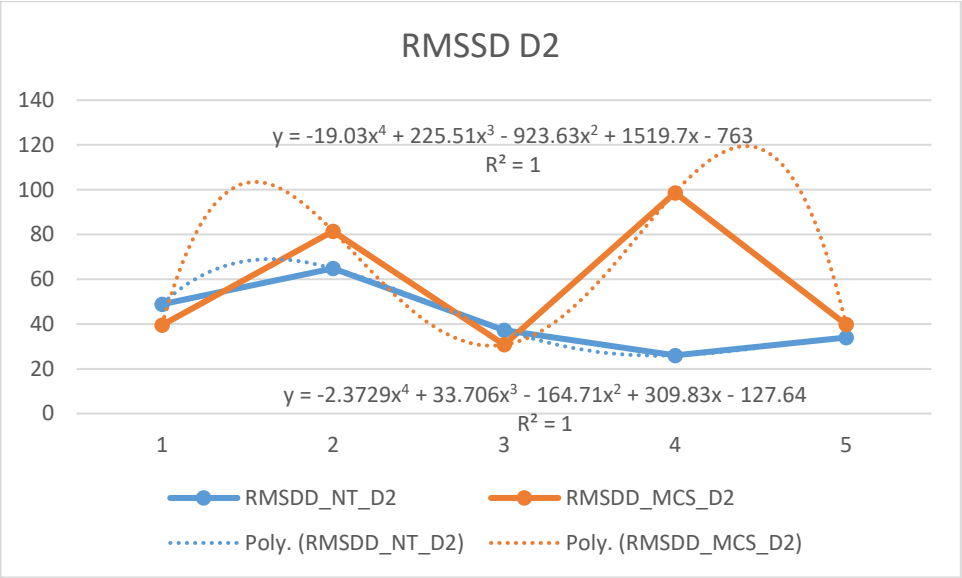

Figure 7.32 Area under the curve for the second Day (D2, Start of Trial) of heart monitoring on a No-stress day. RMSSD by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

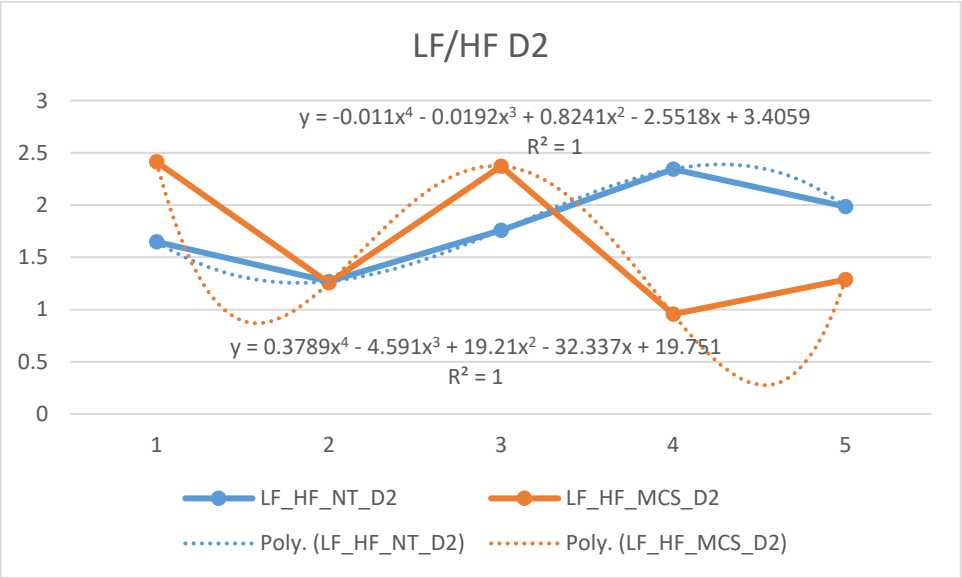

Figure 7.33 Area under the curve for the second Day (D2, Start of Trial) of heart monitoring on a No-stress day. LF/HF by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

7.3.3.3 Day 3

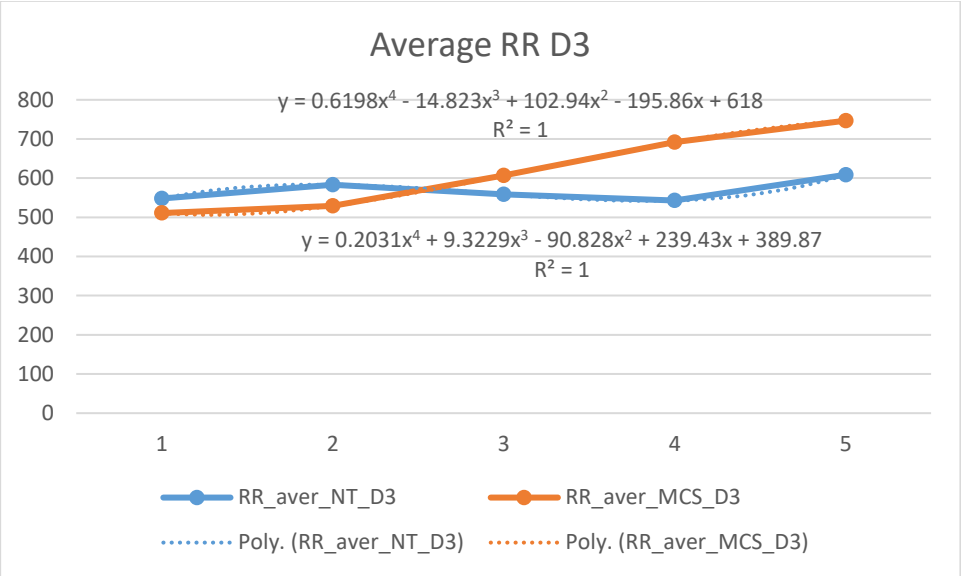

Figure 7.34 Area under the curve for the third Day (D3, Middle of Trial) of heart monitoring on a No-stress day. Average RR by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

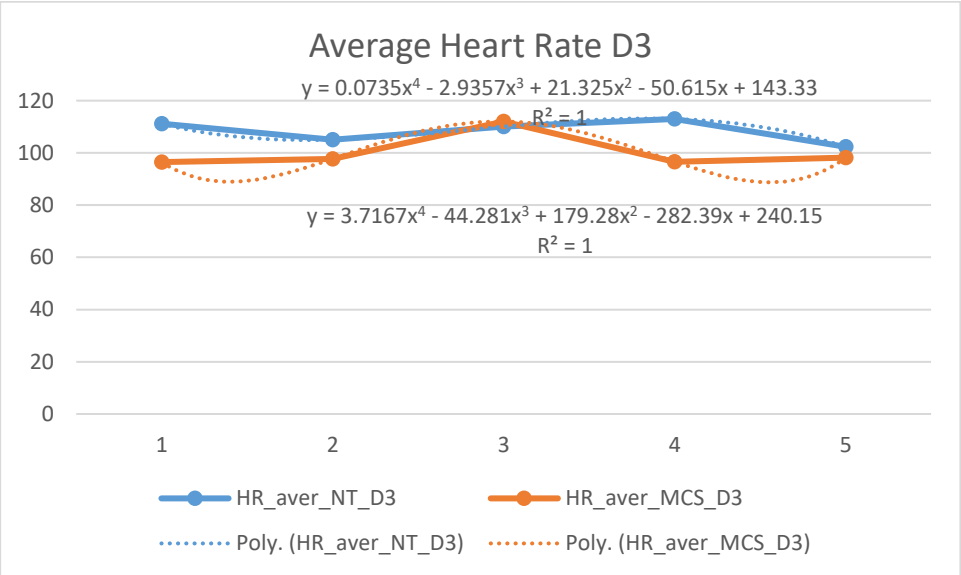

Figure 7.35 Area under the curve for the third Day (D3, Middle of Trial) of heart monitoring on a No-stress day. Average HR by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported

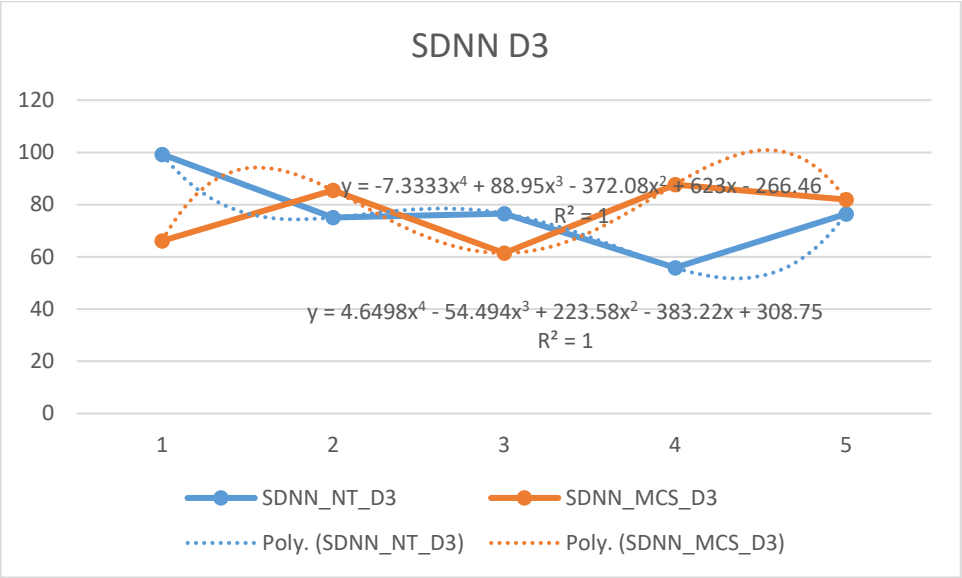

Figure 7.36 Area under the curve for the third Day (D3, Middle of Trial) of heart monitoring on a No-stress day. SDNN by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

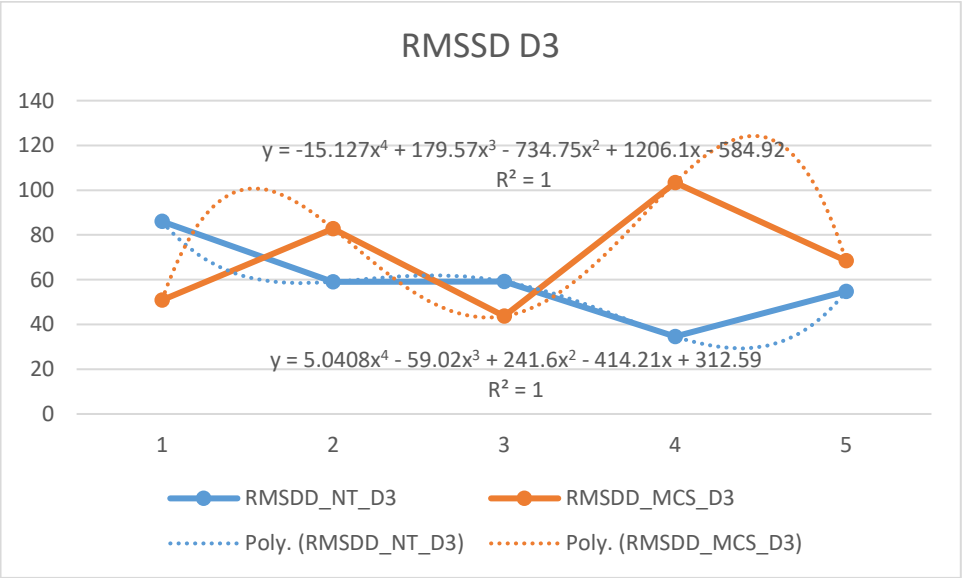

Figure 7.37 Area under the curve for the third Day (D3, Middle of Trial) of heart monitoring on a No-stress day. Average RMSSD by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

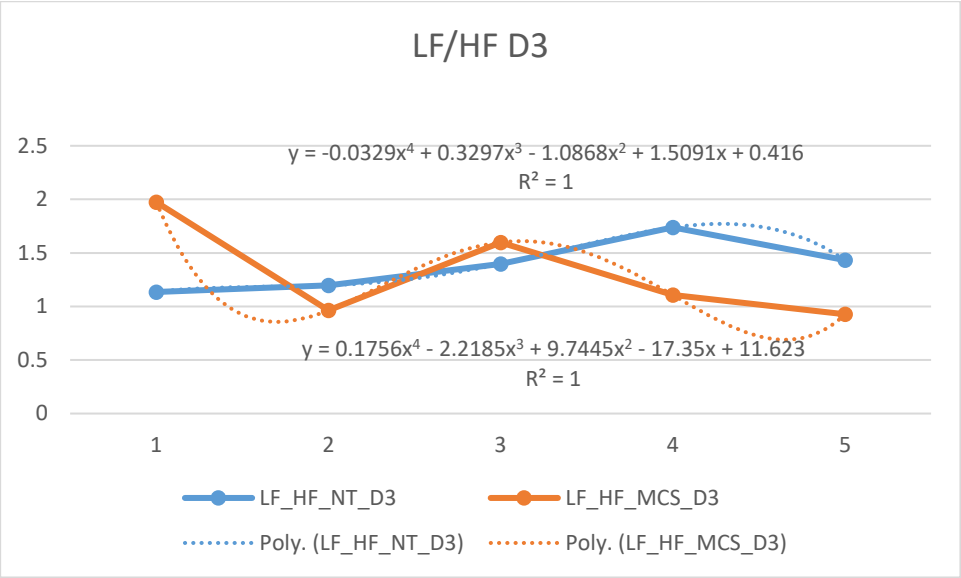

Figure 7.38 Area under the curve for the third Day (D3, Middle of Trial) of heart monitoring on a No-stress day. LF/HF by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

7.3.3.4 Day 4

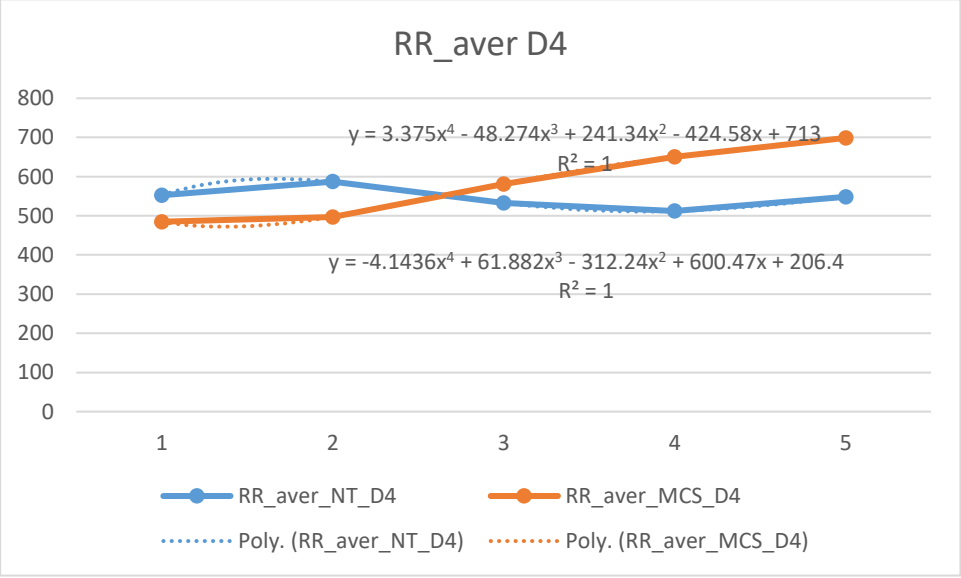

Figure 7.39 Area under the curve for the fourth Day (D4, End of Trial) of heart monitoring on a No-stress day. Average RR by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

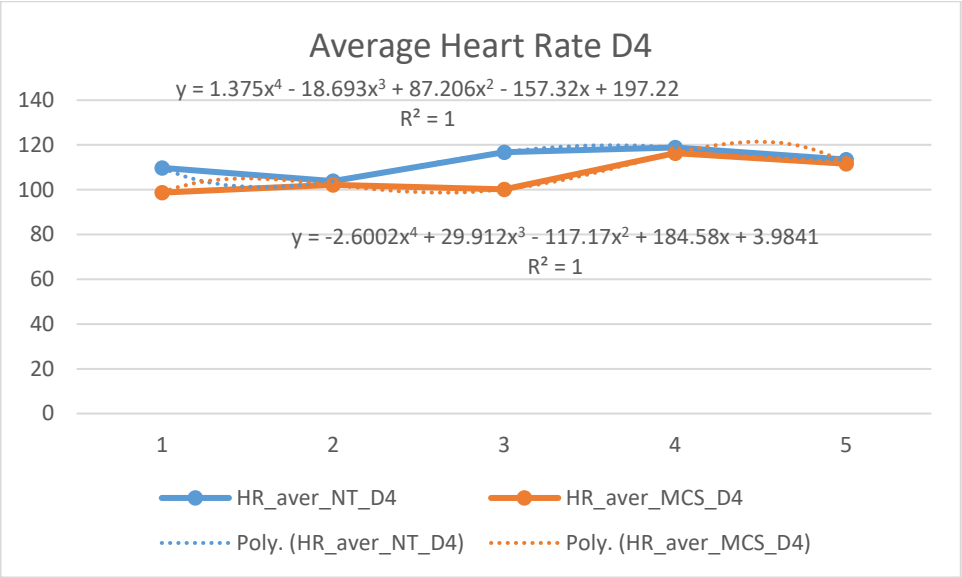

Figure 7.40 Area under the curve for the fourth Day (D4, End of Trial) of heart monitoring on a No-stress day. Average HR by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

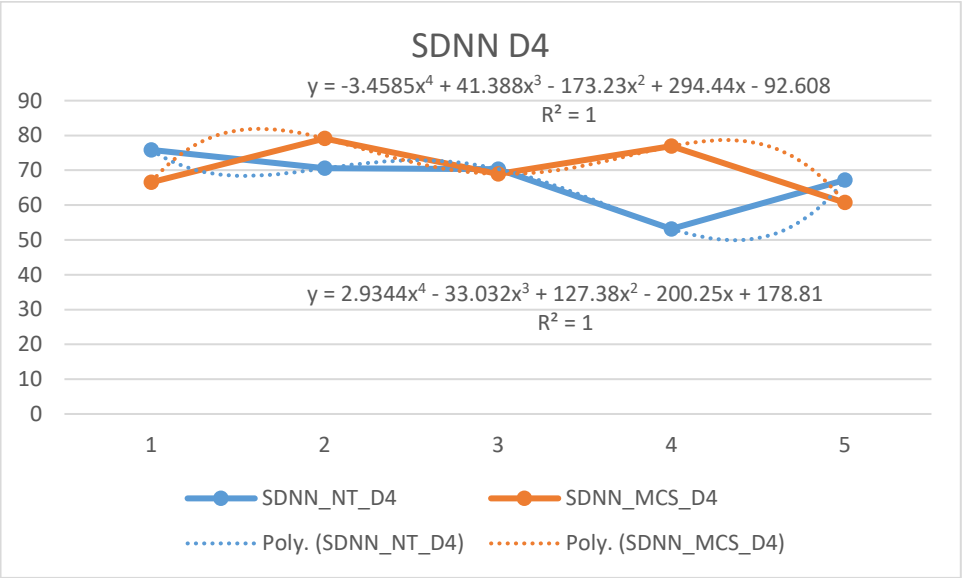

Figure 7.41 Area under the curve for the fourth Day (D4, End of Trial) of heart monitoring on a No-stress day. SDNN by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

