EFFECTS OF COMMERCIAL FEED ADDITIVES ON THE GUT MICROBIOTA OF FOOD ANIMALS

BY

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A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

Major in Animal Science

South Dakota State University

2019

DISSERTATION ACCEPTANCE PAGE

Prakash Poudel

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ACKNOWLEDGEMENT

First of all, I would like to express my thanks to my advisor, Dr. Benoit St-Pierre, for accepting me as a PhD student in your team and providing me the valuable guidance, encouragement, support and knowledge throughout the study of my PhD at South Dakota State University. I extremely felt fortunate to have a professor like you who cared so much about my research activities, always responded my queries, and supported to execute laboratory works in no time. The journey of my study was possible due to your patience and encouragement. You taught me to be an expert on microbiota and a true researcher. Many thanks again for your guidance towards my future career.

I am also indebted to receive a great support from my supervisory committee members, Dr. Crystal Levesque, Dr. Joy Scaria, and Dr. Kunsoon Park. Thank you all for your time, directions, support, advices, and inputs towards my research projects. Special thank goes to Dr. Samuel Ryan for supporting on animal trial on Swine facility at SDSU. At this moment, I would also like to thank my previous advisor Dr. David Casper and fellow graduate students Kelly Froehlich and Subash Acharya for helping me to collect rumen samples from the calves on EO experiment.

I really appreciate the support and assistance I got from my past and present lab colleagues Vinay, Lee, Angie and other graduate student for sampling and data analysis. I would also like to thank swine facility at SDSU for allowing me to carry out the nursery pig research. I would thankful to Vitech Bio-Chem Corporation, CA for providing the funding of two Peptiva experiments carried out on piglets.

Finally, I would like to express my heartful gratitude to my beloved wife Kalpana, daughter Anweshi, and son Ayansh, and other family members for their endless support, patience, understanding, and love throughout my study period without which this great accomplishment would not have been possible. Thank you for all those and love you so much.

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LIST OF ABBREVIATIONS

ADG	Average daily gain
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
ATTD	Apparent total tract digestibility
BLAST	Basic Local Alignment Search Tool
CCA	Canonical Correspondence Analysis
CON	Control diet
CON II	Samples collected at the end of Phase II from animals fed the control diet
CON III	Samples collected at the end of Phase III from animals fed the control diet
d	Day(s)
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
EO	Essential oil
FDA	Food and drug administration
G:F	Gain to feed ratio
GIT	Gastrointestinal tract
kg	kilogram
LPSN	List of Prokaryotic Names with Standing in Nomenclature
NCBI	National center for biotechnology information
NRC	National Research Council
OTU	Operational taxonomical unit
PCoA	Principal Coordinate Analysis

PCR	Polymerase chain reaction
PEP	Control diet supplemented with Peptiva
PEP II	Samples collected at the end of Phase II from animals fed the Peptiva diet
PEP III	Samples collected at the end of Phase III from animals fed the Peptiva diet
PEP10	Control diet containing 90% of the recommended amino acid levels, supplemented with Peptiva
RDP	Ribosomal Database Project
SAS	Statistical analysis system
SCFA	Short chain fatty acid
SEM	Standard error of the mean

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ABSTRACT

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2019

Weaning is a stressful event of newborn animals which can lead to dysbiosis in the GIT causing invasion of pathogens, retarded growth, high incidence of diarrhea, and increased neonatal mortality. Since antibiotics use in livestock production have been regulated, various feed additives have been designed as antibiotic alternatives to use in newborn animal during weaning. This thesis investigated the dynamics of bacterial composition of GIT in weaned animals fed commercial feed additives by analysis of high throughput sequencing data generated from PCR-amplified DNA targeting V1-V3 region of 16S rRNA gene. Experiment 1 investigated the rumen environment of neonatal calves fed concentrate pellet and milk replacer supplemented with a commercial blend of EO. This study demonstrated higher propionate concentrations and higher relative abundance phyla Bacteroidetes in samples from EO fed calves than the control. Two bacterial OTUs were significantly more abundant in EO fed calves; SD_Bt-00966 was found to be a close relative of *Prevotella ruminicola* (97%), while SD_Bt-00978 likely corresponded to an uncharacterized species of Gammaproteobacteria. Experiment 2 evaluated the impact of low inclusion of peptide-based commercial product Peptiva on the performance and fecal microbiome of weaning pigs that were assigned phase diets. Results demonstrated no significant difference in body weight (BW), daily gain, and feed efficiency between control and treatment animals. OTUs analysis revealed that *Lactobacilli,* represented by

