Author:
Meier, Stephanie V

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Meta-analysis of equine gastro-intestinal microbiome research and evaluation of the impact of fructooligosaccharide supplementation on the gastro-intestinal microbiome of thoroughbred youngstock during nutritional stress

Meta-Analysis of Equine Gastro-Intestinal Microbiome Research and Evaluation of the Impact of Fructooligosaccharide Supplementation on the Gastro-Intestinal Microbiome of Thoroughbred Youngstock during Nutritional Stress.

Stephanie Victoria Meier

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Abstract

Horses are trickle feeders and hindgut fermenters, and rely on the microbial fermentation of a fiber-rich diet for their energy. The gastro-intestinal (GI) microbiome is not only vital for digestion, it may also have an important role in the overall health of a horse through interaction with the immune system. Disturbances in the GI microbiome, for example through an inappropriate diet, can lead to gastro-intestinal and systemic diseases. To avoid this, prebiotic and probiotic supplementation has been proposed to stabilize the microbiome during times of dietary change. However, little evidence exists for the efficacy of this approach.
To understand the role of the GI microbiome in horse health and disease, it is important to determine the composition of the 'normal' equine microbiome and what factors can influence it. However, there is large variation between studies investigating the equine GI microbiome, which could be due to technical variation in the scientific methods used and/or small sample sizes that could bias the findings.

To address these issues, two studies were performed that examined the influence of different factors on the equine GI microbiome: The first study was a meta-analysis of equine GI microbiome data, in which we re-analyzed the raw 16S sequencing data from 29 studies and performed a batch mean correction to account for some of the technical variation. The second study was a case-control field study that examined the impact of Fructooligosaccharide supplementation in a group of Thoroughbred yearlings undergoing a dietary change.

The meta-analysis showed that technical factors, such as the sequencing instrument and gene region of the 16S gene used, have a strong impact on the GI microbial composition, such that the influence of biological factors was not visible at a global scale when comparing different studies using multivariate analysis. However, after batch mean correction of some of the technical variation, there was strong variation between different sample types and regions of the GI tract, as well as differential bacterial abundances between age groups, genders, diets and different diseases.

The prebiotic field study showed a stronger shift in microbial composition in control horses than in horses given prebiotics during a time of nutritional stress. This may indicate a stabilizing effect of prebiotic supplementation during dietary change. However, the study was limited by a difference in baseline microbial compositions between horses with prebiotic supplementation and those without, which shows the importance of taking a baseline sample during longitudinal studies.

Overall, these studies highlight the need for a more standardized global approach in investigating the equine GI microbiota if studies are to be comparable. This could be achieved by creating a protocol for equine GI microbiome research and a database where the generated data can be shared and compared to advance research in this field.

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Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: [redacted] DATE: 09.09.2021

COVID Statement

The research plan of this MSc by Research focused on the investigation of the effect of prebiotic supplementation on Thoroughbred yearlings and weanlings. The project was divided into two parts. The first part of the project involved the processing of the fecal samples, including DNA extractions and preparation for sequencing, as well as the analysis of the sequenced bacteria. This experiment was to be repeated in the spring and summer, to get a follow up study from the weanlings. I completed a first study of the yearlings and part of the processing of the weanling samples, but the COVID restrictions came into place just before the sample collection of the follow up study could be implemented. The second part of the study included the development of an equine intestinal enteroid to investigate the interaction of intestinal bacteria in an in vitro model. We washed samples of horse intestine to get the cells required to make an enteroid, and we documented these steps to know how many washings are required to get the right cells, but we discontinued this project due to the pandemic.

Immediately after the start of the lockdown we started to develop an alternative desk-based project, a meta-analysis of current research on the equine microbiome, which has become the central part of my thesis. The meta-analysis was a very good alternative and addition to my existing project, as there are many small studies examining the equine gastro-intestinal microbiome, but they have found strongly diverging results. Consequently, the meta-analysis allowed us to look for overarching factors among these studies instead of producing yet another small-scale study which could be influenced by various confounding factors that may be avoided when comparing the numerous studies that have already been published. Overall, due to the pandemic, parts of the initial project were not completed, while a new opportunity arose which provided a more impactful project.

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1. Introduction: The Equine Gastro-Intestinal Microbiome

The gastro-intestinal (GI) microbiota is a community of bacteria, parasites, viruses, archaea and fungi which are present in the intestine of the host species[1]. The intestinal microbiota has been gaining increased attention in research and medicine due to its potentially large role in the host's health. While the term intestinal microbiota describes the microorganisms in the intestine, the intestinal microbiome is a broader definition that involves these microorganisms including their genetic material and interactions with their environment[1]. The two terms have been commonly used interchangeably and although they encompass all microbial species as mentioned above, most publications focus on the bacterial community in the GI tract. With the development of novel sequencing methods, the investigation of the GI microbiome has changed drastically, since Next Generation Sequencing enables the detailed analysis of the microbiota found in the GI tract within a short amount of time. Previously, bacterial cultivation methods were used to determine species abundances, however this does not show a complete picture as most commensal bacterial species in the intestine cannot be cultivated[2]. Horse breeding has a long tradition globally, alone in Europe the equine industry is an economically important business with a worth of €100 billion per year[3]. In a more recent survey, the British Equestrian Trade Association found the economic value of the equestrian sector to be £4.7 billion in 2019, with an estimated 847'000 horses in the UK[4]. Consequently, there is a strong interest in horse health and research into the equine GI microbiome. As GI diseases are common in horses, understanding the gut microbiota is an especially important aspect of equine health.

Horses are hindgut fermenters and are naturally adapted to high-fiber and low-energy nutrition, hence most of their energy derives from monocarboxylates produced by microbial digestion of plant fiber[5]. In their natural habitat horses spend the majority of the day foraging on fibrous plants, consequently they are used to small amounts of energy intake at a high frequency[5]. Due to this diet mostly of plant fibers, a large part of the equine gastrointestinal tract (GIT) contains commensal microbiota that are responsible for the anaerobic fermentation of carbohydrates and undigestible plant fiber, which results in the production of organic acids, such as acetate,

butyrate, lactate, propionate and succinate[6]. These fatty acids account for over 50% of horses' total energy gain, which stands in contrast to humans, who only get about 10% of their energy from the compounds produced by microbial fermentation[7]. Consequently, the intestinal microbiome of horses has a very important role in horses' metabolism. There are many factors that could have an influence on the equine GI microbiome, including individual horse characteristics, such as age and breed of the horse, as well as external factors, such as the location, management system and possible stress factors[1]. Additionally, there are ways to modulate the GI microbiome using prebiotics, probiotics or fecal microbial transplantations.

2. Literature Review

This literature review is divided into three main parts. The first part focuses on the core microbiome composition and bacterial abundances found in healthy horses, with a specific section on the development of the GI microbiome of foals and the differences in the varying regions of the GIT. The second part of the literature review includes factors that have an influence on the GI microbiome. In healthy horses this includes their management, with a focus on domestication and diet; whereas, in unhealthy horses this includes diseases that have been associated with alterations to the GI microbiome in horses, the most common of which are obesity, laminitis, colic and parasite infections, as well as the antibiotics and anthelmintics with which they are treated. The third and last part involves a review of the data on the directed modulation of the GI microbiota through probiotics, prebiotics and fecal microbial transplants.

2.1. Gastro-Intestinal Microbiome in Healthy Horses

2.1.1. Development in Foals until Weaning

There is a consensus among studies that the GI microbiome at birth is clearly different from the one of adults[1,8,9]. There is a strong shift in the microbiota composition in the first year of life, especially in the time between birth and weaning[1,8–10]. The change of the microbiome during this time can be explained by internal developmental factors and external influencing factors and major events in this period. The first change of the GI microbiota happens in the period during and just after birth, where the foal receives a wide range of microbial species from its mother and environment[11]. The microbiota from the mare is transferred through contact with the skin and vaginal microbiome and through the milk, which contains mostly Enterobacteriaceae and *Enterococcus*, as well as through coprophagy, via which *Prevotella, Blautia* and *Ruminococcus* genera colonize the foals' intestine[12]. During these first few months of life the GI microbiome has been found to be highly diverse and fluctuating, with low alpha diversity between 2 and 30 days

of age[13], and the highest rate of bacterial colonization at around 1 month of age[14]. Several studies found high abundances of Proteobacteria and Firmicutes in the microbiome of young foals: De La Torre et al. (2019) found Proteobacteria to have the highest abundance on the first day after birth, with a subsequent decrease, and an a concurrent increase in Firmicutes and Bacteroidetes between one week after birth and weaning[15]. Similarly, Husso et al. (2020) state that although the foal's GI microbiota is similar to the mare's fecal microbiota immediately after birth, it contains a majority of Firmicutes and Proteobacteria one day after birth[8]. This corresponds with the findings of Schoster et al. (2017), who found Proteobacteria, Firmicutes and Verrucomicrobia phyla to be most abundant at the age of 2-4 weeks, with the highest abundances of *Ruminococcaceae* and *Lachnospiraceae* at bacterial family level[10]. This is in partial agreement with findings from Costa et al. (2016), who state that foals during the first 2-30 days have a high abundance of *Akkermansia* spp. from the Verrucomicrobia phylum[13]. Little is known about the Verrucomicrobia phylum, but species from *Ruminococcaceae* and *Lachnospiraceae* of the Firmicutes phylum may be beneficial for health[13]. Despite this, the instability of the GI microbiome at this young age could have an impact on the development of the immune system, which may be responsible for the frequent occurrence of diarrhea and pneumonia in foals[11,16]. Therefore, the investigation of the GI microbiome in foals is important to find ways to improve their health.

The next clear shift in microbial composition in foals during their first year of life has been found when weaning the foal from the mother at a few months of age[1,9]. This shift was found to increase the prevalence of *Anaerovibrio*, *Lactobacillus*, *Oscillibacter*, *Prevotella* and *Streptococcus*, while decreasing *Fibrobacter*, *Ruminococcus*, *Treponema*, *Clostridium XIVa* and species from *Lachnospiraceae*[9]. The effect of weaning differed depending on whether the weaning took place abruptly or in intervals, with a higher prevalence of *Streptococcus* in abruptly weaned foals. Therefore, it can be assumed that the importance of this event on the GI microbiome is not only due to the dietary change from milk to plant fiber or concentrate, but also the stress of weaning which leads to an increase in cortisol and a change in the GIT microbiota induced by metabolites

that influence the microbial composition. Consequently, these processes could enhance the potential for pathogenic bacteria to colonize the intestine[9].

In summary, although there are differing results between studies, there appear to be two major shifts in the microbiome of foals; and, overall, studies suggest that the microbiome is quite stable by 60 days of age[13]. However, the GI microbiome of foals at 9 months is still different from the adult horses, indicating gradual ongoing development between weaning and adulthood[13]. Consequently, research on the GI microbiome at all ages is highly relevant to prevent diseases in the future, especially during the time when the GI microbiome and immune system are co-developing in foals. However, due to differing scientific methodology the exact changes occurring at different time-points and their functional implications are, as yet, unclear. Consequently, characterization of the development of the GI microbiome in foals needs further, more standardized research in order to determine the microbial changes during this important developmental phase.

2.1.2. Core Microbiota Composition in Healthy Adult Horses

The equine microbiome is mostly studied using fecal samples, as the possibility of taking repeated non-invasive fecal samples allows for changes in the microbiome of an individual horse to be monitored over an extended period of time. Additionally, the bacteria found in the feces are considered representative for the microbiota present in the large colon[17] and in the cecum[18]. As the cecum and colon comprise 70% of the equine gastrointestinal tract[19], and horses rely heavily on their hindgut to gain energy through microbial fiber fermentation[20], the determination of the microbiome in this part of the equine intestine is highly relevant.

The core microbial community that has been at the center of these studies has been defined as the species that are present in all samples used in the study with a relative abundance of at least 0.1%[21]. There is a consensus that this core microbiome of adult horses consists mostly of species

from the Bacteroidetes and Firmicutes phyla, with smaller percentages of Verrucomicrobia, Proteobacteria and Fibrobacteres[3,20,22,23]. Which of the most prevalent phyla, Bacteroidetes or Firmicutes, is more prominent varies between studies. While some studies have found this to be Firmicutes[1,5,22,23], with amounts ranging from 70% of total bacterial species[24] to 50%[23], other studies consider Bacteroidetes as the most common bacterial phylum, with 70% of total species[20]. The abundances of Firmicutes and Bacteroidetes may also be more evenly distributed with 46% resp. 43% of total bacterial species according to a further study[25] or amounting to approximately 80% of bacteria when combined[3]. Apart from external and individual factors that can influence the GI microbiome, the methodological differences between studies examining the equine GI microbiome can have a strong impact on the microbial composition, and thus may account for these differences. Apart from the two most predominant phyla in the microbiome, a number of other bacteria have been found in smaller amounts but consistently over many studies, including Verrucomicrobia, Fibrobacteres, Proteobacteria, Actinobacteria and Spirochaetes[3,22,26,27]. Overall, Dougal et al. (2017) found that the fecal microbiota community in individual adult horses has a high stability with 65% of bacteria preserved after six weeks in a steady environment[21].

Firmicutes is a butyrate-producing bacterial phylum that is decreased after weight-loss[28], while Bacteroidetes contains many starch-fermenting bacterial species[5]. The phylum Proteobacteria is most common in horses on a high starch diet, whilst Verrucomicrobia and Fibrobacteres have been found to be more abundant in horses with a forage-based diet; notably, members of Fibrobacteres, play important role in the fermentation of plant fiber[20]. On the other hand, the overgrowth of gram-negative bacteria, such as species from Bacteroidetes, Proteobacteria and Verrucomicrobia, e.g. *Veillonella* sp. and *Serratia* sp. may lead to colitis and inflammation[23]. The correlation between certain GI microbiota with diseases will be further discussed in the following chapter.

As with the studies in foals there is significant variation in reported composition of the core equine gut microbiota between studies. This variation may be due to different scientific methods,

such as different sequencing techniques and sample types. For instance, Warzecha et al. (2017) and Dougal et al. (2013) used 454 pyrosequencing[20,25], while many other studies used Illumina sequencing[9,15,29–31]. Furthermore, Dougal et al. (2013) and Moreau et al. (2014) used luminal samples from the cecum[23,25], while most commonly fecal samples are used to determine the intestinal microbiome[8,32–34]. This shows that it is necessary to standardize the approach to equine GI microbiome research to get comparable results.

2.1.3. Microbiome in Different Compartments of the GIT

Although most studies concentrate on the hindgut and fecal samples in determining the GI microbiome, the composition of microbiota varies in the different compartments of the GIT due to varying functions in digestion and horse physiology[22]. Therefore, the GIT has been investigated in its entirety to compare the microbiota present in the different GIT regions.

Depending on the microbiota present in the different regions of the equine gut, the GIT can be separated into two parts. The first part is the foregut, or upper GIT, which includes the stomach, jejunum, ileum and duodenum, and shows a higher variability in the microbial community of the different regions. The second part is the hindgut with the cecum, small colon, ventral colon and dorsal colon, all of which had a more uniform microbiome[35,36].

While the most abundant phyla in the foregut have been found to be Firmicutes (65%) and Proteobacteria (23%), Firmicutes dominated less in the hindgut with 45% of sequences, followed by Bacteroidetes with 42%[35]. This corresponds with findings from other studies stating similar abundances of Firmicutes as most common phylum and the high prevalence of Proteobacteria in the foregut compared to the hindgut and vice versa in the case of Bacteroidetes[22,37]. The differences in microbiota between the compartments of the GIT are most likely due to the varying digestive roles of the parts of the GIT and the different availability of substrate. Passage through the upper GIT is relatively fast, and most digestible dietary components are digested in the

jejunum, while the passage through the hindgut is a slower process, as the bacterial fermentation of non-digestible carbohydrates takes place in the hindgut[36].

2.1.4. Interaction of Microbiota and Host Immune System

Although the understanding of the functions of specific GI bacteria is still at its beginnings, the role of the commensal intestinal microbiota in general in host immunity has been investigated in a number of species. Due to the long history of coevolution, the mutualistic relationship has benefited both host and resident microbiota[38]; the host provides a habitat for the microbes, while they aid in digestion and immunity[39,40]. However, not all present bacteria are useful for the host. The host's immune system needs to differentiate between pathogenic and beneficial bacteria, therefore the intestinal microbiota is closely regulated by the host[41].

Most of these interactions occur at the mucosal epithelium (ME), which includes immune cells that cover the inner surface of the GI tract, and is consequently the first immune barrier for pathogens and the site of host contact with commensal microorganisms[42]. Due to this proximate relationship, the commensal bacteria can induce the production of different immune factors in the ME. This includes TGF-beta, which is necessary for the production of Treg lymphocytes; serum amyloid A, which leads to the activation of Th17 cells; and innate lymphoid cells, which support the production of defensins[42]. On the other hand, the level of abundance of Treg cells may also have an impact on the microbial diversity in the intestine. In mice with induced depletion of Treg cells, the phylum Firmicutes was significantly more abundant than in wild type mice [43]. Similarly, another study found that the lack of T cells in mice was correlated with a reduction in gut microbial diversity[44]. This example shows that the relationship between immune cells and gut microbiota may be reciprocal and could also depend on the bacterial species involved. Some of these interactions may be mediated by metabolites produced by the gut microbiota, such as SCFAs and secondary bile acids, which can provide protection against inflammation[41]. Additionally, physiological changes to the host, e.g. those induced by stress can induce changes

to the gut microbiota which, in turn, activate innate immune responses through release of MAMPs[41]. This connection constitutes one aspect of what is termed 'the gut-brain axis', and these interactions are considered increasingly important with regards to their effect on the GI microbiota[9,45].

Apart from the direct influence on the epithelium as mentioned above, the presence of microbiota also has other effects on the host immune system. By occupying the colonization sites, the commensal microbes leave less room for pathogen colonization and increase competition for pathogens due to limited availability of nutrients[42]. This is a proposed mechanism for the negative influence of dysbiosis, or an imbalance in GI microbiota[1], on the equine host immune system and health.

2.2. Factors Influencing the Equine Microbiome

There are a wide range of factors that influence the composition of the equine GI microbiome, with only a small proportion of the observed variation between individuals being hereditary[46]. These include biological factors such as age, gender and diseases, as well as environmental factors such as stress, geography, management system and nutrition[1]. For the purposes of reviewing the literature in this area these factors can be simplified into four broad groups. The first group includes the horse management, such as the level of domestication, the living quarters and especially the diet the horses receive. The second group involves intestinal and metabolic diseases such as colic and colitis, laminitis and obesity; and infectious diseases such as GI parasite infections. The third group includes drugs used for treatment of such diseases, for instance antibiotics and anthelmintics. The last group consists of the ways the GI microbiota may be influenced intentionally, by giving supplements such as prebiotics and probiotics, or by using fecal microbial transplantation techniques.

2.2.1. Management

The management of horses includes their living conditions, social contacts, stress factors and diet. Much of this shows large differences between domestic and feral horses, as feral horses live in bands with clear social structures in large spaces with a typically forage-based diet, while domestic horses are usually kept in stables and pastures with varying degrees of social contact and a diet typically rich in starch.

Although the equine physiology has been preserved over time and the digestive system has remained unchanged in its functions, domestication can nevertheless affect the GI microbiome in a short period of time. This can be seen when comparing the GI microbiome of Przewalski horses with domestic horses, and although factors such as genetics and early life development of the microbiome could partly cause these differences, much evidence points towards the role of diet. In a study comparing captive and re-introduced Przewalski horses, the relative abundances of Firmicutes and Bacteroidetes diverged between the two groups, however they also received a different diet, which could have been partly the reason for this difference[47]. Differences in early life colonization and diet may also partly explain why the Przewalski horses born in zoos before reintroduction to the wild have been found to have a lower microbial diversity than those born in natural reserves[48]. When comparing wild Przewalski horses with domestic horses that lived on adjacent grasslands and therefore had a highly similar nutrient availability, the GI microbiota was still found to be more diverse in the wild horses, which was explained by the preference of the wild horses for a wider variety of plants[48]. These studies show that although the differences in GI microbiota between domestic and wild horses are often explained by the variation in diet, it is likely that genetics, early life and many, as yet uncharacterized, factors are all important influencers of the equine microbiome between domestic and wild horses.

2.2.2. Diet

Diet has been shown to have significant effects on microbiome in horses and is likely to be responsible for a number of diseases, therefore the influence of starch in the GI tract and its effect on the GI microbiota is highly relevant in equine health and in equine GI microbiome research. Horses are hindgut fermenters and trickle feeders, hence their natural diet consists of small portions of forage rich in plant fibers distributed over the whole day. The traditional management of domestic horses involves a diet high in starch and regular feeding times instead of constant grazing, which is contrary to their natural habits of forage with high fiber content[5].

Horses with an acute carbohydrate overload may succumb to endotoxemia, a systemic inflammatory response syndrome resulting from a higher amount of endotoxins produced by gram-negative bacteria in the intestine that then escape into the bloodstream; these processes can lead to laminitis and diarrhea or even death[49,50]. The physiological explanation for this negative effect of starch overload is that, unlike humans, horses cannot hydrolyze high amounts of starch as they have less pancreatic alpha amylase. Consequently, most starch is fermented in the large intestine, which lowers the cecal pH due to the production of lactic acid and CO_2[5]. Although there is a consensus among studies that the diet has a strong role in altering the GI microbiome, there are differences in the findings of what bacteria are most abundant and most affected by dietary changes.

In studies investigating the sudden increase in starch in the diet the intestinal pH and species richness decreased, with a shift from fiber-fermenting bacteria, such as Fibrobacteres, to starch-fermenting bacteria, such as Proteobacteria and lactic acid bacteria[5,20,51]. Specifically, the inclusion of starch has been found to have a large effect on the Firmicutes phylum, with increased abundances in *Veillonellaceae*, *Streptococcaceae* and *Lactobacillaceae* and reduced abundances of *Clostridiaceae* and *Ruminococcaceae*[20]. The large amounts of starch-fermenting bacteria turn carbohydrates into lactic acid and volatile fatty acids, the most common of which are butyric acid, propanoic acid and acetic acid[20]. The adaption of the GI microbiota to a new diet can occur

relatively quickly, as can be seen in a study with horses moving from a hay to grass diet, which are compared to horses constantly on a grass diet. The microbiome of the horses that had a dietary shift resembled the one from the horses without a change in diet after four days[52].

Overall, horses given concentrate feed have been found to have a lower microbial richness and a less stable microbiome than forage fed horses[20,53,54]. Varying abundances of a large number of bacterial species in horses on a high starch diet has also been observed, with a shift in 85 OTUs[55]. The consequences of this may play a role in the pathogenesis of diseases such as laminitis and colitis, as well as behavioral stress responses[45,51,55]. Such diseases that have been correlated with changes in the GI microbiome will be examined more closely in the next chapter.

2.2.3. Handling and Stress

Other potentially important aspects of domestication are different stress factors and exercise patterns. One stressful situation that has been studied in terms of its effect on the GI microbiome is the transportation of horses. In one study, the abundance of *Lactobacillus* spp. was increased in horses after transport[56], while another study found the order Clostridiales to be decreased[57]. The functional implications of these changes are, as yet, unclear. Interestingly, acute intensive exercising was found to influence the microbiota composition in one study[58], but there was no effect in endurance horses that were accustomed to exercising for a long period of time[59]. This may suggest that horses can adapt to the effects of exercise over time; but evidently, the effects of stress and varying exercise on the GI microbiota need to be examined further to get more reliable results. Additionally, as horses naturally live in groups with complex social structures, the impact of the social life on the equine physiology should also not be ignored. A study in semi-feral Welsh Mountain ponies has shown that the social structure within the group influences the GI microbiome, as the close physical contact can lead to a transfer of microbial species and therefore a more similar microbiota[60]. Considering the previously mentioned microbiota transfer from mares to foals, it seems likely that other close relationships have a similar effect.

2.2.4. Microbiome Changes with Disease

A wide range of diseases have been associated with changes in the microbiome of the GI tract, such as varying abundances of different microbial species or reduced diversity of the microbial community. Dysbiosis, or an altered composition of the microbiota, has been considered an important factor in GI diseases[61]. However, the cause and effect of these diseases and the changes in the microbiome are difficult to differentiate, as it could be the altered microbiome that leads to disease or the disease that induces a change in the microbiome. Nevertheless, before exploring cause and effect, the typical microbiome of horses with such diseases needs to be determined. To examine this, horses with a given disease have been compared to healthy counterparts and to the assumed core microbiome of a healthy horse.

2.2.4.1. Metabolic Diseases

2.2.4.1.1. Obesity

Obesity can be the consequence of a prolonged high starch diet, as the high energy density in concentrate feed may exceed the energy required for exercise in domestic horses. Incidence of obesity in domestic horses has become increasingly common and can enhance the risk of other diseases, such as laminitis, insulin resistance and Equine Metabolic Syndrome (EMS)[62].

Obese horses show some differences in GI microbial composition compared to healthy horses. Several studies found an increase in the abundance of bacteria from the Firmicutes phylum[62–64]. However, in some studies Fibrobacteres, Bacteroidetes and Actinobacteria, were decreased in obese horses[62,63], while the opposite was found in a different publication that noted an increase in Bacteroidetes and Actinobacteria in horses with obesity[64]. These contradictory results could

be due to various influencing factors as mentioned in the previous chapter, such as feed mixture and location specific factors or different scientific methods, such as time points of sampling or sequencing techniques. The lack of overarching results in these studies shows the urgency of further investigation of obesity in context with the GI microbiome.

2.2.4.1.2. Laminitis

Laminitis is a common disease of the feet in horses, in which the sensitive laminae are damaged through inflammation that is assumed to stem from bacterial products that escaped from the GI tract into the circulation[23,65]. Carbohydrate overload is assumed to cause laminitis through evoking a shift in the GI microbiota composition leading to a shift towards more gram-positive bacteria, such as *Lactobacillus* and *Streptococcus*, and subsequent GI inflammation[23]. *Streptococcus* have been found as the most predominant after oligofructose during the experimental induction of laminitis. In particular, species from the *Streptococcus bovis/equinus* complex were detected before disease onset, producing large amounts of lactate from the ingested oligofructose, which could show their role in inducing laminitis, as the resulting lower pH cause GI mucosal damage and induce a systemic inflammation[23,66]. The same complex has also been found in higher abundance in horses with a high starch diet in the previous chapter[53]. As the *Streptococci* abundances increase before laminitis onset, the secondary increase of *Lactobacilli* and *Escherichia coli* are considered to be an effect rather than a cause of the dysbiosis leading to laminitis[66]. Another study found a higher diversity in chronic laminitis horses compared to controls and a higher prevalence of *Ruminococcaceae* and *Clostridiaceae*[67]. Consequently, there is still contradiction in the role of different microbial species in the development of laminitis, which needs to be investigated in further research.

2.2.4.2. Gastro-Intestinal Infections

2.2.4.2.1. Bacterial Infections: Colitis, Equine Grass Sickness

Colic and colitis are not specific diseases but rather a description of symptoms from a range of possible sources. Colic describes abdominal pain, while colitis is an inflammation of the intestinal mucosa[68]. A possible change in the microbiome leading to colic could be the reduced overall diversity of microbiota[69]. However, other studies focus not on the overall diversity, but the specific bacteria that have changing abundances: When comparing horses with colitis with healthy horses, Costa et al. (2012) found the core microbiome to consist of Bacteroidetes (40%), Firmicutes (30%) and Proteobacteria (18%) in colitis horses, as opposed to 14%, 68% and 10% respectively in healthy horses[70]. Hence the core phyla were unchanged, but their abundances varied strongly, which implies that relative increases in Proteobacteria and Bacteroidetes and decreases in Firmicutes are associated with GI inflammation[61,70]. Overall no significant reduction in microbial diversity and richness was found in two publications, which contradict the assumption of a lower bacterial diversity in diseased horses, but instead focus on the shift in microbial composition in the GIT[70,71].