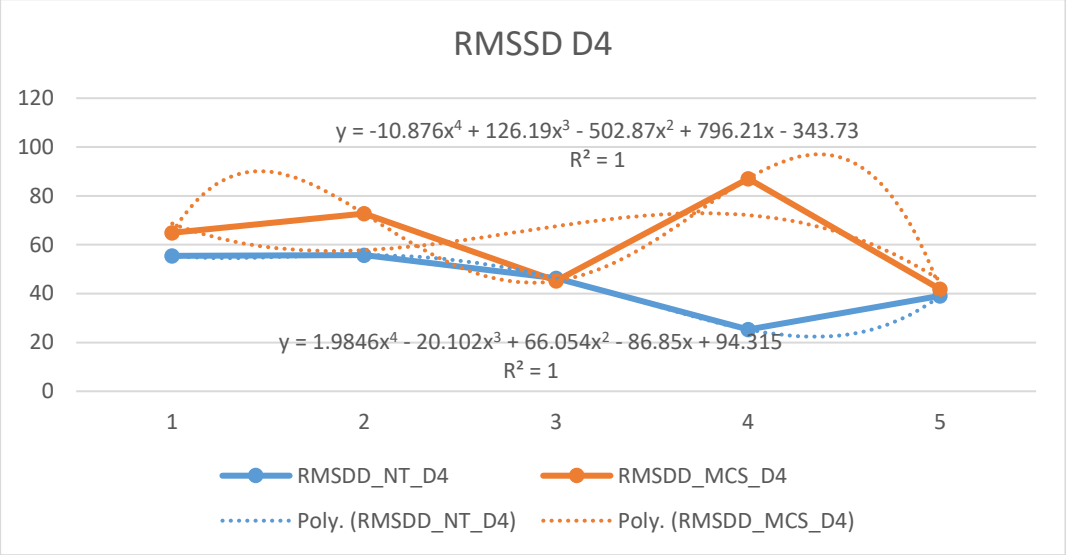

Figure 7.42 Area under the curve for the fourth Day (D4, End of Trial) of heart monitoring on a No-stress day. RMSSD by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

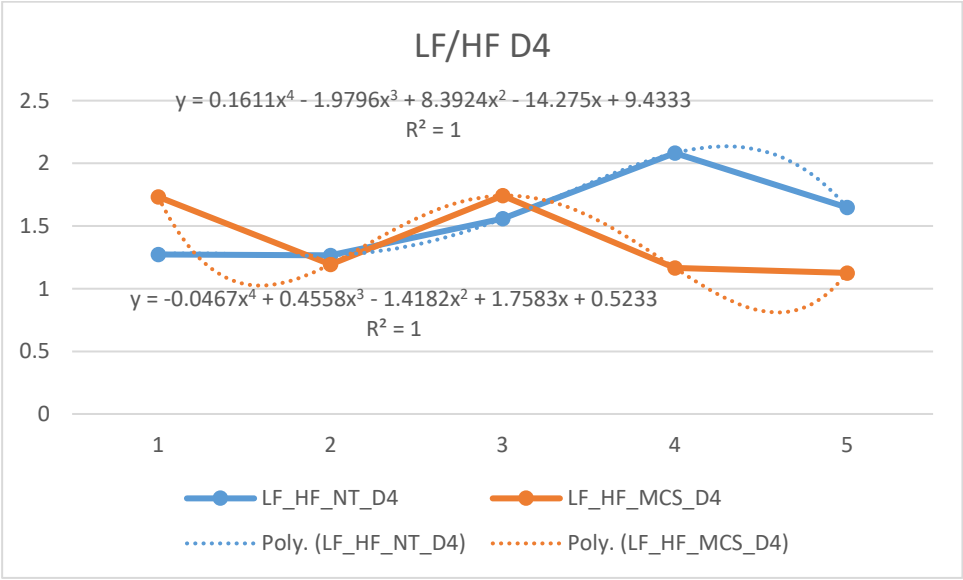

Figure 7.43 Area under the curve for the fourth Day (D4, End of Trial) of heart monitoring on a No-stress day. LF/HF by Treatment Group (NT, MCS) and polynomial equations fitted using Excel are reported.

7.4 Rumen community composition

Table 7.28 Composition of rumen Microbiota community. The most abundant phyla, orders and Genera are presented as a percentage of the total population. The average percentage and minimum and maximum percentages are reported.

Phyla			
	Average%	**Max%**	**Min%**
Bacteroidetes	68.70	81.60	43.00
Firmicutes	22.00	50.10	10.10
Fibrobacteres	4.00	19.90	0.50
Synergistetes	1.10	12.90	0.00
Spirochaetes	0.80	3.20	0.20
None;Other	0.70	2.10	0.30
Proteobacteria	0.60	3.50	0.00
Euryarcheota	0.40	2.70	0.10
Orders			
	Average%	**Max%**	**Min%**
Bacteroidales	68.70	81.60	42.90
Clostridiales	21.10	49.00	10.00
Fibrobacterales	4.00	19.90	0.50
Synergistales	1.10	12.90	0.00
Erysipelotrichales	0.90	5.00	0.10
Spirochaetales	0.80	3.00	0.20
None;Other	0.70	2.10	0.30
Aeromonadales	0.50	3.50	0.00
Genera			
	Average%	**Max%**	**Min%**
Prevotella	50.60	72.70	0.00
Unidentified_Veillonellaceae	5.60	25.30	0.00
Unidentified_Bacteroidales	5.50	19.90	2.20
Fibrobacter	4.00	19.90	0.50
Unidentified_RF16 (o_Bacteroidales)	3.90	31.30	0.10
Unidentified_Clostridiales	2.80	5.60	1.20
Unidentified_S24-7 (o_Bacteroidales)	2.10	7.00	0.20

Selenomonas	1.80	9.20	0.00
YRC22 (f_[Paraprevotellaceae)]	1.60	6.10	0.00
Unidentified__Ruminococcaceae	1.60	22.40	0.40
CF231 (f_[Paraprevotellaceae])	1.50	14.00	0.30
Unidentified_ [Paraprevotellaceae]	1.20	3.80	0.00
Ruminococcus	1.10	2.70	0.30
TG5 (f_Dethiosulfovibrionaceae)	1.10	12.80	0.00
Unidentified_Prevotellaceae	1.00	3.20	0.00
Unidentified_Lachnospiraceae	1.00	4.70	0.30

7.4.1 PLS Rumen relative abundances and cortisol/serotonin

Table 7.29 Rumen PostMCS phyla relationship with "log(x+1)" cortisol values and "log" serotonin cortisol values. VIPs from the PLS analysis, RA, direction of correlation, correlation coefficients (R) and p-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix (Appendix **Figure 7.52. 7.58**).

PostMCS- Cortisol						
	Phylum	**VIP**	**Mean RA**	**Direction**	**R**	**p-value**
A12	*LD1*	1.67	0.001	Positive	0.43	0.04
A16	*Spirochaetes*	1.66	0.60	Positive	0.43	0.04
A4	*Armatimonadetes*	1.62	0.005	Positive	0.41	0.04
A18	*Tenericutes*	1.45	0.30	Positive	0.37	0.08
A13	*Lentisphaerae*	1.33	0.30	Negative	-0.30	0.16
A6	*Chloroflexi*	1.22	0.07	Positive	0.30	0.15
A11	*Fusobacteria*	1.03	0.002	Positive	0.20	0.32
A8	*Elusimicrobia*	1.01	0.008	Positive	0.22	0.30
PostMCS- Serotonin						
	Phylum	**VIP**	**Mean RA**	**Direction**	**R**	**p-value**
A9	*Fibrobacteres*	2.07	4.67	Negative	-0.50	0.01
A10	*Firmicutes*	1.79	21.00	Positive	0.45	0.03
A1	*Euryarchaeota*	1.68	0.38	Positive	0.38	0.07
A19	*Verrucomicrobia*	1.21	0.05	Positive	0.09	0.68
A14	*Planctomycetes*	1.20	0.17	Positive	0.30	0.15
A12	*LD1*	1.01	0.001	Positive	0.23	0.27

Table 7.30 Rumen PostNT phyla relationship with "log(x+1)" cortisol values and "log" serotonin values. VIPs from the PLS analysis, RA, direction of correlation, correlation coefficients (R) and p-

values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix (Appendix **Figure 7.49,7.55**).

PostNT- Cortisol						
	Phylum	**VIP**	**Mean RA**	**Direction**	**R**	**p-value**
A4	*Armatimonadetes*	1.97	0.008	Positive	0.41	0.05
A7	*Cyanobacteria*	1.48	0.30	Positive	0.28	0.19
A13	*Lentisphaerae*	1.21	0.24	Positive	0.04	0.85
A8	*Elusimicrobia*	1.21	<0.001	Positive	0.25	0.23
A3	*Actinobacteria*	1.20	0.23	Negative	-0.26	0.23
A17	*Synergistetes*	1.16	0.54	Positive	0.23	0.27
A10	*Firmicutes*	1.11	23.42	Negative	-0.11	0.61
A19	*Verrucomicrobia*	1.10	0.06	Positive	0.10	0.66
A16	*Spirochaetes*	1.02	1.00	Negative	-0.08	0.71
A5	*Bacteroidetes*	1.01	67.80	Positive	0.08	0.71
PostNT- Serotonin						
	Phylum	**VIP**	**Mean RA**	**Direction**	**R**	**p-value**
A8	*Elusimicrobia*	1.45	<0.001	Negative	-0.30	0.16
A6	*Chloroflexi*	1.36	0.08	Negative	-0.23	0.28
A1	*Euryarchaeota*	1.32	0.50	Positive	0.29	0.18
A9	*Fibrobacteres*	1.29	3.94	Positive	0.10	0.64
A5	*Bacteroidetes*	1.21	67.80	Negative	-0.27	0.21
A10	*Firmicutes*	1.20	23.42	Positive	0.23	0.27
A15	*Proteobacteria*	1.19	0.58	Negative	-0.15	0.49
A7	*Cyanobacteria*	1.16	0.30	Positive	0.18	0.39
A17	*Synergistetes*	1.06	0.55	Positive	0.20	0.35
A14	*Planctomycetes*	1.00	0.15	Positive	0.22	0.30
A4	*Armatimonadetes*	1.00	0.008	Positive	0.20	0.35

Table 7.31 Rumen PostMCS orders relationship with "log(x+1)" cortisol values and "log" cortisol/serotonin values. VIPs from the PLS analysis, RA, direction of correlation, correlation coefficients (R) and p-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix (Appendix **Figure 7.53, 7.59**).

PostMCS- Cortisol						
	Order	**VIP**	**Mean RA**	**Direction**	**R**	**p-value**
A49	*Pseudomonadales*	2.27	0.003	Positive	0.61	0.001
A51	*p_Spirochaetes;c_MVP-15;o_PL-11B10*	2.05	0.005	Positive	0.52	0.01
A30	*Victivallales*	1.98	0.26	Negative	-0.35	0.09
A22	*Unidentified_Firmicutes*	1.81	0.03	Positive	0.48	0.02
A20	*Unidentified_Endomicrobia*	1.75	0.001	Positive	0.43	0.04
A29	*Unidentified_LD1*	1.75	0.001	Positive	0.43	0.04
A9	*Coriobacteriales*	1.52	0.005	Positive	0.41	0.04
PostMCS- Serotonin						
	Order	**VIP**	**Mean RA**	**Direction**	**R**	**P**
A5	*c_Thermoplasmata;o_E2*	2.19	0.05	Positive	0.52	0.01
A21	*Fibrobacterales*	2.18	4,67	Negative	-0.51	0.01
A18	*Streptophyta*	2.06	0.002	Positive	0.48	0.02
A26	*Clostridiales*	1.87	20.25	Positive	0.44	0.03
A53	*Sphaerochaetales*	1.82	0.05	Negative	-0.41	0.04
A38	*Rickettsiales*	1.69	<0.001	Positive	0.37	0.08
A3	*Methanobacteriales*	1.68	0.33	Positive	0.34	0.10

Table 7.32 Rumen PostNT orders relationship with "log(x+1)" cortisol values and "log" serotonincortisol values and serotonin. VIPs from the PLS analysis, RA, direction of correlation, correlation coefficients (R) and p-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix (Appendix **Figure 7.50, 7.56**).

PostNT- Cortisol						
	Order	**VIP**	**Mean RA**	**Direction**	**R**	**p-value**
A34	*Alphaproteobacteria Other*	3.15	<0.001	Positive	0.61	0.002
A45	*Myxococcales*	2.42	0.001	Positive	0.51	0.01
A9	*Coriobacteriales*	1.93	0.008	Positive	0.41	0.05
A53	*Sphaerochaetales*	1.81	0.05	Negative	-0.37	0.07
A46	*Gammaproteobacteria Other*	1.53	0.001	Positive	0.29	0.17
PostNT- Serotonin						
	Order	**VIP**	**Mean RA**	**Direction**	**R**	**p-value**
A44	*Deltaproteobacteria GMD14H09*	2.08	0.007	Positive	0.39	0.06
A42	*Desulfobacterales*	2.05	0.009	Negative	-0.11	0.62
A10	*Bacteroidetes_Other*	1.69	0.03	Negative	-0.28	0.18
A7	*Bifidobacteriales*	1.65	0.001	Positive	0.32	0.13
A3	*Methanobacteriales*	1.51	0.32	Positive	0.30	0.16
A19	*Elusimicrobiales*	1.50	0.007	Negative	-0.30	0.16
A57	*Acholeplasmatales*	1.50	<0.001	Negative	-0.30	0.16
A58	*Anaeroplasmatales*	1.50	0.15	Negative	-0.29	0.17

Table 7.33 Rumen PostMCS genera relationship with "log(x+1)" cortisol values. VIPs from the PLS analysis, RA, direction of correlation, correlation coefficients (R) and p-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix (Appendix **Figure 7.54**).

PostMCS- Cortisol						
	Genus	**VIP**	**Mean RA**	**Direction**	**R**	**p-value**
A164	*Pseudomonas*	2.54	0.03	Positive	0.61	<0.01
A167	*Spirochaetes;c_MVP-15;o_PL-11B10_Unidentified*	2.27	0.004	Positive	0.52	0.01
A70	*Unidentified_ Victivallaceae*	2.08	0.05	Negative	-0.50	0.01
A58	*Firmicutes;Other*	2.02	0.03	Positive	0.48	0.02
A181	*Unidentified_ Anaeroplasmataceae*	1.97	0.01	Positive	0.43	0.40
A132	*Unidentified_ Victivallaceae*	1.96	0.26		-0.35	0.09
A36	*Rikenellaceae_Blvii28*	1.95	0.001	Positive	0.37	0.07
A56	*Unidentified_ Endomicrobia*	1.95	0.001	Positive	0.43	0.04
A133	*Victivallis*	1.95	<0.001	Positive	0.43	0.04
A89	*Filifactor*	1.95	<0.001	Positive	0.43	0.04
A130	*p_LD1_Unidentified*	1.95	0.001	Positive	0.43	0.04
A148	*Alysiella*	1.95	<0.001	Positive	0.43	0.04
A171	*Sphaerochaetaceae_wall-less*	1.89	0.001	Positive	0.46	0.03
A37	*Unidentified_S24-7 Bacteroidales*	1.82	2.02	Negative	-0.44	0.03
A64	*Unidentified Christensenellaceae*	1.81	0.08	Positive	0.40	0.06

A77	*Coprococcus*	1.72	0.22	Positive	0.40	0.05
A41	*Unidentified [Paraprevotellaceae]*	1.71	1.53	Negative	-0.41	0.04
A18	*Unidentified_ RB04_ SJA-176 Armatimonadetes*	1.71	0.005	Positive	0.41	0.04
A140	*Unidentified Bradyrhizobiaceae*	1.64	<0.001	Positive	0.36	0.08
A69	*Anaerofustis*	1.64	0.003	Positive	0.39	0.06
A12	*Other_ Coriobacteriaceae*	1.61	0.005	Positive	0.39	0.06
A173	*Treponema*	1.60	0.53	Positive	0.36	0.08
A174	*Other_ Dethiosulfovibrionaceae*	1.57	0.001	Positive	0.29	0.17
A159	*Ruminobacter*	1.55	0.03	Positive	0.24	0.26
A185	*Unidentified_RF39_ Mollicutes*	1.51	0.13	Positive	0.36	0.08

Table 7.34 Rumen PostMCS genera relationship with "log" serotonin values. VIPs from the PLS analysis, RA, direction of correlation, correlation coefficients (R) and p-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix (Appendix **Figure 7.60**).

PostMCS- Serotonin						
	Genus	**VIP**	**Mean RA**	**Direction**	**R**	**p-value**
A8	*vadinCA11 [Methanomassiliicoccaceae]*	2.95	0.02	Positive	0.66	<0.01
A109	*Mitsuokella*	2.41	0.14	Positive	0.50	0.01
A57	*Fibrobacter*	2.31	4.70	Negative	-0.51	0.01
A160	*Succinimonas*	2.18	0.005	Negative	-0.49	0.02
A53	*Unidentified_ Streptophyta*	2.14	0.001	Positive	0.48	0.02
A147	*Unidentified_ Neisseriaceae*	2.11	<0.001	Negative	-0.47	0.02
A44	*[Prevotella]*	1.96	0.09	Negative	-0.43	0.03
A105	*Unidentified_ Veillonellaceae*	1.91	5.60	Positive	0.43	0.04
A142	*Unidentified_ Rickettsiales*	1.78	<0.001	Positive	0.40	0.05
A20	*Other_ Bacteroidales*	1.78	0.41	Negative	-0.39	0.03
A140	*Unidentified_ Bradyrhizobiaceae*	1.75	<0.001	Negative	-0.39	0.06
A118	*Mogibacterium*	1.73	0.10	Positive	0.38	0.06
A75	*Butyrivibrio*	1.70	0.80	Positive	0.38	0.07
A69	*Anaerofustis*	1.66	0.003	Negative	-0.33	0.12
A95	*Bacteroides*	1.63	<0.001	Negative	-0.33	0.12
A31	*Other _Prevotellaceae*	1.63	0.02	Negative	-0.36	0.09
A4	*Methanosphaera*	1.62	0.02	Positive	0.36	0.08
A165	*Nevskia*	1.62	<0.001	Negative	-0.33	0.12
A32	*Unidentified_*	1.58	0.74	Positive	0.34	0.11

	Prevotellaceae					
A7	*Unidentified_ [Methanomassiliicoccaceae]*	1.51	0.02	Positive	0.33	0.11
A67	*Unidentified_ Dehalobacteriaceae*	1.50	<0.001	Negative	-0.30	0.16

Table 7.35 Rumen PostNT genera relationship with "log(x+1)" cortisol values. VIPs from the PLS analysis, RA, direction of correlation, correlation coefficients (R) and p-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix (Appendix **Figure 7.51**).