four main OTUs (Ssd-00002, Ssd-00019, Ssd-00025, and Ssd-00053), were more abundant at the end of Phase II ($P < 0.05$), while *Streptococci*, mostly represented by OTUs Ssd-00039 and Ssd-00048, were in higher abundance at the end of Phase III ($P < 0.05$). This experiment provided insight that Peptiva can modulate the composition of swine fecal microbiome during a specific window of the nursery stage, potentially by accelerating its maturation. Experiment 3 was aimed to investigate the effects of peptide based commercial product Peptiva along with mannose oligosaccharides (MOS) and protease on growth performance and fecal microbiome composition of weaned piglets on standard phase feeding program. Results revealed no significant difference on body weight on all phases, while pigs fed Peptiva added with MOS and protease at phase II showed higher daily gain and pigs fed Peptiva added with MOS had higher feed efficiency compared to control. At the OTUs level, *Lactobacillus*, represented by two OTUs, Ssd-00001 and Ssd-00123 were most abundant ($P < 0.05$) in phase III, while *Ruminococcus*, represented by one OTU was highly abundant ($P < 0.05$) in phase II. Together, these results showed Peptiva along with MOS and protease can modulate the swine gut microbiome during nursery period.

Chapter 1

LITERATURE REVIEW

1. Meeting the Food Demand of a Growing World Population

1.1 Current Situation and Future Outlook

Global food demand has been rapidly increasing over the last 50 years, as a result of doubling of the world population from 3 billion to more than 6 billion, as well as from the increasing per-capita demand for food (Bodirsky et al., 2015). Per-capita demand is influenced by many factors, including income, standard of living, food prices, industrialization, access to global markets, as well as urbanization (Drewnowski and Popkin, 1997; Bodirsky et al., 2015). By 2050, the global population is expected to reach between 8.1 billion and 10.6 billion people (Godber and Wall, 2014), which will further increase the demand for food of both plant and animal origin. Taking into consideration that 12.5% of the world population is undernourished, it is predicted that food production needs to be increased by 70% to meet the demands of the future global human population (Godber and Wall, 2014). Because of limited land resources and decreasing per-capita land availability from 1.24 hectares/person/year in 1970 to 0.72 hectares/person/year in 2010 (Hurt et al., 2013), increasing productivity rather than just increasing the number of livestock and cropland area is a more feasible strategy (Bodirsky et al., 2015). This must be achieved while protecting the environment and human health, as well as conserving biodiversity and natural resources (McSweeney and Mackie, 2012). In order to successfully meet these challenges, the agriculture sector will require the development of further technological advancements.

1.2 Role of the livestock sector

Due to this increased food demand, the share of livestock based product alone rose from 15.4 to 17.7% from the year 1961 to 2009 (Bodirsky et al., 2015). As a result, there has been a substantial increase in global livestock production between the 1960s and 2010, with doubling of beef production, and a 10-fold increase in chicken meat production. Carcass weight has increased by about 30 percent for both species during this period, compared to approximately 20% for pigs between the early 1960s to mid-2000s. Similarly, dairy production has also increased by approximately 30% during this time (Thornton, 2010). A number of technological advancements were developed to increase feed efficiency and animal performance, including breed and genetic improvement, disease prevention, and nutrition, which directly contributed to increased animal performance (Thornton, 2010).