Another type of bacterial infection of the GIT is Equine Grass Sickness (EGS), a GI disease that is associated with *Clostridium botulinum* presence, specifically the toxin it produces, in the GIT. Leng et al. (2018) found a dysbiosis similar to colitis in EGS horses, with an overall lower microbial diversity, including increased Bacteroidetes and Proteobacteria and reduced Firmicutes and Verrucomicrobia abundances[72], which is a microbial shift comparable to colic and colitis[73]. Thus, is seems that high abundances of Proteobacteria and Bacteroidetes are correlated with a number of disturbances to the GIT and these changes may be a consequence, rather than cause, of these conditions. Further research should focus on the timing of these changes with regards to these diseases, and specific genera within phyla, such as Proteobacteria, which are implicated in specific conditions.

2.2.4.2.2. Parasite Infections

The equine GIT not only harbors commensal microbiota but may also contain parasitic species that share the same ecological niche as bacteria[74]. There are different ways helminths can potentially influence the GI microbiota of their host. The parasites could induce a reaction by the host immune system or directly by the host cells in the intestine, which could then have an impact on the microbiome. On the other hand, the secretory and excretory products of the parasites in the GIT could have a direct influence on the GI microbiome[75]. Additionally, there are a number of parasites that thrive in the same environment in a host species as commensal bacteria. Consequently, it is likely that they share this environment and interact and compete with each other due to their close proximity. Intestinal parasites can influence their niche by changing availability of nutrients and space for other microorganisms[76,77].

Cyathostomins are the most prevalent helminths in horses; especially young horses kept in groups are at risk of infection. In a study of Thoroughbred youngstock, Peachey et al. (2019) found a shift in microbiota composition in acutely infected horses with Cyathostomin infection[34]. When comparing horses with high and low parasite burden, Peachey et al. (2019) found higher abundances of bacterial families *Eubacteriaceae* and *Mogibacteriaceae* from the class Clostridia (phylum Firmicutes) and lower abundances of families *Prevotellaceae* and *Paraprevotellaceae* (phylum Bacteroidetes) in horses with high compared to low parasite burdens[34]. These changes were reversed after anthelmintic treatment. Furthermore, there was a reduction in richness linked to acute infection[34]. Further studies exploring host-helminth interactions in horses support the hypothesis that acute infection may lead to dysbiosis[30,78], but chronic infection has little impact on the gut microbiota[31]. Due to the interaction between helminths, the host immune system and the commensal GI microbiota, Walshe et al. (2020) suggest that the presence of helminths in the equine GIT should be considered a natural part of the GIT environment and may even be beneficial for host health[74].

2.2.4.3. Immune-mediated Diseases: Asthma, Hypersensitivity

In immune-mediated diseases the interaction between GI microbiota and disease is less clear. For example, there are two publications examining asthma and allergies in context of their GI microbiota. In horses with a Culicoides hypersensitivity and severe equine asthma there was no significant difference in gut microbial diversity between cases and healthy controls[79], while horses with asthma who underwent a change in diet did not react to the dietary change in the same way as healthy horses did, as their microbiota composition remained unchanged[80]. However, there is not sufficient evidence to determine a typical asthmatic horse GI microbiome, this requires further research.

2.2.5. Antibiotics and Anthelmintics

Not only diseases can influence the GI microbiome, also the methods of treatment can have an impact. Anthelmintic drugs are commonly used to control parasites in healthy and infected horses, however, additionally to parasites, bacterial communities are affected. Two studies have demonstrated a reduction in bacterial diversity post-anthelmintic administration in healthy horses with worm burdens[74,81]; furthermore, Walshe et al. (2019) reported a concomitant increase in abundance of Proteobacteria and reduction in Bacteroidetes, similar to an inflammatory response of the intestine. However, this was attributed to the removal of helminths from the GIT, not the anthelmintic itself[74]. In further studies, in horses with a low/negligible infection rate, anthelmintic treatment led to minor, but significant, differences in microbiome composition[34,82]. This implies that the reaction of the GI system to anthelmintic treatment may depend on the parasite burden of the horse prior to treatment.

While anthelmintics aim to reduce the parasite burden, antibiotics have a different function in that they target bacteria directly. Several publications have examined the effect of antibiotics on

the GI microbiome and all have found a significant decrease in microbial richness and diversity independent of the type of antibiotic used, although each drug had a different impact[29,73,83]. The strongest impact was seen immediately after treatment and the bacterial communities only started recovering after 25 days post-treatment[73]. Consequently, the administration of antibiotics has a strong influence on the GI microbiome, and despite differences depending on the specific treatment, the effects lasted for an extended time period. Other drug classes may also impact on gut microbial composition; for example, non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to cause a temporary reduction of microbial diversity and dysbiosis, especially a decrease in the Firmicutes phylum[84]. On the other hand, after treatment with omeprazole there was no significant change in the microbial composition[84].

Overall, the effect of drugs on the GI microbiome depends strongly on the specific drugs used and the health of the horse prior to treatment. While anthelmintics induce minor, variable changes of the microbial composition, the administration of antibiotics clearly reduces microbial diversity.

2.3. Microbiota Modulation in Horses

There are different ways to influence the intestinal microbiome intentionally, for example by using prebiotics, probiotics or fecal microbial transplants. The terms ‘prebiotic’ and ‘probiotic’ describe two distinct treatment types with a similar aspired outcome. Both aim to increase the proportion of ‘desirable’ bacteria in the microbiome and by this to impart health benefits on the user. However, while probiotics are living microbes, prebiotics are materials that act as substrates for a type of bacteria[85].

2.3.1. Probiotics

Probiotics have been used in humans to increase the balance of the gut microbiome[86] and to counteract diseases such as diarrhea and gastroenteritis[87]. The bacteria that have been used as probiotics in horses are similar taxa to those used in humans and other animals, such as the lactic acid bacteria *Lactobacillus* and *Bifidobacterium*[88]. The specific species administered depends on the host species, as the probiotic bacteria should be found in the GI tract naturally. This increases the chances of the bacteria fulfilling the criteria of a probiotic, which include three main points. The first criterium includes the capability to survive in the acid environment of the GI tract and to colonize the GIT. The second point is the ability to attach to epithelial cells of the intestinal wall, which inhibits the binding of pathogens and triggers the production of metabolites. The last important function of a probiotic is to produce substances that are beneficial for host health[89]. However, not all species of a bacterial phylum or family have probiotic qualities, therefore each species needs to be tested individually[90]. A difficulty in determining the impact of probiotics on the microbiome is that the measurement of a probiotic bacterial strain in the feces does not necessarily show the effectiveness of the probiotic, but merely shows that the probiotic has not been degraded in the GI tract[90]. Therefore, other parameters proving health benefits are necessary to justify the use of probiotics. Additionally, several postulated mechanisms of probiotics, such as the blockage of pathogenic bacteria through adherence of probiotic bacteria to the intestinal wall, are often tested in vitro or using cell culturing methods, which are limited in their capacity to reflect the mechanisms in vivo[87]. Furthermore, the effects of probiotics in one animal species cannot necessarily be translated directly to another species, hence it is important to test probiotics separately for each target species[87].

In animals, probiotics have been used in livestock production as an alternative to antibiotic treatment to increase production capacities through higher weight gains of the animals[91]. On the contrary to human studies, where the beneficial effect of probiotics is often measured on subjective parameters, such as emotional wellbeing[87], the use of probiotics in animals has more practical implications. For example, the yeast *Saccharomyces cerevisiae* reduced the amount of

pathogenic bacteria in rabbits through competitive exclusion, which had a beneficial effect on the pH in the GIT and increased the production of SCFA's, a major component of the energy gained in rabbits' metabolism, leading to increased weight gain[91]. Another study found a positive effect of *Lactobacillus plantarum* administration on the absorption rate of amlodipine in rabbits, possibly due to increased amounts of red blood cells and hemoglobin in the blood[92]. Consequently, the effect of probiotics is not limited to the GI microbiome but may have further reaching consequences for the immune system of the host. Probiotics have also been tested in their effect on parasite infections, as intestinal parasites interact with the GI microbiota and could therefore be influenced by probiotics and prebiotics. For example, the treatment with *Bifidobacterium animalis* may improve the immune response of mice towards the infection with *Strongyloides venezuelensis* by repairing the intestinal epithelium[93]. Also, when comparing the effects of the probiotic strains *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Lactobacillus casei* on *Trichinella spiralis* infection in mice, each strain showed a different level of efficacy in reducing parasite infection rates[94]. Furthermore, Petkevicius et al. (2004) found that the administration of organic acids reduced worm burdens of *Oesophagostomum dentatum* in pigs, as the lower pH does not provide a suitable environment for these parasites[95]. In a study testing the effect of probiotics on worm infection in mice, the findings varied strongly dependent on the methods used in the study, which shows the difficulty of determining the effect of probiotics, especially in a non-controlled study[93].

In horses, few studies have found probiotics to be beneficial, while more negative effects of probiotics have been found[96]. Although there are studies showing the efficacy of probiotics against pathogenic bacteria *in vitro*[97], evidence *in vivo* is limited and controversial. The administration of *Lactobacillus* and *Bifidobacterium* in foals has led to adverse effects, such as increased diarrhea and no influence on pathogenic bacteria[88,98,99]. Similarly, probiotic administration in colic patients has not had an influence on *Salmonella* shedding[100,101]. On the contrary, Tanabe et al. (2014) made a probiotic by combining different isolated commensal bacteria from horses, including *Lactobacillus* and *Bifidobacterium* spp., and found the administration in foals reduced the incidence and duration of diarrhea. Possibly the use of

probiotics containing bacteria specific to healthy horses is more beneficial than generic probiotic application. The wide variety of available probiotics and their strongly differing impact on the GI microbiota require a cautious approach in probiotic administration and further testing of probiotic species before widespread application in horses.

2.3.2. Prebiotics

Prebiotics have been defined as undigestible dietary carbohydrates that selectively promote supposedly beneficial microbial species[85,104]. In a more recent publication, the International Scientific Association for Probiotics and Prebiotics broadened the definition to include other substances than only carbohydrates and other applications than via the intestine. They define a prebiotic as: "a substrate that is selectively utilized by host microorganisms conferring a health benefit"[105] (p. 491). To be considered a prebiotic, a substance must fulfill the following main criteria: the prebiotic must not be digested by the host directly, but it must be fermented by the intestinal microbiota and it must enhance the growth or activity of specific health-related bacteria[105]. This can include non-digestible carbohydrates, such as oligosaccharides, as well as proteins and lipids[106].

Prebiotics, such as oligosaccharides, can be used to induce the production of short-chain fatty acids (SCFAs), which may have a positive influence on the immune system and metabolism of the host[107]. The most common oligosaccharides that are commercially used as prebiotics for animals include fructooligosaccharides (FOS), a-galacto-oligosaccharides (GOS), transgalacto-oligosaccharides (TOS), mannan-oligosaccharides (MOS) and xilo-oligosaccharides (XOS)[108]. MOS and Isomalto-oligosaccharide (IMO) have been used as prebiotics in rabbits due to their potential in inducing the production of volatile fatty acids, which led to an increased growth rate, amongst other changes[108]. Similarly, MOS and FOS supplementation in pigs led to an increase in food intake and body weight in lactating sows and piglets[109]. Short-chain fructooligosaccharides (scFOS) are commonly used as prebiotics as they are fermented by *Bifidobacterium* and

Lactobacillus spp., which are considered beneficial for host health[110,111]. In horses, there are only few studies to date investigating the effect of prebiotic supplementation on the GIT and on the health of horses. The supplementation with yeast (*Saccharomyces cerevisiae*) and micro-algae (*Aurantiochytrium limacinum*) was found to increase the relative abundances of the family *XIII Clostridiales* and *Veillonellaceae* in the cecum resp. colon and in the feces, but such a change was not detected in the hindgut[102]. A differential effect depending on the GI compartment was also found in Jerusalem artichoke meal supplementation, which contains a fructan similar to FOS and inulin and led to a beneficial effect on the hindgut through an increase of SCFAs but damaged the gastric mucosa[103]. A study using supplementation with scFOS found a reduced effect of carbohydrate overload on the intestinal microbiota in supplemented horses compared to unsupplemented controls. This was visible in the increase in *Lactobacillus* and *Streptococcus* in the control diet and the corresponding unchanged abundances in the supplemented group[110]. This stabilizing effect of FOS on the gut microbiota of horses with carbohydrate overload has also been found in another study[112]. When comparing different amounts of scFOS administered, the fecal concentration of lactate, acetate, propionate and butyrate increased proportionally to scFOS amounts, resulting in a lower pH, but *Lactobacillus* abundances remained the same[113]. Another study using prebiotics found different abundances of some uncommon bacterial species, but little effect on the overall community structure in the GI microbiome of horses[114]. These differences in findings show the importance of gaining more knowledge on the mechanism of prebiotics in horses. In addition to the small number of studies on FOS performed in horses, most of these also used culture-based techniques, which may favor bacteria thriving in culture[103,110,113]. Consequently, larger scale studies with more standardized procedures and modern techniques examining prebiotics in horses are required to reach a consensus on the efficacy of prebiotics in horses and to better understand the effect of prebiotics on the gut microbiome and the overall health of horses.

2.3.3. Fecal Microbial Transplants

Fecal microbial transplantation (FMT) is a concept of transferring fecal material to another host in order to improve gut health by introducing a different set of microbial species[115]. Fecal microbial transplants have served as an established treatment for *Clostridium difficile* infection in humans, and have also been considered for treatment of obesity and metabolic syndrome[115,116]. The treatment of *Clostridium difficile* with FMT may also be of relevance for horses, as diarrhea resulting from *Clostridium difficile* infection is especially common in foals[98].

Furthermore, treatment with FMT has been considered for various other diseases, such as neurologic diseases and cancer, but the evidence for the efficacy of this is limited[117]. A study on geriatric horses suffering from colitis and diarrhea found a reduction in diarrhea prevalence and an increase in alpha diversity following FMT treatment[118]. However, studies investigating the effect of FMT on the GI microbiome of horses are still rare [119] and to date there is no standardized protocol for the FMT procedure[120]. Consequently, the use of FMT requires further research in horses before it can be used in clinical practice.

3. Aims and Hypotheses

There is large variation between studies in their findings with regards to the composition of the GI microbiome, which could be, in part, due to small sample sizes and different methodologies used, and which makes the interpretation of the found bacterial composition and correlation with diseases difficult. To address this issue, we aimed to perform a meta-analysis of equine GI microbiome raw data; to find overarching factors that influence the microbiome, and the exact nature of those effects. Secondly, since Fructooligosaccharide (FOS) prebiotics may have a stabilizing effect on the GI microbiome[110], but have been insufficiently researched to date, we aimed to establish the impact of prebiotic treatment on the gut microbiota of Thoroughbred youngstock during dietary change.

Hypotheses:

1) The composition of the equine gut microbiota is significantly associated with biological factors (e.g. age, sex, diet), disease states (e.g. intestinal, metabolic disease) and technical factors across studies.
2) FOS supplementation reduces compositional change in the equine gut microbiota caused by diet change in horses.

The aims of this thesis were therefore:

1) To perform a meta-analysis of equine GI microbiome literature, based on re-analysis of existing 16S rRNA sequencing data from equine gut microbial samples, with a focus on identifying the impact of biological, environmental and technical factors on equine microbiome composition.
2) To measure the effect of FOS supplementation on the fecal microbiome of a cohort of Thoroughbred yearlings undergoing a dietary change from pasture to stabling and hay to concentrate feed.

4. Meta-analysis of Current Equine Gastro-intestinal Microbiome Research

The large variation between studies investigating the effect of biological and environmental factors on the equine GI microbiome highlights that, in addition to inter-individual variation between horses, methodological differences in sampling and analysis may have a strong impact on the results. For example, fecal samples have been commonly used to determine the intestinal microbial composition as they represent the distal section of the hindgut and can be taken from living animals. However, limited conclusions can be drawn about the bacterial population of the proximal GIT from the fecal microbial composition[26,35–37,121]. An additional difficulty in determining the effects of the various influencing factors on the equine gut microbial composition may be the small sample sizes of the studies, as this increases the likelihood that the results will be confounded by technical or individual variation, leading to diverging results between studies. Combining existing raw data from previous horse GI microbiome studies to increase the power of the analyses, and reduce the impact of confounding variation, could be a powerful tool to gain a better understanding of the equine microbiome.

To test the hypothesis of this meta-analysis, namely that the equine gut microbiome is significantly associated with biological and technical factors as well as disease states in a larger number of studies, the aim of this meta-analysis was to compile and re-analyze the 16S sequencing data from equine GI microbiome studies. With the resulting larger sample size, the meta-analysis aimed to find overarching patterns in the microbial composition and the factors influencing it.

4.1. Methods

4.1.1. Study Selection

The search for publications for the meta-analysis was performed using different databases (PubMed, CABI, Google Scholar, ScienceDirect) using the search terms 'horse' AND/OR 'equine' and 'microbiota' AND/OR 'microbiome' AND/OR 'intestinal' AND/OR '16s' according to the PRISMA protocol (Preferred Reporting Items for Systematic review and Meta-Analysis Protocols[122]. In order to include only papers with 16S data, the publications were included if the microbiome data was determined using 16S rRNA sequencing, including Illumina, Ion Torrent and 454 pyrosequencing. This selection was chosen as the vast majority of recent publications in this field use 16S rRNA sequencing, and it was necessary to use the same type of data to standardize the downstream bioinformatics and biostatistics pipeline. The searches using the above search terms were performed on 23.04.2021, resulting in 69 studies chosen according to the described selection criteria.

After the first selection of eligible studies, the studies were filtered for their data availability. Studies were included if their raw sequencing data and metadata were already publicly available, or if their corresponding authors were willing to share their data upon request. If there was no response from the authors after following up twice, the study was excluded. Due to the large number of unavailable metadata or sequencing data, as well as the lacking responses from the authors, there were only 40 studies that could be included after this process. This included the data from an unpublished study from Laura Peachey and the unpublished data of the prebiotic study (part II of this thesis). The studies with available sequencing data and metadata were uploaded to the web-based bioinformatics program MGnify for further analysis, during which 10 had to be excluded due to incompatible format of the data and delays and technical issues in the analysis pipeline. Furthermore, one study was excluded at the beginning of the statistical analysis due to strong deviance of the data from the rest of the studies. This resulted in 2796 samples from 29 studies that were finally included in the meta-analysis. The pipeline of study selection and bioinformatic analysis is visualized in a flow chart (Fig. 1). A complete overview of the studies

included in the meta-analysis and the studies excluded during the selection process can be found in the appendix (Tables 11 and 12).

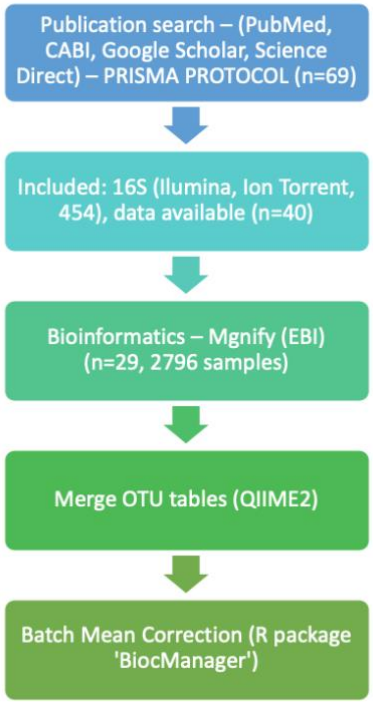

Figure 1: Flow diagram of meta-analysis pipeline including publication selection with number of studies (n) and samples, as well as bioinformatic analysis.

4.1.2. Bioinformatics

The quality filtering or raw sequence reads and subsequent taxonomic classification was performed on MGnify, a bioinformatics program of the European Bioinformatics Institute (EBI)[123] (https://www.ebi.ac.uk/meta genomics/).

This data analysis platform was used to get a standardized procedure which is the same for all studies and can be replicated in future studies. Furthermore, MGnify had been previously used to re-analyze 16S rRNA sequencing data in a meta-analysis of studies investigating helminth-microbiota interactions[124]. The pipelines used were SeqPrep, Trimmomatic, Biopython, Infernal,

cmsearch deoverlap script, FragGeneScan, Prodigal, InterProScan and MAPseq (https://www.ebi.ac.uk/metagenomics/pipelines/4.0).

The availability of data on the European Nucleotide Archive (ENA) was a prerequisite for the analysis on MGnify. Studies that had already published their raw sequencing data in ENA were requested to be analyzed in MGnify directly. Studies for which we got the sequencing data from the authors were uploaded to ENA and then analyzed by MGnify. The analysis results of each study are publicly available on the MGnify website (accession numbers listed in the appendix, table 11). The OTU tables and taxonomies of all studies were then merged using QIIME2 for statistical analysis (source code available in appendix chapter 2).

4.1.2.1. Metadata

The metadata was structured to account for biological variables and possible confounding factors in the data (see appendix table 10). These factors were divided into two groups: technical and biological factors. The technical factors involved variation in scientific methods, such as instrument model, gene region and library layout. The instrument model included sequencing machines for 454 sequencing, Ion Torrent and Illumina MiSeq. The sequencing was either single or paired end and the sequenced gene regions varied as V1-V2, V1-V3, V3, V3-V4, V4, V3-V5 or V4-V5 of the bacterial 16S gene.

The metadata included environmental factors, such as diet, management, location and season, as well as biological factors, such as age, sex, breed and disease, as well as sample type and sampling location. However, several external factors, including management, location and season, proved difficult to control, as there were too many possible confounders associated with each study and most studies did not describe the details of these factors in their metadata. Therefore, making categories within the factors management, location and season would have led to small sample sizes and unspecific groups, which would not have given reliable results, hence these three factors were excluded from analysis. Further detail was included in the sample

type, which involved fecal, mucosal and luminal samples. The mucosal and luminal samples were from different parts of the GIT, namely the rectum, cecum, colon, duodenum, ileum, jejunum, small intestine and stomach. The diet of the horses was very variable between the studies, including a wide range of feed brands and forage-grain combinations. Therefore, the assessment of diet was divided according to the fraction of Non-Structural Carbohydrates (NSC), which is a level for the sugar and starch content of the diet, into low (forage or balancer only), restricted (up to 25% starch) and high (over 25% starch). The age of the horses was examined through forming age ranges to account for the development of the GI microbiome over time, based on the timepoints of microbial variation in horses found in the literature. This included the age categories of 0-2 months, 2-6 months, 6 months to 2 years, 2-20 years and 20-30 years. The breed of the horses was briefly considered but then excluded from analysis due to the high number of crossbreeds and missing breed information. The sex was divided into male and female. The disease factor of the horses was divided into intestinal diseases (diarrhea, Equine Grass Sickness (EGS), colic and colitis) and metabolic diseases (obesity, laminitis, insulin dysregulation and Equine Metabolic Syndrome (EMS)); as well as antibiotic and anthelmintic treatment and healthy controls.

4.1.2.2. Statistical Analysis

The statistical analysis was performed in Calypso[125] and R[126]. In Calypso, data filtering and normalization was set to remove samples with less than 1000 sequence reads and to include only the top 3000 taxa which have a relative abundance of at least 0.01% and the data was normalized by total sum normalization and transformed by square root. The data from one study was removed as it appeared as a major outlier on Principle Coordinates Analysis (PCoA), thus skewing the data (Fig. 2).

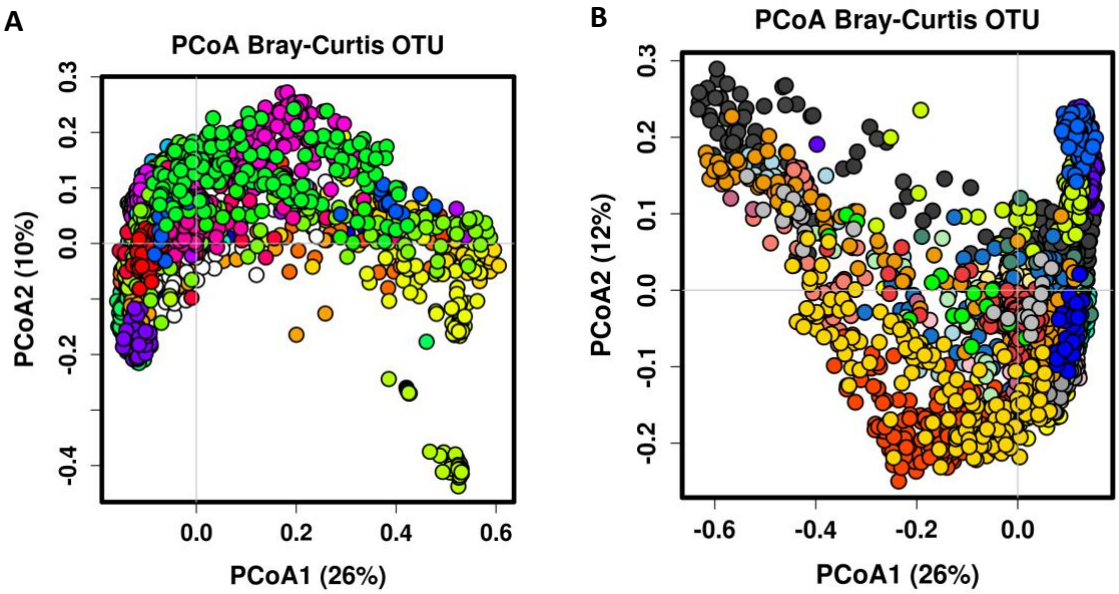

Figure 2: Principle Coordinates Analyses (PCoA) of the fecal microbial profiles of horses from the meta-analysis showing the similarity of microbiome composition of each horse: comparison of PCoA with 30 studies included (A) and one outlier study removed (B). A: the extreme outlier study is visible in light green (bottom right of the plot). B: The PCoA with this study excluded showed improved resolution.

The multivariate tests and visualizations, unsupervised Principal Coordinates Analysis (PCoA) and supervised Canonical Correspondence Analysis (CCA), were performed to compare the beta diversity between in the samples grouped according to different technical and biological factors. To determine statistical differences in different phyla between groups while taking into account the biological relevance of these bacteria, a Linear Discriminant Analysis (LDA) Effect Size (LEfSe[127]) was performed. The LDA score is used in the LEfSe to determine the degree of difference in relative microbial abundance between features of different groups, hence it can be seen as a ranking of significant differences between groups based on their biological relevance[127]. Additionally, the microbial alpha diversity was measured by the bacterial richness, evenness and the combination of the two (the Shannon index).

After initial data analysis it was noted that there was a large variation between samples due to the different gene regions and instrument models used for sequencing (Fig 3, 4). After evaluating the different options to reduce the impact of this technical variation statistically in several

publications[128–130], we found that a Batch Mean Correction (BMC) would be most appropriate to account for technical variation. We based this on the structure of our data that corresponded to the criteria for using a BMC, namely that the batch, in this case the sequencing instrument model and the gene region sequenced, is known and systematic[128]. The BMC to correct for gene region and instrument model (instrument-gene) was performed on the data from different studies using the R package "BiocManager"[131] (see R code in appendix chapter 1). In this case, the Total Sum Scaling (TSS) and square root normalization were also performed in R before the BMC, as well as the removal of blanks and the faulty study, hence the filtering and normalization in Calypso was set to zero (see R code in appendix chapter 1). The statistical analysis pipeline, as described above, was then performed on the corrected data. To reduce the effect of confounders, the data was filtered for fecal samples and healthy adult horses when comparing the effect of factors age and diet; when comparing the effect of sample type the data was filtered for healthy adults; and when comparing diseased/treated with healthy control samples the data was filtered for fecal samples in adults.