PostNT- Cortisol						
	Genus	**VIP**	**Mean RA**	**Direction**	**R**	**p-value**
A160	*Succinimonas*	3.03	<0.001	Positive	<0.01	0.99
A11	*Bifidobacterium*	3.03	<0.001	Positive	0.61	<0.01
A137	*Other Alphaproteobacteria*	3.03	<0.001	Positive	0.15	0.49
A84	*Shuttleworthia*	2.69	0.15	Negative	-0.49	0.02
A191	*Unidentified_ WCHB1-25_ WCHB1-41 Verruco-5*	2.64	0.004	Positive	0.45	0.03
A155	*Unidentified Myxococcales*	2.50	0.001	Positive	0.51	0.01
A183	*gut Anaeroplasmataceae*	2.50	<0.001	Positive	0.51	0.01
A69	*Anaerofustis*	2.28	0.005	Negative	-0.46	0.02
A112	*Schwartzia*	2.24	0.03	Negative	-0.27	0.20
A18	*Unidentified _RB046 SJA-176 Armatimonadetes*	2.00	0.008	Positive	0.41	0.05
A71	*Other_ Lachnospiraceae*	1.98	1.10	Negative	-0.40	0.05
A76	*Clostridium*	1.77	0.13	Negative	-0.37	0.07
A101	*Sporobacter*	1.68	0.003	Positive	0.34	0.10

A170	*Sphaerochaeta*	1.59	0.035	Negative	-0.22	0.30
A90	*Peptostreptococcus*	1.58	<0.001	Positive	0.32	0.12
A97	*Clostridium*	1.52	0.03	Negative	-0.32	0.13
A25	*BF311_Bacteroidaceae*	1.51	0.17	Positive	0.30	0.15
A109	*Mitsuokella*	1.51	0.17	Negative	-0.19	0.37
A98	*Oscillospira*	1.51	0.06	Positive	0.33	0.12
A22	*Unidentified BS11 Bacteroidales*	1.50	0.05	Positive	0.30	0.16
A23	*Unidentified Bacteroidaceae*	1.50	0.01	Positive	0.29	0.16

Table 7.36 Rumen PostNT genera relationship with "log" serotonin values. VIPs from the PLS analysis, RA, direction of correlation, correlation coefficients (R) and p-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix (Appendix **Figure 7.57**).

PostNT- Serotonin						
	Genus	**VIP**	**Mean RA**	**Direction**	**R**	**p-value**
A127	*p-75a5_ Erysipelotrichaceae*	2.76	0.006	Negative	-0.53	0.01
A12	*Other_Coriobacteriaceae*	2.62	0.003	Positive	0.51	0.01
A36	*Blvii28_Rikenellaceae*	2.29	<0.001	Positive	0.44	0.03
A17	*Slackia*	2.29	<0.001	Positive	0.44	0.03
A30	*Porphyromonas*	2.27	0.003	Positive	0.44	0.03
A103	*Syntrophomonas*	2.25	<0.001	Positive	0.43	0.04
A182	*Anaeroplasma*	2.10	0.11	Negative	-0.41	0.05
A145	*Other_Comamonadaceae*	2.09	<0.001	Positive	0.35	0.10
A154	*Unidentified_ GMD14H09 Deltaproteobacteria*	2.03	0.01	Positive	0.39	0.06
A15	*Atopobium*	1.78	0.03	Negative	-0.34	0.10
A124	*RFN20_*	1.77	0.15	Positive	0.34	0.10

	Erysipelotrichaceae					
A4	*Methanosphaera*	1.74	0.02	Positive	0.34	0.11
A147	*Unidentified_ Neisseriaceae*	1.71	<0.001	Negative	-0.33	0.11
A78	*Dorea*	1.64	0.001	Positive	0.32	0.13
A110	*Phascolarctobacterium*	1.60	<0.001	Positive	0.30	0.16
A10	*Unidentified_ Bifidobacteriaceae*	1.57	0.001	Positive	0.30	0.15
A54	*Unidentified_ Elusimicrobiaceae*	1.57	<0.001	Negative	-0.30	0.16
A179	*Unidentified_ Acholeplasmatales*	1.57	<0.001	Negative	-0.30	0.16
A106	*Acidaminococcus*	1.56	0.004	Positive	0.30	0.15
A140	*Unidentified_ Bradyrhizobiaceae*	1.56	<0.001	Positive	0.30	0.15
A165	*Nevskia*	1.56	<0.001	Positive	0.30	0.15
A144	*Sutterella*	1.55	0.003	Negative	-0.30	0.16
A81	*Moryella*	1.55	0.12	Negative	-0.30	0.16
A111	*Propionispira*	1.54	0.001	Negative	0.29	0.17
A126	*[Eubacterium]*	1.52	0.03	Positive	0.27	0.19
A25	*BF311_Bacteroidaceae*	1.52	0.18	Positive	0.29	0.17

7.4.2 Correlation matrices for rumen phyla orders and genera with serotonin

7.4.2.1 Correlations between relative abundances for NT group and serotonin

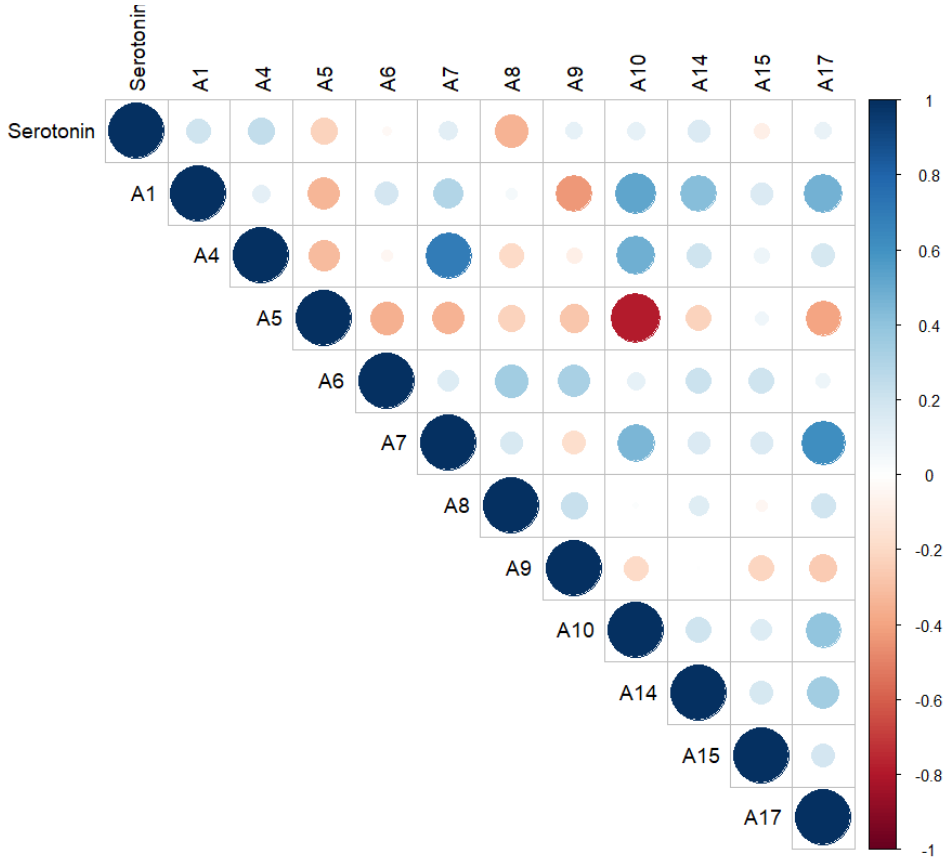

Figure 7.44 Spearman's rank correlation matrices for SRT rumen NT phylum abundances and serotonin values. The matrix depicts relationships among phyla, and between phyla and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two phyla. Phyla and VIPs obtained from the PLS regression are also shown in **Table 7.37.**

Table 7.37 Rumen NT phyla with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT phyla relative abundances and serotonin. The VIP score from the PLS analysis, mean relative abundance of each phyla and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.45**

	Phylum	**VIP**	**Mean RA**
A8	*Elusimicrobia*	1.45	<0.001
A6	*Chloroflexi*	1.36	0.08
A1	*Euryarchaeota*	1.32	0.50
A9	*Fibrobacteres*	1.29	3.94
A5	*Bacteroidetes*	1.21	67.80
A10	*Firmicutes*	1.20	23.42
A15	*Proteobacteria*	1.19	0.58
A7	*Cyanobacteria*	1.16	0.30
A17	*Synergistetes*	1.06	0.55
A14	*Planctomycetes*	1.00	0.15
A4	*Armatimonadetes*	1.00	0.008

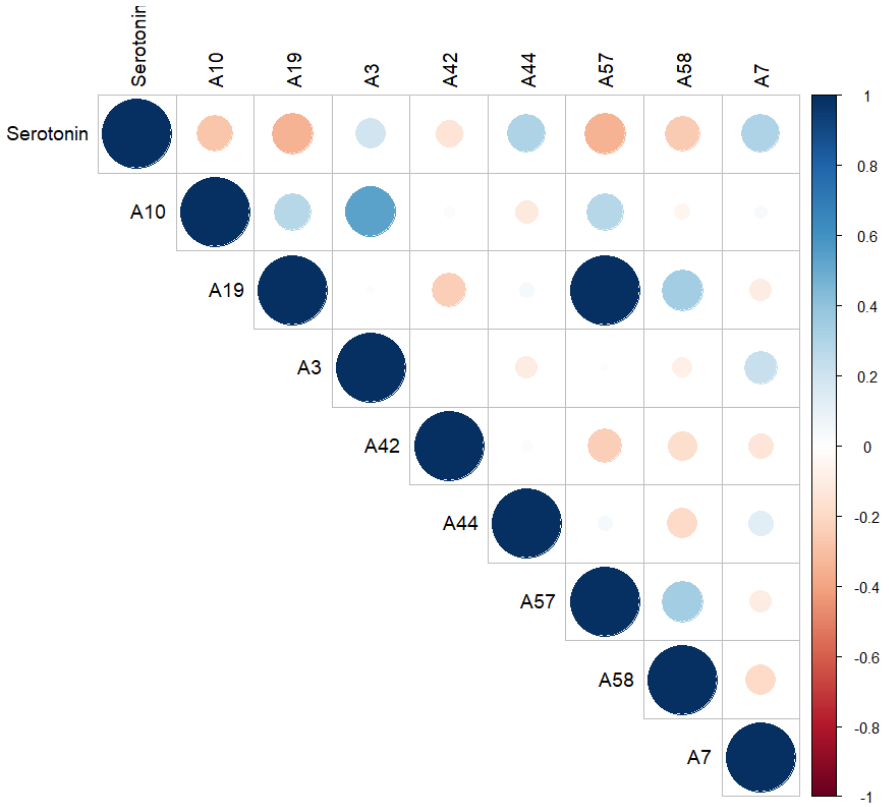

Figure 7.45 Spearman's rank correlation matrices for SRT rumen NT order abundances and serotonin values. The matrix depicts relationships among orders, and between orders and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two orders. Orders and VIPs obtained from the PLS regression are also shown in **Table 7.38.**

Table 7.38 Rumen NT orders with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT order relative abundances and serotonin. The VIP score from the PLS analysis, mean relative abundance of each order and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.46.**

	Order	**VIP**	**Mean RA**
A44	*Deltaproteobacteria GMD14H09*	2.08	0.007
A42	*Desulfobacterales*	2.05	0.009
A10	*Bacteroidetes_Other*	1.69	0.03
A7	*Bifidobacteriales*	1.65	0.001
A3	*Methanobacteriales*	1.51	0.32
A19	*Elusimicrobiales*	1.50	0.007
A57	*Acholeplasmatales*	1.50	<0.001
A58	*Anaeroplasmatales*	1.50	0.15

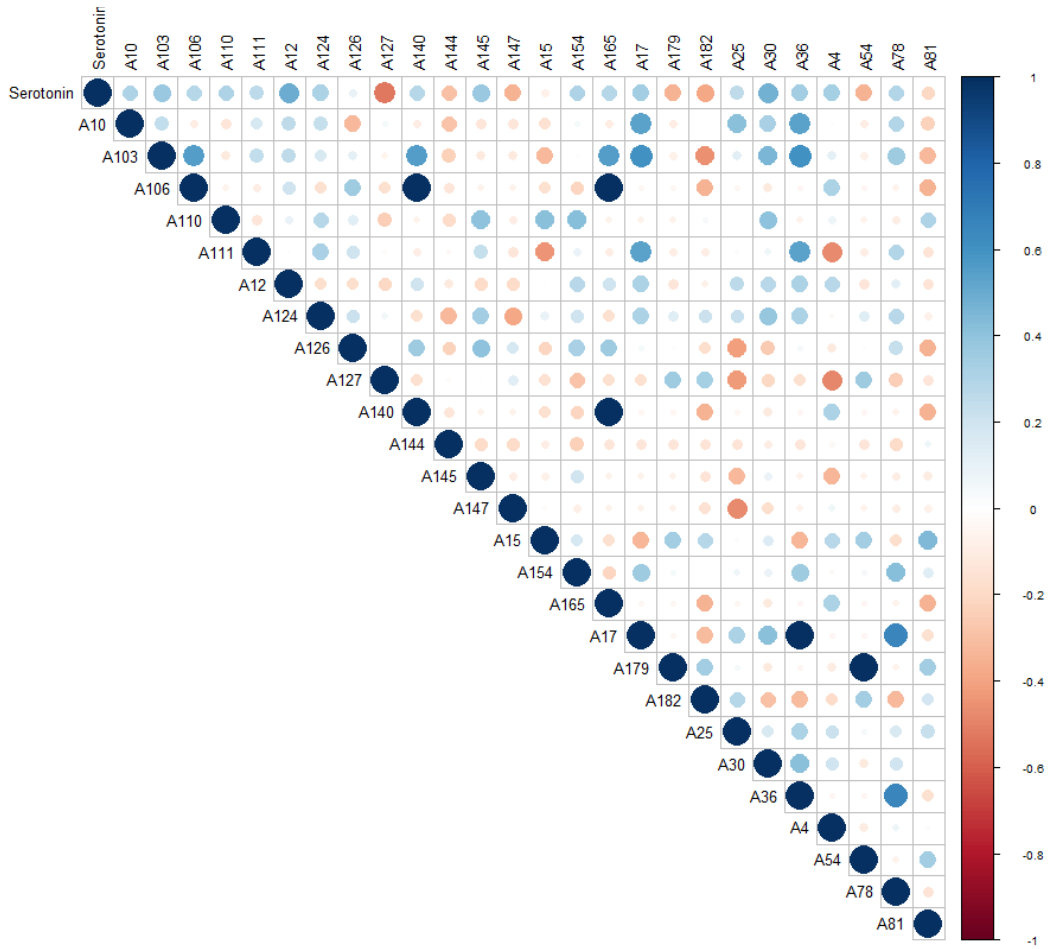

Figure 7.46 Spearman's rank correlation matrices for SRT rumen NT genus abundances and serotonin values. The matrix depicts relationships among genera, and between genera and serotonin on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two genera. Genera and VIPs obtained from the PLS regression are also shown in **Table 7.39.**

Table 7.39 Rumen NT genera with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT genus relative abundances and serotonin. The VIP score from the PLS analysis, mean relative abundance of each genus and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.47.**

	Genus	**VIP**	**Mean RA**
A127	*p-75-a5_Erysipelotrichaceae*	2.76	0.006
A12	*Other_Coriobacteriaceae*	2.62	0.003
A36	*Blvii28_Rikenellaceae*	2.29	<0.001
A17	*Slackia*	2.29	<0.001
A30	*Porphyromonas*	2.27	0.003
A103	*Syntrophomonas*	2.25	<0.001
A182	*Anaeroplasma*	2.10	0.11
A145	*Other_Comamonadaceae*	2.09	<0.001
A154	*Unidentified_ GMD14H09 Deltaproteobacteria*	2.03	0.01
A15	*Atopobium*	1.78	0.03
A124	*RFN20_ Erysipelotrichaceae*	1.77	0.15
A4	*Methanosphaera*	1.74	0.02
A147	*Unidentified_ Neisseriaceae*	1.71	<0.001
A78	*Dorea*	1.64	0.001
A110	*Phascolarctobacterium*	1.60	<0.001
A10	*Unidentified_ Bifidobacteriaceae*	1.57	0.001
A54	*Unidentified_ Elusimicrobiaceae*	1.57	<0.001

A179	*Unidentified_ Acholeplasmatales*	1.57	<0.001
A106	*Acidaminococcus*	1.56	0.004
A140	*Unidentified_ Bradyrhizobiaceae*	1.56	<0.001
A165	*Nevskia*	1.56	<0.001
A144	*Sutterella*	1.55	0.003
A81	*Moryella*	1.55	0.12
A111	*Propionispira*	1.54	0.001
A126	*[Eubacterium]*	1.52	0.03
A25	*BF311_Bacteroidaceae*	1.52	0.18

7.4.2.2 Correlation between abundances for MCS group and serotonin

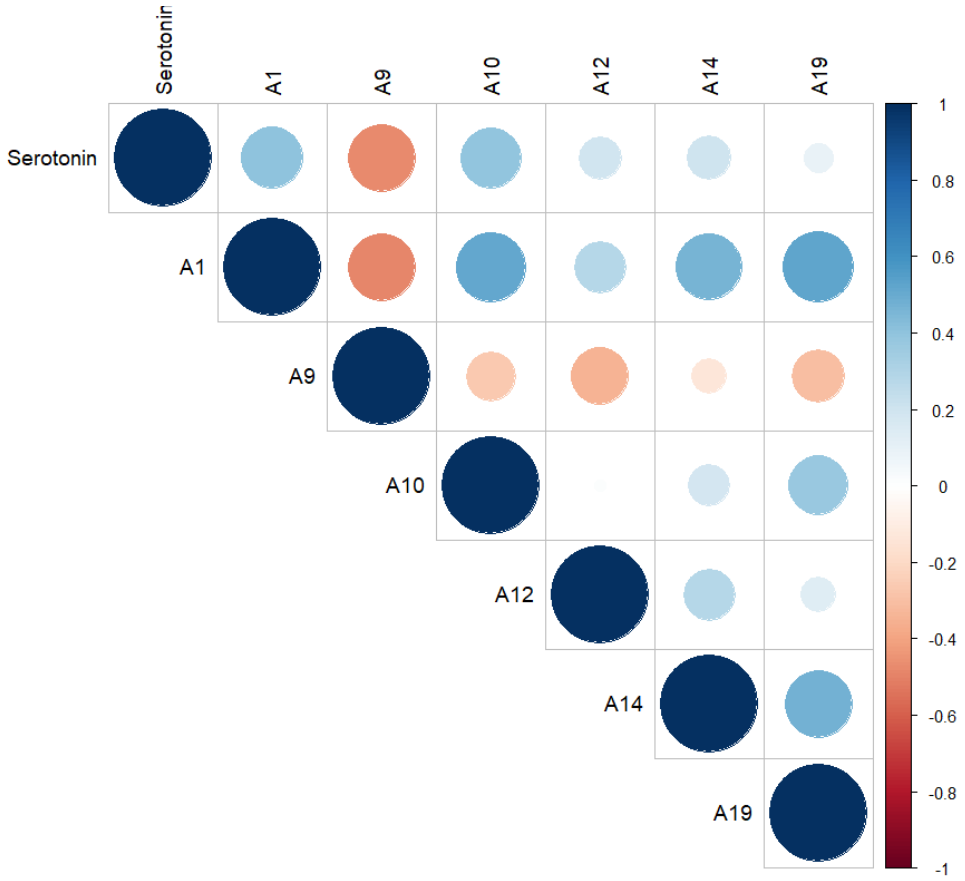

Figure 7.47 Spearman's rank correlation matrices for SRT rumen MCS phylum abundances and serotonin values. The matrix depicts relationships among phyla, and between phyla and serotonin on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two phyla. Phyla and VIPs obtained from the PLS regression are also shown in **Table 7.40.**