Food animals are important for human nutrition compared to plant sources. Animal products are nutritionally dense sources of energy, protein, and various essential micronutrients. A variety of micronutrients like vitamin A, vitamin B-12, riboflavin, calcium, iron, and zinc are provided from animal source foods and are difficult to obtain in adequate quantities from plant source foods alone (Murphy and Allen, 2003). Inadequate intake of these nutrients has negative outcomes for human health, such as anemia, poor growth, rickets, impaired cognitive performance, blindness, neuromuscular deficits, and eventually death (Murphy and Allen, 2003). Plant based diets are deficient in one or more essential amino acids such as lysine, methionine, and threonine (Young and Pellett, 1994). On the other hand, foods from animal sources are rich in these nutrients, and only small amounts added to any vegan diet can substantially increase the nutrient

availability of the plant-based food sources. For instance, Dutch infants consuming strictly vegan macrobiotic diets had poorer nutritional status and likely to have rickets as well as vitamin B-12 and iron deficiency (Dagnelie et al., 1989; Dagnelie et al., 1990). Similarly, there was lower serum ferritin concentrations in vegan diets consumed by US men, with a marginal deficit for vitamin B-12 in 10 out of 25 vegans (Haddad et al., 1999).

A study by Mottet et al. (Mottet et al., 2017) demonstrated that 86% of livestock feed is not suitable for human consumption. Their use for livestock thus alleviates a burden for the environment as the increasing human population consumes more food. The same study also showed that out of 2.5 billion ha of land used by livestock, 77% consist of grassland and pastures, which could not be converted into croplands, but are suitable for animal grazing. Livestock production therefore plays a major role in food systems by making use of uncultivated land, turning by-products of human food into edible foods, and contributing to land fertility (Mottet et al., 2017). Obviously, the increased demand for food from animal sources will have a major effect on the global food system and land use, so there is a critical need to better inform policy makers and consumers about feed use and feed use efficiency in the livestock sector (Capper et al., 2013). Therefore, steps to improve feed efficiency and animal productivity through better feed formulations, genetic selection, health management, and improved understanding of digestive physiology, which have already been undertaken dating back from few decades ago, are very crucial to meet the increasing global food demand.

In addition to these areas of improvement, there has been growing interest in the microbiomes of food animals, because of their potential to further improve animal health

and performance. However, there remain major gaps in our knowledge and understanding of gut microbiomes and their interaction with their respective host animals.

2. Challenges for livestock production: health and welfare of young animals

2.1 Overview of challenges

There has been a dramatic change in animal husbandry practices over the course of a few decades, developing from small, exposed (outdoor), labor-dependent enterprises into large, secured (indoor), capital dependent, and mechanized production systems (Stull and Reynolds, 2008; Kittawornrat and Zimmerman, 2011). This has led to a number of concerns, including the maintenance of livestock health and welfare, including their living conditions. There are many factors that contribute to the wellbeing of young animals on commercial farms, including housing and environment, nutritional and health programs, animal handling and caretaker interactions, herd dynamics, as well as common management practices during transportation, euthanasia, and dehorning (Stull and Reynolds, 2008). The concerns about animal welfare can be summarized in the form of three main questions: is the animal functioning well (i.e. good health and productivity); is the animal feeling well (i.e. absence of pain); and is the animal able to find comfort naturally (Appleby, 1999; von Keyserlingk et al., 2009). Assessing and ensuring the welfare of young animals in a commercial setting is a challenge, as it is a complex and dynamic process (Curtis, 1987). One reason is that animal physiological processes, such as development, immune and hormonal responses, growth, as well as stress, may fluctuate in response to normal patterns of behavior or circadian rhythm (Curtis, 1987). For instance, the assessment of welfare during farrowing is a unique challenge for swine

producers, veterinarians, and animal scientists, as they deal with individuals, the sow and her piglets, that are at different stages of their development, and have different requirements regarding their thermal, social and physical environments. Therefore, high standard animal welfare programs are important for sustainable improvements in animal production.