4.2. Results

A total of 69 papers were selected to be checked for raw sequencing data and metadata, of which 40 were requested for analysis in MGnify after ensuring the availability of the data, either from publication on the databases NCBI, ENA, MG-Rast, Mendeley or figshare, or from personal contact and sharing of the data. Of these 40 studies, 29 were finally included in the meta-analysis.

4.2.1. Comparison Pre and Post Batch Mean Correction

The PCoA of gene region and instrument model combined showed strong clustering of each group of different sequencing instrument models (454 pyrosequencing, Ion Torrent and Illumina sequencing) combined with the sequenced gene regions (V1-V2, V3-V4, V4, V3-V5 and V4-V5)

(Fig. 3A). This was especially visible when filtering out other influencing factors, by only including fecal samples from healthy adult horses (Fig. 4A). The samples from Illumina V1-V2, Ion Torrent V1-V2 and 454 sequencing V1-V2 gene regions were clearly split from the other samples, forming separate groups, whereas samples from the V3-V5 regions were more closely clustered regardless of platform in the PCoA plot (Fig. 3A).

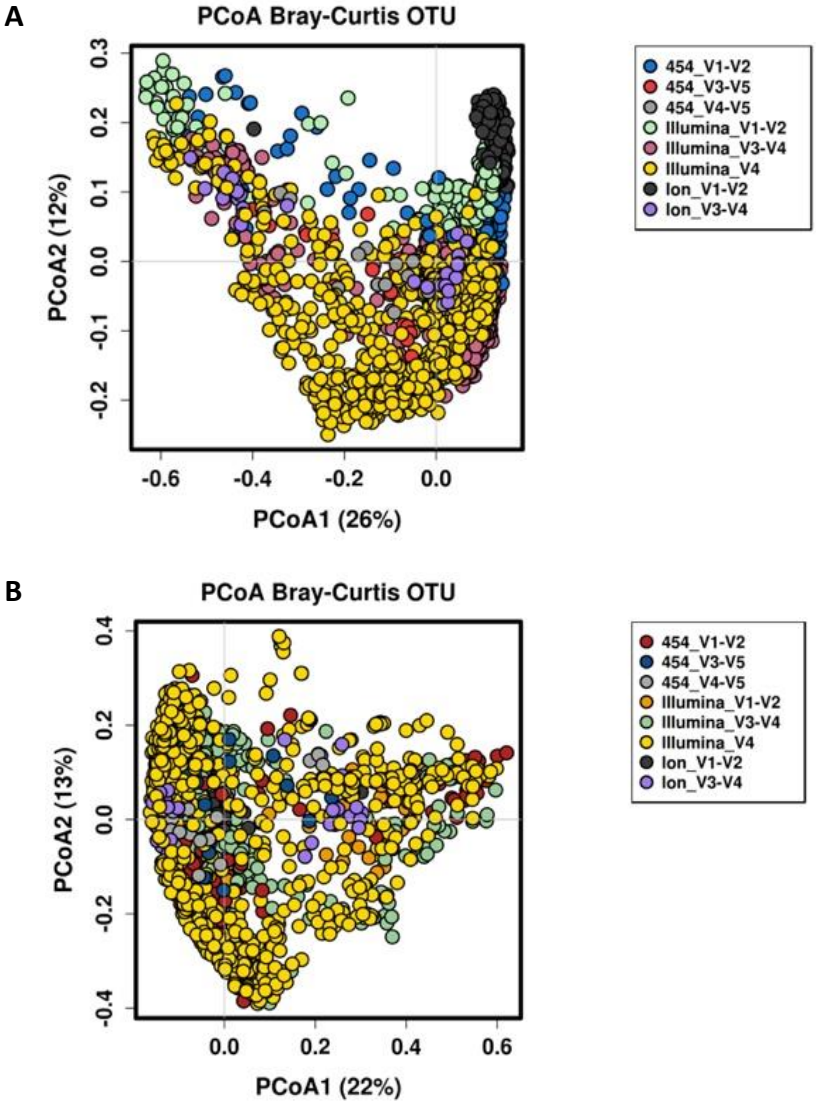

Figure 3: Principle Coordinates Analyses (PCoA) comparing similarities of the gastro-intestinal microbial profiles of horses in the meta-analysis grouped by sequenced gene region (V1-V2, V3-V4, V3-V5, V4, V4-V5) and sequencing machine used (454 pyrosequencing, Ion Torrent and Illumina sequencing) before and after Batch Mean Centering (BMC). A: Principle Coordinates Analysis (PCoA) plots clustered by gene region + instrument before BMC.B: PCoA post BMC with less clustering is visible after correction.

The technical variation strongly skewed the data, especially due to the large differences between the instrument models and gene regions. To determine the impact of biological factors and to compare all samples from the different gene regions and instrument models, a batch mean correction (BMC) was performed to correct for the combination of gene region and instrument model. After the BMC, the distribution of instrument model and gene region combined was clearly more regular on a smaller scale, hence there was a higher similarity between these groups (Fig. 3, 4). All subsequent analysis was performed on BMC corrected data.

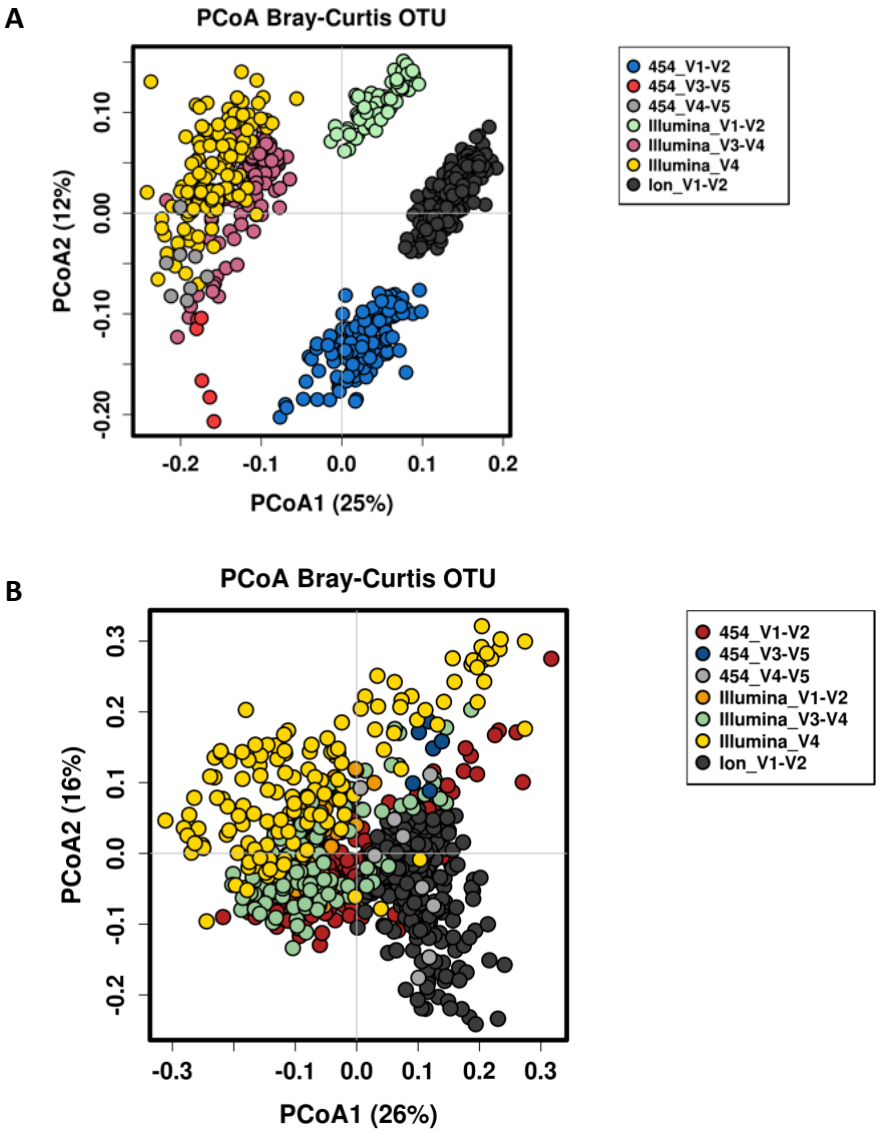

Figure 4: Principle Coordinates Analyses (PCoA) of the fecal microbial profiles of horses from the meta-analysis showing the similarity of microbiome composition of each horse grouped by sequenced gene region (V1-V2, V3-V4, V3-V5, V4, V4-V5) and sequencing machine used (454 pyrosequencing, Ion Torrent and Illumina sequencing). Comparison of adult fecal samples in the Principle Coordinates Analysis (PCoA) plots clustered by gene region and sequencing instrument pre Batch Mean Centering (BMC) and post BMC. A: Microbial profiles are clearly clustered according to gene region and sequencing instrument before BMC. B: Much less clustering of microbiome compositions is visible after BMC correction.

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4.2.2. Sample type and GIT region

In the comparison of sample types feces, mucosa and lumina, a larger variation was observed in luminal and mucosal samples than in fecal samples (Fig. 5). Significant differences in microbial composition were observed between all groups in the CCA (P=0.001) (Fig. 5A).

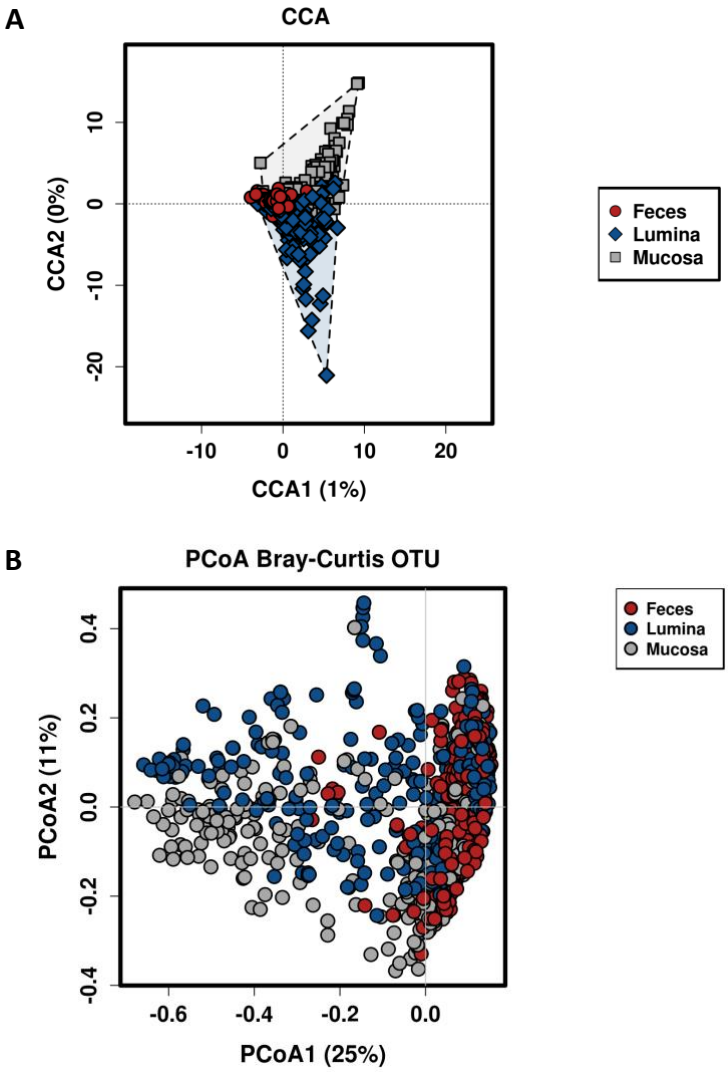

Figure 5: Beta diversity analyses of the gastro-intestinal microbial profiles of healthy adult horses in the meta-analysis grouped by sample type (fecal, mucosal and luminal). A: Canonical Correspondence Analysis (CCA) plot shows less clustering in mucosal and luminal samples than feces. B: Principle Coordinates Analysis (PCoA) plot using Bray Curtis distance matrices shows less variation in feces than in luminal and mucosal samples.

Since the mucosal and luminal sample types diverged in the above PCoA plot (Fig 5B), the microbial composition of the different regions of the GIT were subsequently examined in luminal and mucosal samples separately (Fig. 6).

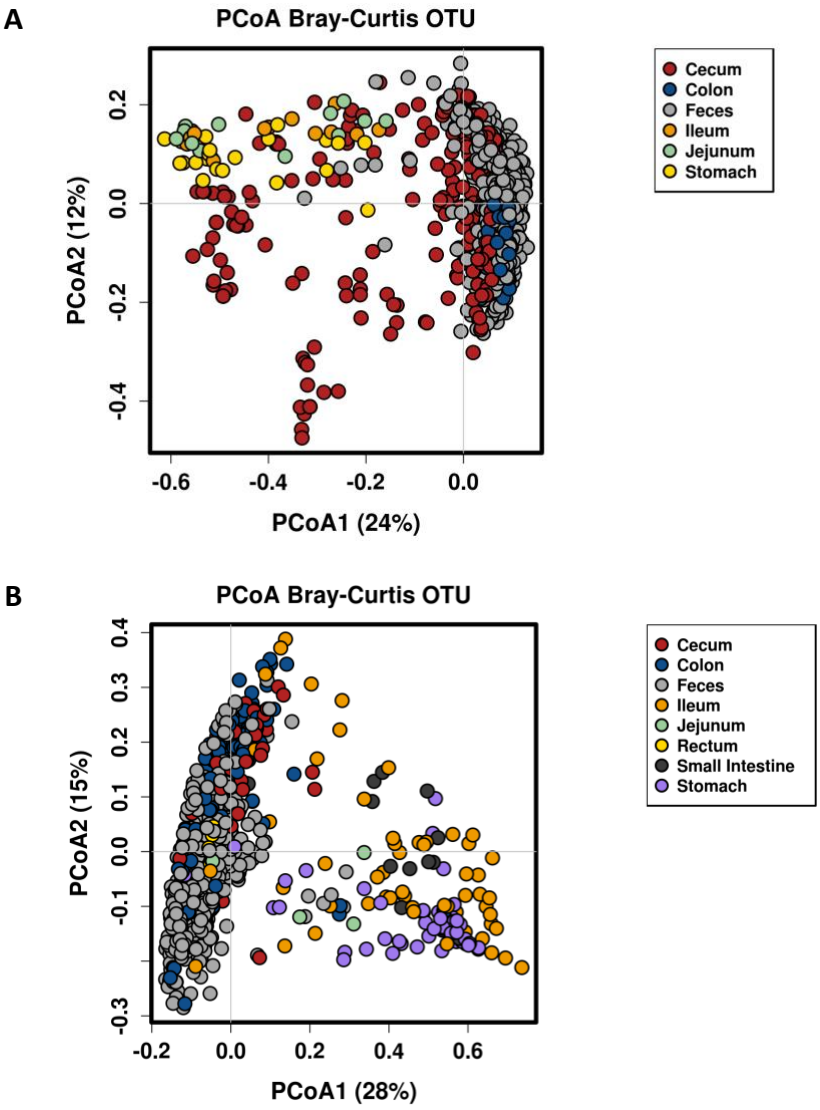

Figure 6: Principle Coordinates Analyses (PCoA) of the fecal microbial profiles of horses from the meta-analysis showing the similarity of microbiome composition of each horse grouped by sample region (stomach, ileum, jejunum, small intestine, cecum, colon, rectum and feces). Comparison of mucosal and luminal samples in different regions of the gastro-intestinal tract using Principle Coordinates Analysis (PCoA) of luminal samples and feces (A) or of mucosal samples and feces (B). Larger variation in microbiome composition is visible in luminal samples from the stomach, jejunum and cecum (A), as well as in mucosal samples from the stomach and ileum (B) compared to samples from other gut regions.

In the luminal samples, which were available from the stomach, jejunum, ileum, cecum and colon, as well as the feces, the LefSe analysis showed 8 bacterial phyla and 17 bacterial families that were differentially abundant between GIT regions (Table 1). The phyla Cyanobacteria, Tenericutes, Euryarcheaota, Kiritimatiellaeota were most abundant in the feces, as well as

Fibrobacteres (in particular the bacterial family *Fibrobacteraceae)* and Spirochaetes (family *Spirochaetaceae*). However, the largest difference in bacterial abundance levels (with Linear Discriminant Analysis (LDA) scores >5) were found between Proteobacteria, which was most abundant in the ileum; and Bacteroidetes, which was most abundant in the colon. A very strong association was made between the bacterial family Enterobacteriaceae (phylum Proteobacteria) and the ileum (LDA=5.465), showing the highest abundance in this location compared to the other regions. Further associations with an LDA score >5 were found with the highest abundance of the bacterial family *Pasteurellaceae* (phylum Proteobacteria) in the ileum and the highest abundance of *Lactobacillaceae* (phylum Firmicutes) in the stomach compared to the rest of the GIT (Table 1).

Table 1: Linear Discriminant Analysis Effect Size (LEfSe) analysis of luminal and fecal samples of healthy adult horses from the meta-analysis comparing taxonomic abundances of gut microbiota between sample regions (stomach, ileum, jejunum, small intestine, cecum, colon, rectum and feces). The table below shows the Linear Discriminant Analysis (LDA) score on phylum and family taxonomic level, with the intermediary taxonomic levels included to show the relationship between them. A score of 3-3.5 describes a low association, 3.5-4 a medium, 4-4.5 a high and >4.5 a very high association between the taxa and the investigated group.

Phylum	Class	Order	Family	Group	LDA Score
Actinobacteria				Feces	3.809
	Coriobacteriia				
		Eggerthellales			
			Eggerthellaceae		
Bacteroidetes				Colon	5.216
	Bacteroidia				
		Bacteroidales			
			Rikenellaceae	Colon	4.727
			Muribaculaceae	Cecum	3.589
			Bacteroidaceae	Cecum	3.906
Cyanobacteria				Feces	4.28
Euryarchaeota				Feces	3.998
Fibrobacteres				Feces	4.166
	Fibrobacteria				
		Fibrobacterales			
			Fibrobacteraceae	Feces	4.098
Firmicutes					
	Erysipelotrichia				
		Erysipelotrichales			
			Erysipelotrichaceae	Feces	3.748
	Negativicutes				
		Acidaminococcales			
			Acidaminococcaceae	Colon	3.589
	Clostridia				
		Clostridiales			
			Clostridiaceae	Stomach	4.48
			Ruminococcaceae	Colon	4.889
			Hungateiclostridiaceae	Colon	3.641
	Bacilli				
		Lactobacillales			
			Lactobacillaceae	Stomach	5.067
			Streptococcaceae	Ileum	4.727
Kiritimatiellaeota				Feces	4.392
Proteobacteria				Ileum	5.273
	Betaproteobacteria				
		Neisseriales			
			Neisseriaceae	Stomach	3.756
	Gammaproteobacteria				
		Pasteurellales			
			Pasteurellaceae	Ileum	5.098
		Enterobacterales			
			Enterobacteriaceae	Ileum	5.465
		Xanthomonadales			

			Xanthomonadaceae	Stomach	4.169
Spirochaetes				Feces	4.316
	Spirochaetales				
		Spirochaetales			
			Spirochaetaceae	Feces	4.222
Tenericutes				Feces	3.889
Unclassified				Cecum	4.248

In a second step, the relative bacterial abundance in mucosal samples from the stomach, jejunum, ileum, small intestine, cecum, colon and rectum, as well as from the feces, was compared. The strongest difference at phylum level was a higher abundance of Firmicutes in the stomach (LDA=5.301). In this phylum, the bacterial families *Veillonellaceae* (class Negativicutes), as well as *Lactobacillaceae* and *Streptococcaceae* (both class Bacilli) were most abundant in the stomach. In the cecum, the phylum Bacteroidetes was most abundant (LDA=5.105), however, within this phylum only the bacterial family *Prevotellaceae* was most abundant in the cecum, while other families were highest in the feces (*Rikenellaceae* and *Paludibacteraceae*), the colon (*Bacteroidaceae*) and the jejunum (*Muribaculaceae*). The phylum Proteobacteria was considerably higher in the jejunum than in the other locations (LDA=5.206), which was also reflected on family level, with *Rhizobiaceae*, *Burkholderiaceae*, *Pasteurellaceae*, *Xanthomonadaceae* and *Desulfovibrionaceae* most abundant in the jejunum (Table 2).

In contrast to the luminal samples, the LEfSe analysis of mucosal samples showed significant differences in 14 bacterial phyla and 38 bacterial families between the GIT compartments. Furthermore, there was a difference in bacterial abundances between the luminal and mucosal sample type of the same gut region; for example, *Clostridiaceae* (class Clostridia) was most abundant in the ileum in mucosal samples, but most abundant in the stomach in luminal samples. In some rare cases, the region of the highest abundance of a bacterial species was the same in both sample types, for example, Enterobacteriaceae (phylum Proteobacteria) was most

abundant in the ileum and *Lactobacillaceae* (phylum Firmicutes) was most abundant in the stomach compared to other GIT regions (Table 2).

Table 2: Linear Discriminant Analysis Effect Size (LEfSe) analysis of mucosal and fecal samples of healthy adult horses from the meta-analysis comparing taxonomic abundances of gut microbiota between sample regions (stomach, ileum, jejunum, small intestine, cecum, colon, rectum and feces). The table below shows the Linear Discriminant Analysis (LDA) score on phylum and family taxonomic level, with the intermediary taxonomic levels included to show the relationship between them. A score of 3-3.5 describes a low association, 3.5-4 a medium, 4-4.5 a high and >4.5 a very high association between the taxa and the investigated group.

Phylum	Class	Order	Family	Group	LDA Score
Actinobacteria				Ileum	4.25
	Actinobacteria				
		Propionibacteriales			
			Propionibacteriaceae	Jejunum	3.53
	Coriobacteriia				
		Eggerthellales			
			Eggerthellaceae	Colon	3.321
		Corynebacteriales			
			Corynebacteriaceae	Ileum	3.808
Bacteroidetes				Cecum	5.105
	Bacteroidia				
		Bacteroidales			
			Prevotellaceae	Cecum	4.649
			Rikenellaceae	Feces	4.556
			Muribaculaceae	Jejunum	4.38
			Paludibacteraceae	Feces	3.334
			Bacteroidaceae	Colon	3.644
Cyanobacteria				Colon	3.904
Euryarchaeota				Feces	3.926
Fibrobacteres				Rectum	4.52
	Fibrobacteria				
		Fibrobacterales			
			Fibrobacteraceae	Rectum	4.462
Firmicutes				Stomach	5.301
	Erysipelotrichia				
		Erysipelotrichales			
			Erysipelotrichaceae	Rectum	4.123
	Negativicutes				
		Vellionellales			
			Veillonellaceae	Stomach	3.648
		Acidaminococcales			
			Acidaminococcaceae	Cecum	3.721
	Clostridia				
		Clostridiales	*Eubacteriaceae*	Rectum	3.646
			Clostridiaceae	Ileum	4.263
			Lachnospiraceae	Cecum	4.849
			Ruminococcaceae	Rectum	4.808

			Christensenellaceae	Rectum	4.495
			Peptococcaceae	Rectum	3.531
			Hungateiclostridiaceae	Rectum	3.562
			Defluviitaleaceae	Jejunum	3.299
	Bacilli				
		Lactobacillales			
			Lactobacillaceae	Stomach	5.277
			Streptococcaceae	Stomach	4.631
			Carnobacteriaceae	Ileum	3.95
			Aerococcaceae	Ileum	3.898
		Bacillales	*Planococcaceae*	Ileum	3.331
			Staphylococcaceae	Ileum	4.08
Kiritimatiellaeota				Rectum	5.079
Lentisphaerae				Rectum	4.458
	Oligosphaeria				
		Oligosphaerales			
			Oligosphaeraceae	Rectum	3.138
Plactomycetes				Rectum	4.395
	Planctomycetia				
		Pirellulales			
			Pirellulaceae	Rectum	3.215
Proteobacteria				Jejunum	5.206
	Alphaproteobacteria				
		Rhizobiales			
			Rhizobiaceae	Jejunum	3.517
	Betaproteobacteria				
		Burkholderiales			
			Burkholderiaceae	Jejunum	3.417
	Gammaproteobacteria				
		Pasteurellales			
			Pasteurellaceae	Jejunum	5.071
		Enterobacterales			
			Enterobacteriaceae	Ileum	4.041
		Xanthomonadales			
			Xanthomonadaceae	Jejunum	3.531
	Deltaproteobacteria				
		Desulfovibrionales			
			Desulfovibrionaceae	Jejunum	3.825
Spirochaetes				Rectum	4.531
	Spirochaetales				
		Spirochaetales			
			Spirochaetaceae	Rectum	4.463
Synergistetes				Cecum	4.094
	Synergistia				
		Synergistales			
			Synergistaceae	Cecum	3.404
Tenericutes				Rectum	4.213
	Mollicutes				
		Anaeroplasmatales			
			Anaeroplasmataceae	Rectum	3.164
Unclassified				Cecum	4.256
	Unclassified				
		Unclassified			

			Unclassified	Cecum	5.05
Verrucomicrobia				Rectum	4.431
	Verrucomicrobiae				
		Verrucomicrobiales			
			Akkermansiaceae	Rectum	3.806

4.2.3. Age

To look at the impact of age on equine gut microbial composition, the largest influencing factors were filtered, hence the difference between different age ranges was investigated in the feces and healthy control horses.

The PCoA and CCA plots show large variation in the group of foals <2 months of age, while the older age groups show a smaller variation between samples (Fig. 7). In the CCA there was a significant difference in microbial populations between the different age ranges (P=0.001) (Fig. 7B). Similarly, the Shannon index showed significant differences in alpha diversity between groups (P<0.001), with the groups of younger horses (≤2 years) having a lower diversity index; whilst in the <2 months old foals the variation in alpha diversity between animals was very large (Fig. 8).

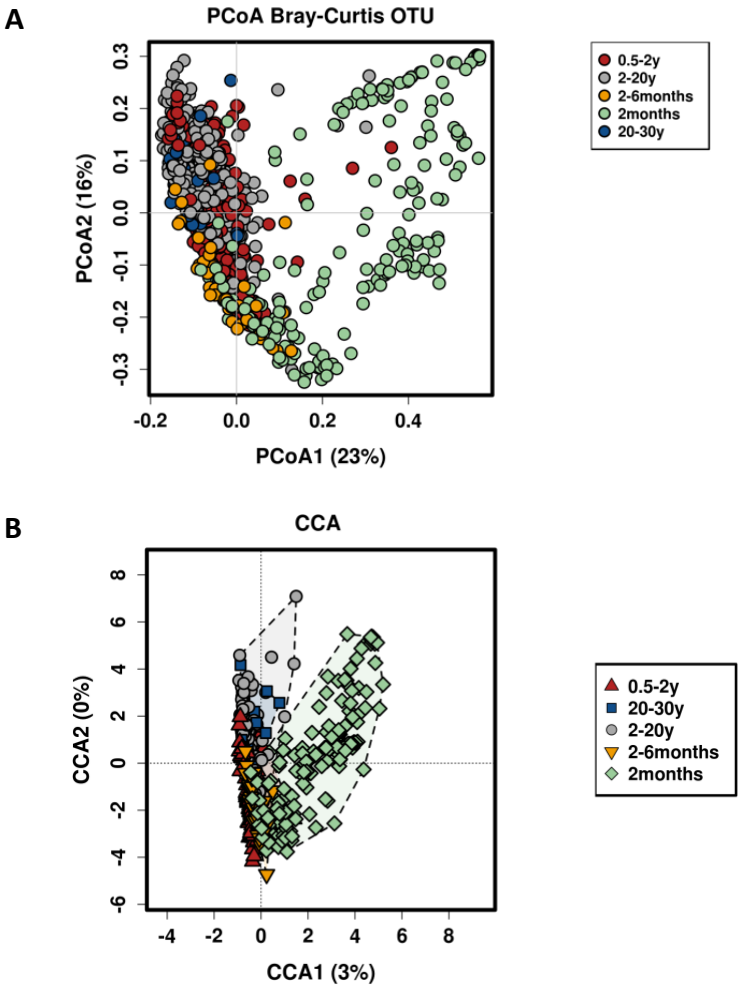

Figure 7: Beta diversity analyses of the fecal microbial profiles of healthy horses in the meta-analysis grouped by age (≤2 months, 2-6 months, 0.5-2 years, 2-20 years and 20-30 years). Both the Principle Coordinates Analysis (PCoA) (A) and Canonical Correspondence Analysis (CCA) (B) plots of age range show the largest variation of microbial composition in ≤2 months old horses compared to other age groups.