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Table 7.40 Rumen MCS phyla with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT phylum relative abundances and serotonin. The VIP score from the PLS analysis, mean relative abundance of each phylum and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.48.**

	Phylum	**VIP**	**Mean RA**
A9	*Fibrobacteres*	2.07	4.67
A10	*Firmicutes*	1.79	21.00
A1	*Euryarchaeota*	1.68	0.38
A19	*Verrucomicrobia*	1.21	0.05
A14	*Planctomycetes*	1.20	0.17
A12	*LD1*	1.01	0.001

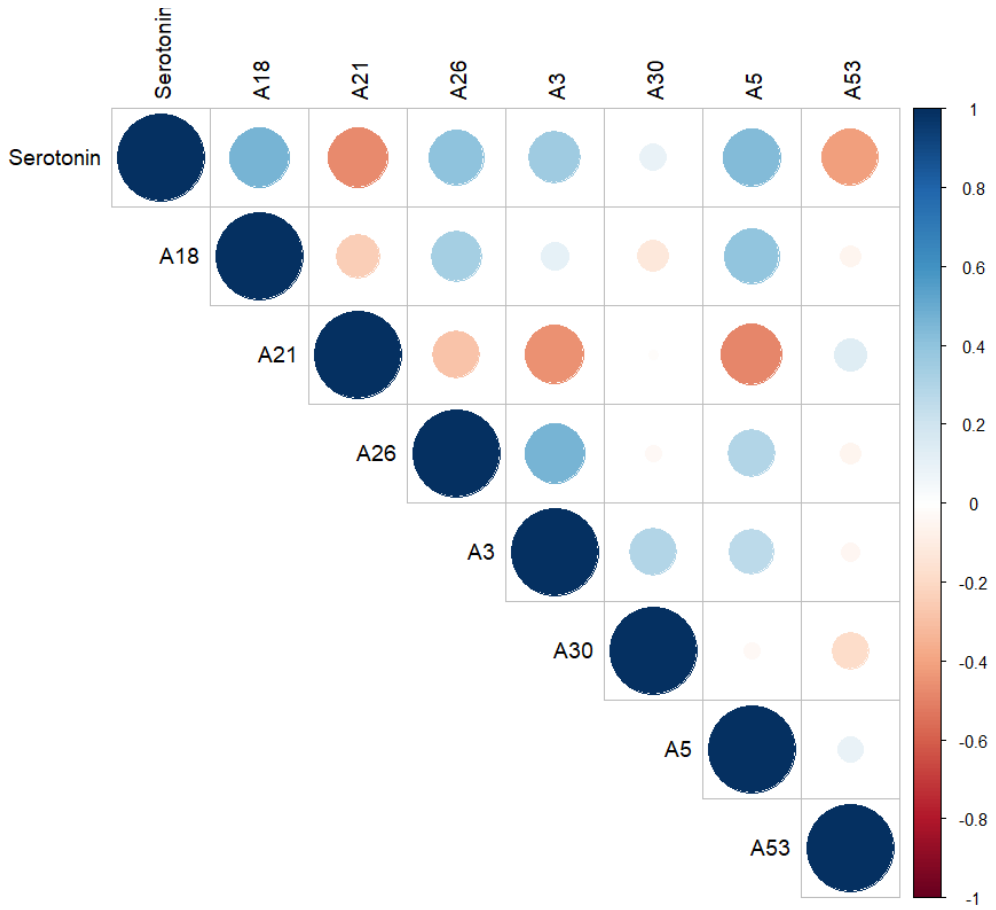

Figure 7.48 Spearman's rank correlation matrices for SRT rumen MCS order abundances and serotonin values. The matrix depicts relationships among orders, and between orders and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two orders. Orders and VIPs obtained from the PLS regression are also shown in **Table 7.41.**

Table 7.41 Rumen MCS orders with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT order relative abundances and serotonin. The VIP score from the PLS analysis, mean relative abundance of each order and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.49.**

	Order	**VIP**	**Mean RA**
A5	*c_Thermoplasmata;o_E2*	2.19	0.05
A21	*Fibrobacterales*	2.18	4,67
A18	*Streptophyta*	2.06	0.002
A26	*Clostridiales*	1.87	20.25
A53	*Sphaerochaetales*	1.82	0.05
A38	*Rickettsiales*	1.69	>0.001
A3	*Methanobacteriales*	1.68	0.33

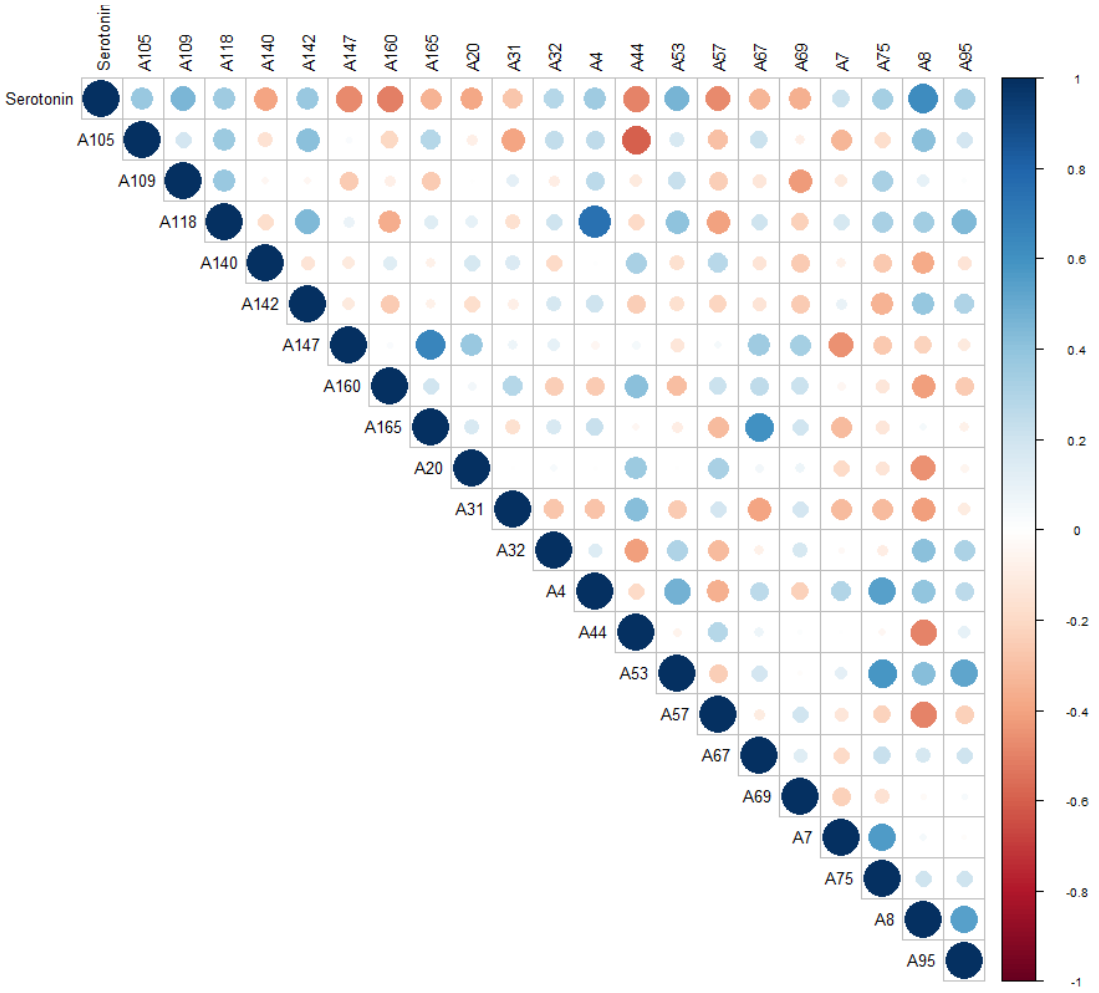

Figure 7.49 Spearman rank correlation matrices for SRT rumen MCS genus abundances and serotonin values. The matrix depicts relationships among genera, and between genera and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two genera. Genera and VIPs obtained from the PLS regression are also shown in **Table 7.42.**

Table 7.42 Rumen MCS genera with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT genus relative abundances and serotonin. The VIP score from the PLS analysis, mean relative abundance of each genus and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.50.**

	Genus	**VIP**	**Mean RA**
A8	*vadinCA11 [Methanomassiliicoccaceae]*	2.95	0.02
A109	*Mitsuokella*	2.41	0.14
A57	*Fibrobacter*	2.31	4.70
A160	*Succinimonas*	2.18	0.005
A53	*Unidentified_ Streptophyta*	2.14	0.001
A147	*Unidentified_ Neisseriaceae*	2.11	<0.001
A44	*[Prevotella]*	1.96	0.09
A105	*Unidentified_ Veillonellaceae*	1.91	5.60
A142	*Unidentified_ Rickettsiales*	1.78	<0.001
A20	*Other_ Bacteroidales*	1.78	0.41
A140	*Unidentified_ Bradyrhizobiaceae*	1.75	<0.001
A118	*Mogibacterium*	1.73	0.10
A75	*Butyrivibrio*	1.70	0.80
A69	*Anaerofustis*	1.66	0.003
A95	*Bacteroides*	1.63	<0.001
A31	*Other _Prevotellaceae*	1.63	0.02
A4	*Methanosphaera*	1.62	0.02
A165	*Nevskia*	1.62	<0.001
A32	*Unidentified_Prevotellaceae*	1.58	0.74
A7	*Unidentified_ [Methanomassiliicoccaceae]*	1.51	0.02
A67	*Unidentified_*	1.50	<0.001

	Dehalobacteriaceae		

7.4.3 Correlation matrices for rumen phyla orders and genera with cortisol

7.4.3.1 Correlations between relative abundances for NT group and cortisol

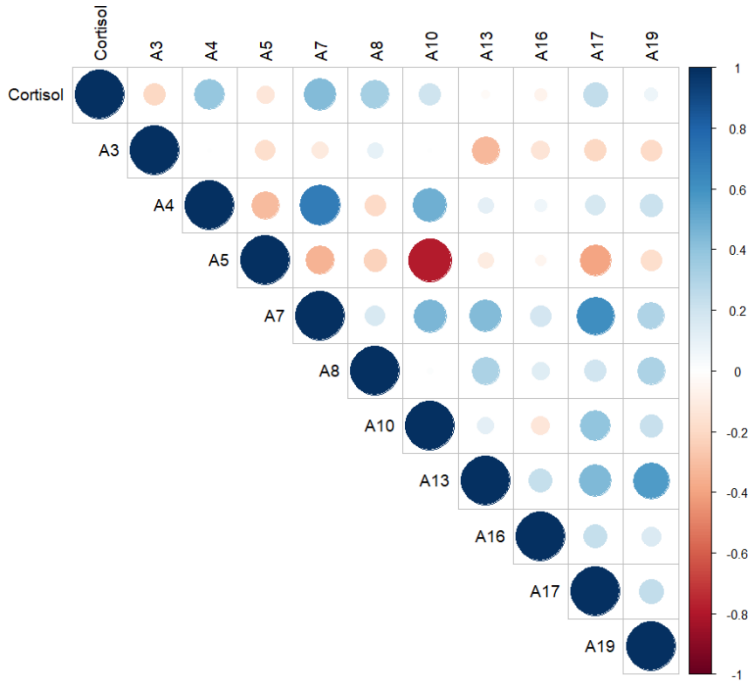

Figure 7.50 Spearman's rank correlation matrices for SRT rumen phyla NT abundances and cortisol values. The matrix depicts relationships among phyla, and between phyla and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two phyla. Phyla and VIPs obtained from the PLS regression are also shown in **Table 7.43**.

Table 7.43 Rumen NT phyla with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT phyla relative abundances and cortisol. The VIP score from the PLS analysis, mean relative abundance of each phylum and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.51..**

	Phylum	**VIP**	**Mean RA**
A4	*Armatimonadetes*	1.97	0.008

A7	*Cyanobacteria*	1.48	0.30
A13	*Lentisphaerae*	1.21	0.24
A8	*Elusimicrobia*	1.21	<0.001
A3	*Actinobacteria*	1.20	0.23
A17	*Synergistetes*	1.16	0.54
A10	*Firmicutes*	1.11	23.42
A19	*Verrucomicrobia*	1.10	0.06
A16	*Spirochaetes*	1.02	1.00
A5	*Bacteroidetes*	1.01	67.80

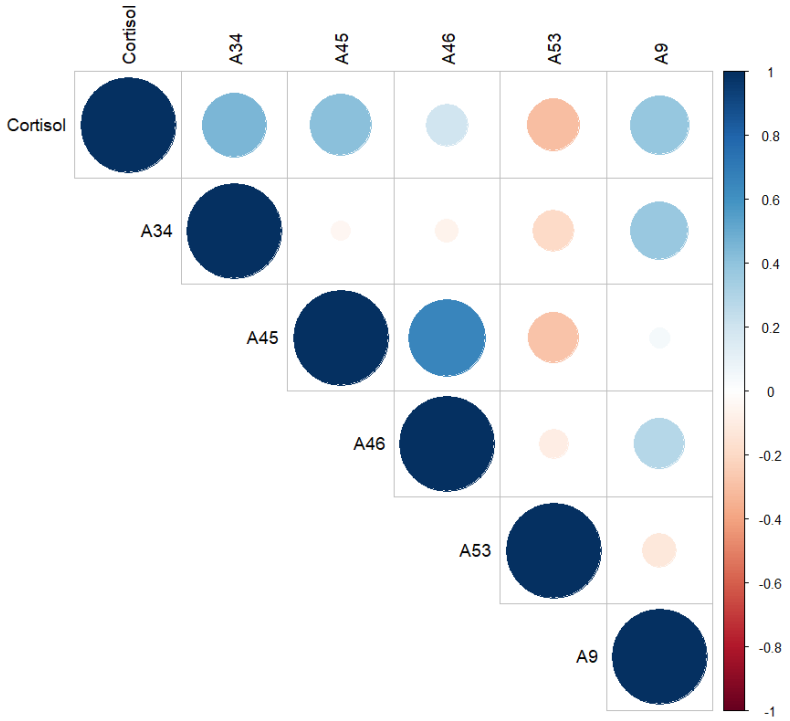

Figure 7.51 Spearman's rank correlation matrices for SRT rumen order NT abundances and cortisol values. The matrix depicts relationships among orders, and between order and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two orders. orders and VIPs obtained from the PLS regression are also shown in **Table 7.44.**

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Table 7.44 Rumen NT orders with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT order relative abundances and cortisol. The VIP score from the PLS analysis, mean relative abundance of each order and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.52**.

	Order	VIP	Mean RA
A34	*Alphaproteobacteria Other*	3.15	<0.001
A45	*Myxococcales*	2.42	0.001
A9	*Coriobacteriales*	1.93	0.008
A53	*Sphaerochaetales*	1.81	0.05
A46	*Gammaproteobacteria Other*	1.53	0.001

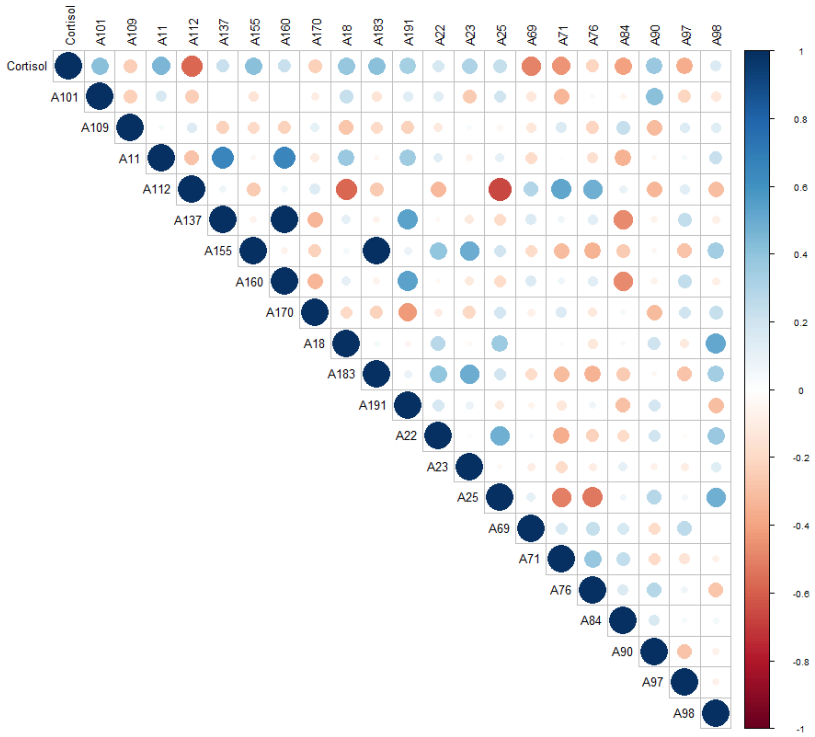

Figure 7.52 Spearman's rank correlation matrices for SRT rumen NT genus abundances and cortisol values. The matrix depicts relationships among genera, and between genera and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two genera. Genera and VIPs obtained from the PLS regression are also shown in **Table 7.45.**

Table 7.45 Rumen NT genera with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT genus relative abundances and cortisol. The VIP score from the PLS analysis, mean relative abundance of each genus and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.53**

	Genus	**VIP**	**Mean RA**
A160	*Succinimonas*	3.03	<0.001
A11	*Bifidobacterium*	3.03	<0.001
A137	*Other Alphaproteobacteria*	3.03	<0.001
A84	*Shuttleworthia*	2.69	0.15
A191	*Unidentified_ WCHB1-25_ WCHB1-41 Verruco-5*	2.64	0.004
A155	*Unidentified Myxococcales*	2.50	0.001
A183	*gut Anaeroplasmataceae*	2.50	<0.001
A69	*Anaerofustis*	2.28	0.005
A112	*Schwartzia*	2.24	0.03
A18	*Unidentified _RB046 SJA-176 Armatimonadetes*	2.00	0.008
A71	*Other_ Lachnospiraceae*	1.98	1.10
A76	*Clostridium*	1.77	0.13
A101	*Sporobacter*	1.68	0.003
A170	*Sphaerochaeta*	1.59	0.035
A90	*Peptostreptococcus*	1.58	<0.001
A97	*Clostridium*	1.52	0.03
A25	*BF311_Bacteroidaceae*	1.51	0.17
A109	*Mitsuokella*	1.51	0.17
A98	*Oscillospira*	1.51	0.06
A22	*Unidentified BS11 Bacteroidales*	1.50	0.05
A23	*Unidentified Bacteroidaceae*	1.50	0.01