2.2 Dairy production

2.2.1 Rumen development in young calves

The foregut of adult ruminants consists of four compartments: the rumen, the reticulum, and the omasum, which are followed by the abomasum. The rumen and reticulum function as a fermentation vat, while the omasum absorbs water and minerals from the digesta leaving the rumen, and the abomasum performs the functions of a glandular stomach, as is typical of the stomach of mono-gastric animals. Ingested feed material is first hydrolyzed and fermented in the rumen by symbiotic microorganisms, then rumen contents moves posteriorly to the abomasum to undergo gastric digestion.

Since newborn calves start life as simple stomached animals, their rumen is rudimentary and nonfunctional (Warner et al., 1956). The reticulo-rumen is about one third of the total stomach capacity during birth, and it needs to increase in size to about 85% by the time adulthood is reached. Promoting optimal rumen development can therefore benefit calf producers, by allowing early weaning through shortening the time required to feed milk replacer. Inadequate rumen development has also been associated with increased health problem, which also ultimately contributes to a delayed weaning age (Beharka et al., 1998). The metabolic activities of rumen microorganisms are

essential for ruminants to digest plant fiber material into short chain fatty acids (SCFAs), which are used as a source of energy by the host animal, and to synthesize proteins from non-protein sources of nitrogen (Warner et al., 1956), the development of the rumen in newborn calves is greatly influenced by the consumption of dry feed and the end-products formed from its digestion (Anderson et al., 1987). Early intake of solid feed by young ruminants leads to the establishment of rumen fermentation, thereby promoting the physical and metabolic development of the rumen by increasing rumen mass and papillae growth (Baldwin et al., 2004). Forage consumption has also been found to promote muscular development of the rumen, and to stimulate the rumination-induced flow of saliva into the rumen (Tamate et al., 1962; Hodgson, 1971). The composition of starter feed and forage has also been found to greatly influence development of the rumen. Indeed, while microbial fermentation of forages in the developing rumen of calves is not yet efficient enough to provide sufficient concentrations of SCFAs, especially butyrate, for stimulation of rumen papillae development (Nocek et al., 1980), controlling hay particle size in starter feed can help increase intake and improve feed efficiency to compensate (Coverdale et al., 2004). Young ruminants fed solely on milk during their first month of life exhibit limited ruminal development compared to grain and hay fed animals, which is likely due to shunting of milk directly to the abomasum by closure of the esophageal groove, preventing substrates from entering the rumen and initiating ruminal fermentation (Tamate et al., 1962). Indeed, papillary growth was found to be stimulated by SCFA production when milk was infused directly into the rumen (Tamate et al., 1962).

2.2.2 Rumen physiology

Understanding the function of the rumen and its physiology is important, as rumen dynamics are ultimately essential in shaping the rumen microbiome so that it can provide nutrients to the host animal. Ruminants are capable of utilizing structural polysaccharides from indigestible fibers, such as cellulose and hemicellulose, and convert them into products for human consumption, thanks to the microbial enzymes that perform anaerobic fermentation. Rumination triggers saliva flow to maintain an optimum pH for ruminal microorganisms, as well as muscular contractions that mix ingested feed with microorganisms and expose SCFAs to the ruminal wall for absorption (Russell and Rychlik, 2001). The host animal is then able to extract energy from fibrous materials, and also be provided with microbially synthesized amino acids and vitamin B-complex (Krehbiel, 2014). Other end products include fermentation gases, such as methane and carbon dioxide, which are expelled by eructation. The type of feedstuffs, as well as the types and activities of microorganisms present in the rumen, affect the proportion of end products generated, which directly impacts nutrient output and animal performance (Mackie and White, 1990). Maintaining stable conditions in the rumen is very important for its proper function. The temperature is usually maintained between 38-41°C, with pH ranging from 7 on forage-based diets to 4.6 on high grain diets. Among the SCFAs produced, acetate is by far the most abundant followed by propionate and butyrate.

2.2.3 State of problem and current management

Newborn calf management is not only an important aspect of dairy cattle operations but it is also critical for the economic sustainability of the industry as a whole.