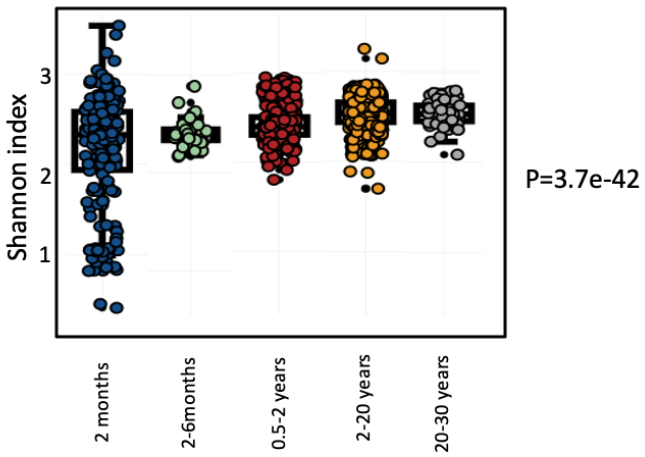

Figure 8: The Shannon Index of alpha diversity between fecal samples of healthy horses of different age ranges of the meta-analysis grouped by age (<2 months, 2-6 months, 0.5-2 years, 2-20 years and 20-30 years) shows a large variation in alpha diversity in the age group of ≤2 months old foals.

When looking at differences in phyla using LEfSe, <2 months old foals had the highest abundances of Actinobacteria, Verrucomicrobia, Proteobacteria, Euryarchaeota, Fusobacteria and Chloroflexi (Table 3). The geriatric horses aged 20-30y had the highest abundances of Kiritimatiellaeota, Lentisphaerae, Tenericutes and Fibrobacteres. The age groups 2-20y and 20-30y had similar abundances of many phyla, including Fibrobacteres, Spirochaetes, Tenericutes, Cyanobacteria and Bacteroidetes, with only three phyla with different abundances between the two groups. On the contrary, when comparing the microbiome of 2-20y old horses to <2mo old foals there were 14 phyla with differences in abundance; then comparing them to 2-6mo old horses there were 6 different phyla and comparing them to 6mo-2y there were 9 different phyla. Consequently, the age groups 2-20y and 20-30y were considered to be similar, and to represent adults for the rest of the analysis, while the foals and youngsters up to 2y of age were excluded when looking at other factors, e.g. disease, due to the possibility of skewing the results due to age associated differences between samples.

Table 3: Linear Discriminant Analysis Effect Size (LEfSe) analysis of fecal samples of healthy horses from the meta-analysis comparing taxonomic abundances of gut microbiota between age groups (<2 months, 2-6 months, 0.5-2 years, 2-20 years and 20-30 years). The table below shows the Linear Discriminant Analysis (LDA) score on phylum and family taxonomic level, with the intermediary taxonomic levels included to show the relationship between them. A score of 3-3.5 describes a low association, 3.5-4 a medium, 4-4.5 a high and >4.5 a very high association between the taxa and the investigated group: here there is a large number of differential abundances, especially many taxa associated with <2 months old foals.

Phylum	Class	Order	Family	Group	LDA Score
Actinobacteria				<2m	3.841
	Coriobacteriia				
		Eggerthellales			
			Eggerthellaceae	2-6m	3.546
Bacteroidetes				2-20y	4.969
	Bacteroidia				
		Bacteroidales			
			Prevotellaceae	0.5-2y	4.475
			Rikenellaceae	20-30y	4.496
			Muribaculaceae	0.5-2y	3.123
			Paludibacteraceae	20-30y	3.478
			Tannerellaceae	<2m	3.661
	Sphingobacteria				
		Sphingomonadales			
			Sphingomonadaceae	<2m	3.305
Chloroflexi				<2m	3.76
Cyanobacteria				2-20y	3.977
Euryarchaeota				<2m	3.758
	Methanomicrobia				
		Methanomicrobiales			
			Methanocorpusculaceae	20-30y	3.091
Fibrobacteres				20-30y	4.254
	Fibrobacteria				
		Fibrobacterales			
			Fibrobacteraceae	20-30y	4.279
Firmicutes				2-6m	4.947
	Erysipelotrichia				
		Erysipelotrichales			
			Erysipelotrichaceae	20-30y	3.82
	Negativicutes				
		Acidaminococcales			
			Acidaminococcaceae	2-20y	3.447
		Selenomonadales			
			Selenomonadaceae	0.5-2	3.224
	Clostridia				
		Clostridiales			
			Eubacteriaceae	2-6m	3.366
			Clostridiaceae	<2m	3.694
			Lachnospiraceae	2-6m	4.548
			Ruminococcaceae	2-6m	4.74
			Christensenellaceae	2-6m	4.18
			Peptostreptococcaceae	<2m	3.683
			Hungateiclostridiaceae	0.5-2y	3.565
	Bacilli				

		Lactobacillales			
			Lactobacillaceae	<2m	3.997
			Streptococcaceae	<2m	4.072
			Enterococcaceae	<2m	3.444
Fusobacteria				<2m	3.975
	Fusobacteriia				
		Fusobacteriales			
			Fusobacteriaceae	<2m	3.788
Kiritimatiellaeota				20-30y	4.482
Lentisphaerae				20-30y	3.87
Proteobacteria				<2m	5.032
	Alphaproteobacteria				
		Rhizobiales			
			Methylobacteriaceae	<2m	3.24
	Betaproteobacteria				
		Burkholderiales			
			Burkholderiaceae	<2m	4.617
	Gammaproteobacteria				
		Pasteurellales			
			Pasteurellaceae	2-20y	3.876
		Pseudomonadales			
			Moraxellaceae	<2m	4.714
			Pseudomonadaceae	<2m	3.314
		Enterobacterales			
			Enterobacteriaceae	<2m	4.238
	Deltaproteobacteria				
		Desulfovibrionales			
			Desulfovibrionaceae	<2m	3.257
Spirochaetes				2-20y	4.305
	Spirochaetales				
		Spirochaetales			
			Spirochaetaceae	2-20y	4.264
Tenericutes				20-30y	3.899
	Mollicutes				
		Anaeroplasmatales			
			Anaeroplasmataceae	2-20y	3.041
Unclassified				2-20y	4.037
Verrucomicrobia				<2m	3.713

The results of LEfSe at family level showed that *Ruminococcaceae* and *Lachnospiraceae* (both class Clostridia) had significantly higher abundances in 2-6 months old foals (LDA>4.5) than in other groups. Further strong associations (LDA>4.5) were found in *Moraxellaceae* and *Burkholderiaceae* (both phylum Proteobacteria), which were highest in <2 months old foals compared to the other groups. This corresponds with the finding that the phylum Proteobacteria was most abundant in <2 months old foals (LDA=5.032) and the most significant finding in the LEfSe of age group comparisons (Table 3).

The age group from 0.5-2 years had very few bacteria that were more abundant than at other ages, one of which was the family *Prevotellaceae* from the phylum Bacteroidetes. In the oldest group of 20-30y old horses, the families *Rikenellaceae*, *Fibrobacteraceae*, *Paludibacteraceae*, *Erysipelotrichaceae* and *Methanocorpusculaceae* were found to be most abundant (Table 3).

4.2.4. Sex

There was no clear clustering visible in the PCoA between male and female horses (Fig. 9A). However, the alpha diversity was significantly higher in female horses, as can be seen in the Shannon index (p=0.001; Fig. 9C). Also, the females have a more varied GI microbiome in the CCA plot, however, this could be due to an outlier (Fig. 9B).

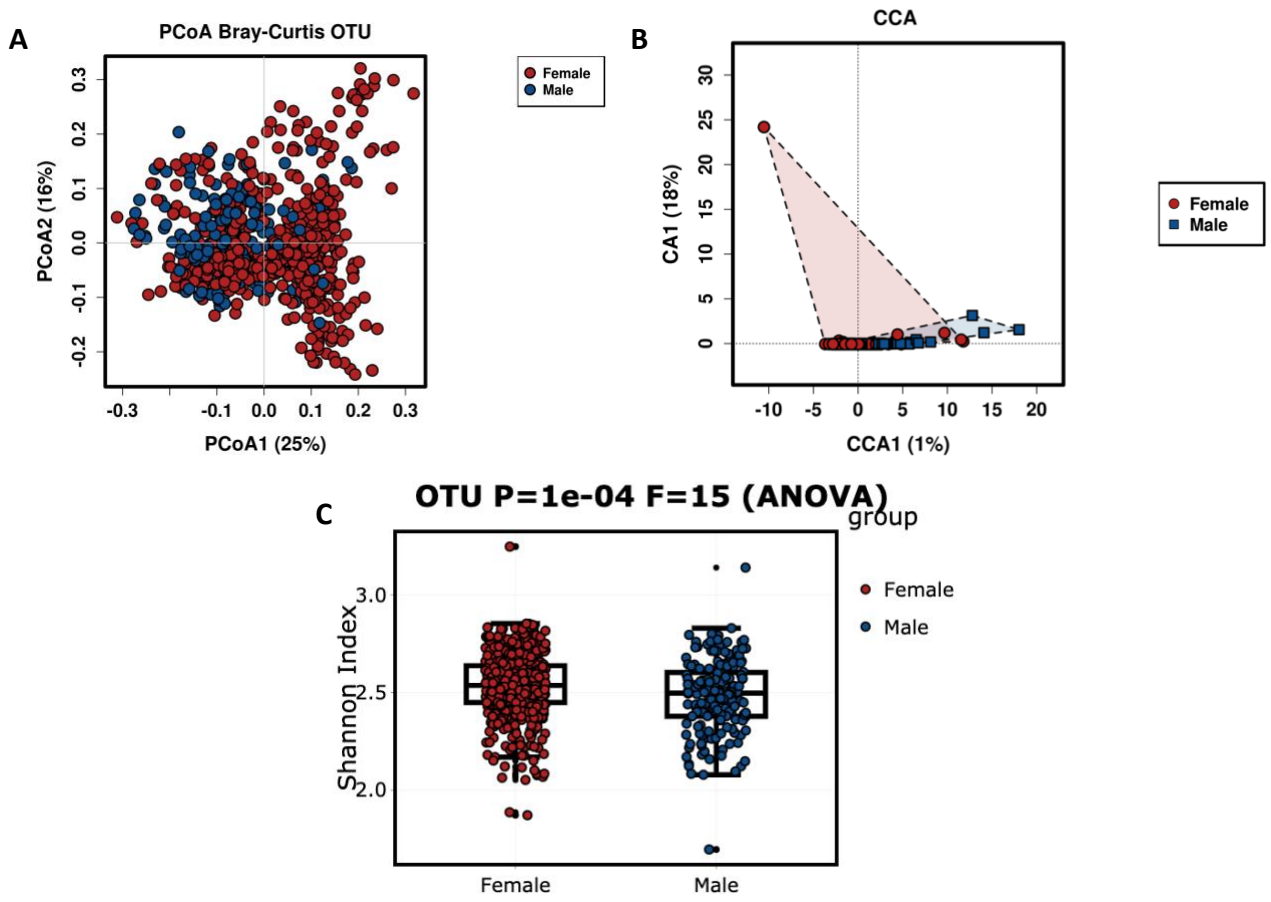

Figure 9: Comparison of alpha and beta diversities of the fecal microbial profiles of healthy adult horses in the meta-analysis grouped by sex (male/female). Principle Coordinates Analysis (PCoA) (A), Canonical Correspondence Analysis (CCA) (B) and Shannon diversity plot (C) comparing fecal samples of healthy adults, by gender. No clear clustering of different genders in the PCoA plot (A) was observed, but a significant difference (p=0.0001) in bacterial alpha diversity was observed between the groups, of which the females had the highest average Shannon index (C). This may also be reflected in the CCA plot showing a larger variety in the female samples (B).

Overall, the differences between the microbiota compositions of females and males was not as pronounced as those associated with other biological factors, with the highest LDA score being 4.353 (phylum Firmicutes in females) (Table 4). From the phylum Firmicutes a large number of bacterial families was associated with female horses, including 4 families from the class Clostridia (*Clostridiaceae*, *Lachnospiraceae*, *Ruminococcaceae* and *Christensenellaceae*) and 2 families from the class Bacilli (*Lactobacillaceae* and *Streptococcaceae*). Furthermore, the phylum Proteobacteria was most abundant in females, especially the families *Pasteurellaceae*,

Enterobacteriaceae and *Moraxellaceae*. Additionally, the phyla Actinobacteria, Planctomycetes and Verrucomicrobia (family *Akkermansiaceae*) were most abundant in females. On the other hand, the phyla Fibrobacteres (family *Fibrobacteriaceae*) and Spirochaetes (family *Spirochaetaceae*) were associated with male horses, as well as the bacterial family *Rikenellaceae* from the phylum Bacteroidetes, two families from the order Bacillales (*Bacillaceae* and *Planococcaceae*) as well as the family *Veillonellaceae* from the class Negativicutes (Table 4).

Table 4: Linear Discriminant Analysis Effect Size (LEfSe) analysis of fecal samples of healthy adult horses from the meta-analysis comparing taxonomic abundances of gut microbiota between sexes (male/female). The table below shows the Linear Discriminant Analysis (LDA) score on phylum and family taxonomic level, with the intermediary taxonomic levels included to show the relationship between them. A score of 3-3.5 describes a low association, 3.5-4 a medium, 4-4.5 a high and >4.5 a very high association between the taxa and the investigated group.

Phylum	Class	Order	Family	Group	LDA Score
Actinobacteria				Female	3.547
Bacteroidetes				Male	4.300
	Bacteroidia				
		Bacteroidales			
			Prevotellaceae	Female	3.847
			Rikenellaceae	Male	4.258
			Muribaculaceae	Female	2.816
			Bacteroidaceae	Female	3.047
		Marinilabiliales			
			Marinifilaceae	Male	2.700
Euryarchaeota				Female	3.445
	Methanomicrobia				
		Methanomicrobiales			
			Methanocorpusculaceae	Male	2.935
Fibrobacteres				Male	3.241
	Fibrobacteria				
		Fibrobacterales			
			Fibrobacteraceae	Male	3.134
Firmicutes				Female	4.353
	Negativicutes				
		Vellionellales			
			Veillonellaceae	Male	2.686
		Acidaminococcales			

			Acidaminococcaceae	Female	2.719
	Clostridia				
		Clostridiales			
			Clostridiaceae	Female	3.389
			Lachnospiraceae	Female	3.935
			Ruminococcaceae	Female	3.426
			Christensenellaceae	Female	3.681
			Hungateiclostridiaceae	Male	3.147
	Bacilli				
		Lactobacillales			
			Lactobacillaceae	Female	3.771
			Streptococcaceae	Female	3.399
			Leuconostocaceae	Male	2.925
		Bacillales			
			Bacillaceae	Male	2.888
			Planococcaceae	Male	2.443
Planctomycetes				Female	3.766
Proteobacteria				Female	3.431
	Gammaproteobacteria				
		Pasteurellales			
			Pasteurellaceae	Female	3.106
		Pseudomonadales			
			Moraxellaceae	Female	3.142
		Enterobacterales			
			Enterobacteriaceae	Female	3.056
		Aeromonadales			
			Succinivibrionaceae	Male	2.616
		Xanthomonadales			
			Xanthomonadaceae	Male	2.806
Spirochaetes				Male	4.162
	Spirochaetales				
		Spirochaetales			
			Spirochaetaceae	Male	4.023
Tenericutes					
	Mollicutes				
		Anaeroplasmatales			
			Anaeroplasmataceae	Female	2.665
Unclassified				Female	3.506
	Unclassified				
		Unclassified			
			Unclassified	Male	3.845
Verrucomicrobia				Female	4.112
	Verrucomicrobiae				
		Verrucomicrobiales			
			Akkermansiaceae	Female	3.255

4.2.5. Diet

The diet was grouped into high, restricted or low NSC/dietary starch content to enhance the comparability of the various diets. While the samples were rather evenly distributed on the PCoA plot, the CCA plot showed clear differences between the three diet types ($p=0.001$) (Fig. 10A,B). The alpha diversity of the samples was significantly higher in horses with a low NSC diet ($p<0.001$), as could be seen in the Shannon index (Fig. 10C).

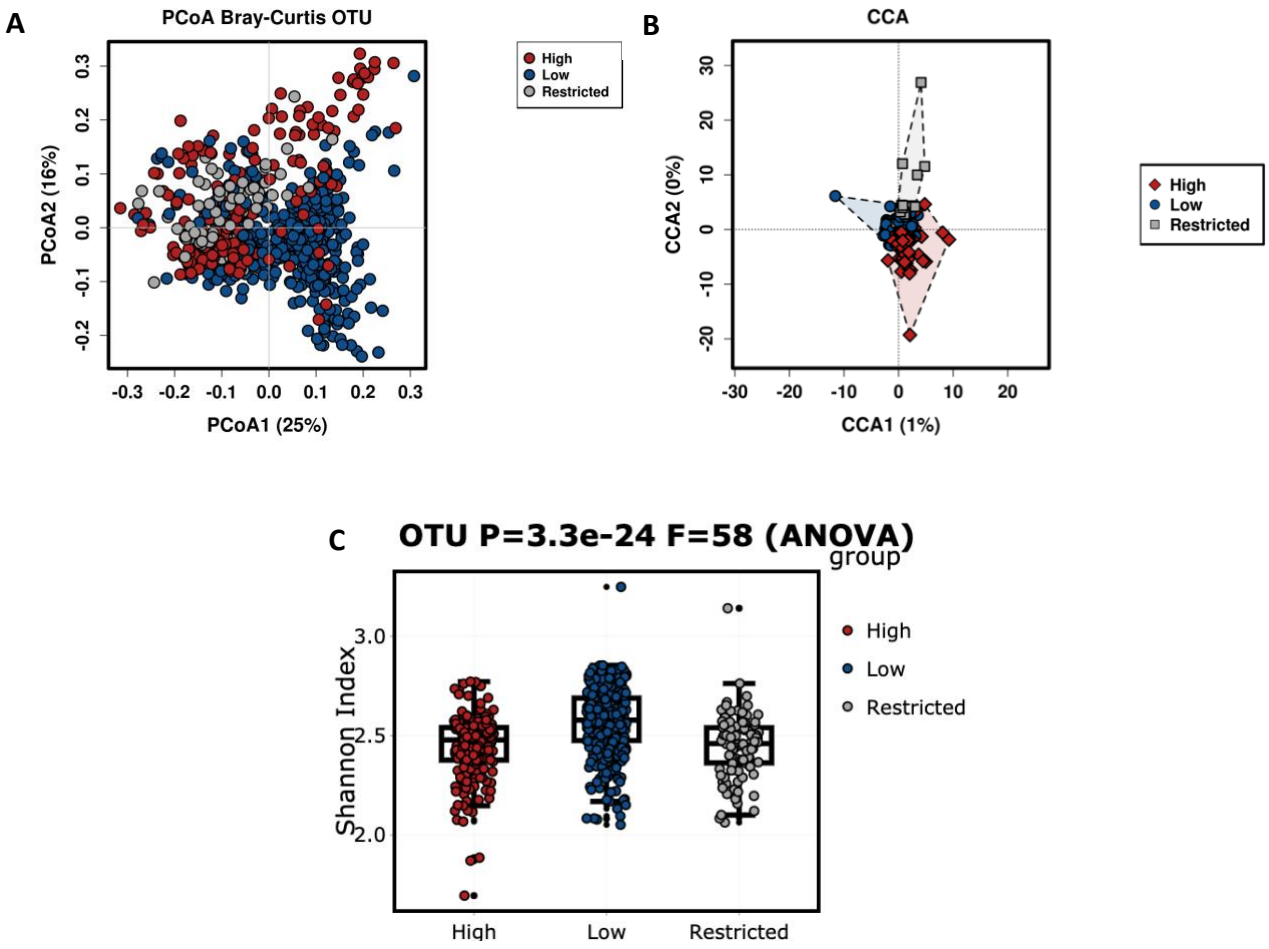

Figure 10: Comparison of alpha and beta diversities of the fecal microbial profiles of horses in the meta-analysis grouped by diet (high, medium and low dietary starch content). A: Principle Coordinates Analysis (PCoA) plot showing clustering of healthy adult fecal samples according to dietary starch levels; B: The Canonical Correspondence Analysis (CCA) plot of healthy adult fecal samples according to different starch contents of the diet; C: The Shannon Index plot of healthy adult fecal samples grouped by dietary starch levels.

When considering bacterial phyla, the LEfSe found significant differences in 12 phyla between the groups with different levels of starch content, with the phyla Verrucomicrobia, Proteobacteria, Firmicutes and Fibrobacteres being most abundant in horses with a low NSC diet (Table 5). Additionally, Fibrobacteres had a gradient from the highest abundance in the low NSC diet to the lowest abundance in the high NSC diet and the bacterial family *Fibrobacteraceae* was most abundant in the low starch diet. From the phylum Verrucomicrobia, the family *Akkermansiaceae* was most abundant in horses with a low starch diet, while in the phylum Actinobacteria it was the family *Eggerthellaceae*. Several bacterial families from the phylum

Firmicutes were most abundant in the low NSC diet: *Veillonellaceae* (class Negativicutes); *Clostridiaceae*, *Lachnospiraceae* and *Christensenellaceae* (class Clostridia); *Lactobacillaceae* and *Streptococcaceae* (class Bacilli). On the other hand, Bacteroidetes, Lentisphaerae and Spirochaetes were most common in horses with a restricted NSC diet. Correspondingly, the bacterial families *Oligosphaeraceae* and *Victivallaceae* (both phylum Lentisphaerae), as well as *Spirochaetaceae* (phylum Spirochaetes) were most abundant in horses with a restricted NSC diet. Horses with a high NSC diet were found to have higher abundances of Synergistetes, Euryarchaeota and Kiritimatiellaeota compared to the groups with lower dietary NSC content. From the phylum Euryarchaeota, the family *Methanobacteriaceae* was associated with the high starch diet, while the bacterial family *Methanocorpusculaceae* from the same phylum was associated with the restricted diet. Furthermore, although the phylum Proteobacteria was most common in horses with a low NSC diet, the family *Succinivibrionaceae* from this phylum was most abundant in horses with a high NSC diet (Table 5).

Overall, the results of the LEfSe showed less pronounced differences between groups in comparison to the sample region or age group comparisons, as none of the LDA scores reached a value above 4.5, with Firmicutes and the low NSC group having the strongest association (LDA=4.498) (Table 5).

Table 5: Linear Discriminant Analysis Effect Size (LEfSe) analysis of fecal samples of healthy adult horses from the meta-analysis comparing taxonomic abundances of gut microbiota between different levels of dietary starch content (high, restricted and low starch content). The table below shows the Linear Discriminant Analysis (LDA) score on phylum and family taxonomic level, with the intermediary taxonomic levels included to show the relationship between them. A score of 3-3.5 describes a low association, 3.5-4 a medium, 4-4.5 a high and >4.5 a very high association between the taxa and the investigated group.

Phylum	Class	Order	Family	Group	LDA Score
Actinobacteria				Low	3.895
	Coriobacteriia				
		Eggerthellales			
			Eggerthellaceae	Low	3.169
Bacteroidetes				Restricted	4.014
	Bacteroidia				
		Bacteroidales			
			Rikenellaceae	Restricted	4.06
			Barnesiellaceae	Low	2.704
			Bacteroidaceae	Low	3.664
		Marinilabiliales			
			Marinifilaceae	Low	2.865
	Sphingobacteria				
		Sphingobacteriales			
			Sphingobacteriaceae	Restricted	2.761
Cyanobacteria				Low	3.476
Euryarchaeota				High	3.36
	Methanomicrobia				
		Methanomicrobiales			
			Methanocorpusculaceae	Restricted	3.221
	Methanobacteria				
		Methanobacteriales			
			Methanobacteriaceae	High	3.133
Fibrobacteres				Low	4.043
	Fibrobacteria				
		Fibrobacterales			
			Fibrobacteraceae	Low	4.031
Firmicutes				Low	4.498
	Erysipelotrichia				
		Erysipelotrichales			
			Erysipelotrichaceae	High	3.224
	Negativicutes				
		Vellionellales			
			Veillonellaceae	Low	2.595
		Acidaminococcales			
			Acidaminococcaceae	High	2.954
	Clostridia				
		Clostridiales			
			Eubacteriaceae	High	3.037
			Clostridiaceae	Low	3.481
			Lachnospiraceae	Low	3.966
			Christensenellaceae	Low	3.691
			Peptococcaceae	High	3.081
	Bacilli				
		Lactobacillales			

			Lactobacillaceae	Low	4.001
			Streptococcaceae	Low	3.39
			Carnobacteriaceae	High	2.803
			Leuconostocaceae	High	3.073
		Bacillales			
			Bacillaceae	Restricted	2.792
			Planococcaceae	High	2.882
Kiritimatiellaeota				High	4.451
Lentisphaerae				Restricted	3.607
	Oligosphaeria				
		Oligosphaerales			
			Oligosphaeraceae	Restricted	2.707
	Lentisphaeria				
		Victivallales			
			Victivallaceae	Restricted	2.805
Proteobacteria				Low	4.041
	Betaproteobacteria				
		Burkholderiales			
			Burkholderiaceae	Low	3.428
	Gammaproteobacteria				
		Pasteurellales	*Pasteurellaceae*	Low	3.773
		Pseudomonadales	*Moraxellaceae*	Low	3.535
		Enterobacterales	Enterobacteriaceae	Low	3.649
		Aeromonadales	*Succinivibrionaceae*	High	2.957
		Xanthomonadales	*Xanthomonadaceae*	Restricted	3.013
Spirochaetes				Restricted	4.044
	Spirochaetales				
		Spirochaetales			
			Spirochaetaceae	Restricted	3.998
Synergistetes				High	3.965
Tenericutes					
	Mollicutes				
		Anaeroplasmatales			
			Anaeroplasmataceae	Low	2.77
		Mycoplasmatales			
			Mycoplasmataceae	Low	3.135
Unclassified				Low	3.39
	Unclassified				
		Unclassified			
			Unclassified	Restricted	4.46
Verrucomicrobia				Low	3.838
	Verrucomicrobiae				
		Verrucomicrobiales			
			Akkermansiaceae	Low	3.364

4.2.6. Disease Factor

The disease factor was divided into controls, intestinal diseases, metabolic diseases and antibiotic (labeled as drugs in Fig. 11) and anthelmintic treatment. These groups were distributed evenly in the PCoA (Fig. 11A), however, the antibiotic treatment and intestinal disease groups deviated from the controls and had larger variation in the CCA plot (Fig. 11B), with a significant difference between all groups (p=0.001). The alpha diversity was significantly lower in the antibiotic treated horses compared to the other groups (p<0.001), as showed in the Shannon index (Fig. 11C).