7.4.3.2 Correlations between Relative Abundances for MCS group and cortisol

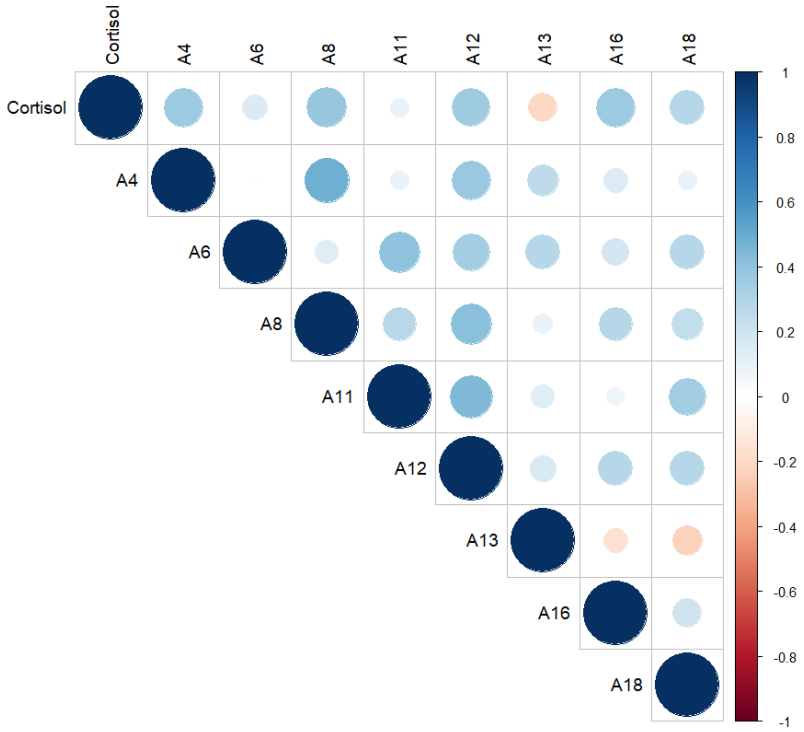

Figure 7.53 Spearman's rank correlation matrices for SRT rumen phyla MCS abundances and cortisol values. The matrix depicts relationships among phyla, and between phyla and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two phyla. Phyla and VIPs obtained from the PLS regression are also shown in **Table 7.46.**

Table 7.46 Rumen MCS phyla with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT phylum relative abundances and cortisol. The VIP score from the PLS analysis, mean relative abundance of each phylum and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.54.**

	Phylum	**VIP**	**Mean RA**
A12	*LD1*	1.67	0.001
A16	*Spirochaetes*	1.66	0.60
A4	*Armatimonadetes*	1.62	0.005
A18	*Tenericutes*	1.45	0.30

A13	*Lentisphaerae*	1.33	0.30
A6	*Chloroflexi*	1.22	0.07
A11	*Fusobacteria*	1.03	0.002
A8	*Elusimicrobia*	1.01	0.008

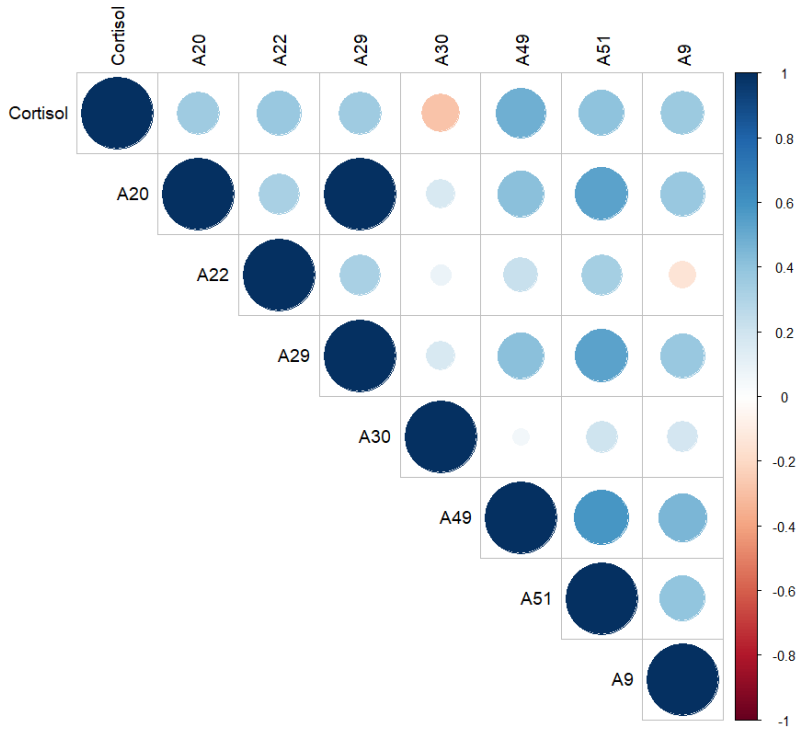

Figure 7.54 Spearman's rank correlation matrices for SRT rumen MCS order abundances and cortisol values. The matrix depicts relationships among orders, and between orders and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two orders. Orders and VIPs obtained from the PLS regression are also shown in **Table 7.47.**

Table 7.47 Rumen MCS orders with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT order relative abundances and cortisol. The VIP score from the PLS analysis, mean relative abundance of each order and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.55.**

	Order	**VIP**	**Mean RA**
A49	*Pseudomonadales*	2.27	0.003
A51	*p_Spirochaetes;c_MVP-15;o_PL-11B10*	2.05	0.005
A30	*Victivallales*	1.98	0.26
A22	*Unidentified_Firmicutes*	1.81	0.03
A20	*Unidentified_Endomicrobia*	1.75	0.001
A29	*Unidentified_LD1*	1.75	0.001
A9	*Coriobacteriales*	1.52	0.005

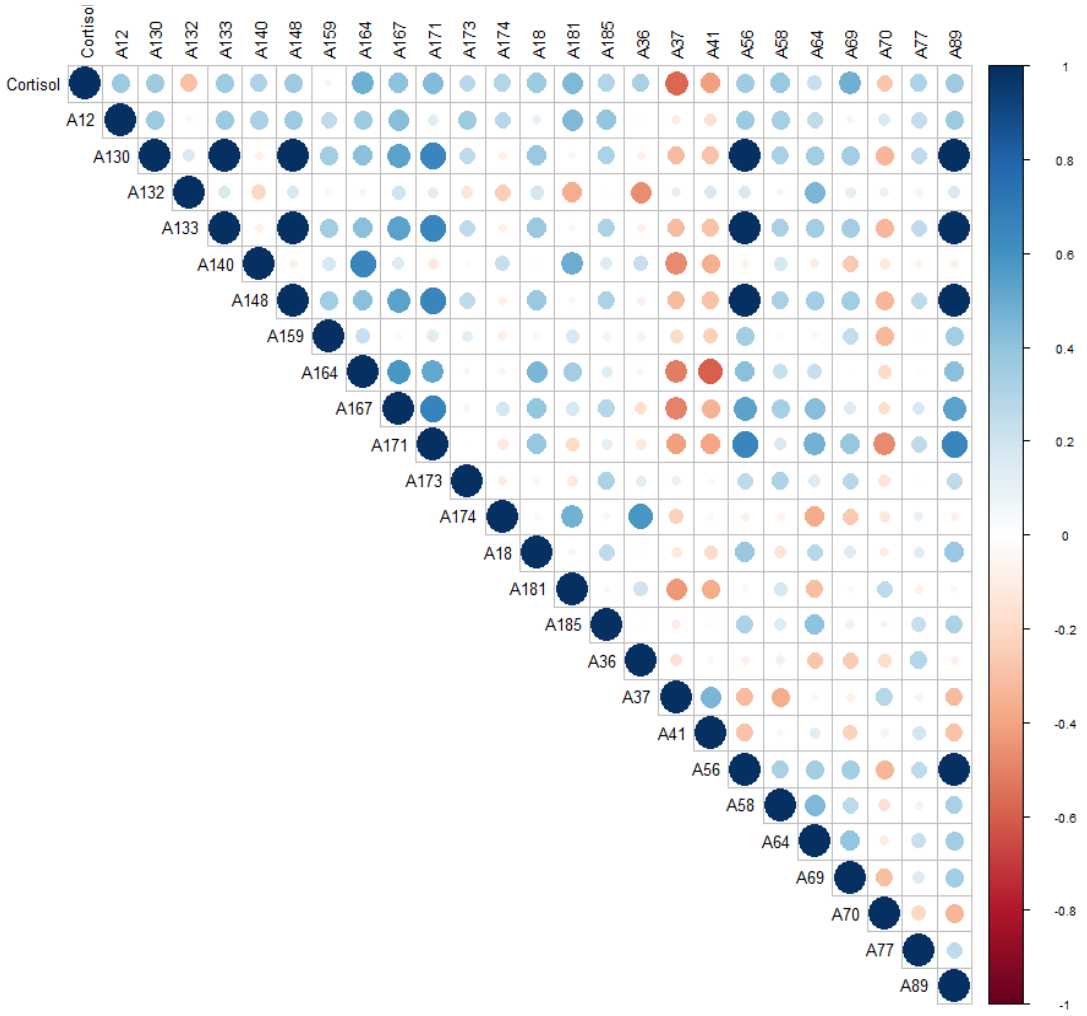

Figure 7.55 Spearman's rank correlation matrices for SRT rumen MCS genus abundances and cortisol values. The matrix depicts relationships among genera, and between genera and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two genera. Genera and VIPs obtained from the PLS regression are also shown in **Table 7.48.**

Table 7.48 Rumen MCS genera with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT genus relative abundances and cortisol. The VIP score from the PLS analysis, mean relative abundance of each genus and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.56.**

	Genus	**VIP**	**Mean RA**
A164	*Pseudomonas*	2.54	0.03
A167	*Spirochaetes;c_MVP-15;o_PL-11B10_Unidentified*	2.27	0.004
A70	*Unidentified_ Victivallaceae*	2.08	0.05
A58	*Firmicutes;Other*	2.02	0.03
A181	*Unidentified_ Anaeroplasmataceae*	1.97	0.01
A132	*Unidentified_ Victivallaceae*	1.96	0.26
A36	*Rikenellaceae_Blvii28*	1.95	0.001
A56	*Unidentified_ Endomicrobia*	1.95	0.001
A133	*Victivallis*	1.95	<0.001
A89	*Filifactor*	1.95	<0.001
A130	*p_LD1_Unidentified*	1.95	0.001
A148	*Alysiella*	1.95	<0.001
A171	*Sphaerochaetaceae_wall-less*	1.89	0.001
A37	*Unidentified_S24-7 Bacteroidales*	1.82	2.02
A64	*Unidentified Christensenellaceae*	1.81	0.08
A77	*Coprococcus*	1.72	0.22
A41	*Unidentified [Paraprevotellaceae]*	1.71	1.53
A18	*Unidentified_ RB04_*	1.71	0.005

	SJA-176 *Armatimonadetes*		
A140	*Unidentified Bradyrhizobiaceae*	1.64	<0.001
A69	*Anaerofustis*	1.64	0.003
A12	*Other_* *Coriobacteriaceae*	1.61	0.005
A173	*Treponema*	1.60	0.53
A174	*Other_* *Dethiosulfovibrionaceae*	1.57	0.001
A159	*Ruminobacter*	1.55	0.03
A185	*Unidentified_RF39_* *Mollicutes*	1.51	0.13

7.5 Faecal community composition

Table 7.49 Composition of Faecal microbiota community. The most abundant phyla, orders and genera are presented as a percentage of the total population. The average percentage and minimum and maximum percentages are reported.

Phyla			
	Average%	**Max%**	**Min%**
Firmicutes	56.3	57.20	42.40
Bacteroidetes	35.50	50.10	10.10
Spirochaetes	2.20	4.00	0.20
None;Other	1.90	12.90	0.00
Tenericutes	1.10	3.20	0.20
Cyanobacteria	0.50	0.60	0.00
Euryarcheota	1.10	3.50	0.00
Orders			

	Average%	Max%	Min%
Clostridiales	55.60	56.80	42.20
Bacteroidales	35.40	49.50	10.00
Spirochaetales	1.80	4.00	0.2
Methanomicrobiales	1.00	3.2	0.00
Genera			
	Average%	**Max%**	**Min%**
Unidentified_Ruminococcaceae	23.60	25.60	10.00
Bacteroidaceae_ J_7N15	8.40	20.40	4.60
Unidentified_Bacteroidales	8.60	19.70	3.20
Unidentified_ Bacteroidaceae	6.20	10.80	0.40
Ruminococcus	5.70	22.30	0.10
Unidentified_Rikenellaceae	4.50	5.30	0.9
Oscillospira	3.70	6.70	0.10
Unidentified_Lachnospiraceae	3.50	7.20	0.00
Treponema	1.80	5.10	0.00
Other__Ruminococcaceae	1.70	6.40	0.00
Unidentified_Clostridiales	1.40	.00	0.00
Other_Lachnospiraceae	1.20	3.60	0.00
Coprococcus	1.10	2.80	0.00
Bacteroides	1.60	13.60	0.00
Methanocorpusculaceae	1.00	3.20	0.00

7.5.1 PLS phylum, order and *genus level*

Table 7.50 Faecal PostMCS phyla relationship with "log(x+1)" cortisol values and "log" cortisol/serotonin values. VIPs from the PLS analysis, RA, direction of correlation, correlation

coefficients (R) and P-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix in the Appendix (**Figure 7.64, 7.70)**.

PostMCS- Cortisol						
	Phylum	**VIP**	**Mean RA**	**Direction**	**R**	**P**
A5	*Bacteroidetes*	2.03	35.88	Negative	-0.58	<0.01
A10	*Firmicutes*	1.64	52.90	Positive	0.47	0.02
A11	*Lentisphaerae*	1.62	0.32	Positive	0.46	0.02
A16	*Tenericutes*	1.43	1.12	Positive	0.40	0.05
A18	*WPS-2*	1.20	0.001	Positive	0.30	0.16
PostMCS- Serotonin						
	Phylum	**VIP**	**Mean RA**	**Direction**	**R**	**P**
A11	*Lentisphaerae*	1.91	0.32	Negative	-0.41	0.05
A2	*Euryarchaeota*	1.75	1.07	Negative	-0.38	0.07
A13	*Proteobacteria*	1.24	0.43	Positive	0.10	0.64
A17	*Verrucomicrobia*	1.16	0.11	Negative	-0.13	0.54
A18	*WPS-2*	1.11	0.001	Negative	-0.23	0.29
A14	*Spirochaetes*	1.10	1.90	Positive	0.24	0.27

Table 7.51 Faecal PostNT phyla relationship with "log(x+1)" cortisol values and "log" cortisol/serotonin values. VIPs from the PLS analysis, relative abundance, direction of correlation, correlation coefficients (R) and P-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix in the Appendix (**Figure 7.61, Figure 7.67)**.

PostNT- Cortisol						
	Phylum	**VIP**	**Mean RA**	**Direction**	**R**	**P**
A8	*Elusimicrobia*	1.77	0.04	Negative	-0.37	0.07
A12	*Planctomycetes*	1.72	0.35	Positive	0.40	0.05
A17	*Verrucomicrobia*	1.27	0.09	Positive	0.29	0.17
A15	*Synergistetes*	1.24	0.001	Positive	0.28	0.19
A13	*Proteobacteria*	1.13	0.46	Positive	0.26	0.22
A9	*Fibrobacteres*	1.10	0.61	Negative	-0.07	0.75
A11	*Lentisphaerae*	1.01	0.32	Positive	0.23	0.27
PostNT- Serotonin						
	Phylum	**VIP**	**Mean RA**	**Direction**	**R**	**P**
A9	*Fibrobacteres*	2.33	0.61	Negative	-0.45	0.03
A13	*Proteobacteria*	1.35	0.46	Positive	0.14	0.53
A12	*Planctomycetes*	1.30	0.35	Positive	0.16	0.46
A6	*Chloroflexi*	1.10	0.002	Negative	-0.21	0.33
A4	*Actinobacteria*	1.05	0.24	Positive	0.15	0.48
A17	*Verrucomicrobia*	1.01	0.09	Negative	-0.02	0.92

Table 7.52 Faecal PostMCS orders relationship with "log(x+1)" cortisol values and "log" serotonin values. VIPs from the PLS analysis, relative abundance, direction of correlation, correlation coefficients (R) and P-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix in the Appendix (**Figure 7.65, Figure 7.71)**.

PostMCS- Cortisol						
	Order	**VIP**	**Mean RA**	**Direction**	**R**	**P**
A11	*Bacteroidales*	2.49	35.85	Negative	-0.57	0.003
A27	*Victivallales*	2.02	0.31	Positive	0.47	0.02
A24	*Clostridiales*	1.98	54.16	Positive	0.46	0.02
A59	*ML615J-28_ RF3_ Tenericutes*	1.80	0.68	Positive	0.42	0.04
A35	*Rhodospirillales*	1.74	0.007	Negative	-0.40	0.05
A57	*Mycoplasmatales*	1.72	0.01	Positive	0.40	0.05
A60	*HA64_ Opitutae*	1.57	0.005	Positive	0.36	0.09
A30	*Other_Proteobacteria*	1.53	0.03	Positive	0.36	0.09
A55	*Other_Mollicutes*	1.52	0.002	Positive	0.35	0.09
PostMCS- Serotonin						
	Order	**VIP**	**Mean RA**	**Direction**	**R**	**P**
A38	*Burkholderiales*	2.48	0.13	Positive	0.51	0.01
A3	*Methanomicrobiales*	2.10	0.95	Negative	-0.44	0.03
A27	*Victivallales*	1.96	0.31	Negative	-0.41	0.05
A20	*Bacillales*	1.76	<0.001	Positive	0.35	0.09
A18	*Other_Firmicutes*	1.63	0.008	Positive	0.34	0.11
A5	*E2_Thermoplasmata*	1.53	0.03	Positive	0.32	0.13
A2	*Methanobacteriales*	1.52	0.08	Positive	0.30	0.15

Table 7.53 Faecal PostNT orders relationship with "log(x+1)" cortisol values and "log" serotonin values. VIPs from the PLS analysis, RA, direction of correlation, correlation coefficients (R) and P-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix in the Appendix (**Figure 7.62, Figure 7.68)**.