Calf health is important for the long-term success of producers, as heifer calves typically have better genetics and represent the future of the dairy herd (USDA, 2010). However, the dairy industry still faces challenges due to high calf morbidity and mortality rates, with highest incidence risk during the first 3 weeks of life. These result in loss of value for calves, and loss of genetic potential towards herd improvement, which ultimately lead to economic losses for producers (Wells et al., 1996). In addition to the cost of treating sick calves and loses due to mortality, there is an economic burden due to reduced growth rate, as well as increased first calving age and difficulty at first calving after reaching maturity (Sivula et al., 1996; Østerås et al., 2007; Windeyer et al., 2014). Neonatal calf diarrhea and respiratory diseases are the most common causes of morbidity and mortality in young dairy cattle (Windeyer et al., 2014), and their annual costs have been estimated at $33.46 and $14.71, respectively, per pre-weaned calf at risk (Kaneene and Scott Hurd, 1990). Accounting for more than half of all calf mortality in dairy calves (Foster and Smith, 2009), diarrhea remains problematic because of its multi-factorial nature, as it can be caused by either pathogenic agents or non-pathogenic factors. Pathogens involved typically include enterotoxigenic *Escherichia coli* (ETEC), *Salmonella enterica*, *Clostridium perfringens*, *Cryptosporidium parvum*, rotavirus, and coronavirus (Bergman, 1990; Foster and Smith, 2009), while non-pathogenic factors include handling during birth, colostrum management, calf housing, feeding, and hygiene (Klein-Jöbstl et al., 2014). Pathogenic and non-pathogenic factors are not mutually exclusive; for instance, oral exposure to fecal coliforms at birth, which can lead to gut colonization, implicates both types of factors in increasing the risk of diarrhea incidence.

2.3 Swine production

2.3.1 Current management practices

Nursery pig husbandry practices in the swine industry involve not only nutrition but also a number of management practices, including maintenance of hygiene, disease prevention, and animal welfare, with the ultimate goal of providing adequate space and environmental conditions, such as an optimal ambient temperature, to minimize losses and allow nursery pigs to thrive. Husbandry practices and gut health are interconnected, as they both have a direct effect on gut structure and function. (Jayaraman and Nyachoti, 2017). The term gut health is used in reference to gut structure, function, microbial composition, and incidences of diarrhea (Lallès et al., 2007). As in other livestock species, weaning is one of the most challenging phases of a pig's development in commercial facilities, and it is typically associated with reduced growth performance and increased rate of diarrhea (Lallès et al., 2007). Abrupt changes in diet composition, crowding stress, sanitation and other conditions favorable for disease onset are recognized as major factors responsible for reduced growth and increased diarrhea that are typically observed during the weaning phase (Dong and Pluske, 2007; Opapeju et al., 2009; Khafipour et al., 2014). Reduced feed intake during this period can lead to disrupted physiological activities in immature digestive and immune systems (Vente-Spreeuwenberg et al., 2003; Jayaraman and Nyachoti, 2017). Indeed, the digestive tract in weaned pigs is at that point in a transition phase, with enzyme activity specific to plant -based diets and hence digestion of these nutrient sources, while changes in intestinal morphology and enteric microbial community composition are ongoing (Hampson, 1986; Boudry et al., 2004; Konstantinov et al., 2004). Similarly, the immaturity of the nursey

pig immune system can lead to higher incidences of inflammation, which can increase the risk of diarrhea and reduced intake. As the histological, microbiological, and immunological components of the gastrointestinal tract each contribute interactively to gut health, effective strategies need to be further improved or developed in order to minimize the adverse effects of weaning and their subsequent consequences (Jayaraman and Nyachoti, 2017).