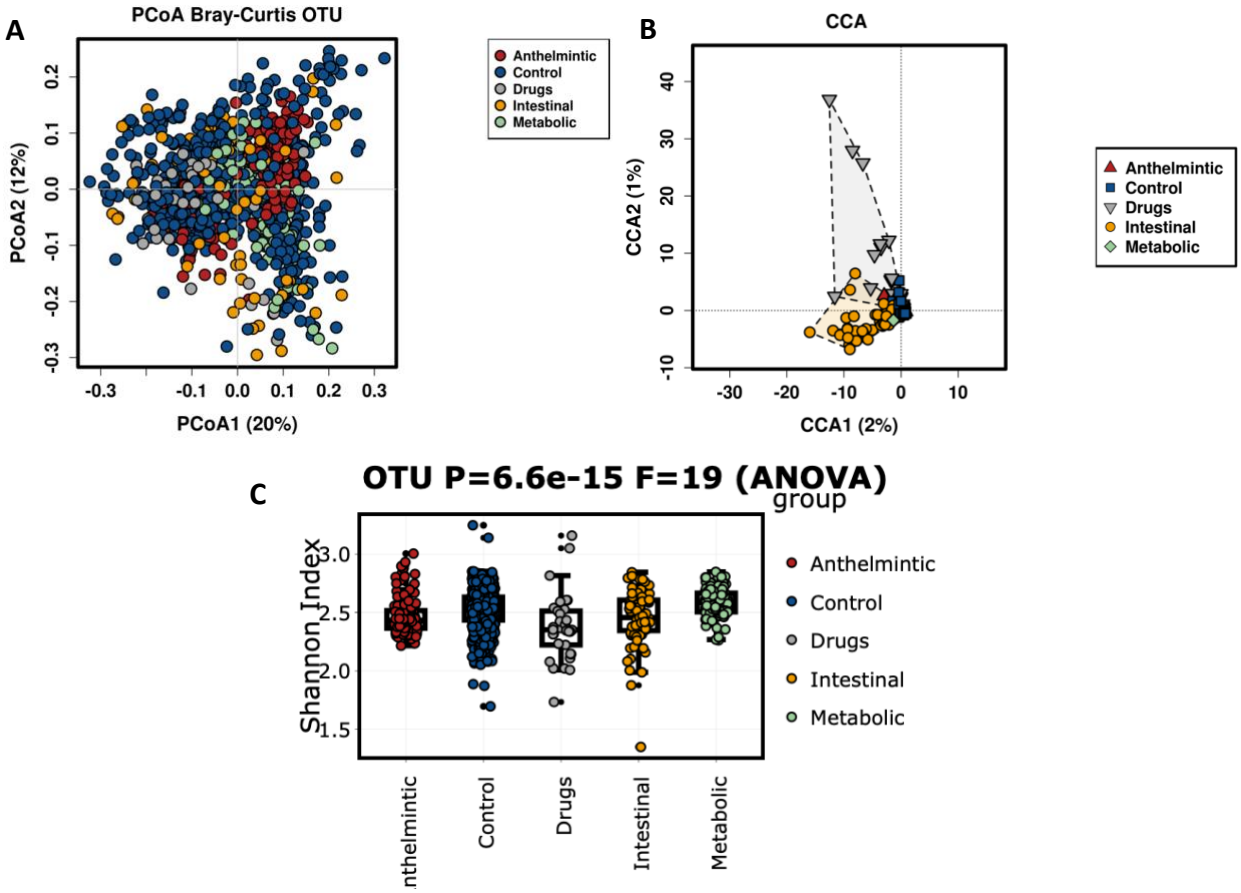

Figure 11: Comparison of alpha and beta diversities of the fecal microbial profiles of horses in the meta-analysis grouped by disease/treatment (anthelmintic, drugs, controls and intestinal and metabolic diseases). A: Principle Coordinates Analysis (PCoA) of adult fecal samples ordered according to disease, control and drug treatment groups; B: The Canonical Correspondence Analysis (CCA) plot of adult fecal samples ordered according to disease and control groups; C: Shannon index comparison between of adult fecal samples of disease and control groups.

The LEfSe analysis showed significant differences in bacterial abundance between groups in 12 bacterial phyla and 30 families and association with intestinal disease of the phyla Lentisphaerae, Verrucomicrobia (family *Akkermansiaceae*) and Fusobacteria (family *Fusobacteriaceae*) (Table 6). Furthermore, several bacterial families from the phylum Firmicutes were most abundant in horses with intestinal disease, including *Veillonellaceae* (class Negativicutes) and *Erysipelotrichaceae* (class Erysipelotrichia) (Table 6).

Different bacterial species were associated with metabolic diseases, namely the phyla Cyanobacteria and Euryarchaeota, as well as the bacterial families *Pasteurellaceae* (class Gammaproteobacteria), *Clostridiaceae* (class Clostridia) and *Lactobacillaceae* and *Streptococcaceae* (both order Lactobacillales) (Table 6).

The horses treated with antibiotics or anthelmintics had the largest amount of differentially abundant bacteria in the LEfSe analysis: A large number of bacterial species from the class Clostridia (phylum Firmicutes) were most abundant in horses treated with anthelmintics, namely *Lachnospiraceae, Christensenellaceae, Hungateiclostridiaceae* and *Ruminococcaceae.* Furthermore, several species from the order Bacteroidales (phylum Bacteroidetes) were most abundant in anthelmintic treated horses, including *Prevotellaceae, Muribaculaceae* and *Paludibacteraceae.* On the other hand, the phylum Proteobacteria was associated with the antibiotic treated horses, with a very large number of bacteria being most abundant in this group, including *Burkholderiaceae, Sutterellaceae, Neisseriaceae, Moraxellaceae, Xanthomonadaceae, Succinivibrionaceae* and *Aeromonadaceae.* Furthermore, the phylum Spirochaetes (with family *Spirochaetaceae*) was associated with antibiotic treatment, as well as the families *Actinomycetaceae* and *Corynebacteriaceae* (class Actinobacteria) (Table 6).

On the contrary to the disease groups, the healthy control horses had the highest abundances of just a few bacterial phyla, including Cyanobacteria and Kiritimatiellaeota (Table 6).

Table 6: Linear Discriminant Analysis Effect Size (LEfSe) analysis of fecal samples of adult horses from the meta-analysis comparing taxonomic abundances of gut microbiota between horses with different diseases and treatments (anthelmintic, drugs (antibiotics), controls and intestinal and metabolic diseases). The table below shows the Linear Discriminant Analysis (LDA) score on phylum and family taxonomic level, with the intermediary taxonomic levels included to show the relationship between them. A score of 3-3.5 describes a low association, 3.5-4 a medium, 4-4.5 a high and >4.5 a very high association between the taxa and the investigated group.

Phylum	**Class**	**Order**	**Family**	**Group**	**LDA Score**
Actinobacteria					
	Actinobacteria				
		Actinomycetales			
			Actinomycetaceae	Drugs	3.289
		Corynebacteriales			
			Corynebacteriaceae	Drugs	3.200
	Coriobacteriia				
		Eggerthellales			
			Eggerthellaceae	Anthelmintic	3.279
Bacteroidetes				Drugs	4.741
	Bacteroidia				
		Bacteroidales			
			Prevotellaceae	Anthelmintic	4.131
			Rikenellaceae	Drugs	4.385
			Muribaculaceae	Anthelmintic	3.387
			Paludibacteraceae	Anthelmintic	3.329
			Barnesiellaceae	Intestinal	3.011
		Marinilabiliales			
			Bacteroidaceae	Intestinal	4.318
Cyanobacteria				Control	3.535
Euryarchaeota				Metabolic	3.502
	Methanomicrobia				
		Methanomicrobiales			
			Methanocorpusculaceae	Intestinal	3.139
Firmicutes				Metabolic	4.865
	Erysipelotrichia				
		Erysipelotrichales			
			Erysipelotrichaceae	Intestinal	3.791
	Negativicutes				
		Vellionellales			
			Veillonellaceae	Intestinal	3.459
	Clostridia				
		Clostridiales			
			Clostridiaceae	Metabolic	3.608
			Lachnospiraceae	Anthelmintic	4.522
			Christensenellaceae	Anthelmintic	4.085
			Hungateiclostridiaceae	Anthelmintic	3.281
			Ruminococcaceae	Anthelmintic	4.356
	Bacilli				
		Lactobacillales			

			Lactobacillaceae	Metabolic	4.003
			Streptococcaceae	Metabolic	3.744
			Aerococcaceae	Drugs	3.638
	Tissierellia				
		Tissierellales			
			Peptoniphilaceae	Drugs	3.350
Fusobacteria				Intestinal	3.964
	Fusobacteriia				
		Fusobacteriales			
			Fusobacteriaceae	Intestinal	3.989
Kiritimatiellaeota				Control	4.270
Lentisphaerae				Intestinal	3.454
Proteobacteria				Drugs	4.395
	Betaproteobacteria				
		Burkholderiales			
			Burkholderiaceae	Drugs	3.709
			Sutterellaceae	Drugs	3.137
		Neisseriales			
			Neisseriaceae	Drugs	3.299
	Gammaproteobacteria				
		Pasteurellales			
			Pasteurellaceae	Metabolic	3.623
		Pseudomonadales			
			Moraxellaceae	Drugs	3.920
		Xanthomonadales			
			Xanthomonadaceae	Drugs	3.221
		Aeromonadales			
			Succinivibrionaceae	Drugs	3.754
			Aeromonadaceae	Drugs	3.718
	Deltaproteobacteria				
		Desulfovibrionales			
			Desulfovibrionaceae	Anthelmintic	2.980
Spirochaetes				Drugs	4.275
	Spirochaetales				
		Spirochaetales			
			Spirochaetaceae	Drugs	4.241
Tenericutes				Drugs	3.355
	Mollicutes				
		Anaeroplasmatales			
			Anaeroplasmataceae	Intestinal	3.099
Unclassified				Control	3.882
	Unclassified				
		Unclassified			
			Unclassified	Control	4.466
Verrucomicrobia				Intestinal	4.282
	Verrucomicrobiae				
		Verrucomicrobiales			
			Akkermansiaceae	Intestinal	4.237

4.4. Discussion

This meta-analysis aimed to determine the influence of various biological and technical factors on the equine GI microbiome in a large dataset by combining and re-analyzing the data of previous studies. Overall, however, the technical variation between studies had the strongest impact on the found microbiota composition, which made the findings from different studies incomparable to each other. This was accounted for by correcting for gene region and sampling instrument using a batch mean correction (BMC). This shows the importance of correcting for different sequencing techniques used in studies when comparing findings from different studies. Without correcting for these differences or standardizing the scientific methodology in equine microbiome research, the findings of studies cannot be easily compared. After performing the BMC, the sample type and region of the GI tract, as well as the age range, dietary starch content and diseases were found to be the most important factors influencing the GI microbiome in terms of microbiota composition (beta diversity) and alpha diversity. One aspect that was not investigated in this meta-analysis is the effect of research methodology beyond the instrument model and gene region sequenced, such as DNA extraction protocols and sample storage time and conditions. Due to the importance of fecal samples in intestinal microbiome studies, the storage of the fecal samples until DNA extraction and the procedure of DNA extraction itself has increasingly been studied to compare the effects of different scientific methods. For example, a study in humans found a difference in the abundances of *Bifidobacteria* and *Bacteroides* genera when using a commercially available kit for extraction of fecal DNA compared to using kits for tissue or blood DNA[132]. This finding was shared with a study in fish which considered the fecal DNA extraction kit to work better than a tissue kit[133]. On the contrary to the extraction kit, the best practice for sample storage conditions is less established. A study in pigs found differences in bacterial compositions when fecal samples were used fresh or after frozen storage for 3 months before DNA extraction[134], while another study in cats found no effect of keeping the feces at room temperature for up to four days[135]. Apart from the storage temperature, also the preservatives used for storing fecal samples may have an effect on microbial abundance, as the storage of fecal samples from monkeys without preservatives reduced microbial diversity

compared to samples stored with ethanol or RNAlater[136]. These findings show that the inclusion of sample storage conditions such as DNA extraction methods as technical variants between datasets could be an important extension of this study.

4.4.1. Sample Type and Region

Due to the substantial difference between the luminal, mucosal and fecal sample type, the differences between luminal and mucosal samples from different gut regions were examined separately and compared to fecal samples. The similarity in microbial composition of the feces and hindgut in this study is in accordance with previous studies that found the microbiome of the feces to resemble the hindgut, which gave rise to the widespread use of fecal samples for the determination of the GI microbiome[18,22,25,121]. Consequently, although fecal samples cannot be used to identify the microbial composition of the foregut, this sample type has been most commonly used to investigate the equine intestinal microbiome[22]. Therefore, whilst acknowledging the limitations with regards to inferring effects in foregut microbiota, the analysis of other factors of this meta-analysis was done on fecal samples alone to avoid confounding these analyses by gut region.

However, to compare the different parts of the GIT to each other and to fecal samples, mucosal and luminal samples from different compartments were used. There was a smaller variation in microbiota composition in samples in the cecum and colon compared to the stomach, jejunum and ileum, a finding which has also been detected in a previous study[36].

Furthermore, previous studies have found differential bacterial abundances in the different compartments of the GI tract. The high abundances of Proteobacteria in the jejunum (mucosa) and ileum (lumina) found here are comparable to previous findings of large amounts of Proteobacteria found in the ileum compared to the cecum and colon[37]. Similarly, Su et al. (2020) found a higher proportion of Proteobacteria and less Bacteroidetes in the foregut compared to the hindgut[35]. This difference between foregut and hindgut microbiome has also been found by

Costa et al. (2015), who determined Proteobacteria and Firmicutes to be dominant in the foregut, while Bacteroidetes was most common in the hindgut[22]. This is corresponding to the findings of this meta-analysis, which also detected larger amounts of Firmicutes in the stomach than in other parts of the GIT in mucosal samples, as well as a strong association of Bacteroidetes with the cecum (mucosal samples) and colon (luminal samples).

At bacterial family level, *Ruminococcaceae, Spirochaetaceae, Lachnospiraceae* and *Prevotellaceae* were highly abundant in the colon, cecum and rectum. Similarly, a previous study found *Ruminococcaceae, Lachnospiraceae, Rikenellaceae, Prevotellaceae* to be dominant in the hindgut[22]. *Lachnospiraceae* and *Ruminococcaceae* are fiber-fermenting bacteria, explaining their abundance in the hindgut where most of the fiber fermentation takes place[22,137]. Consequently, even at a lower taxonomic level the microbial differences between the parts of the GIT corresponded with previous findings. The differences in GI microbiota composition can be explained by the varying functions the compartments have in digestion and horse physiology[22].

The differences between luminal and mucosal samples in the same GIT region when examining demonstrate that these sample types cannot be used interchangeably when looking for the bacterial composition of a gut compartment. Consequently, when examining the equine GI microbiome, the sample type and location in the gut must be chosen according to the specific research question investigated. The correlation of these results with previous studies (several of which were included in the meta-analysis) allows us to confirm some of the findings of these studies by increasing the power of the analysis and taking into account the technical variation between them. Furthermore, the similarity between the meta-analysis output and previous studies of the GI microbiota confirms the relevance of the data after BMC; thus validating this technique.

4.4.2. Age

As has been shown in previous studies, there are significant differences in bacterial diversity and abundances of specific phyla and families between age groups. In general, there was a trend towards increasing alpha diversity with age; with a slight drop in the elderly population; this is in accordance with previous literature[26]. Notably, there was a high variation in alpha and beta diversity in <2 months old foals, which can be attributed to the microbial colonization of the GIT still being in process[14]. The high variation in beta diversity reflects the instability of the foal microbiome, which may render the foals more susceptible to disease during this period, and could explain the high prevalence of diarrhea in neonatal foals[10,11].

The high abundances of Proteobacteria in foals <2 months old is in accordance with findings of Husso et al. (2020) and De La Torre et al. (2019), who found high levels of Proteobacteria after birth, which then decreased with age and were replaced by Firmicutes and Bacteroidetes as dominating phyla by the time they reached weaning[8,15]. This may explain the relatively low abundance of Bacteroidetes in young foals, which subsequently reaches a peak in horses aged 2-20y. The high abundance of Firmicutes in foals aged 2-6mo, suggests that the Firmicutes phylum expands more quickly than Bacteroidetes and reaches its peak before/during weaning; this may reflect relatively low fiber levels at this age.

The bacterial families *Ruminococcaceae* and *Lachnospiraceae* from the phylum Firmicutes were found to be most common in 2-4 weeks old foals in a previous study[10], however, in this study the same bacterial families were most abundant in older foals at 2-6 months age. Due to the role of these bacteria in fiber fermentation[5,20] it would make more sense to find them in higher abundance in foals that are shifting their diet from milk to fiber. Previous studies in foals have found similarly high abundances of phyla as were found in foals of <2 months in this meta-analysis: The high abundances of Verrucomicrobia in foals has been found by Schoster et al. (2017) and the increased abundances of Fusobacteria in foals up to 1 month old compared to older foals was found by Costa et al. (2016)[10,13]. In weanlings and yearlings from 0.5-2 years old there were few bacteria that were highly associated with this group, possibly because they had only marginal differences to the 2-20-year-old group. However, *Prevotellaceae* was most

abundant in this age group, a bacterial family that has been previously found increased in horses shifting from a grass to hay based diet[80]. This may be due to the change in diet in horses after weaning. These findings show that the GI microbiome changes with age, with the compositional shift being strongest in foals and weanlings and then gradually becoming more stable with age. Additionally, the alpha diversity is lowest and most variable in foals and gradually increases with age.

4.4.3. Sex

Although the differences between male and female GI microbiota composition were not as strong as in the other biological factors, females had a significantly higher alpha diversity than males and higher abundances of several bacteria, including the families *Clostridiaceae*, *Lachnospiraceae*, *Ruminococcaceae* and *Christensenellaceae* (phylum Firmicutes) and *Pasteurellaceae*, *Enterobacteriaceae* and *Moraxellaceae* (phylum Proteobacteria). *Lachnospiraceae* and *Ruminococcaceae* have been considered important in fiber fermentation[5,62], while the order Clostridiales has been associated with intestinal disease[15] and high parasite burdens[9]. However, none of the bacteria associated with sex in this study have previously been associated with the sex of the horse. The reason for this may be that the microbial composition of horses with different genders has not been intensively studied, at least not by considering the gender as a factor that could have an effect on the microbiome. If it was included, as in a previous study by Dong et al. (2016), no significant difference was found between groups[138]. Consequently, it is likely that the differences due to gender are small, and that the increased power associated with the meta-analysis has facilitated their detection. On the other hand, the results found here may be due to other confounding factors that may be correlated with gender, such as different management systems and diets of female and male horses. This topic requires larger scale investigations of uniform populations of male and female horses that are matched for age, diet and management to avoid other biological factors to interfere with the findings.

4.4.4. Diet/NSC

The comparison of horses with a high starch diet, a restricted starch diet and a forage diet showed a reduced alpha diversity in horses with a high starch diet compared to the other groups and varying bacterial abundances in each group. This is in line with findings from previous studies of horses given a high starch diet, in which the carbohydrate rich diet has been associated with a reduction in microbial alpha diversity[20,102], although the abundances of some individual taxa increased[32]. The reduced stability of the GI microbiome of horses on a concentrate diet, as mentioned in several studies[53,54], could not be investigated here since this is not a longitudinal study and differences over time could not be measured. However, a relative reduction in alpha diversity was found in the high starch group; this could potentially lead to a loss of beneficial bacteria and an overgrowth of pathogenic bacteria in the GIT, which can be associated with a number of diseases[1,61].

The abundance of *Clostridiaceae* was higher in horses with a low starch diet than in the other groups in this meta-analysis. Similarly, in a previous study the abundance of *Clostridiaceae* was decreased after inclusion of starch in the diet[20]. Kaiser-Thom et al. (2020) found that higher amounts of starch led to increase the abundances of Bacteroidetes and decrease the abundances of Verrucomicrobia[79]. Furthermore, Daly et al. (2012) found higher amounts of Bacteroidetes and *Lachnospiraceae*, but a reduction in Fibrobacteres in horses with a starch rich diet. This is not surprising given the important role of Fibrobacteres in fiber fermentation[5]. Interestingly, also in this study the abundances of Verrucomicrobia and Fibrobacteres were lower, while the abundances of Bacteroidetes were higher in the high starch diet compared to the low starch diet. However, in this metanalysis the bacterial families of *Lachnospiraceae* and *Streptococcaceae* were more abundant in horses with a low starch diet, which is contrary also to the findings of Warzecha et al. (2017)[20]. Additionally, the phylum Proteobacteria was associated with a low starch diet, while a previous study found Proteobacteria to be most abundant in horses receiving a starch rich diet[20]. The differences seen between this study and previous, more specific studies

(e.g. no association between *Streptococcaceae* and high starch levels were seen here) could be due to the grouping of starch levels in this meta-analysis, as the diets of the horses in the different studies were highly variable, with a large number of different products involved and the amounts of each part of the diet was not always described in detail. To examine the exact effect of carbohydrate levels on the equine GI microbiome, large scale studies with horses receiving a precisely monitored diet are necessary to avoid the variation of different dietary options and management systems present in a meta-analysis.

4.4.5. Disease Factor

The disease categories of the horses were divided into intestinal diseases, such as colic and colitis, Equine Grass Sickness (EGS) and diarrhea; metabolic diseases, such as laminitis, obesity, insulin dysregulation and Equine Metabolic Syndrome (EMS). The reduced bacterial diversity in horses with intestinal diseases compared to healthy controls, which was found in this study, has also been found in previous studies in horses with colic[69] and EGS[72]. The phyla Cyanobacteria and Kiritimatiellaeota which were associated with healthy horses in this meta-analysis, have been found in small amounts in previous studies[20,52,67]. However, they have not been specifically associated with healthy horses thus far.

The findings of bacterial abundances in intestinal and metabolic diseases in this study correspond in part with previous results: The increased abundance of Fusobacteria, as was found in this study in horses with intestinal diseases, was also found in horses with colitis by Costa et al. (2012), as well as in horses with diarrhea[139]. Also, bacterial families that have been increased in laminitis, such as *Clostridiaceae*[67], *Lactobacillaceae* and *Streptococcaceae*[23] were increased in metabolic diseases in this study. However, in many cases the abundances of bacteria in the GI microbiome in horses with intestinal and metabolic diseases frequently overlap when comparing the results from this meta-analysis to previous studies. Horses with Equine Metabolic Syndrome (EMS) have been shown to have an increased abundance of Verrucomicrobia and a decrease in Fibrobacteres [140], which correspond with our findings in horses with intestinal disease. Additionally, the high

abundance of *Streptococcaceae* in the metabolic group correlates with the increase in abundance of *Streptococcus* found in horses with colic in a previous study[69]. Furthermore, the increased abundances of *Clostridiaceae* in metabolic diseases found in this meta-analysis can potentially be explained by previous findings where the bacterial order Clostridiales was associated with GI inflammation[15]. The similarities of findings for horses with metabolic and intestinal diseases may be due to the fact that some metabolic diseases could, in theory, be linked with dysbiosis of the gut microbiota or change in diet.

We also compared horses that had received antibiotics with the control group. The antibiotic treatment group had a lower alpha diversity than the control group. This could be due to the direct bactericidal effect of antibiotics on bacteria in the GIT, as was found in previous studies: In a previous study of horses given TMS, there was a significant decrease in bacterial richness and evenness, in particular the phylum Verrucomicrobia was markedly reduced[73]. The same decrease in diversity was the case in horses given Metronidazole[29].

The increased abundances of seven bacterial families from the phylum Proteobacteria in horses receiving antibiotics found in this study may be due to the reduction of 'healthy' gut bacteria due to antibiotic treatment allowing proliferation of pathobionts, as was the case three days after metronidazole administration in a previous study[29]. This could lead to the proliferation of species from Proteobacteria, a phylum which has been associated with GI inflammation[61,70,72,141]. Furthermore, similar to the findings of this study, Arnold et al. (2020) found a higher abundance of Actinobacteria in horses receiving antibiotic treatment[29].

In horses treated with anthelmintic drugs, the alpha diversity was lower than in healthy controls, but comparable to horses with intestinal disease and higher than in horses treated with antibiotics. Although a decrease in alpha diversity after treatment has been found in a previous study[81], the findings here show the different effect of antibiotic and anthelmintic treatment on GI microbiota. The increase in Proteobacteria and decrease in Bacteroidetes in horses treated with anthelmintics in a previous study[74] was not as clear in this meta-analysis, however, several

species from the phylum Bacteroidetes were most abundant in the anthelmintic group (*Prevotellaceae, Muribaculaceae* and *Paludibacteraceae*). Similarly, Peachey et al. (2019) found a decrease in *Prevotellaceae* in horses with high parasite burdens, a trend which was reversed after anthelmintic treatment, which would explain the increase in *Prevotellaceae* after treatment in this meta-analysis[34].

Although it was not fully clear from previous studies whether the effect of anthelmintics on the gut microbiota was due to inflammation resulting from the death of the parasite, or the drug itself, there is evidence to suggest that the effects are due to the removal of the parasite. To investigate this further, the effect of anthelmintic and antibiotic treatment needs to be examined in more studies.

4.5. Conclusion

Overall, the findings of this meta-analysis regarding the influence of biological factors on the GI microbiome overlap with findings from previous studies. However, in these previous studies there was large variation in their findings, which shows the strength of combining data in a meta-analysis; and furthermore, the necessity of further research with standardized methodology to reduce technical variation, especially in terms of sample collection and laboratory methods, such as the type of samples collected, as well as sequencing methods and machinery.

Apart from these specific factors that can have an influence on the equine GI microbiome, which were accounted for in this meta-analysis, there are still many sources of variation in each study that lead to non-quantifiable changes in the microbiome. This may include handling of samples, DNA extraction methods, as well as varying characteristics of the machines used. An indicator of this were the significant differences between studies even after BMC correction. These differences may have led to inaccuracies in our results. Consequently, it is of high importance to generate a standardized protocol for the methodology of determining the bacterial composition of the GI microbiome for samples to be better comparable. This could involve the sample

collection, such as location of the samples in the fecal mass, or the handling of samples after collection, such as freezing temperature and duration before DNA extraction.

Additionally, the difficulty of collecting the raw sequencing data due to lacking public availability of data, faulty or incomplete data if available or lacking responses from authors highlight the need of a database specifically for equine GI microbiome data. It would be desirable for authors to upload their data in a central database in order to maintain transparency and the advancement of this field of research.

5. The Effect of Prebiotic Supplementation on the Gastro-Intestinal Microbiome of Horses Undergoing Dietary Change

5.1. Background, Aims & Hypotheses

In the meta-analysis we concluded that the diet had a measurable effect on the GI microbiome; furthermore, intestinal diseases were correlated with differential bacterial abundances. Additionally, previous studies have shown the connection of diet and disease through the GI microbiome, which highlights the importance of gut health on the overall health of a horse and the large potential of beneficially modulating the microbial composition with supplements. In particular, it has been reported that FOS prebiotics may be able to stabilize the gut microbiota in the face of carbohydrate overload[110], suggesting a role for prevention of some GI and metabolic diseases.

Only a handful of studies have reported the effects of prebiotics on the equine gut microbiota. Most of these were performed using culture-based techniques, which may bias the results towards bacteria thriving in culture[103,110,113]. With next generation sequencing becoming increasingly common in microbiome studies, more studies are necessary using this technique to investigate the impact of prebiotics on the microbiome. Furthermore, prebiotics are commercially available and are commonly used in horses, despite limited evidence regarding their efficacy[103]. The availability of a supplementation with lacking scientific evidence can have negative effects, as has been seen when treating foals with probiotics: in some cases, the foals had higher incidence[142] or severity[98] of diarrhea or other adverse effects[88]. Therefore, it is all the more important to investigate prebiotics supplements further to gain more knowledge on their function and effect.

Therefore, in the second part of this project we aimed to determine whether there were any differences in intestinal microbiome composition between horses that received scFOS prebiotic supplementation compared to non-supplemented controls in a population of Thoroughbred yearlings during a period of nutritional change to a carbohydrate rich diet. This was a longitudinal study of a group of horses with highly similar demographics in order to reduce interindividual variation.