PostNT- Cortisol						
	Order	**VIP**	**Mean RA**	**Direction**	**R**	**P**
A13	*Flavobacteriales*	2.18	0.009	Positive	0.51	0.01
A18	*Other_Firmicutes*	1.84	0.008	Negative	-0.42	0.04
A22	*Turicibacterales*	1.81	0.10	Negative	-0.42	0.04
A29	*Pirellulales*	1.70	0.35	Positive	0.40	0.05
A16	*Elusimicrobiales*	1.59	0.04	Negative	-0.37	0.07
A4	*Methanosarcinales*	1.51	<0.001	Positive	0.25	0.23
PostNT- Serotonin						
	Order	**VIP**	**Mean RA**	**Direction**	**R**	**P**
A25	*SHA-98_ Clostridia*	2.43	0.01	Positive	0.50	0.01
A17	*Fibrobacterales*	2.15	0.62	Negative	-0.45	0.03
A57	*Mycoplasmatales*	1.71	0.007	Negative	-0.28	0.18
A28	*Z20_ [Lentisphaeria]*	1.69	0.001	Positive	0.35	0.09
A23	*Other_ Clostridia*	1.64	0.02	Positive	0.34	0.10
A47	*Xanthomonadales*	1.56	0.008	Positive	0.31	0.15

Table 7.54 Faecal PostMCS genera relationship with "log(x+1)" cortisol values. VIPs from the PLS analysis, RA, direction of correlation, correlation coefficients (R) and P-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix in the Appendix (**Figure 7.66).**

PostMCS- Cortisol						
	Genus	**VIP**	**Mean RA**	**Direction**	**R**	**P**
A85	*Clostridium*	2.79	0.04	Negative	-0.60	<0.01
A150	*Unidentified _Victivallaceae*	2.21	0.28	Positive	0.48	0.02
A73	*02d06_ Clostridiaceae*	2.18	0.20	Positive	0.47	0.02
A42	*Butyricimonas*	2.16	0.02	Negative	-0.46	0.02
A143	*PSB-M-3_ Erysipelotrichaceae*	2.09	0.008	Positive	0.44	0.03
A160	*Bradyrhizobium*	2.06	<0.001	Positive	0.44	0.03
A60	*Bacillus*	1.98	<0.001	Positive	0.43	0.04
A136	*ph2_[Tissierellaceae]*	1.98	<0.001	Positive	0.43	0.04
A194	*Unidentified_ Mycoplasmataceae*	1.98	0.001	Positive	0.43	0.04
A108	*Anaerotruncus*	1.97	0.10	Positive	0.42	0.04
A197	*Unidentified_ML615J-28_ RF3_Tenericutes*	1.93	0.68	Positive	0.42	0.04
A163	*Unidentified_Acetobacteraceae*	1.88	0.007	Negative	-0.40	0.05
A34	*Other_ Rikenellaceae*	1.85	0.01	Negative	-0.40	0.05
A97	*Syntrophococcus*	1.77	<0.001	Positive	0.38	0.07
A41	*Barnesiella*	1.70	0.004	Positive	0.34	0.10
A68	*Unidentified_ Clostridiales*	1.68	5.97	Positive	0.34	0.11
A191	*Other_Mollicutes*	1.68	0.002	Positive	0.35	0.09
A20	*Other_Bacteroidales*	1.66	0.67	Positive	0.36	0.08
A198	*Unidentified_ HA64_ Opitutae*	1.65	0.005	Positive	0.36	0.09
A154	*Other_Proteobacteria*	1.64	0.03	Positive	0.36	0.09
A195	*Mycoplasma*	1.64	0.008	Positive	0.35	0.09

A23	*Other_ Bacteroidaceae*	1.62	0.07	Negative	-0.35	0.09
A140	*Anaerorhabdus*	1.61	0.004	Positive	0.35	0.09
A171	*Unidentified_ Desulfovibrionaceae*	1.59	0.02	Positive	0.34	0.10
A12	*Bifidobacterium*	1.55	0.02	Negative	-0.33	0.11
A48	*YRC22_ [Paraprevotellaceae]*	1.53	0.01	Positive	0.33	0.12
A199	*Unidentified_ [Cerasicoccaceae]*	1.51	0.01	Negative	-0.29	0.16

Table 7.55 Faecal PostMCS genera relationship with "log" serotonin values. VIPs from the PLS analysis, RA, direction of correlation, correlation coefficients (R) and P-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix in the Appendix (**Figure 7.72)**.

PostMCS- Serotonin						
	Genus	**VIP**	**Mean RA**	**Direction**	**R**	**P**
A41	*Barnesiella*	2.75	0.004	Negative	-0.58	<0.01
A168	*Sutterella*	2.41	0.007	Positive	0.54	0.01
A11	*Unidentified_ Bifidobacteriaceae*	2.25	0.003	Negative	-0.50	0.01
A118	*Subdoligranulum*	2.24	0.04	Negative	-0.49	0.02
A103	*Unidentified_ Peptostreptococcaceae*	2.24	0.32	Positive	0.51	0.01
A99	*Other_ Peptococcaceae*	2.23	0.03	Positive	0.50	0.01
A107	*Unidentified_ Ruminococcaceae*	2.00	23.26	Negative	-0.45	0.03
A4	*Unidentified_ Methanocorpusculaceae*	1.96	0.95	Negative	-0.44	0.03
A96	*Shuttleworthia*	1.92	<0.001	Positive	0.41	0.05
A146	*[Eubacterium]*	1.79	0.04	Positive	0.39	0.06

A33	*Unidentified _RF16_Bacteroidales*	1.76	0.83	Negative	-0.35	0.09
A149	*Other_ Victivallaceae*	1.73	0.001	Negative	-0.39	0.06
A38	*Unidentified_S24-7_ Bacteroidales*	1.69	0.52	Positive	0.38	0.07
A139	*Unidentified_ Erysipelotrichaceae*	1.69	0.16	Positive	0.32	0.12
A150	*Unidentified _Victivallaceae*	1.68	0.26	Negative	-0.37	0.08
A86	*Coprococcus*	1.66	1.00	Positive	0.36	0.08
A104	*Clostridium*	1.63	0.02	Negative	-0.32	0.12
A102	*Other_ Peptostreptococcaceae*	1.57	0.001	Positive	0.34	0.10
A113	*Faecalibacterium*	1.57	0.006	Positive	0.35	0.09
A47	*Paraprevotella*	1.57	0.01	Negative	-0.33	0.12
A141	*Coprobacillus*	1.56	0.01	Positive	0.31	0.14
A56	*Elusimicrobium*	1.55	0.004	Positive	0.30	0.16
A58	*Other_Firmicutes*	1.51	0.008	Positive	0.34	0.11
A3	*Methanosphaera*	1.50	0.008	Positive	0.30	0.16

Table 7.56 Faecal PostNT genera relationship with "log(x+1)" cortisol values. VIPs from the PLS analysis, RA, direction of correlation, correlation coefficients (R) and P-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix in the Appendix (**Figure 7.63)**.

PostNT- Cortisol						
	Genus	**VIP**	**Mean RA**	**Direction**	**R**	**P**
A159	*Afipia*	2.41	<0.001	Positive	0.54	0.01
A51	*Other_ Flavobacteriales*	2.35	0.01	Positive	0.51	0.01
A104	*Clostridium*	2.30	0.02	Negative	-0.51	0.01
A150	*Unidentified _Victivallaceae*	2.09	0.23	Positive	0.47	0.02
A23	*Other_ Bacteroidaceae*	2.04	0.09	Positive	0.46	0.02
A28	*Paludibacter*	1.98	0.19	Negative	-0.43	0.03
A58	*Other_Firmicutes*	1.94	0.01	Negative	-0.42	0.04
A65	*Turicibacter*	1.89	0.10	Negative	-0.42	0.04
A121	*Unidentified_ Veillonellaceae*	1.82	0.03	Negative	-0.40	0.05
A64	*Streptococcus*	1.82	0.003	Positive	0.39	0.06
A153	*Unidentified_ Pirellulaceae*	1.81	0.35	Positive	0.40	0.05
A193	*Anaeroplasma*	1.80	0.02	Positive	0.37	0.07
A105	*[Clostridium]*	1.80	0.04	Negative	-0.39	0.06
A67	*Other_ Clostridiales*	1.74	1.26	Positive	0.39	0.06
A115	*Papillibacter*	1.71	0.38	Negative	-0.38	0.07
A70	*Unidentified_ Christensenellaceae*	1.71	0.46	Positive	0.36	0.08
A131	*Unidentified_ [Mogibacteriaceae]*	1.71	0.93	Positive	0.36	0.09
A119	*Syntrophomonas*	1.71	0.001	Positive	0.38	0.06
A37	*Alistipes*	1.69	0.06	Negative	-0.38	0.07

A81	*Unidentified_ Lachnospiraceae*	1.64	3.38	Positive	0.37	0.08
A30	*Porphyromonas*	1.63	0.004	Negative	-0.26	0.22
A78	*Anaerofustis*	1.61	0.08	Positive	0.35	0.09
A142	*L7A_E11_ Erysipelotrichaceae*	1.60	0.01	Positive	0.35	0.09
A103	*Unidentified_ Peptostreptococcaceae*	1.58	0.29	Negative	-0.35	0.10
A201	*Akkermansia*	1.55	0.07	Positive	0.34	0.10
A15	*Adlercreutzia*	1.53	0.03	Positive	0.34	0.10
A198	*Unidentified_ HA64_ Opitutae*	1.52	0.007	Negative	-0.34	0.11

Table 7.57 Faecal PostNT genera relationship with "log" serotonin values. VIPs from the PLS analysis, RA, direction of correlation, correlation coefficients (R) and P-values from the correlation analysis are reported. Coding is also available to relate with the correlation matrix in the Appendix (**Figure 7.69)**.

PostNT- Serotonin						
	Genus	**VIP**	**Mean RA**	**Direction**	**R**	**P**
A82	*Anaerostipes*	2.58	0.07	Negative	-0.56	<0.01
A98	*[Ruminococcus]*	2.32	0.01	Negative	-0.50	0.01
A137	*Unidentified_ SHA-98_ Clostridia*	2.31	0.01	Positive	0.50	0.01
A57	*Fibrobacter*	2.08	0.62	Negative	-0.45	0.03
A128	*Succiniclasticum*	2.01	<0.001	Positive	0.41	0.05
A70	*Unidentified_ Christensenellaceae*	1.87	0.46	Positive	0.39	0.06
A37	*Alistipes*	1.84	0.06	Positive	0.39	0.06
A38	*Unidentified_S24-7_ Bacteroidales*	1.77	0.52	Negative	-0.38	0.07
A144	*RFN20_ Erysipelotrichaceae*	1.76	0.001	Negative	-0.37	0.07
A112	*Ethanoligenens*	1.76	0.005	Positive	0.38	0.07
A129	*Succinispira*	1.75	0.001	Negative	-0.37	0.08
A67	*Other_ Clostridiales*	1.72	1.26	Positive	0.36	0.08
A141	*Coprobacillus*	1.69	0.03	Negative	-0.37	0.08
A152	*Unidentified_ [Lentisphaeria]*	1.66	0.001	Positive	0.35	0.09
A45	*Unidentified_ [Paraprevotellaceae]*	1.66	0.22	Negative	-0.36	0.09
A9	*Arcanobacterium*	1.63	0.003	Positive	0.33	0.11
A195	*Mycoplasma*	1.62	0.007	Negative	-0.28	0.18
A25	*5-7N15_ Bacteroidaceae*	1.61	7.89	Negative	-0.35	0.09

A66	*Other_ Clostridia*	1.60	0.02	Positive	0.34	0.10
A131	*Unidentified_ [Mogibacteriaceae]*	1.55	0.93	Positive	0.30	0.16
A11	*Unidentified_ Bifidobacteriaceae*	1.52	0.005	Positive	0.32	0.12
A32	*Prevotella*	1.50	0.48	Positive	0.32	0.13
A46	*CF231_ [Paraprevotellaceae]*	1.50	0.23	Positive	0.23	0.29

7.5.2 Correlation matrices for faecal phyla orders and genera with cortisol

7.5.2.1 Correlations for NT group abundances and cortisol

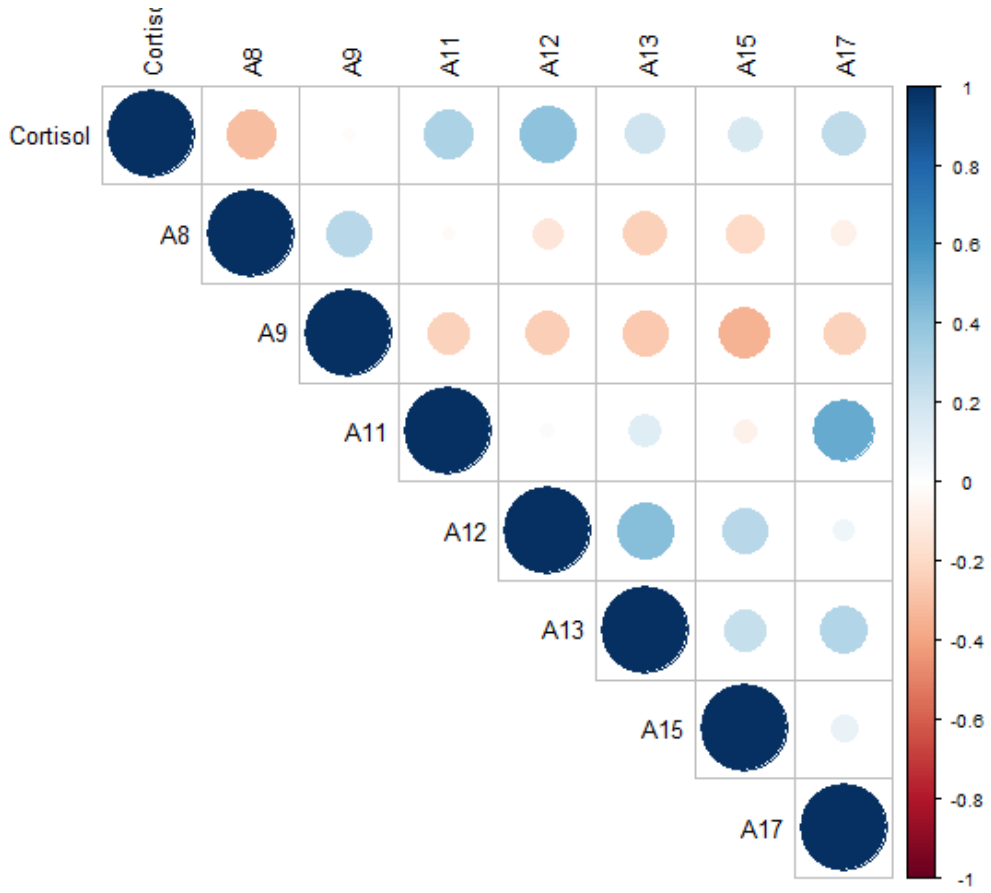

Figure 7.56 Spearman's rank correlation matrices for SRT faecal NT phylum abundances and cortisol values. The matrix depicts relationships among phyla, and between phyla and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two phyla. Phyla and VIPs obtained from the PLS regression are also shown in **Table 7.58**.

Table 7.58 Faecal NT phyla with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT phylum relative abundances and cortisol. The VIP score from the PLS analysis, mean relative abundance of each phylum and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.57.**

Phyla		VIP	Mean RA
A8	*Elusimicrobia*	1.77	0.04
A12	*Planctomycetes*	1.72	0.35
A17	*Verrucomicrobia*	1.27	0.09
A15	*Synergistetes*	1.24	0.001
A13	*Proteobacteria*	1.13	0.46
A9	*Fibrobacteres*	1.10	0.61
A11	*Lentisphaerae*	1.01	0.32

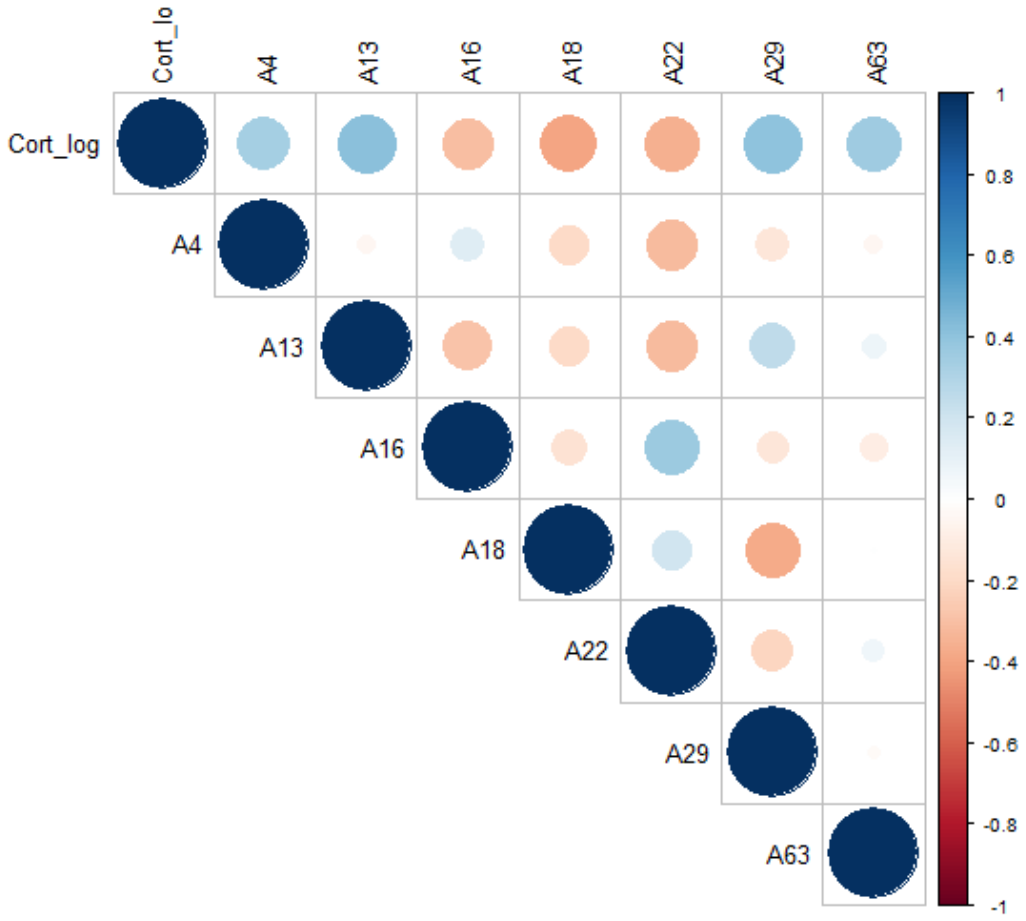

Figure 7.57 Spearman's rank correlation matrices for SRT faecal NT order abundances and cortisol values. The matrix depicts relationships among orders, and between orders and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two orders. Orders and VIPs obtained from the PLS regression are also shown in **Table 7.59.**

Table 7.59 Faecal NT orders with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT order relative abundances and cortisol. The VIP score from the PLS analysis, mean relative abundance of each order and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.58.**

	Order	**VIP**	**Mean RA**
A13	*Flavobacteriales*	2.18	0.009
A18	*Other_Firmicutes*	1.84	0.008
A22	*Turicibacterales*	1.81	0.10
A29	*Pirellulales*	1.70	0.35
A16	*Elusimicrobiales*	1.59	0.04
A4	*Methanosarcinales*	1.51	<0.001