2.3.2 Swine gut development

The development of the swine gastro-intestinal tract during the prenatal period is a complex process that results in the formation of specialized epithelial layers that can digest and absorb nutrients as well as perform endocrine and immunological functions (Barszcz and Skomiał, 2011). Just a few weeks before parturition, the swine intestine undergoes a period of intensive development, as it grows at a faster rate than the rest of the animal (Sangild et al., 2000). Within three days after birth, following ingestion of colostrum, the small intestine doubles its weight and increases its length by 30% (Xu et al., 1992), while intestinal crypts depth and villi height augment by 40% and 35%, respectively (Godlewski et al., 2005). Weaning is another critical phase of gastro-intestinal tract development in young animals. Among the stressors experienced during this period, the abrupt replacement of highly digestible maternal milk with solid feed that can contain plant-based ingredients directly affects the gastro-intestinal environment. Adjustments to a dramatically different diet involves changes in enzyme secretion and activity rates, as well as transitions in the composition of symbiotic bacterial communities. The transition from milk to solid feed also results in dramatic changes in

histology parameters, as shown by villous atrophy and crypt hyperplasia, which contribute to decreased nutrient digestion and absorption, as well as increased incidence of diarrhea, ultimately compromising growth rates as well as the ability to fight off infection by pathogens (Odle et al., 1996; Pluske et al., 1997).

At birth, the GIT is colonized by microorganisms from exposure to the dam, its milk and the environment. Microbial colonization, as well as ingestion of milk, are important stimulants for the development of the intestinal immune system. The mucus layer covering the intestinal epithelium consists of mucin and glycoproteins, and it plays an important role in intestinal permeability and barrier function during intestinal development. The mucin creates a favorable environment for the colonization of specific symbiotic microorganisms, which act in combination as a protective barrier in the intestine. SCFAs, the end products of bacterial fermentation, consisting mainly of acetate, propionate and butyrate, constitute key energy for host epithelial cells and other tissues. Additionally, by lowering the pH of digesta, symbiotic microorganisms facilitate the absorption of mineral complexes by the colonic mucosa (Younes et al., 1996), and prevent the growth of pathogenic bacteria (Younes et al., 1996). Therefore, GIT microbial communities and their end products play a very important role during development of the intestinal epithelium and its protective barrier function.

3. The gut microbiome

3.1 Importance

The gut microbiota encompasses the complex communities of microorganisms that each inhabit a particular environment along the GIT. They typically consist of

bacteria, archaea, fungi, protozoa (Turner, 2018), with bacteria representing the most diverse and abundant group. The gut contains the most abundant and complex microbiota of any area of the host, of which an estimated 10% of microbial species or less have been identified, with many belonging to novel phylogenetic lineages whose functions remain poorly understood even after intensive research that has been ongoing for more than a decade (Spor et al., 2011). The enteric microbiota is thought to play a significant role in maintaining the health of their host animal or human, participating in nutrient digestion and absorption, in the synthesis of SCFAs, amino acids, and vitamins, in the maintenance of intestinal mucosal integrity and gut peristalsis, as well as in the development of the gut immune system (Berg, 1996; Clarke et al., 2014). The GIT microbiome has profound effects on the anatomical, physiological, and immunological development of the host (Berg, 1996), thus showing great potential towards improving productivity in food animals. It contributes to the health of the host by stimulating its immune system to respond more quickly to pathogen challenge, and, through bacterial antagonism, by inhibiting colonization of the GIT by opportunistic pathogens. In addition to reducing the incidence of infectious diseases, it also contributes to lowering the risks of inflammatory and other immune diseases (Guevarra et al., 2018). The intestinal microbiota is also capable of communicating with other organ systems, including the brain, lungs, skin, and liver, thereby modulating their respective functions (Kamada et al., 2013). Alterations in the human gut microbiota have been linked to many diseases and adverse effects including obesity, Crohn’s disease, diabetes mellitus, ulcerative colitis, and some types of neoplasia (Turner, 2018). In food animals, gut microbiota dysbiosis during weaning in pigs has emerged as a leading cause of post-weaning diarrhea and other associated

infections, causing high mortality and reduced growth performance (Gresse et al., 2017). Similarly, adverse conditions for the rumen microbiota in dairy cows can have severe consequences, including rumen papillae damage, sudden drops in pH, acidosis, loss of appetite, lower milk production, diarrhea, shock and death of animal (Fecteau et al., 2016; Khafipour et al., 2016).