5.2. Materials & Methods

5.2.1. Ethical Approval

This study received the ethical approval by the University of Bristol with the reference VIN/19/**018** and the sample collection was performed with written consent by the stud farm.

5.2.2. Experimental Design

This study included 12 male Thoroughbred yearlings. All of the horses were between 16-20 months old and were bred for the racing industry and reared at the same UK stud farm. They were kept in groups on the pasture when the baseline sample was taken and for the rest of the study they were stabled. When they were stabled the yearlings received hay *ad libitum*, 5kg Alfalfa and three different types of grain feed, resulting in a total of 2 scoops (5kg) of feed type 1, 2 scoops of feed type 2 and 4 cups of feed type 3 per horse and day. Feed type 1 consisted of 18.5% crude fiber, 13% crude protein, 8% starch and 6% sugar; Feed type 2 contained 13% crude fiber, 14% crude protein, 16.5% starch and 7% sugar and feed type 3 contained 6.5% crude fiber, 12.5% crude protein, 20% starch and 9% sugar. The detailed nutritive information and ingredients of each type of feed can be found in the appendix (Table 13). The yearlings were randomly

assigned treatments, with half of the horses receiving prebiotics and the other half serving as negative controls with no prebiotics. The prebiotic consisted of scFOS from dried chicory root reduced to a powder (99.5%) and an anti-caking agent (0.5%) with a high fraction of inulin (at least 65%). A detailed account of the nutritive values of the product can be found in the appendix (Table 15). The horses in the prebiotic groups received 30g FOS dietary supplement per day in their feed, as has been done previously in horses[110] and was calculated based on 0.07g supplement per kg bodyweight. The supplement was given as part of their feed for a period of 8 weeks, while those in the control group did not receive any feed supplements.

5.2.3. Sample Collection

Freshly voided fecal samples were collected starting with a baseline sample (D0 – before the animals had received prebiotics), a sample after 1 week, and then after further 2 weeks. The sample collection took place between June and October 2019. Samples were collected immediately after defecation and taken from the center of the fecal mass. Samples for microbiome analysis were frozen in liquid nitrogen immediately after collection and stored at -80°C until DNA extraction, which was performed within 6 months of collection.

5.2.4. Laboratory Analysis

A total of 36 yearling samples, as well as one blank control (without DNA) were processed and analyzed. Genomic DNA was extracted from the fecal samples using a PowerSoil DNA Isolation Kit (Qiagen, Carlsbad, CA, USA) following the manufacturer's instructions. The V3-V4 region of the 16S rRNA gene was sequenced using Illumina sequencing. This sequencing method was chosen as it is a cost-effective tool to examine the different bacterial taxa present in the horse feces and it has been used frequently in other equine microbiome studies[8,9,30,31,34,37,74].

Between laboratory steps the DNA was then quantified with a Qubit Quant-iTTM dsDNA Broad-Range Assay Kit (Life Technologies, Carlsbad, California, USA) to ensure sufficient amounts of DNA were available to continue the process. This measure to monitor the DNA concentration was repeated after every step of the following laboratory procedure. The V3-V4 region of the 16S rRNA gene was amplified by PCR using universal primers: Forward, 5⁰-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3⁰; Reverse, 5⁰-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3⁰. After the first PCR (settings: 98°C for 2 minutes; then 20 cycles of 98°C for 15 seconds, 63°C for 30 seconds and 72°C for 30 seconds; finally 72°C for 5 minutes), the samples were run through an electrophoresis gel and viewed with a UV transilluminator to ensure the PCR successfully amplified the targeted 16S region. After a successful PCR, DNA purification was performed to purify the amplicons using AMPure XP beads (Beckman Coulter, Brea, California, USA). The indexing PCR was performed according to the manufacturer's instructions (New England Biolabs Inc, Massachusetts, USA) with the following cycles: 95°C for 3 minutes; then 8 cycles of each 30 seconds at 95°C, 55°C and 72°C; 72°C for 5 minutes; reduced to 4°C until removed. After the indexing PCR, the samples were purified once again with AMPure XP beads and DNA concentrations were determined, as described above, using the Qubit. The DNA library was prepared according to the Illumina 16S protocol(https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf) and high-throughput sequencing was performed using an Illumina MiSeq platform with 2x 300 bp paired-end reads.

5.2.5. Bioinformatic Analysis

In order to perform the statistical analysis, sequence reads were trimmed and quality filtered, and the sequences compared to a taxonomic database to determine what bacterial species were present in the samples. This was performed using the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) software (version 2019.10)[143]. Qiime2 is a Python-based microbiome data analysis

platform that allows the upstream processing of microbiome data, as well as statistical analysis and visualization tools[143], and has been commonly used for microbiome analyses[33,34,43,144]. First, the raw paired-end sequence reads were joined and then filtered and trimmed based on their quality and the length of their primers. Second, a 99% classifier was trained to the primers to generate the taxonomy by comparing these high-quality sequences to bacterial sequences from the SILVA database[145] (https://www.arb-silva.de/). Based on the found similarity, the sequences were grouped into Operational Taxonomic Units (OTUs) that distinguish the present bacterial species. As filtering was performed further downstream in the analysis, the sampling depth cut-off for quality filtering in Qiime2 was initially kept low to allow inclusion of blanks, and to screen for significant contamination (see appendix chapter 3 for Qiime2 analysis code).

The corresponding metadata file included the sample ID of each animal, timepoint of sampling (1, 2 or 3), treatment group of each horse (prebiotic or control) and a combination of timepoint and treatment group (two-way: A, B, C, D, E or F) to determine changes in the bacterial populations in treatment groups over time (Table 7; for complete metadata see appendix, Table 14)

Table 7: Key for groupings of samples from fecal microbiota measurements in 12 male Thoroughbred yearlings. Timepoints of sampling (1: baseline; 2: week 1; 3: week 3) and treatment group (prebiotic or control) combined into two-way analysis (A-E).

Timepoint	**Treatment**	**Two-way**
1	Prebiotic	A
2	Prebiotic	B
3	Prebiotic	C
1	Control	D
2	Control	E
3	Control	F

The second part of the bioinformatic analysis was performed on Calypso, a web based graphical interface specifically designed for microbiome data analysis [125].

First, output files from QIIME2 were imported into Calypso, including the taxonomy file (taxonomy.tsv), a metadata file with blanks still included (metadata.csv), a featuretable BIOM file (feature-table.biom) and a UniFrac distance matrix (distance-matrix.tsv). Data filtering and normalization was set to remove samples with less than 1000 sequence reads and to include only taxa which have more than 0.01% relative abundance and maximally the top 3000 taxa. This led to one sample being excluded for analysis. The data underwent a Hellinger transformation[146], hence it was normalized by total sum normalization and transformed by square root. To ensure the sequencing depth of the samples was sufficient, the rarefaction curve of operational taxonomic units (OTU's) was visualized. Two main measures of diversity were investigated in this study: the alpha diversity, which describes the species number (richness) & distribution (evenness) in each sample, as well as the beta diversity, which shows a similarity score between the microbial populations of different samples. The alpha diversity was measured using the Shannon diversity index, which combines the richness and evenness of species. This was determined in a two-way ANOVA to account for time points and supplementation. The beta diversity was determined using a weighted UniFrac distance matrix to estimate how related the different samples were in terms of their OTU's. The following multivariate tests and visualizations were performed to examine the differences in bacterial populations based on the beta diversity of the samples: Principal Coordinates Analysis (PCoA), Canonical Correspondence Analysis (CCA) and Redundancy Analysis (RDA).

Further, a Linear Discriminant Analysis Effect Size (LefSe) was performed, which is a method developed for assessing differential abundance in metagenomic research that considers biological information in significance testing and therefore measures the difference in relative abundance between groups in terms of statistical and biological relevance[127]. Additionally, a network analysis and the multivariate analyses ANOSIM[147] and PERMDISP2[148] were performed to compare the differences between the groups.

5.3. Results

A total of 37 samples were analyzed, of which 36 were from yearlings and 1 was a blank for quality control. After demultiplexing, there were 1'649'482 of each forward and reverse reads in total, with a mean of 44'580.6 reads (see in appendix, Table 16B). After quality filtering and trimming the forward and reverse read ends, 1,009,800 sequences were retained. The rarefaction analysis based on OTU's showed a flattening of the curve of bacterial richness in relation to the reads sampled, hence the sequencing depth of the samples was sufficient. One sample (61) was excluded due to very low forward and reverse sequence counts of 194 (see in appendix, Table 16A). The bacterial sequences were assigned to 24 bacterial phyla, 9 of which had an abundance >1%. Over all samples the most dominant of these were Firmicutes (42.67% and Bacteroidetes (28.57%), followed by Kiritimatiellaeota (8.41%), Verrucomicrobia (7.87%), Spirochaetes (3.40%), Euryarchaeota (2.00%), Fibrobacteres (1.58%), Actinobacteria (1.45%) and Planctomycetes (1.06%). Smaller amounts (0.1%-1%) were found of Proteobacteria, Cyanobacteria, Synergistetes, Armatimonadetes, Lentisphaerae and Patescibacteria.

To compare the difference between yearlings receiving a prebiotic treatment and untreated controls over time, a two-way analysis was performed to account for treatment and timepoint. First, a multivariate analysis was performed on OTU level to investigate whether there were any significant differences in alpha and beta diversity (Fig. 12). The Principle Coordinate Analysis (PCoA) with Uni Frac distance metric showed a slightly larger degree of clustering in prebiotic samples compared to controls (Fig. 12A). In the CCA plot less variation can be seen between the different time-points of the prebiotic group compared to the control group (Fig. 12B); although this was not statistically significant (p=0.541). Notably the prebiotic and control groups were statistically different at D0, suggesting that the control and prebiotic groups had differences prior to the study. Whilst this has limited the strength of conclusions we could draw from this data, it was considered of value to evaluate the longitudinal effect of diet change with and without prebiotic on these groups individually.

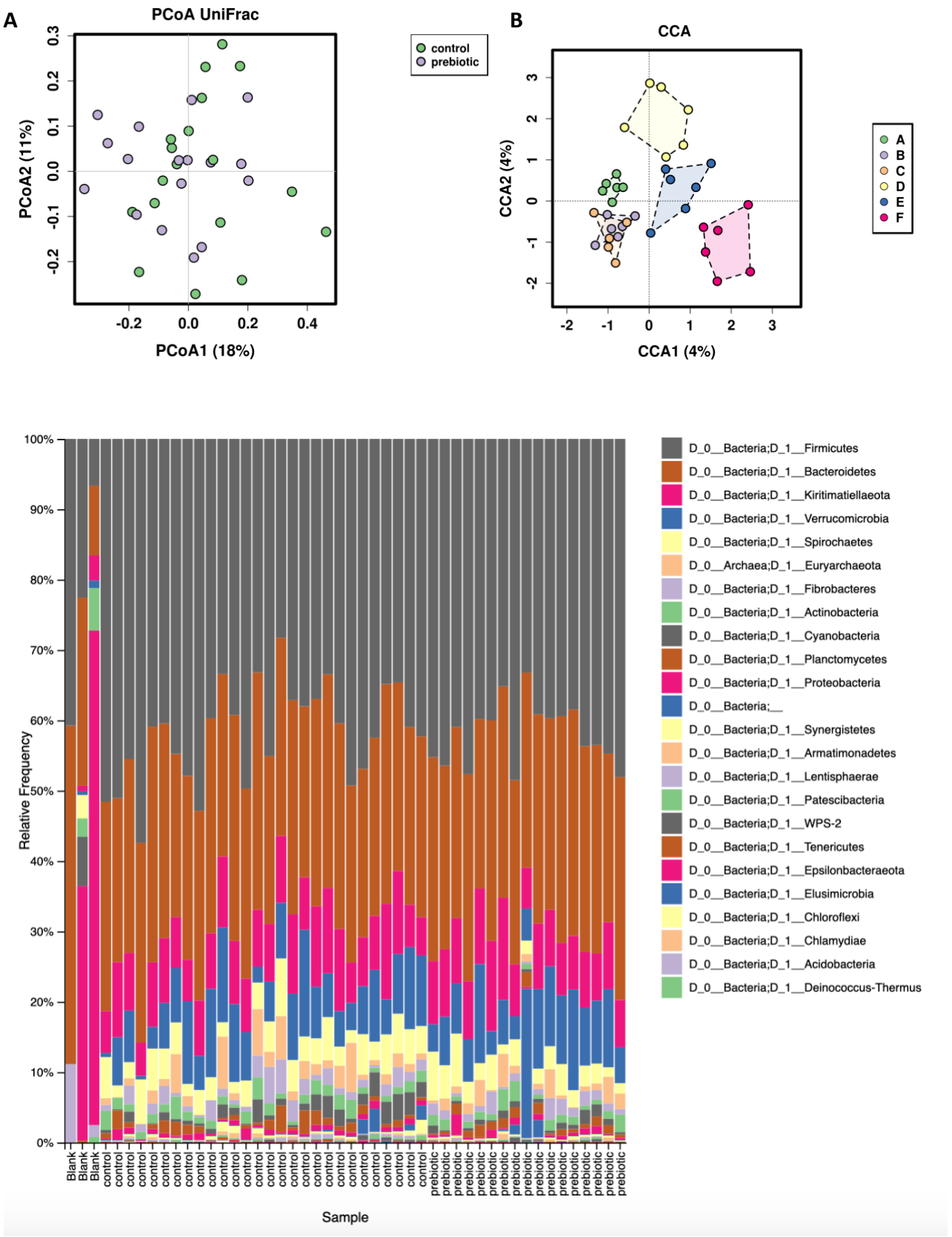
A
PCoA UniFrac
PCoA2 (11%)
PCoA1 (18%)
control
prebiotic
B
CCA
CCA2 (4%)
CCA1 (4%)
A
B
C
D
E
F
C
Relative Frequency
Sample
D_0__Bacteria;D_1__Firmicutes
D_0__Bacteria;D_1__Bacteroidetes
D_0__Bacteria;D_1__Kiritimatiellaeota
D_0__Bacteria;D_1__Verrucomicrobia
D_0__Bacteria;D_1__Spirochaetes
D_0__Archaea;D_1__Euryarchaeota
D_0__Bacteria;D_1__Fibrobacteres
D_0__Bacteria;D_1__Actinobacteria
D_0__Bacteria;D_1__Cyanobacteria
D_0__Bacteria;D_1__Planctomycetes
D_0__Bacteria;D_1__Proteobacteria
D_0__Bacteria;__
D_0__Bacteria;D_1__Synergistetes
D_0__Bacteria;D_1__Armatimonadetes
D_0__Bacteria;D_1__Lentisphaerae
D_0__Bacteria;D_1__Patescibacteria
D_0__Bacteria;D_1__WPS-2
D_0__Bacteria;D_1__Tenericutes
D_0__Bacteria;D_1__Epsilonbacteraeota
D_0__Bacteria;D_1__Elusimicrobia
D_0__Bacteria;D_1__Chloroflexi
D_0__Bacteria;D_1__Chlamydiae
D_0__Bacteria;D_1__Acidobacteria
D_0__Bacteria;D_1__Deinococcus-Thermus

Figure 12: Beta diversity analyses and the fecal microbial profiles of fecal samples from 12 male Thoroughbred yearlings, half of which received prebiotic supplementation. A: Canonical Correspondence Analysis (CCA) plot of the prebiotic group (A=D0, B=Week1, C=Week3) and control group (D=D0, E=Week1, F=Week3) with three different time points each: more clustering of timepoints in prebiotic group than controls. B: Principle Coordinates Analysis (PCoA) plot of the prebiotic and control groups. C: Bar chart of taxonomic abundances of bacterial phyla in prebiotic supplemented groups and controls.

Differences between pre-treatment groups could be seen in the Shannon diversity index: the prebiotic group had a trend towards higher diversity values already at baseline sample, although this difference was not significant (p=0.26) (Fig. 13). However, the shift in shift in alpha diversity during the change in diet shows the same pattern in both groups, as can be seen between the timepoints in the Shannon index as well as the richness and evenness indices. In both groups, the alpha diversity had a tendency to reduce after the horses were stabled and their diet was changed from grass to hay and concentrate, although this difference was not significant.

Therefore, although the differences at baseline between the prebiotic and control groups meant that these two groups were not comparable, the prebiotic did not seem to have a strong effect on the microbial diversity.

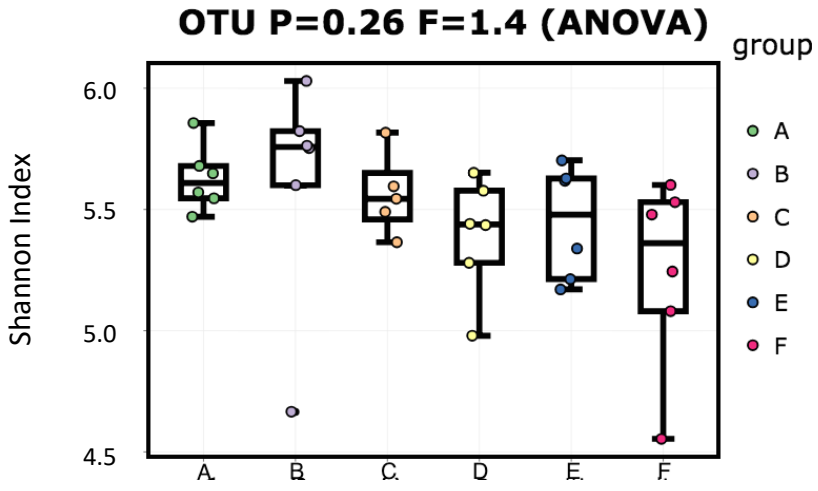

Figure 13: The Shannon Index of alpha diversity in fecal samples of 12 male Thoroughbred yearlings, half of which received prebiotic supplementation. Shannon Diversity Index of prebiotic and control groups over time: A: prebiotic timepoint 1 (baseline sample); B: prebiotic timepoint 2; C: prebiotic timepoint 3; D: control timepoint 1; E: control timepoint 2; F: control timepoint 3.

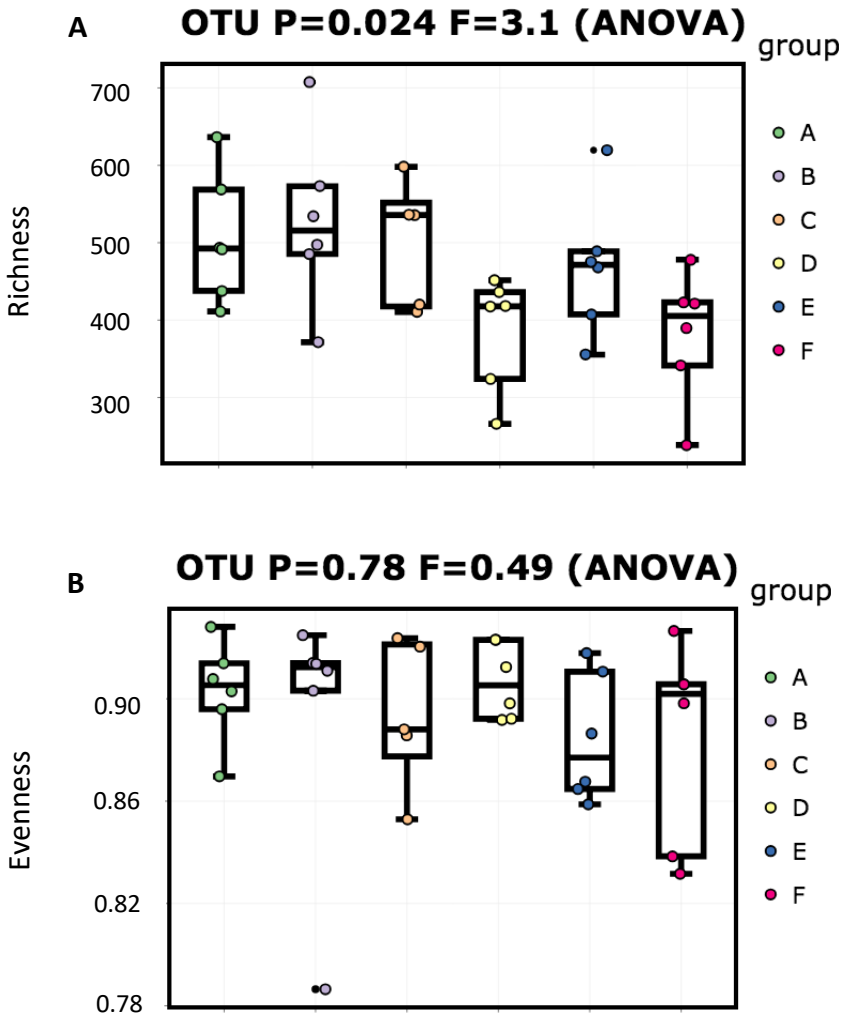

Figure 14: Alpha diversity analyses of fecal samples from 12 male Thoroughbred yearlings, half of which received prebiotic supplementation. Richness and Evenness of alpha diversity of all groups, two-way with treatment and timepoint combined (A: prebiotic timepoint 1 (baseline sample); B: prebiotic timepoint 2; C: prebiotic timepoint 3; D: control timepoint 1; E: control timepoint 2; F: control timepoint 3) show higher richness and evenness of prebiotic group at baseline.

To examine the effect of dietary change in more detail, a multivariate and univariate analyses of the prebiotic group and the control group were done separately to investigate the difference in bacterial composition over time of each group.

In the **control** group, there is a visible, albeit not significant (p=0.1 and p=0.595), difference between the three timepoints in both the ANOSIM and the PERMDISP2 distance matrices (Fig. 15A,B). Furthermore, the network analysis of bacterial families associated with the three timepoints shows clustering of bacterial co-correlating taxa according to the timepoints, especially in timepoint 3 (Fig. 16).

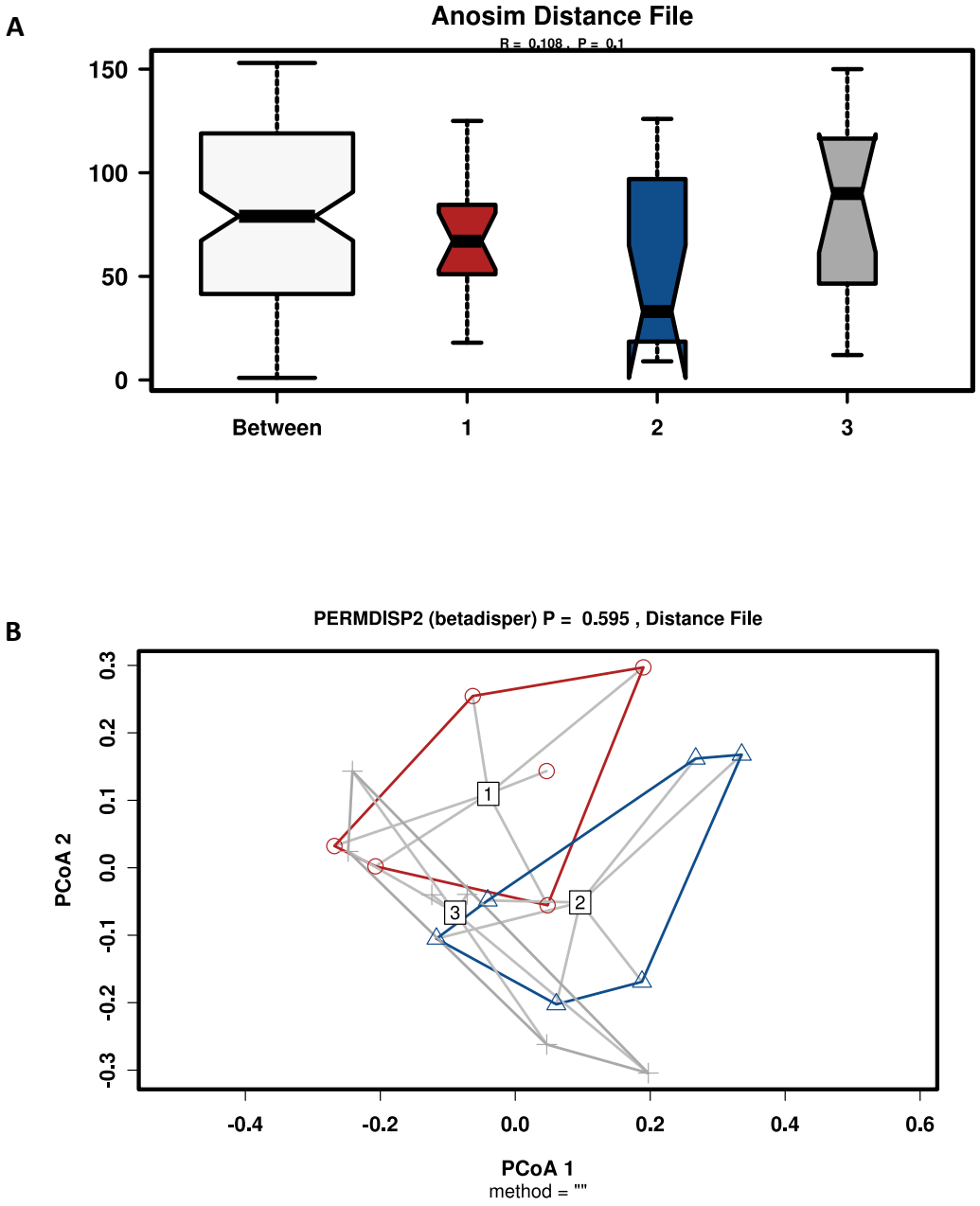

Figure 15: The multivariate analyses of fecal microbial profiles of samples from 6 healthy control male Thoroughbred yearlings show visible, but not significant, differences between the timepoints (timepoint 1 (baseline sample); timepoint 2 (week 1); timepoint 3 (week 3)); A: The ANOSIM distance file of the control group over time shows some variation between the three timepoints (P=0.1); B: The Permdisp2 distance file shows only little overlap between timepoints. (P=0.595)

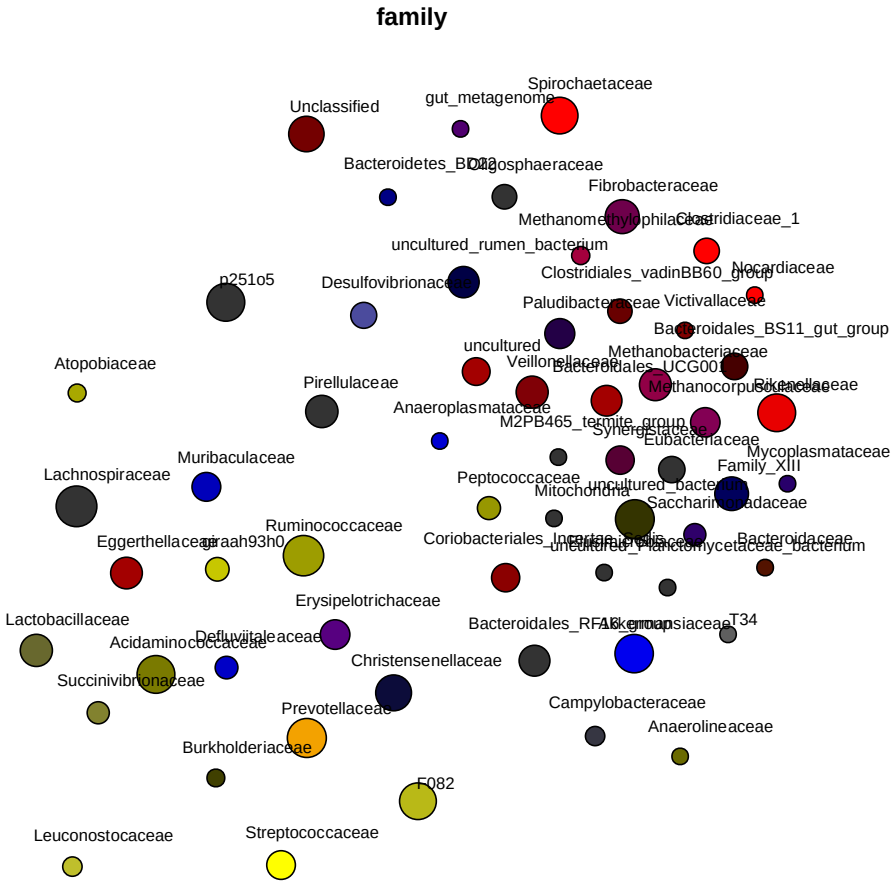

Figure 16: Comparison of the fecal microbial abundances in samples from 6 healthy control male Thoroughbred yearlings. The network analysis of bacterial families associated with different timepoints (timepoint 1 (baseline sample); timepoint 2 (week 1); timepoint 3 (week 3)) in the control group shows clustering of taxa from each timepoint (timepoint 1 (baseline sample): red; timepoint 2: blue; timepoint 3: yellow).