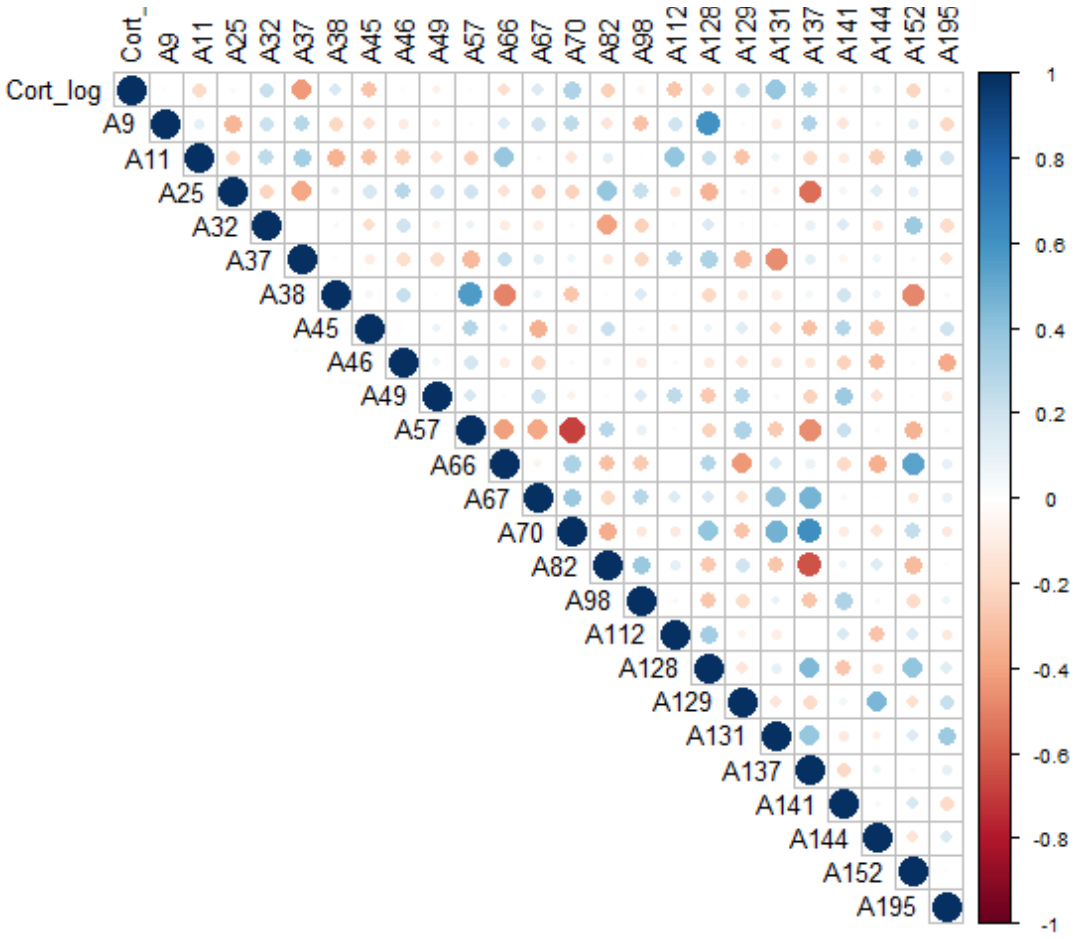

Figure 7.58 Spearman's rank correlation matrices for SRT faecal NT genus abundances and cortisol values. The matrix depicts relationships among genera, and between genera and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two genera. Genera and VIPs obtained from the PLS regression are also shown in **Table 7.60.**

Table 7.60 Faecal NT genera with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT genus relative abundances and cortisol. The VIP score from the PLS analysis, mean relative abundance of each genus and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.59.**

	Genus	**VIP**	**Mean RA**
A159	*Afipia*	2.41	<0.001
A51	*Other_ Flavobacteriales*	2.35	0.01
A104	*Clostridium*	2.30	0.02
A150	*Unidentified _Victivallaceae*	2.09	0.23
A23	*Other_ Bacteroidaceae*	2.04	0.09
A28	*Paludibacter*	1.98	0.19
A58	*Other_Firmicutes*	1.94	0.01
A65	*Turicibacter*	1.89	0.10
A121	*Unidentified_ Veillonellaceae*	1.82	0.03
A64	*Streptococcus*	1.82	0.003
A153	*Unidentified_ Pirellulaceae*	1.81	0.35
A193	*Anaeroplasma*	1.80	0.02
A105	*[Clostridium]*	1.80	0.04
A67	*Other_ Clostridiales*	1.74	1.26
A115	*Papillibacter*	1.71	0.38
A70	*Unidentified_ Christensenellaceae*	1.71	0.46
A131	*Unidentified_ [Mogibacteriaceae]*	1.71	0.93
A119	*Syntrophomonas*	1.71	0.001
A37	*Alistipes*	1.69	0.06
A81	*Unidentified_ Lachnospiraceae*	1.64	3.38
A30	*Porphyromonas*	1.63	0.004
A78	*Anaerofustis*	1.61	0.08
A142	*L7A_E11_ Erysipelotrichaceae*	1.60	0.01

A103	*Unidentified_ Peptostreptococcaceae*	1.58	0.29
A201	*Akkermansia*	1.55	0.07
A15	*Adlercreutzia*	1.53	0.03
A198	*Unidentified_ HA64_ Opitutae*	1.52	0.007

7.5.2.2 Correlations for MCS group abundances and cortisol

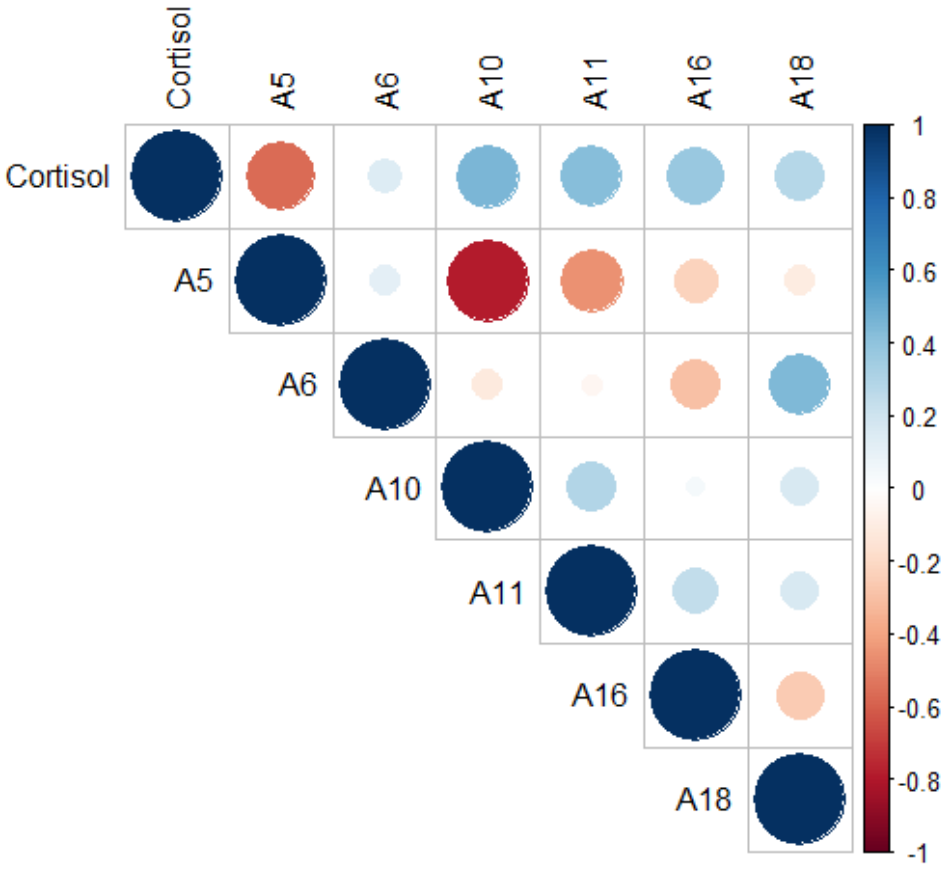

Figure 7.59 Spearman's rank correlation matrices for SRT faecal MCS phylum abundances and cortisol values. The matrix depicts relationships among phyla, and between phyla and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two phyla. Phyla and VIPs obtained from the PLS regression are also shown in **Table 7.61.**

Table 7.61 Faecal MCS phyla with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT phylum relative abundances and cortisol. The VIP score from the PLS analysis, mean relative abundance of each phylum and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.60.**

	Phylum	**VIP**	**Mean RA**
A5	*Bacteroidetes*	2.03	35.88
A10	*Firmicutes*	1.64	52.90
A11	*Lentisphaerae*	1.62	0.32
A16	*Tenericutes*	1.43	1.12
A18	*WPS-2*	1.20	0.001

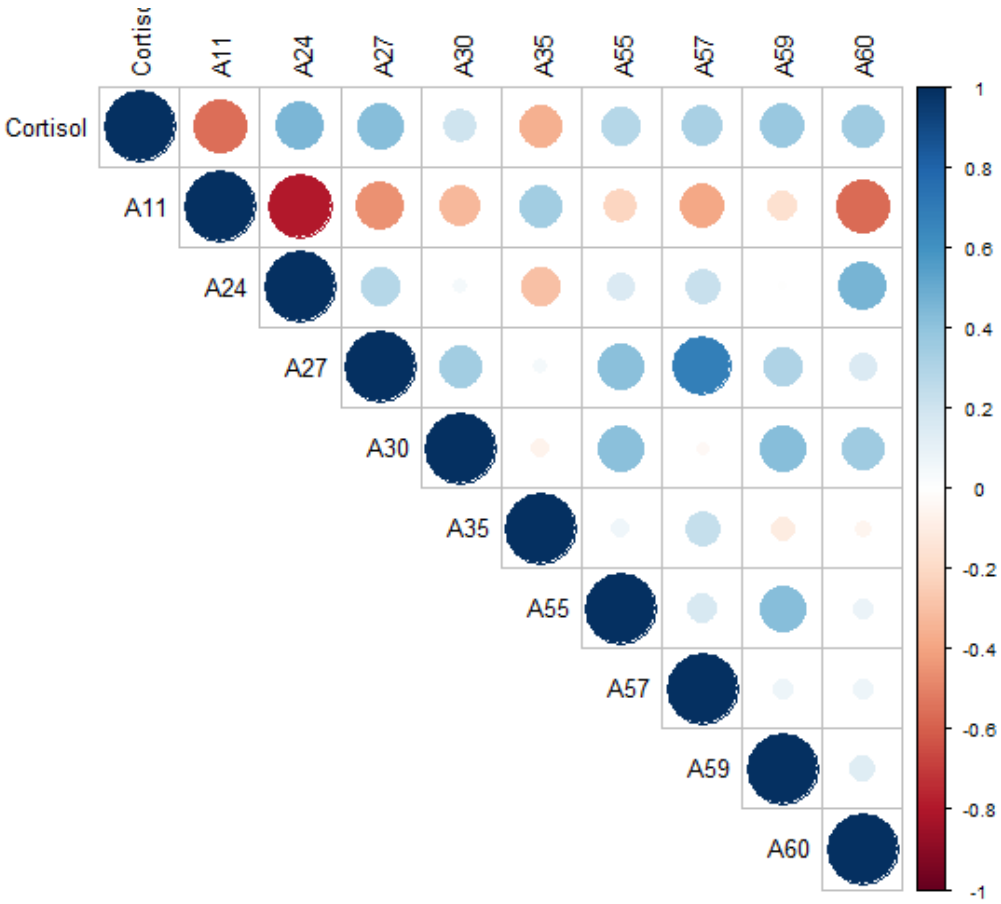

Figure 7.60 Spearman's rank correlation matrices for SRT faecal MCS order abundances and cortisol values. The matrix depicts relationships among orders, and between orders and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two orders. Orders and VIPs obtained from the PLS regression are also shown in **Table 7.62.**

Table 7.62 Faecal MCS orders with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT order relative abundances and cortisol. The VIP score from the PLS analysis, mean relative abundance of each order and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.61.**

	Order	**VIP**	**Mean RA**
A11	*Bacteroidales*	2.49	35.85
A27	*Victivallales*	2.02	0.31
A24	*Clostridiales*	1.98	54.16
A59	*ML615J-28_ RF3_ Tenericutes*	1.80	0.68
A35	*Rhodospirillales*	1.74	0.007
A57	*Mycoplasmatales*	1.72	0.01
A60	*HA64_ Opitutae*	1.57	0.005
A30	*Other_Proteobacteria*	1.53	0.03
A55	*Other_Mollicutes*	1.52	0.002

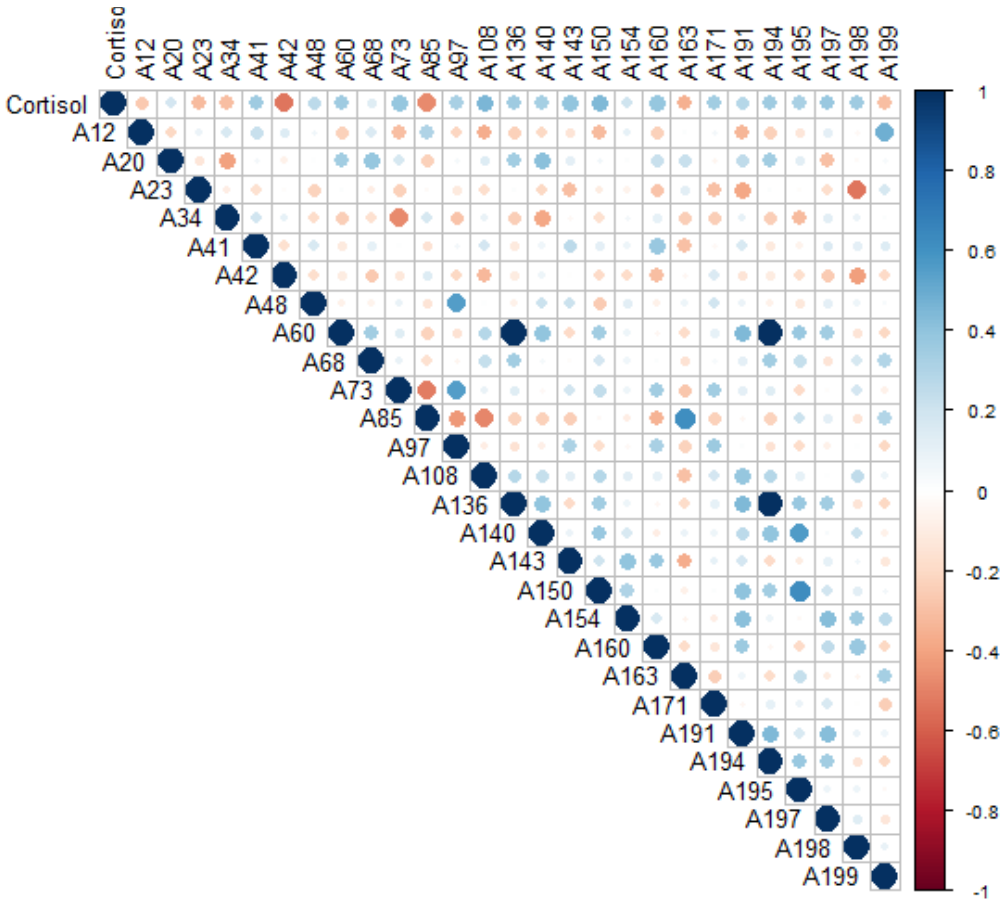

Figure 7.61 Spearman's rank correlation matrices for SRT faecal MCS genus abundances and cortisol values. The matrix depicts relationships amongst genera, and between genera and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two genera. Genera and VIPs obtained from the PLS regression are also shown in **Table 7.63.**

Table 7.63 Faecal MCS genera with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT genus relative abundances and cortisol. The VIP score from the PLS analysis, mean relative abundance of each genus and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.62.**

	Genus	**VIP**	**Mean RA**
A85	*Clostridium*	2.79	0.04
A150	*Unidentified _Victivallaceae*	2.21	0.28

A73	*02d06_ Clostridiaceae*	2.18	0.20
A42	*Butyricimonas*	2.16	0.02
A143	*PSB-M-3_ Erysipelotrichaceae*	2.09	0.008
A160	*Bradyrhizobium*	2.06	>0.001
A60	*Bacillus*	1.98	>0.001
A136	*ph2_[Tissierellaceae]*	1.98	>0.001
A194	*Unidentified_ Mycoplasmataceae*	1.98	0.001
A108	*Anaerotruncus*	1.97	0.10
A197	*Unidentified_ML615J-28_ RF3_Tenericutes*	1.93	0.68
A163	*Unidentified_Acetobacteraceae*	1.88	0.007
A34	*Other_ Rikenellaceae*	1.85	0.01
A97	*Syntrophococcus*	1.77	>0.001
A41	*Barnesiella*	1.70	0.004
A68	*Unidentified_ Clostridiales*	1.68	5.97
A191	*Other_Mollicutes*	1.68	0.002
A20	*Other_Bacteroidales*	1.66	0.67
A198	*Unidentified_ HA64_ Opitutae*	1.65	0.005
A154	*Other_Proteobacteria*	1.64	0.03
A195	*Mycoplasma*	1.64	0.008
A23	*Other_ Bacteroidaceae*	1.62	0.07
A140	*Anaerorhabdus*	1.61	0.004
A171	*Unidentified_ Desulfovibrionaceae*	1.59	0.02
A12	*Bifidobacterium*	1.55	0.02
A48	*YRC22_ [Paraprevotellaceae]*	1.53	0.01
A199	*Unidentified_ [Cerasicoccaceae]*	1.51	0.01

7.5.3 Correlation matrices for faecal phyla orders and genera with serotonin

7.5.3.1 Correlations between abundances for NT group and serotonin

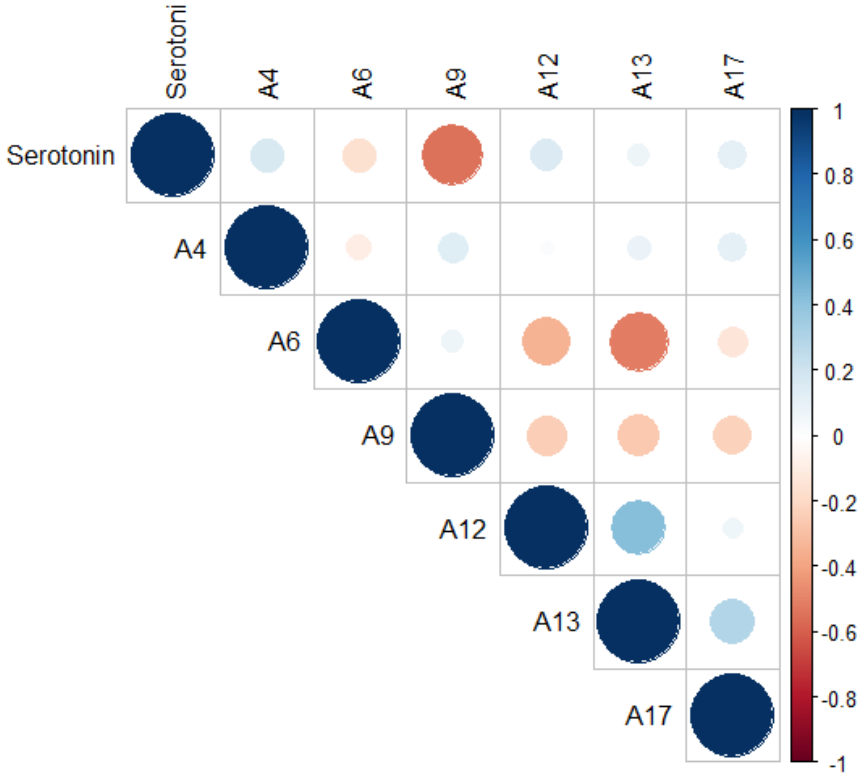

Figure 7.62 Spearman's rank correlation matrices for SRT faecal NT phylum abundances and serotonin values. The matrix depicts relationships amongst phyla, and between phyla and serotonin on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two phyla. Phyla and VIPs obtained from the PLS regression are also shown in **Table 7.64.**

Table 7.64 Faecal NT phyla with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT phylum relative abundances and serotonin. The VIP score from the PLS analysis, mean relative abundance of each phylum and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.63.**

	Phylum	**VIP**	**Mean RA**
A9	*Fibrobacteres*	2.33	0.61
A13	*Proteobacteria*	1.35	0.46
A12	*Planctomycetes*	1.30	0.35
A6	*Chloroflexi*	1.10	0.002
A4	*Actinobacteria*	1.05	0.24
A17	*Verrucomicrobia*	1.01	0.09

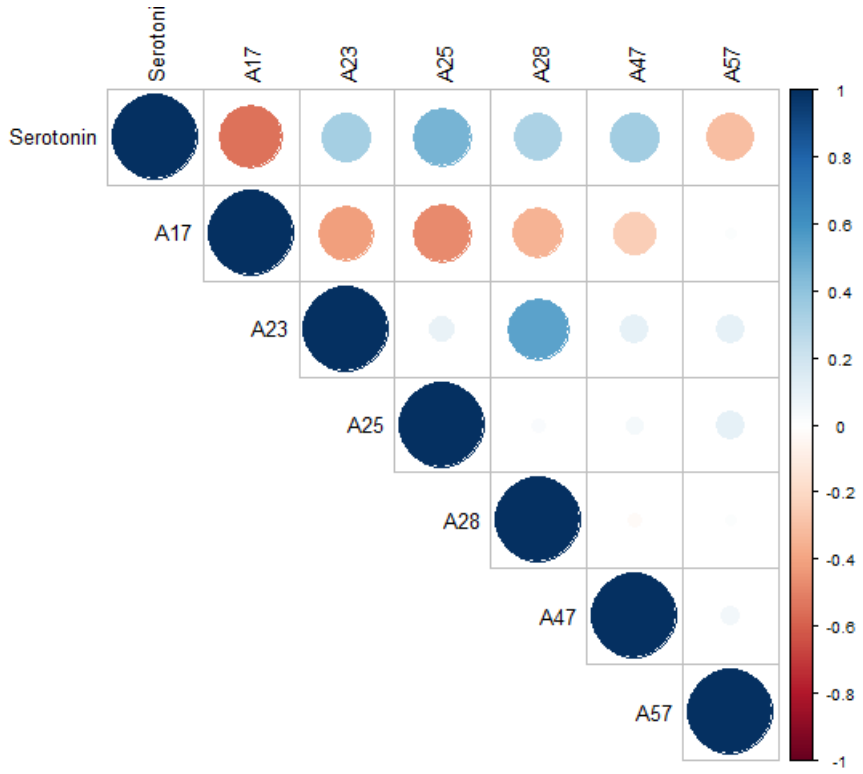

Figure 7.63 Spearman's rank correlation matrices for SRT faecal NT order abundances and serotonin values. The matrix depicts relationships amongst orders, and between orders and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two orders. Orders and VIPs obtained from the PLS regression are also shown in **Table 7.65**.