In the LEfSe analysis of the control group, the baseline sample had the highest abundance of the families *Nocardiaceae* (phylum Actinobacteria) and *Clostridiaceae_1* (phylum Firmicutes), as well as the genera *Sarcina* and *Lachnospiraceae_XPB1014_group*, both from the order Clostridiales. At the second timepoint two genera from the family *Ruminococcaceae* were most abundant (*Oscillospira* and *Ruminococcaceae_UCG008*), as well as the genus *Fretibacterium* from the phylum Synergistetes. The last timepoint had the highest abundance of the bacterial phylum Proteobacteria and the genus *Lachnospiraceae_FCS020_group* from the Firmicutes phylum (Table 8).

Table 8: Linear Discriminant Analysis Effect Size (LEfSe) analysis of fecal samples of healthy control Thoroughbred yearlings comparing taxonomic abundances of gut microbiota between yearlings at three timepoints before and after dietary shift (timepoint 1 (baseline sample); timepoint 2 (week 1); timepoint 3 (week 3)). The table below shows the Linear Discriminant Analysis (LDA) score on phylum and family taxonomic level, with the intermediary taxonomic levels included to show the relationship between them. A score of 3-3.5 describes a low association, 3.5-4 a medium, 4-4.5 a high and >4.5 a very high association between the taxa and the investigated group.

Phylum	Class	Order	Family	Genus	Group	LDA Score
Actinobacteria						
	Coriobacteriia					
		Corynebacteriales				
			Nocardiaceae		1	3.751
				Oscillospira	2	3.436
Firmicutes						
	Clostridia					
		Clostridiales				
			Ruminococcaceae			
				Ruminococcaceae_UCG008	2	3.384
			Lachnospiraceae			
				Lachnospiraceae_FCS020_group	3	3.339
				Lachnospiraceae_XPB1014_group	1	3.647
			Clostridiaceae_1		1	3.868
			Clostridiaceae			
				Sarcina	1	3.593
Proteobacteria					3	4.097
Synergistetes						
	Synergistia					
		Synergistales				
			Synergistaceae			
				Fretibacterium	2	3.419

In the prebiotic group, the multivariate analyses of ANOSIM and PERMDISP2 between timepoints was not significantly different (p=0.206 and p=0.843 respectively) (Fig. 17A,B). Also, no clear links were seen between correlations between taxa and association with time-point in the network analysis of bacterial families (Fig. 18).

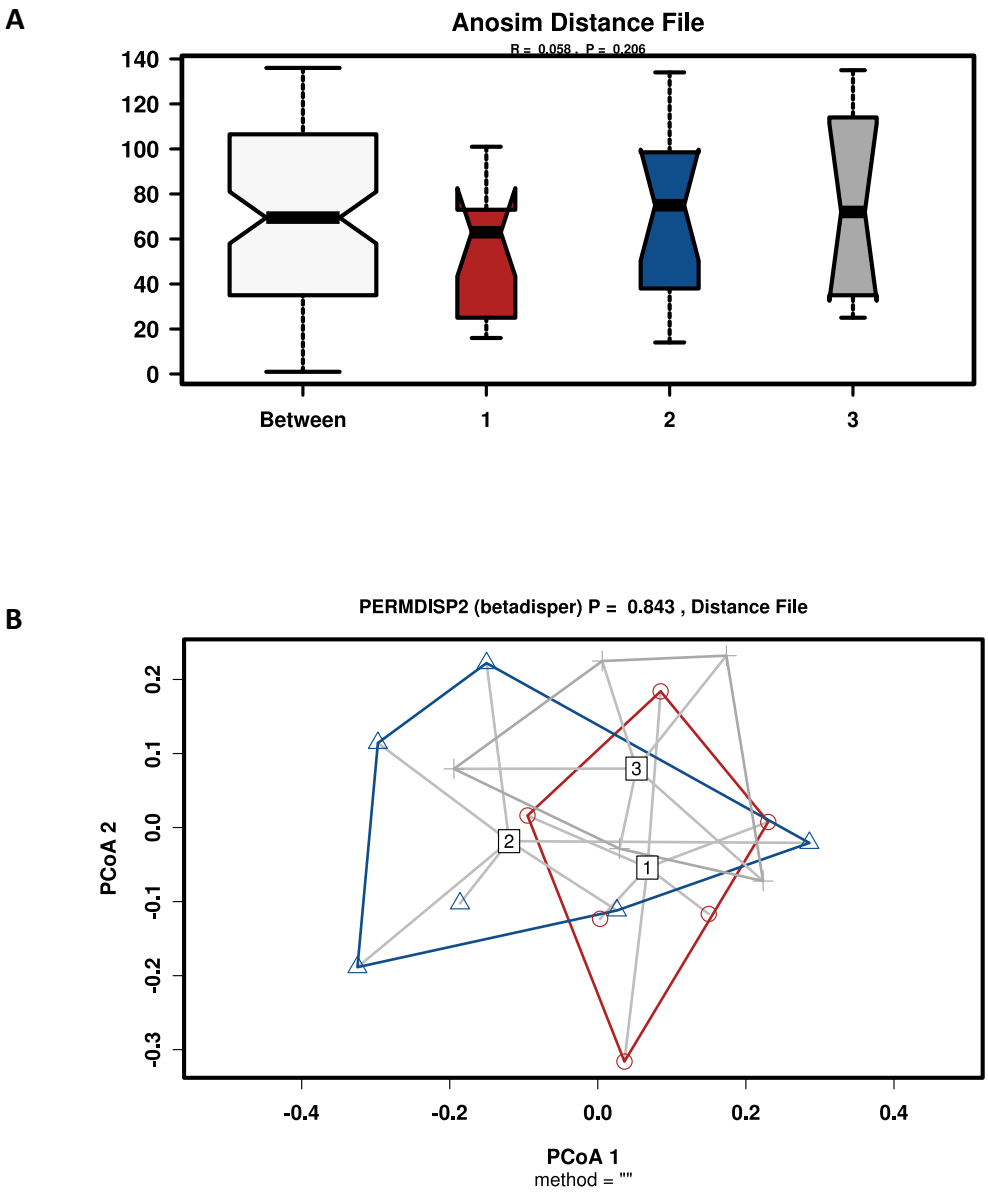

Figure 17: The multivariate analyses of fecal microbial profiles of samples from 6 male Thoroughbred yearlings receiving prebiotic supplementation show no significant difference over time. A: The ANOSIM distance file of the prebiotic group over time shows no significant difference between the timepoints (timepoint 1 (baseline sample); timepoint 2 (week 1); timepoint 3 (week 3)) (p=0.206); B: The Permdisp2 distance file shows large overlap between timepoints (p=0.843).

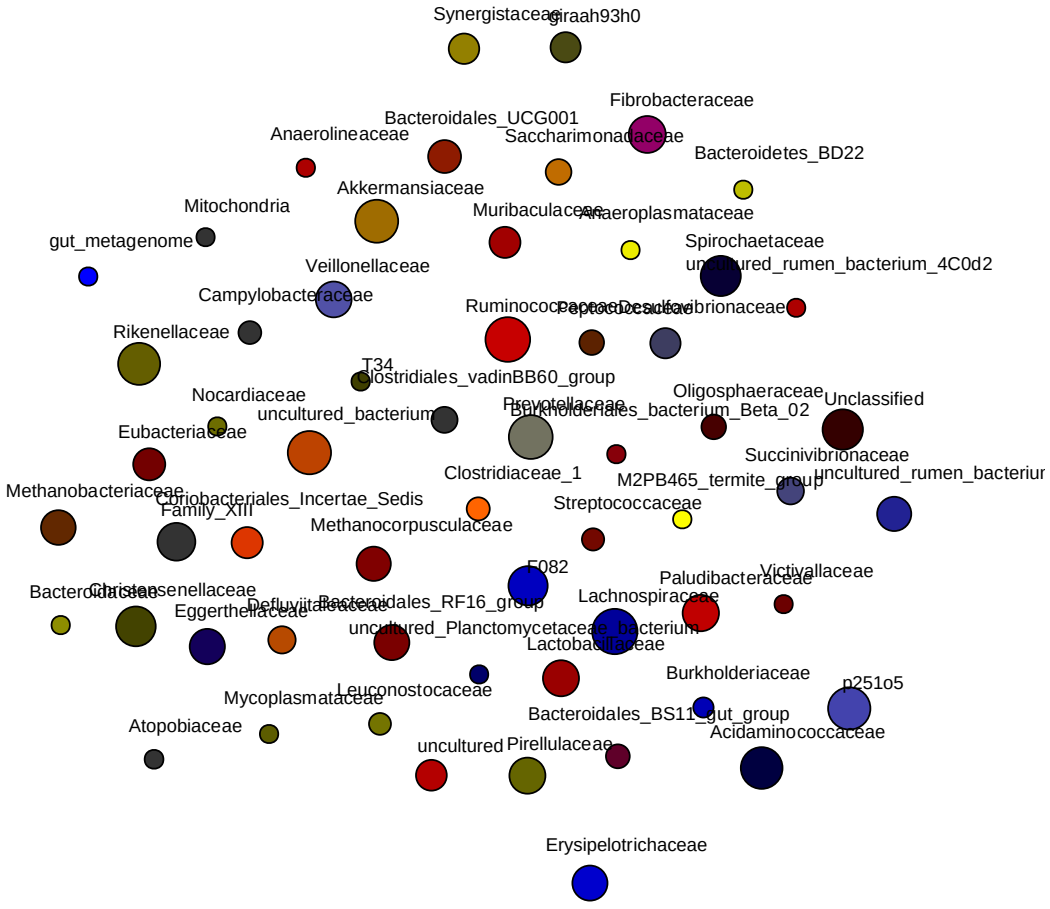

Figure 18: Comparison of the fecal microbial abundances in samples from 6 male Thoroughbred yearlings receiving prebiotic supplementation. A network analysis of bacterial families shows no clustering between taxa associated with different timepoints (timepoint 1 (baseline sample): red; timepoint 2: blue; timepoint 3: yellow) in the yearlings treated with prebiotics.

In the prebiotic group, there were fewer differences in bacterial abundances between the timepoints, with one genus from the phylum Firmicutes associated with the last timepoint (*Lachnospiraceae_ND3007_group*) (Table 9).

Table 9: Linear Discriminant Analysis Effect Size (LEfSe) analysis of fecal samples of prebiotic supplemented Thoroughbred yearlings comparing taxonomic abundances of gut microbiota between yearlings at three timepoints before and after dietary shift (timepoint 1 (baseline sample); timepoint 2 (week 1); timepoint 3 (week 3)). The table below shows the Linear Discriminant Analysis (LDA) score on phylum and family taxonomic level, with the intermediary taxonomic levels included to show the relationship between them. A score of 3-3.5 describes a low association, 3.5-4 a medium, 4-4.5 a high and >4.5 a very high association between the taxa and the investigated group.

Phylum	Class	Order	Family	Genus	Group	LDA Score
Firmicutes						
	Clostridia					
		Clostridiales				
			Lachnospiraceae			
				Lachnospiraceae_ND3007_group	3	3.588
				gut_metagenome	2	3.422

5.4. Discussion

Determination of the composition of the equine GI microbiome under different circumstances and in various cohorts is an important step in the advancement of equine health and welfare. Here we evaluated the impact of prebiotic administration in a group of yearlings undergoing a change from pasture feeding to stabling and high levels of carbohydrate. In general, the phyla Bacteroidetes and Firmicutes were most abundant in all samples; this is in accordance with the general consensus of previous findings in healthy horses[3,20,21,24–27]. Here we also showed relatively large abundances of Verrucomicrobia and Kirimatiellaeota in the fecal microbiota; this is in accordance with findings in other studies, e.g. Costa et al. (2015) and Moreau et al. (2014). The phylum Kiritimatiellaeota has been previously considered a subtype of Verrucomicrobia and was therefore only described as a phylum in newer publications, with similar measured relative abundances of up to 7%[8,32,149]. The abundances of Fibrobacteres, Spirochaetes and Actinobacteria found in this study were also in the range of relative abundances found in healthy horses of previous studies[3,21,26,27]. Proteobacteria, with a relative abundance of 0.75%, is slightly lower compared to findings of previous studies, where this phylum was found at 1-2%[20,27]. 16S rRNA sequencing was chosen for this study as this is a cost-effective tool to examine the different taxa present in the feces and this method has been frequently used in previous

publications[31,34,144]. However, other methods, such as shotgun metagenomics, have been shown to detect a larger amount of bacterial species with a higher accuracy[150,151]. While we appreciate the importance of shotgun metagenomics for functional prediction of microbiota, it was not possible to implement this method in this study due to funding limitations. A more in-depth analysis of these samples using shotgun metagenomics could be an interesting extension of the current study.

When comparing horses supplemented with prebiotics and control, there was a difference in beta diversity in the samples at baseline sample between the prebiotic and control groups, therefore these two groups were not directly compared in their microbial composition in the rest of the analysis. These differences in the microbiome composition in the first samples show the importance of taking a baseline sample when investigating the effect of a variable external factor. The large inter-individual differences in microbial composition of the samples before prebiotic administration could be due to farm management factors and could influence the results of the measured effect of prebiotics. Although taking baseline samples before prebiotic administration has been done in previous studies[101,152], it is still common that studies measure the impact of supplementation during administration, without previously examining the normal microbial composition of each individual[98,99,153]. However, results of comparing two groups with a specific treatment are only relevant if there is evidence that the groups were not different from the beginning.

In this study, however, not only the difference between two groups based on prebiotics was investigated, but also the microbial composition during a dietary change and the effect of prebiotics on this microbial shift was examined over three timepoints. The alpha diversity analyses showed a similar pattern in both groups of initial increase in microbial diversity and a decrease following the change from pasture to stable. This reduced bacterial diversity after shift to a higher carbohydrate diet is in accordance with previous studies investigating the change of diet from forage to concentrate[20,102], as well as the findings from the meta-analysis discussed

previously, where the horses on a high starch diet had a lower bacterial diversity than those on a forage diet.

When measuring the changes over time in the control group, higher abundances of Proteobacteria where found in the last timepoint, which is corresponding to previous studies which found increased Proteobacteria in horses with high starch diets due to its role in starch fermentation[20]. Additionally, the high abundance of *Clostridiaceae* in the baseline samples is according to findings of horses on forage diet in these same studies[20,26]. In the prebiotic group, there was an increase in abundance of a genus from *Lachnospiraceae* and a species from *Lactobacillaceae* over the three timepoints. The increase in *Lactobacillaceae* is in line with findings from previous studies that found increased *Lactobacillaceae* in horses on a high carbohydrate diet[23].

Overall, there was a larger change in beta diversity over time in the control group than in the prebiotic group when comparing the different timepoints before and after dietary change, although this was not statistically significant. This was visible in the higher variation between timepoints in the ANOSIM and PERMDISP2 distance matrices, as well as in the stronger clustering in the network analysis and the larger amounts of differentially abundant bacteria in the LEfSe analysis of the control group compared to the prebiotic group. No difference in diversity was evident over time and no clustering could be seen in the network analysis of bacterial families associated with timepoints in the prebiotic group. This may imply an effect of the prebiotic supplementation in stabilizing the GI microbiome during a time of nutritional change. A stabilizing effect of prebiotics has been found in previous studies of horses with prebiotic supplementation during dietary change, with less variation in GI microbiota after the dietary shift when given prebiotics[102,110], which supports the findings here. However, given the limitations of this study with regards to the difference between groups prior to supplementation, it is necessary to repeat this work on a larger scale to verify the data. The expansion of this study in the future could be achieved by additional techniques, for example, nuclear magnetic resonance (NMR) spectroscopy could be used to determine metabolites produced by bacteria in biofluids of

horses[144]. This could be done using either fecal water or urine samples, as has been done previously when investigating the impact of diet and age[64] or in correlation with disease[72]. Furthermore, blood samples could be analyzed with NMR[65], however, this would then require an invasive treatment. Although these other sample types were not collected in this study, the analysis of different sample types combined with the examination of the GI microbiome in fecal samples would be a promising continuation of this study.

5.5. Conclusion

This is one of the first studies to investigate the impact of prebiotics and the effect of diet change in Thoroughbred youngstock on the GI microbiome using Next Generation Sequencing methods. The differing microbial compositions between the prebiotic and control groups at the first sampling point show the importance of taking a baseline sample before starting with a treatment, as varying microbiomes in the beginning can be due to other factors which can distort the findings on the effects of prebiotics. Furthermore, the smaller change in GI microbiota abundances after dietary shift in horses receiving prebiotics compared to controls may implicate a stabilizing effect of prebiotics on the GI microbiome during nutritional stress. Consequently, large scale follow-up studies are needed to determine the effect of confounding factors and the corresponding microbial changes in the investigation of the effect of prebiotic supplementation during dietary change on the equine GI microbiome.

6. Overall Summary

Determination of the composition of the gastro-intestinal (GI) microbiome is highly important for equine health, as horses rely heavily on microbial fermentation in their hindgut to gain energy. The GI microbiota are not only required for fermentation of undigestible carbohydrates and fibers, they also interact with the host immune system[41–43]. Changes in the GI microbiome, for example through a concentrate-rich diet, can lead to diseases such as colitis[70], obesity and laminitis[62]. There is a consensus that the phyla Firmicutes and Bacteroidetes form the majority of the bacterial species present in the GIT of healthy horses, however, the relative abundances range from 20-70% of each[1,3,20–22,24–27,64]. Further bacterial species found in several studies, but at lower abundances than the above, with abundances ranging from 2-15% include Proteobacteria, Actinobacteria, Verrucomicrobia, and Fibrobacteres[3,20,21,23,26,27]. The same core microbiome was also found in the meta-analysis and the prebiotic study. However, despite similarities in the species of bacteria that are present in healthy horse intestines, there is much variation in abundance measurements between studies. The varying abundances of bacterial species can be due to biological external and individual factors, including, but not limited to, diet, management and location or age, sex, breed and health respectively[1]. However, this meta-analysis showed that the largest difference between studies is due to technical variation; in this case, the different sequencing methods and gene regions had a strong effect on the data. Furthermore, with the batch mean correction we showed that there is a way to reduce the effect of technical variation to compare biological factors when looking at large amounts of data. With this, the effects of sample type and region, age, sex, diet and disease on the GI microbiome could be investigated.

The sample type and location in the GIT was shown to have the strongest impact on the GI microbiome in the meta-analysis, as luminal and mucosal samples of the same GIT compartment had differential bacterial abundances and the different compartments from the same sample type also varied strongly in their bacterial composition. These differences between parts of the GIT have been found in previous studies, with feces being most similar to the hindgut[22,35,36]. The

findings from the meta-analysis show the importance of using the same sample type and closely connected GIT regions tailored to the problem investigated when looking at the GI microbiome, as samples from different types and regions are not comparable with each other. Age is also a factor that was responsible for differential abundances of a large number of bacterial species. Similar to previous studies, we found the largest variation in foals and youngstock up to 2 years of age. This correlates with an important developmental period during which the GI microbiome and the immune system develop in tandem[8,9]. Furthermore, we found the alpha diversity to be lowest in foals less than 2 months old and increasing with age throughout life, as has been shown in previous studies[13]. Consequently, further research should focus on the health implications associated with the high levels of variation seen between young foals at this crucial time. While the age groups had differential abundances in a large number of bacteria, the sex did not result in as strong differences in the microbial composition. This is in accordance with findings from a previous study in which no significant difference was found between males and females[138]. The factor diet had a visible influence on the GI microbiome in both the meta-analysis and the prebiotic study. The horses on high starch diet had a lower alpha diversity than those on a forage diet in the meta-analysis, and also the Thoroughbred yearlings shifting from a forage-based to concentrate-based diet had a reduction in alpha diversity after the dietary shift in both prebiotic supplemented horses and healthy controls. Furthermore, previous studies with horses receiving a starch rich diet have shown a different microbial composition with a lower diversity and higher fluctuations in bacterial abundances compared to forage fed controls[20,28,53,102]. The effect of diet is especially important in disease prevention, as most horses in domesticated conditions receive starch-based feed at given feeding times, which is the opposite of natural grazing habits in feeding amounts and starch content. This imbalance can lead to gastro-intestinal and systemic diseases, such as colic, laminitis and obesity[51,62,70]. This meta-analysis found the correlation of diseases with bacterial species in the GIT overlapping between intestinal and metabolic diseases. However, also in previous studies the microbial changes found in horses suffering from diseases were not uniform, with differences in bacterial diversity and abundances of species varying with each study.

In the meta-analysis the alpha diversity was not significantly different between metabolic and intestinal diseases, however, it was significantly lower in horses with drug treatment. This reduced diversity in horses under drug treatment, especially antibiotics, has also been found in previous studies[29,73,83]. The overlapping findings in terms of bacterial abundances in horses with intestinal and metabolic diseases indicates the need to investigate the connection of diseases and the GI microbiome in more standardized, large-scale studies in order to reach a consensus and possibly better differentiate the diseases in their relationship with the GI microbiota.

Due to the correlation of diseases with the GI microbiome and to improve equine health, feed supplements, such as probiotics and prebiotics, have been used to modulate the microbiome in a beneficial way. However, proof of their efficacy in horses is still rather limited, as previous studies have found varying results[98,100,110,114,152,154,155]. When investigating the effect of prebiotics on equine yearlings during a dietary shift in this study, the horses receiving prebiotics had a more stable microbiome compared to the control group, which may imply a supporting role of prebiotics during a time of nutritional stress.

Overall, there are still many controversial issues surrounding the bacterial diversity and role of the GI microbiome in health and disease, which is additionally complicated by the unclear role of cause and effect of microbial changes. This study showed that the large variation between studies may in large part be due to the different scientific methods used, especially the type of sequencing and gene region sequenced renders the data almost not comparable due to the strong differences in found bacteria. Furthermore, while the scientific procedure was uniform throughout the prebiotic study, as the fecal sample collection and storage were following the same protocol and the DNA extraction was performed using the same kit, this may be more of an issue in a meta-analysis combining different studies or in comparing results to previous literature, as there is to date no common scientific protocol for microbiome studies, which may be an additional confounding factor in determining the microbial composition of the GIT. Therefore, it is vital for future research to coordinate on a global scale, possibly by creating a protocol that is followed in equine GI research in order to gain uniform information that can be

compared and shared among groups. Additionally, the meta-analysis could be expanded in a future study to include more studies, for example by collaborating with authors to obtain the necessary sequencing data and metadata, in order to provide a more in-depth analysis of potential influencing factors of the GI microbiome. Furthermore, additional parameters could be included in the meta-analysis, such as the sample storage and handling, as well as the lab equipment used for DNA extraction. Also, the prebiotic part of this thesis could be expanded in a future study including a range of different sample types and analysis methods in combination with the microbiome determination of the fecal samples, for example by doing metabolomics on fecal waters or blood samples to determine their metabolites.

7. Conclusion

This thesis showed with a meta-analysis and a prebiotic field study how the biological factors sample type and region, age, sex, diet, disease and prebiotic supplementation can influence the GI microbiome. Furthermore, the meta-analysis showed that technical factors, such as gene regions of the 16S rRNA sequenced, as well as the sequencing instrument used, were accountable for the strongest variation in bacterial composition, more so than biological factors. This effect was so dominant that it required a batch mean correction for the impact of biological factors to become evident. These findings highlight the necessity of standardizing scientific methodology in equine GI microbiome research if any comparable results are to be achieved. Additionally, the challenges of compiling raw data from equine GI microbiome studies show the potential for a central database to enhance global collaboration and advancement in the field of horse microbiome research.

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Appendix

1. R Script for Batch Mean Correction

```
setwd("/Users/stephaniemeier/Desktop/BMC_tss_sqrt/BMC_TSS_sqrt_new_metadata")
# Set Metadata PCA grouping variable here...

grouping = "Adult_Foal"          # This is the variable to colour the PCA by (i.e. your variable of interest)
batch_to_correct = "Instrument_Gene"  # This is the variable to correct for (i.e. the confounder)

# Install all packages locally
 #if (!requireNamespace("BiocManager", quietly = TRUE))
 #  install.packages("BiocManager")
 #BiocManager::install(version = "3.11")
 #BiocManager::install("factoextra")
 #BiocManager::install("matrixStats")
 #BiocManager::install("viridis")
 #install.packages("preputils")

 ####BiocManager::install("biomformat")

 #install.packages("bapred")
 #BiocManager::install("sva")
 #BiocManager::install("affyPLM")

#################### Set up working env ####################

library(factoextra) #ggplot2 based PCA graphics
```

```
library(matrixStats)
library(preputils)
library(viridis)
library(bapred)

#################### Import converted biom file ####################

# Convert in BASH using following command
#biom convert -i feature-table.biom -o feature-table.tsv --to-tsv
biom_import = read.table("feature-table.tsv", sep = "\t", header = T, comment.char = "", skip =
1, row.names = 1)
head(biom_import)
matrix = as.matrix(biom_import)
head(matrix)

#################### Attempt TSS Normalisation ####################

#BiocManager::install("hilldiv")
library("hilldiv")
?tss()
tss_tranform = tss(matrix)

#################### Attempt Square Root Normalisation ####################

#BiocManager::install("expm")
library("expm")
sqr_rt_transform = sqrt(tss_tranform)

#################### Import Metadata ####################
```

```
metadata_in = "metadata_noblank_NA_nofernandes_new.csv"
metadata = read.csv(metadata_in, header = TRUE)

#################### Order Samples ####################

order1_all = as.character(metadata$SampleID)
iso_table_all = tss_tranform[, match(order1_all, colnames(tss_tranform))]
head(iso_table_all)
colnames(iso_table_all)
metadata$SampleID
head(iso_table_all)
nrow(iso_table_all)
ncol(iso_table_all)

iso_table_all = tss_tranform[, match(order1_all, colnames(tss_tranform), nomatch = 0)]

# Remove all samples with 0 Sd
#non_var2=iso_table_all2[!rowSds(iso_table_all2)==0,]
non_var = iso_table_all[rowSds(iso_table_all, na.rm=TRUE) != 0,]
head(non_var)

#################### PCA - PRE BMC ####################
#Transpose to make sample names rownames
df_alex_t = t(non_var)
ncol(df_alex_t)
nrow(df_alex_t)
# # Filter on output
# keep_lrt <- colSums(df_alex_t) >= 1
```

```
# keep = df_alex_t[,keep_lrt]

res.pca_norm_out_pre <- prcomp(df_alex_t, scale. = T)
fviz_eig(res.pca_norm_out_pre)
groups_phase <- as.factor(metadata[[grouping]])
pca_ind_phase_pre = fviz_pca_ind(res.pca_norm_out_pre,
                     col.ind = groups_phase,
                     repel = F, # Avoid text overlapping
                     addEllipses = F, # Concentration ellipses
                     ellipse.type = "confidence",
                     legend.title = "Grouping",
                     label = "none",
) + scale_color_viridis(discrete = T, option = "viridis")
pca_ind_phase_pre

ggsave("pre_norm_PCA_age_twoway_tss_sqrt.png")

#################### Batch Mean Centering ####################
# Create levels for batches
batch_factor = as.factor(metadata[[batch_to_correct]])
batch_numeric = as.numeric(batch_factor)
batch_numeric_factor = as.factor(batch_numeric)
nlevels(batch_numeric_factor)
nrow(batch_numeric_factor)

# # Batch correct using BMC (bapred) - Samples as rownames
# batch_correct = meancenter(df_alex_t, batch = batch_numeric_factor)
# batch_corrected_bmc = batch_correct$xadj
```

```
# write.table(batch_corrected_bmc, file =
paste0("BMC_Batch_corrected_by_",batch_to_correct,".tsv"), sep = "\t", col.names = NA)

# Batch correct using rmbat - Samples as colnames
batch_corrected_rmbat = rmbat(non_var, batches = batch_numeric_factor)

batch_corrected_rmbat[batch_corrected_rmbat<0] = 0

write.table(batch_corrected_rmbat, file =
paste0("RMBat_Batch_corrected_by_",batch_to_correct,".tsv"), sep = "\t", col.names = NA)

#################### PCA - POST BMC ###################

res.pca_norm_out_post <- prcomp(t(batch_corrected_rmbat), scale. = T)
fviz_eig(res.pca_norm_out_post)
#PCA for Phase of timepoint
groups_phase <- as.factor(metadata[[grouping]])
pca_ind_phase_post = fviz_pca_ind(res.pca_norm_out_post,
                  col.ind = groups_phase,
                  repel = F, # Avoid text overlapping
                  addEllipses = F, # Concentration ellipses
                  ellipse.type = "confidence",
                  legend.title = "Grouping",
                  label = "none",
) + scale_color_viridis(discrete = T, option = "viridis")
pca_ind_phase_post

ggsave("post_norm_PCA_rmbat_age_twoway_tss_sqrt.png")
```

2. Source Code for Merging Taxonomies

```
for dir in ./*/*/*ssu*;
        do
                echo $dir
                for file in $dir/E*_FASTQ_SSU_OTU.tsv;
                        do echo "Input file: $file"
                        filename="$(basename $file)"
                        filename_no_ext="$(basename $file .tsv)"
                        echo "Basename (without extension): $filename_no_ext"
                        if [ -f "$dir/clean_$filename" ]; then
                                echo "Clean file exists. Doing nothing"
                        else
                                echo "Clean file does not exist. Using python script to parse and
clean"
                                python format_mapseq_old.py "$file" > "$dir/clean_$filename"
                        fi
                        sed s/\taxid/\ /g "$dir/clean_$filename" > "$dir/new_$filename"
                        echo "Using Biom to convert to OTU table"
                        biom convert -i $dir/new_$filename -o
$dir/${filename_no_ext}_steph.biom --table-type="OTU table" --process-obs-metadata
taxonomy --to-hdf5
                        echo "Importing file: $dir/${filename_no_ext}_steph.biom to qiime2 as
Frequency Table"
                        qiime tools import --input-path $dir/${filename_no_ext}_steph.biom --
type 'FeatureTable[Frequency]' --input-format BIOMV210Format --output-path
$dir/${filename_no_ext}.qza
                        echo "Importing file: $dir/${filename_no_ext}_steph.biom to qiime2 as
Taxonomy Table"
```

```
                        qiime tools import --input-path $dir/${filename_no_ext}_steph.biom --
type "FeatureData[Taxonomy]" --input-format BIOMV210Format --output-path
$dir/${filename_no_ext}.taxonomy.qza
                    done
          done
echo "Running Ruby script to merge all studies"
ruby merge_table_taxonomy.rb
echo "Exporting merged OTU.qza to readable OTU table"
qiime tools export   --input-path merged_table.qza   --output-path merged_otu_table_qiime2
echo "Exporting merged taxonomy.qza to readable Taxonomy table"
qiime tools export   --input-path merged_taxonomy.qza   --output-path
merged_taxonomy_qiime2
sed  's/Feature ID/#OTUID/g' merged_taxonomy_qiime2/taxonomy.tsv >
merged_taxonomy_qiime2/biom-taxonomy.tsv
```

3. Code for Qiime2 Analysis of Prebiotic Study

```
mkdir Desktop/ChasemoreYearlings
cd Desktop/ChasemoreYearlings
qiime tools import \
  --type 'SampleData[PairedEndSequencesWithQuality]' \
  --input-path casava-18-paired-end-demultiplexed-ChasemoreYearlings \
  --input-format CasavaOneEightSingleLanePerSampleDirFmt \
  --output-path demux-paired-end.qza
qiime demux summarize \
  --i-data demux-paired-end.qza \
  --o-visualization demux-paired-end.qzv

qiime tools view demux-paired-end.qzv
qiime dada2 denoise-paired \
  --i-demultiplexed-seqs demux-paired-end.qza \
  --p-trim-left-f 35 \
  --p-trim-left-r 35 \
  --p-trunc-len-f 290 \
  --p-trunc-len-r 256 \
  --o-representative-sequences rep-seqs-dada2.qza \
  --o-table table-dada2.qza \
  --o-denoising-stats stats-dada2.qza
qiime metadata tabulate \
  --m-input-file stats-dada2.qza \
  --o-visualization stats-dada2.qzv
qiime feature-table tabulate-seqs \
  --i-data rep-seqs-dada2.qza \
  --o-visualization rep-seqs.qzv
```

```
qiime feature-table summarize \
  --i-table table-dada2.qza \
  --o-visualization table.qzv \
  --m-sample-metadata-file sample-metadata_chasemore_yearlings.tsv
qiime phylogeny align-to-tree-mafft-fasttree \
  --i-sequences rep-seqs-dada2.qza \
  --o-alignment aligned-rep-seqs.qza \
  --o-masked-alignment masked-aligned-rep-seqs.qza \
  --o-tree unrooted-tree.qza \
  --o-rooted-tree rooted-tree.qza
qiime diversity core-metrics-phylogenetic \
  --i-phylogeny rooted-tree.qza \
  --i-table table-dada2.qza \
  --p-sampling-depth 190 \
  --m-metadata-file sample-metadata_chasemore_yearlings.tsv \
  --output-dir core-metrics-results
qiime feature-classifier classify-sklearn \
  --i-classifier classifier.qza \
  --i-reads rep-seqs-dada2.qza \
  --o-classification taxonomy.qza
qiime metadata tabulate \
  --m-input-file taxonomy.qza \
  --o-visualization taxonomy.qzv
qiime tools export \
--input-path table-dada2.qza \
--output-path exported
qiime tools export --input-path taxonomy.qza --output-path exported
qiime tools export --input-path /Users/nv19845/Desktop/ChasemoreYearlings/core-metrics-
results-new/weighted_unifrac_distance_matrix.qza --output-path exported
```

4. Figures and Tables Appendix

Table 10: Excerpt of metadata used for the meta-analysis of studies on the equine gastro-intestinal microbiome of horses with the following factors included: Sample ID, ENA and MGnify accession numbers, subject, age range, sex, disease/treatment, dietary starch content (NSC), study, sample type and region of the gastro-intestinal tract, instrument model used for sequencing, gene region sequenced, instrument model and gene region combined and library layout used.

SampleID	ENA	Mgnify	Subject	Age_Range	Female_Male	Disease	NSC	Study	Sample_Type	Sample_Region	Instrument_Model	Gene_Region	Instrument_Gene	Library_Layout
SRR10397380_FASTQ_SSU_OTU	SRP119693	MGYS00005549	Horse 1	20-30y	Female	Control	Low	Arnold 2020	Feces	Feces	Illumina MiSeq	V4	Illumina_V4	SINGLE
ERR4319613_MERGED_FASTQ_SSU_OTU	PRJEB39250	MGYS00005591	1	2-20y	Female	Drugs	High	Clark 2018	Feces	Feces	Illumina MiSeq	V3-V4	Illumina_V3-V4	PAIRED
SRR518639_FASTQ_SSU_OTU	SRP012927	MGYS00005570	Healthy 2	20-30y	Female	Control	Low	Costa2012	Feces	Feces	454	V3-V5	454_V3-V5	SINGLE
SRR3131283_MERGED_FASTQ_SSU_OTU	PRJNA254186	MGYS00005558	2	20-30y	Female	Control	Low	Costa 2015	Mucosa	Other	Illumina MiSeq	V4	Illumina_V4	PAIRED
SRR8878229_FASTQ_SSU_OTU	PRJNA475435	MGYS00005552	51	No_Answer	Female	Control	High	DeLa Torre 2019	Feces	Feces	Illumina MiSeq	V4	Illumina_V4	SINGLE
ERR227180_FASTQ_SSU_OTU	ERP002202	MGYS00005566	1	2-20y	Male	Control	Low	Dougal 2013	Mucosa	Ileum	454	V1-V2	454_V1-V2	SINGLE
ERR1981259_FASTQ_SSU_OTU	PRJEB20876	MGYS00005565	1	2-20y	Male	Control	Low	Dougal2017	Feces	Feces	454	V1-V2	454_V1-V2	SINGLE
SRR5937773_MERGED_FASTQ_SSU_OTU	PRJNA322656	MGYS00005555	1	2-20y	Male	Control	No_Answer	Ericsson 2016	Lumina	Colon	Illumina MiSeq	V4	Illumina_V4	PAIRED
ERR4336949_MERGED_FASTQ_SSU_OTU	PRJEB39375	MGYS00005577	1	2-20y	Female	Metabolic	Restricted	Fitzgerald2020	Feces	Feces	Illumina MiSeq	V3-V4	Illumina_V3-V4	PAIRED
ERR3226043_MERGED_FASTQ_SSU_OTU	PRJEB31758	MGYS00005603	No_Answer	20-30y	Female	Control	High	Glatter 2019	Mucosa	Cecum	Illumina MiSeq	V1-V2	Illumina_V1-V2	PAIRED
ERR3289278_MERGED_FASTQ_SSU_OTU	PRJEB32017	MGYS00005551	Foal1	<2months	No_Answer	Control	Low	Husso 2020	Feces	Feces	Illumina MiSeq	V3-V4	Illumina_V3-V4	PAIRED
SRR6677874_MERGED_FASTQ_SSU_OTU	PRJNA433202	MGYS00005553	gs	0.5-2y	Male	Drugs	Low	Kunz 2019	Feces	Feces	Illumina MiSeq	V4	Illumina_V4	PAIRED
ERR1110230_MERGED_FASTQ_SSU_OTU	PRJEB11642	MGYS00000570	EGS7	No_Answer	Female	Intestinal	No_Answer	Leng 2018	Feces	Feces	Illumina MiSeq	V4	Illumina_V4	PAIRED
ERR3464875_MERGED_FASTQ_SSU_OTU	PRJEB33830	MGYS00005546	11	2-20y	Female	Control	No_Answer	Lindenberg 2019	Mucosa	Ileum	Illumina MiSeq	V3-V4	Illumina_V3-V4	PAIRED
ERR4318532_MERGED_FASTQ_SSU_OTU	PRJEB39226	MGYS00005590	METAFOAL00	0.5-2y	Female	Control	Low	Mach 2017	Feces	Feces	Illumina MiSeq	V3-V4	Illumina_V3-V4	PAIRED
ERR3316853_FASTQ_SSU_OTU	PRJEB32490	MGYS00005604	Far	2-20y	Female	Control	High	McKinney2020	Feces	Feces	Illumina MiSeq	V1-V2	Illumina_V1-V2	SINGLE
ERR2888048_FASTQ_SSU_OTU	PRJEB29667	MGYS00005564	13	2-20y	Female	Control	Low	Morrison 2018	Feces	Feces	Ion Torrent	V1-V2	Ion_V1-V2	SINGLE
ERR3604501_FASTQ_SSU_OTU	PRJEB34659	MGYS00005557	7	2-20y	Female	Intestinal	Low	Morrison 2020	Feces	Feces	Ion Torrent	V1-V2	Ion_V1-V2	SINGLE
ERR4192219_FASTQ_SSU_OTU	PRJEB38664	MGYS00005560	HS	No_Answer	Female	Control	High	Peachey 2018	Feces	Feces	Illumina MiSeq	V3-V4	Illumina_V3-V4	PAIRED
ERR4195125_MERGED_FASTQ_SSU_OTU	PRJEB38717	MGYS00005559	ED1	0.5-2y	Female	Control	High	Peachey 2019	Feces	Feces	Illumina MiSeq	V3-V4	Illumina_V3-V4	PAIRED
ERR4309764_MERGED_FASTQ_SSU_OTU	PRJEB39150	MGYS00005579	Bo	2-20y	Female	Control	Restricted	Perry 2018	Lumina	Cecum	Illumina MiSeq	V3	Illumina_V3	PAIRED
SRR2132339_FASTQ_SSU_OTU	PRJNA279335	MGYS00005562	2	2-20y	Female	Intestinal	Low	Rodriguez 2015	Feces	Feces	454	V1-V2	454_V1-V2	SINGLE
SRR831198_FASTQ_SSU_OTU	PRJNA177883	MGYS00005583	L1	2-20y	Male	Metabolic	High	Steelman 2012	Feces	Feces	454	V4-V5	454_V4-V5	SINGLE
SRR8631927_FASTQ_SSU_OTU	PRJNA524207	MGYS00005545	3	2-20y	Male	Control	Low	Su 2019	Lumina	Cecum	Ion Torrent	V3-V4	Ion_V3-V4	SINGLE
ERR4193967_MERGED_FASTQ_SSU_OTU	PRJEB38701	MGYS00005563	1	0.5-2y	Male	Control	No_Answer	Walshe 2019	Feces	Feces	Illumina MiSeq	V3-V4	Illumina_V3-V4	PAIRED
SRR2098014_FASTQ_SSU_OTU	60764, PRJNA28	MGYS00005550	101	<2months	No_Answer	Other	No_Answer	Whitfield 2015	Feces	Feces	Illumina MiSeq	V4	Illumina_V4	SINGLE

Table 11: Studies included in the meta-analysis of studies on the equine gastro-intestinal microbiota: 29 studies included in the final analysis: Name of study as in the metadata with ENA and MGnify accession numbers.

Included studies			
Number	**Study**	**ENA**	**MGnify**
1	Arnold AB 2020[29]	SRP119693	MGYS00005549
2	Arnold 2020	SRP228480	MGYS00005589
3	Clark 2018[30]	PRJEB39250	MGYS00005591
4	Costa 2012[70]	SRP012927	MGYS00005570
5	Costa 2015[22]	PRJNA254186	MGYS00005558
6	Dela Torre 2019[15]	PRJNA475435	MGYS00005552
7	Dougal 2013[25]	ERP002202	MGYS00005566
8	Dougal 2017[21]	PRJEB20876	MGYS00005565
9	Ericsson 2016[36]	PRJNA322656	MGYS00005555
10	Fitzgerald 2020[32]	PRJEB39375	MGYS00005577
11	Glatter 2019[137]	PRJEB31758	MGYS00005603
12	Husso 2020[8]	PRJEB32017	MGYS00005551
13	Kunz 2019[33]	PRJNA433202	MGYS00005553
14	Leng 2018[72]	PRJEB11642	MGYS00000570
15	Lindenberg 2019[37]	PRJEB33830	MGYS00005546
16	Mach 2017[9]	PRJEB39226	MGYS00005590
17	McKinney 2020[118]	PRJEB32490	MGYS00005604
18	Morrison 2018[64]	PRJEB29667	MGYS00005564
19	Morrison 2020[28]	PRJEB34659	MGYS00005557
20	Peachey 2018[31]	PRJEB38664	MGYS00005560
21	Peachey 2019[156]	PRJEB38717	MGYS00005559
22	Perry 2018[56]	PRJEB39150	MGYS00005579
23	Laura Exmoors (unpublished)	PRJEB39336	MGYS00005578
24	Stephanie yearlings (unpublished)	PRJEB38804	MGYS00005567
25	Rodriguez 2015[139]	PRJNA279335	MGYS00005562
26	Steelman 2012[67]	PRJNA177883	MGYS00005583
27	Su 2020[35]	PRJNA524207	MGYS00005545
28	Walshe 2019[74]	PRJEB38701	MGYS00005563
29	Whitfield-Cargile 2015[16]	SRP060764, PRJNA288488	MGYS00005550

Table 12: Studies excluded from the meta-analysis of studies on the equine gastro-intestinal microbiota: 42 studies excluded from the final analysis, either during the data collection process (before upload to the web-based bioinformatics program MGnify) or during the analysis with MGnify (after upload to MGnify). Columns include study name, reason and timepoint of exclusion.

Excluded studies			
Number	**Study**	**Reason for exclusion**	**Timepoint of exclusion**
1	Almeida et al. 2016[58]	Data not available	Before upload to MGnify
2	Alvarez-Narvaez et al. 2020[83]	Data not available	Before upload to MGnify
3	Biddle et al. 2018[62]	Data not available	Before upload to MGnify
4	Bordin et al. 2013[157]	Data not available	Before upload to MGnify
5	Bulmer et al. 2019[55]	Data not available	Before upload to MGnify
6	Costa et al. 2016[13]	Data not available	Before upload to MGnify
7	Dong et al. 2016[138]	Data not available	Before upload to MGnify
8	Grimm et al. 2020[102]	Data not available	Before upload to MGnify
9	Hansen et al. 2015[158]	Data not available	Before upload to MGnify
10	Kristoffersen et al. 2016[54]	Lacking permission to upload to ENA	Before upload to MGnify
11	Langner et al. 2020[63]	Data not available	Before upload to MGnify
12	Li et al. 2019[47]	Metadata incomplete	Before upload to MGnify
13	Massacci et al. 2020[159]	Data not available	Before upload to MGnify
14	Moreau et al. 2014[23]	Data not available	Before upload to MGnify
15	O'Donnell et al. 2013[3]	Data not available	Before upload to MGnify
16	Park et al. 2019[160]	Data not available	Before upload to MGnify
17	Plancade et al. 2019[59]	Data not available	Before upload to MGnify
18	Proudman et al. 2015[161]	Metadata incomplete	Before upload to MGnify
19	Quercia et al. 2018[12]	Metadata incomplete	Before upload to MGnify
20	Schoster et al. 2016[99]	Data not available	Before upload to MGnify
21	Schoster et al. 2015[98]	Data not available	Before upload to MGnify
22	Schoster et al. 2017[10]	Data not available	Before upload to MGnify
23	Schoster et al. 2019[162]	Data not available	Before upload to MGnify
24	Shepherd et al. 2012[19]	Metadata incomplete	Before upload to MGnify
25	Stewart et al. 2018[163]	Data not available	Before upload to MGnify
26	Stewart et al. 2019[69]	Data not available	Before upload to MGnify
27	Tang et al. 2020[164]	Data not available	Before upload to MGnify
28	Tyma et al. 2019[165]	Data not available	Before upload to MGnify
29	Warzecha et al. 2017[20]	Data not available	Before upload to MGnify
30	Weese et al. 2014[61]	Data not available	Before upload to MGnify
31	Zhao et al. 2016[166]	Data not available	Before upload to MGnify
32	Antwis et al. 2018[60]	MGnify delay	After upload to MGnify
33	Coleman et al. 2019[167]	MGnify delay	After upload to MGnify
34	Costa, Stämpfli 2015[73]	Data format incompatible	After upload to MGnify
35	Dougal et al. 2014[26]	NCBI issues	After upload to MGnify
36	Fernandes et al. 2014[52]	Extreme outlier	After upload to MGnify
37	Kaiser-Thom et al. 2020[79]	MGnify delay	After upload to MGnify
38	Leclere et al. 2020[80]	NCBI issues	After upload to MGnify
39	Metcalf et al. 2017[48]	MGnify delay	After upload to MGnify
40	Salem et al. 2018[27]	Format incompatible	After upload to MGnify
41	Salem et al. 2019[71]	Format incompatible	After upload to MGnify
42	Whitfield-Cargile et al. 2018[84]	MGnify delay	After upload to MGnify

Table 13: Ingredients and nutritive information of the three feed types administered to the 12 Thoroughbred yearlings from the prebiotic study. Each yearling received two scoops of feed 1, two scoops of feed 2 and 4 cups of feed 3 per day during the study period.

Component	**Feed type 1**	**Feed type 2**	**Feed type 3**
Crude Fibre	18.50%	13.0%	6.5%
Crude Protein	13.0%	14.0%	12.5%
Lysine	0.79%	0.70%	1.40%
Methionine	0.19%	0.20%	0.38%
Crude Oil	9.0%	6.5%	18.0%
Crude Ash	9.8%	-	-
Starch	8.0%	16.5%	20.0%
Sugar	6.0%	7.0%	9.0%
Digestible Energy	12.9 MJ/kg	12.5 MJ/kg	18 MJ/kg
Vitamin A	12100 iu/kg	14000 iu/kg	-
Vitamin D	1200 iu/kg	1400 iu/kg	-
Vitamin E	319 iu/kg	280 iu/kg	440 iu/kg
Vitamin C	170 mg/kg	200 mg/kg	-
Vitamin B1	8.0 mg/kg	9.0 mg/kg	-
Vitamin B2	8.0 mg/kg	8.8 mg/kg	-
Vitamin B6	3.0 mg/kg	3.5 mg/kg	-
Vitamin B12	0.03 mg/kg	0.03 mg/kg	-
Pantothenic Acid	11 mg/kg	13 mg/kg	-
Niacin	29 mg/kg	33 mg/kg	-
Folic Acid	3 mg/kg	3.5 mg/kg	-
Biotin	0.3 mg/kg	0.3 mg/kg	-
Calcium	1.25%	1.35%	2.25%
Phosphorus	0.50%	0.60%	1.50%
Magnesium	0.35%	0.37%	0.72%
Sodium	0.48%	0.25%	1.00%
Choride	0.80%	0.55%	-
Potassium	1.20%	1.00%	-
Iron	320 mg/kg	230 mg/kg	-
Iodine	0.44 mg/kg	0.88 mg/kg	-
Copper	44 mg/kg	78 mg/kg	-
Zinc	140 mg/kg	243 mg/kg	-
Manganese	61 mg/kg	117 mg/kg	-
Selenium	0.53 mg/kg	0.98 mg/kg	1.0%
Ingredients	Soya Hulls, Dried Sugar Beet Pulp, Pea Flakes, Soya Flakes, Molasses, Grass Pellets, Soya Oil, Sunflower Seed Meal, Dicalcium Phosphate, Sodium Chloride, Maerl (calcareous marine algae), Vitamins & Minerals, Mixture of flavouring compounds	Crushed Oats, Lucerne Pellets, Soya Hulls, Molasses, Soya Bean Meal, Maize Flakes, Dried Sugar Beet Pulp, Soya Flakes, Soya Oil, Dicalcium Phosphate, Sunflower Seed Meal, Maerl (calcareous marine algae), Wheat, Vitamins & Minerals, Sodium Chloride, Wheatfeed, Mixture of flavouring compounds	Stabilised Rice Bran, Calcium Carbonate, Maerl (calcareous marine algae), Vitamin E, Selenium

Table 14: Complete metadata from prebiotic study: data from fecal samples of 12 male Thoroughbred yearlings undergoing dietary change with a total of 36 samples from three different timepoints (1, 2, 3) and two treatment groups (prebiotic/control) and the combination of timepoint and treatment group (Two-way).

SampleID	Label	Subject	Include	Timepoint	Treatment	Two-way	Sex
34	34	Y1	1	1	prebiotic	A	M
35	35	Y1	1	2	prebiotic	B	M
36	36	Y1	1	3	prebiotic	C	M
37	37	Y2	1	1	control	D	M
38	38	Y2	1	3	control	F	M
39	39	Y3	1	3	control	F	M
40	40	Y4	1	3	control	F	M
41	41	Y5	1	1	control	D	M
42	42	Y5	1	3	control	F	M
43	43	Y6	1	3	prebiotic	C	M
44	44	Y7	1	1	prebiotic	A	M
45	45	Y7	1	2	prebiotic	B	M
46	46	Y7	1	3	prebiotic	C	M
47	47	Y8	1	1	prebiotic	A	M
48	48	Y8	1	2	prebiotic	B	M
49	49	Y8	1	3	prebiotic	C	M
50	50	Y9	1	1	control	D	M
51	51	Y9	1	2	control	E	M
52	52	Y9	1	3	control	F	M
53	53	Y10	1	1	prebiotic	A	M
54	54	Y10	1	2	prebiotic	B	M
55	55	Y10	1	3	prebiotic	C	M
56	56	Y11	1	1	control	D	M
57	57	Y11	1	2	control	E	M
58	58	Y11	1	3	control	F	M
59	59	Y12	1	1	prebiotic	A	M
60	60	Y12	1	2	prebiotic	B	M
61	61	Y12	1	3	prebiotic	C	M
62	62	Blank	1	Blank	Blank	Blank	Blank
63	63	Y2	1	2	control	E	M
64	64	Y3	1	1	control	D	M
65	65	Y3	1	2	control	E	M
66	66	Y4	1	1	control	D	M
67	67	Y4	1	2	control	E	M
68	68	Y5	1	2	control	E	M
69	69	Y6	1	1	prebiotic	A	M
70	70	Y6	1	2	prebiotic	B	M

Table 15: Nutritive information of prebiotic supplementation administered to 6 from 12 male Thoroughbred yearlings.

Fructooligosaccharide Prebiotic Supplement: Nutritive Values			
Dry matter	90%	Lysine	1,5 g/kg
Crude protein	5%	Meth.	0,4 g/kg
Crude fat	0,6 %	THR.	1,4 g/kg
Crude fibre	5%	TRP.	0,5 g/kg
Carbohydrates	75%	CYS.	0,3 g/kg
Inulin	65%	ARG.	7,3 g/kg
Pectin	5,6 %	GLU.	6,4 g/kg
Sugars	10%	ASP.	4 g/kg
Ashes	5%	Salmonella	Absent CSFU/25g
ADF	55 g/kg	WHA (water retention)	4:1
ADL	4,6 g/kg	Ca	17,44 g/kg
NDF	62 g/kg	Na	8,9 g/kg
TDF	118 g/kg	P	1,95 g/kg
EW porc (energy pigs)	0,890	Cl	1,78 g/kg
NE porc	1870 kCal	K	20,7 g/kg
DE porc	2751 kCal	Mg 2,67g/kg	2,67 g/kg
ME porc All.	1804 kCal	Iron	+/-50 ppm

Table 16: Sequence and read counts in raw sequencing data of fecal samples from 12 male Thoroughbred yearlings undergoing dietary change with a total of 36 samples of which there was one group supplemented with scFOS prebiotics and a control group. A: Sequence counts of all samples from the prebiotic study. B: Forward and reverse reads of 16S rRNA sequencing data from prebiotic study.

A

Sample ID	Sequence	Sequence
70	107554	107554
51	80692	80692
55	75443	75443
45	68104	68104
59	67706	67706
53	66598	66598
64	57624	57624
47	55597	55597
60	54112	54112
57	51571	51571
46	49551	49551
65	48386	48386
41	48295	48295
63	47755	47755
44	47618	47618
54	46476	46476
49	45175	45175
43	44403	44403
52	44248	44248
48	43682	43682
37	41417	41417
50	41140	41140
68	38789	38789
39	38025	38025
35	37971	37971
69	36751	36751
67	36598	36598
36	33906	33906
66	32384	32384
38	29401	29401
42	29288	29288
34	29108	29108
58	27588	27588
40	25176	25176
56	19392	19392
Blank	1764	1764
61	194	194

B

	Forward Reads	Reverse Reads
Minimum	194	194
Median	44248	44248
Mean	44580.6	44580.6
Maximum	107554	107554
Total	1649482	1649482