Table 7.65 Faecal NT orders with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT order relative abundances and serotonin. The VIP score from the PLS analysis, mean relative abundance of each order and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.64.**

	Phylum	**VIP**	**Mean RA**
A9	*Fibrobacteres*	2.33	0.61
A13	*Proteobacteria*	1.35	0.46
A12	*Planctomycetes*	1.30	0.35
A6	*Chloroflexi*	1.10	0.002
A4	*Actinobacteria*	1.05	0.24
A17	*Verrucomicrobia*	1.01	0.09

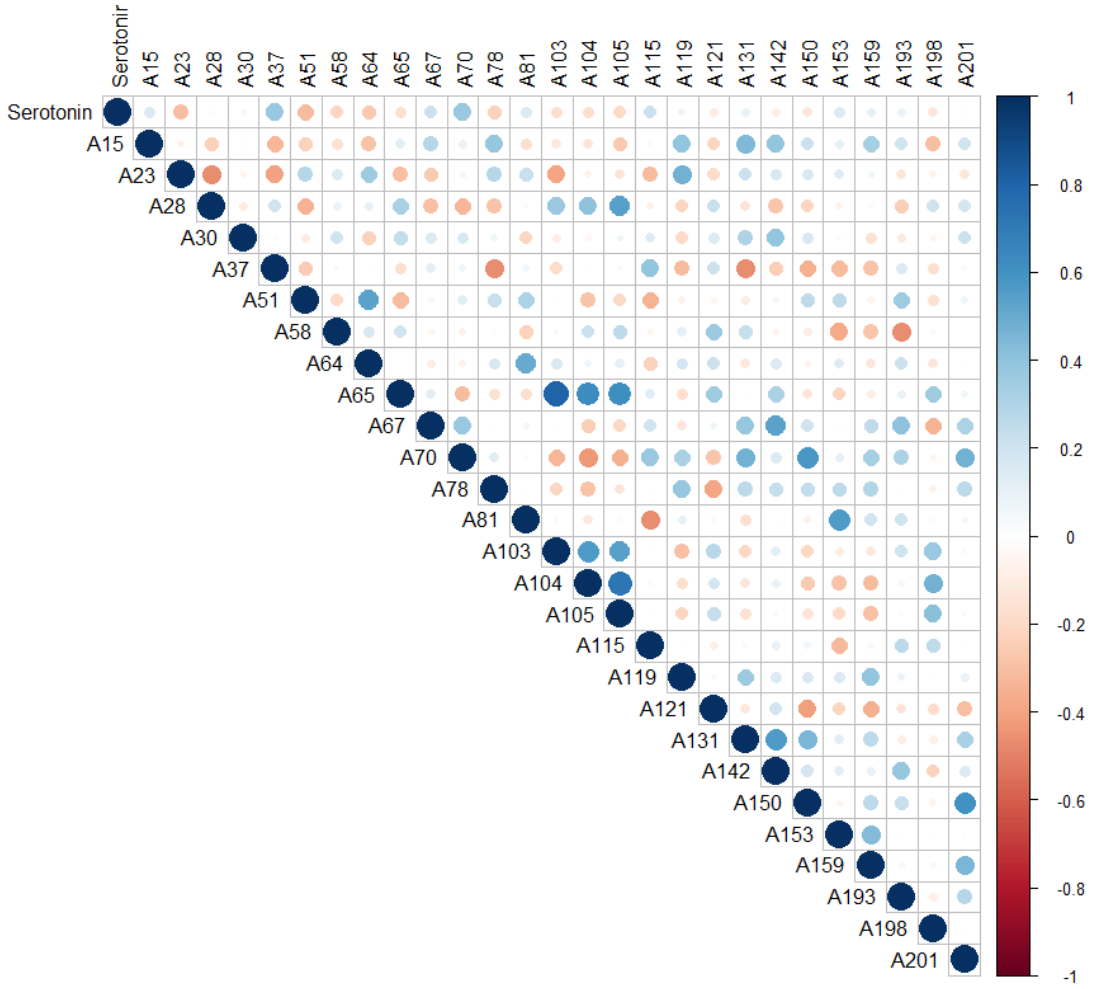

Figure 7.64 Spearman's rank correlation matrices for SRT faecal NT genus abundances and serotonin values. The matrix depicts relationships amongst genera, and between genera and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two genera. Genera and VIPs obtained from the PLS regression are also shown in **Table 7.66**.

Table 7.66 Faecal NT genera with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT genus relative abundances and serotonin. The VIP score from the PLS analysis, mean relative abundance of each genus and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.65.**

	Genus	**VIP**	**Mean RA**
A82	*Anaerostipes*	2.58	0.07
A98	*[Ruminococcus]*	2.32	0.01
A137	*Unidentified_ SHA-98_ Clostridia*	2.31	0.01
A57	*Fibrobacter*	2.08	0.62
A128	*Succiniclasticum*	2.01	<0.001
A70	*Unidentified_ Christensenellaceae*	1.87	0.46
A37	*Alistipes*	1.84	0.06
A38	*Unidentified_S24-7_ Bacteroidales*	1.77	0.52
A144	*RFN20_ Erysipelotrichaceae*	1.76	0.001
A112	*Ethanoligenens*	1.76	0.005
A129	*Succinispira*	1.75	0.001
A67	*Other_ Clostridiales*	1.72	1.26
A141	*Coprobacillus*	1.69	0.03
A152	*Unidentified_ [Lentisphaeria]*	1.66	0.001
A45	*Unidentified_ [Paraprevotellaceae]*	1.66	0.22
A9	*Arcanobacterium*	1.63	0.003
A195	*Mycoplasma*	1.62	0.007
A25	*5-7N15_ Bacteroidaceae*	1.61	7.89
A66	*Other_ Clostridia*	1.60	0.02
A131	*Unidentified_ [Mogibacteriaceae]*	1.55	0.93
A11	*Unidentified_ Bifidobacteriaceae*	1.52	0.005

A32	*Prevotella*	1.50	0.48
A46	*CF231_ [Paraprevotellaceae]*	1.50	0.23

7.5.3.2 Faecal relative abundances for MCS group and serotonin

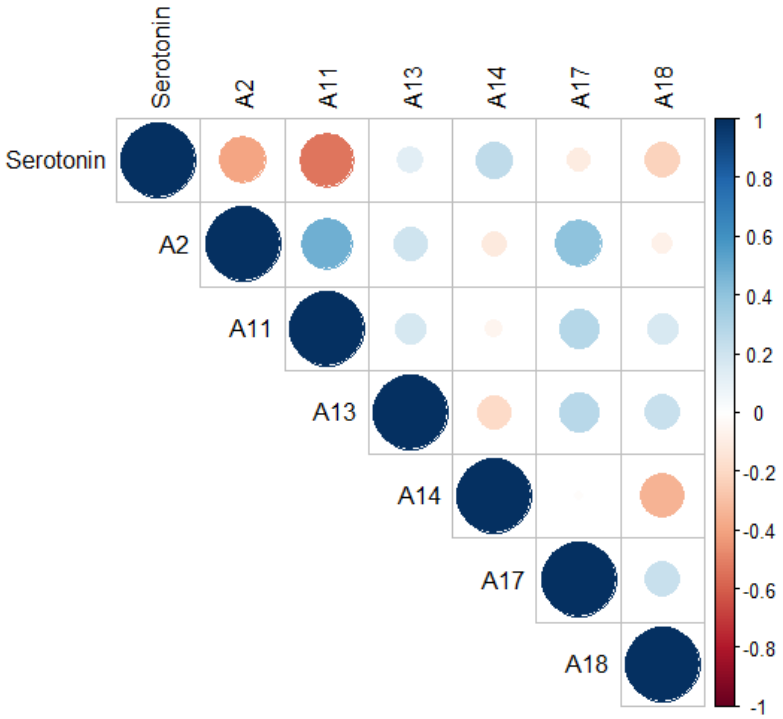

Figure 7.65 Spearman's rank correlation matrices for SRT faecal MCS phylum abundances and serotonin values. The matrix depicts relationships amongst phyla, and between phyla and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two phyla. Phyla and VIPs obtained from the PLS regression are also shown in **Table 7.67.**

Table 7.67 Faecal MCS phyla with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT phylum relative abundances and serotonin. The VIP score from the PLS analysis, mean relative abundance of each phylum and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.66.**

	Phylum	**VIP**	**Mean RA**

A11	*Lentisphaerae*	1.91	0.32
A2	*Euryarchaeota*	1.75	1.07
A13	*Proteobacteria*	1.24	0.43
A17	*Verrucomicrobia*	1.16	0.11
A18	*WPS-2*	1.11	0.001
A14	*Spirochaetes*	1.10	1.90

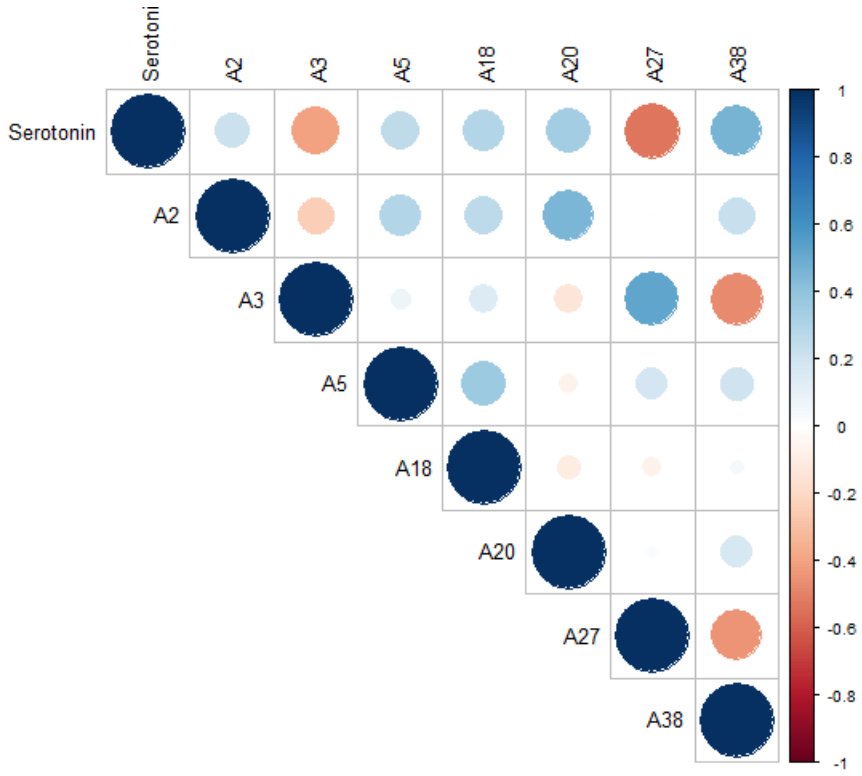

Figure 7.66 Spearman's rank correlation matrices for SRT faecal MCS order abundances and serotonin values. The matrix depicts relationships amongst orders, and between orders and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two orders. Orders and VIPs obtained from the PLS regression are also shown in **Table 7.68**.

Table 7.68 Faecal MCS orders with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT order relative abundances and serotonin. The VIP score from the PLS analysis, mean relative abundance of each order and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.67.**

	Order	**VIP**	**Mean RA**
A38	*Burkholderiales*	2.48	0.13
A3	*Methanomicrobiales*	2.10	0.95
A27	*Victivallales*	1.96	0.31
A20	*Bacillales*	1.76	<0.001
A18	*Other_Firmicutes*	1.63	0.008
A5	*E2_Thermoplasmata*	1.53	0.03
A2	*Methanobacteriales*	1.52	0.08

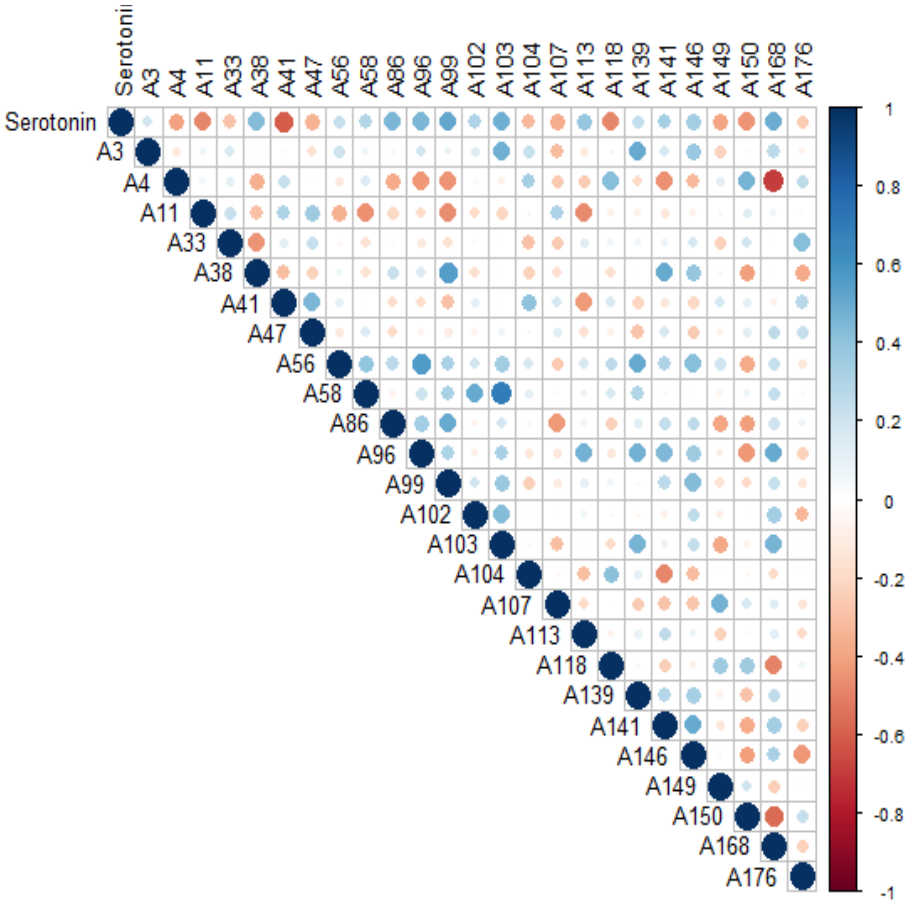

Figure 7.67 MCS genera over 1.5 Spearman's rank correlation matrices for SRT faecal MCS genus abundances and serotonin values. The matrix depicts relationships amongst genera, and between genera and cortisol on the top row. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colours of the scale bar denote the nature of the correlation with 1 indicating perfect positive correlation (dark blue) and -1 indicating perfect negative correlation (dark red) between two genera. Genera and VIPs obtained from the PLS regression are also shown in **Table 7.69.**

Table 7.69 Faecal MCS genera with VIP scores higher than 1, as reported by the PLS regression used to explore the relationship of SRT genus relative abundances and serotonin. The VIP score from the PLS analysis, mean relative abundance of each genus and correlation coefficients (R) are reported. Coding is also available to relate with Correlogram **Figure 7.68.**

	Genus	**VIP**	**Mean RA**
A41	*Barnesiella*	2.75	0.004
A168	*Sutterella*	2.41	0.007
A11	*Unidentified_ Bifidobacteriaceae*	2.25	0.003
A118	*Subdoligranulum*	2.24	0.04
A103	*Unidentified_ Peptostreptococcaceae*	2.24	0.32
A99	*Other_ Peptococcaceae*	2.23	0.03
A107	*Unidentified_ Ruminococcaceae*	2.00	23.26
A4	*Unidentified_ Methanocorpusculaceae*	1.96	0.95
A96	*Shuttleworthia*	1.92	<0.001
A146	*[Eubacterium]*	1.79	0.04
A33	*Unidentified _RF16_Bacteroidales*	1.76	0.83
A149	*Other_ Victivallaceae*	1.73	0.001
A38	*Unidentified_S24-7_ Bacteroidales*	1.69	0.52
A139	*Unidentified_ Erysipelotrichaceae*	1.69	0.16
A150	*Unidentified _Victivallaceae*	1.68	0.26
A86	*Coprococcus*	1.66	1.00
A104	*Clostridium*	1.63	0.02
A102	*Other_ Peptostreptococcaceae*	1.57	0.001
A113	*Faecalibacterium*	1.57	0.006
A47	*Paraprevotella*	1.57	0.01
A141	*Coprobacillus*	1.56	0.01
A56	*Elusimicrobium*	1.55	0.004

A58	*Other_Firmicutes*	1.51	0.008
A3	*Methanosphaera*	1.50	0.008