3.2 Rumen Microbial ecology

The rumen, the largest compartment of the ruminant stomach, acts as a fermentation vat for ingested feed. It is the habitat for some of the most diverse and dense known microbial communities, and they are responsible for metabolizing plant biomass into SCFAs and providing their host with other nutrients such as amino acids in the form of microbial proteins and vitamins (Russell and Rychlik, 2001; Khiaosa-ard and Zebeli, 2014). Ruminal microbial communities consist of bacteria, archaea, protozoa, fungi, and viruses, which associate into complex microbial ecosystems that play a vital role in the nutritional, physiological, and immunological functions of the host (McSweeney and Mackie, 2012). The ingested plant material is hydrolyzed then fermented by bacteria, fungi and protozoa, then microbial cells and undigested plant particles move downstream to the abomasum where host digestion takes place. While methanogens do not participate directly in metabolizing feed, they play an essential role by utilizing the hydrogen gas (H_2) produced from fermentation of feed, a function crucial to maintaining the functional efficiency of the other members of ruminal communities (Martin et al., 2010).

3.3 Microbial succession in the gastrointestinal tract

A series of microbial succession events take place in the gut of young animals, starting with a limited number of colonizing species that are sequentially replaced by microbial communities of increasing cellular complexity and density (Isaacson and Kim, 2012; Guevarra et al., 2019). While the mechanisms involved still remain to be elucidated, microbial succession is thought to take place in conjunction with the growth and development of the host (Isaacson and Kim, 2012). Indeed, a number of studies have indicated that microbial succession events can have short term and long term impacts on the health and productivity of the host (Petri et al., 2010). Microbiota development can be influenced by a number of factors, including the genotype of the host, exposure to maternal associated microbiota (gut, reproductive tract, udder / nipple and milk) as well as changes in diet composition (Bauer et al., 2006; Lallès et al., 2007). Transition between successional phases, as well as their respective stability, can also be influenced by the physiology of the host (e.g. intestinal pH, peristalsis), host-symbiont interactions, use of antimicrobials or other drugs, stress level, as well as ambient conditions, such as temperature (Sghir et al., 1999).

A number of studies have reported the existence of microorganisms in the placenta and meconium, suggesting that microbial colonization of the gut may begin before birth, and that the gut of newborns is not sterile prior to parturition. Indeed, non-pathogenic commensal microbial species belonging to the phyla *Firmicutes*, *Tenericutes*, *Proteobacteria*, *Bacteriodetes*, and *Fusobacteria* have been reported in human placenta; these intriguingly share some similarities with the human oral microbiome (Aagaard et al., 2014). However, regardless of its status prior to birth, the newborn gut undergoes

rapid changes after birth, as it transitions from a germ free / low diversity state to more complex microbial communities. Colonization of the gut is initiated by ingestion of microorganisms from the maternal and immediate environment. Intriguingly, gut microbial succession during the first few weeks of life is very similar amongst human, chicks, piglets, and calves, although newborns from each species would be expected to be exposed to distinct fecal and environmental bacteria (Sghir et al., 1999; Konstantinov et al., 2004). Within a few days of birth, early colonizers such as Coliforms, *Streptococcus*, and *Enterococcus* dominate the gut of newborn animals, where they utilize the available oxygen to create anaerobic conditions in the gut that are more suitable for strict anaerobic species belonging to *Bifidobacterium* and *Bacteriodetes* (Konstantinov et al., 2004; Malmuthuge et al., 2015). Notably, members of these genera have been shown to be beneficial for the mucosal immune system of human infants (Mazmanian et al., 2008). Species of *Clostridia* and *Lactobacilli* may also be present during a short period of time (Sghir et al., 1999), and they are thought to play an important role in the maintenance of gut homeostasis (Lopetuso et al., 2013) and preventing proliferation of pathogenic bacteria (Gritz and Bhandari, 2015), respectively. Petri et al. (Petri et al., 2010) demonstrated a clear succession pattern in pre-weaned piglets, transitioning from high abundance of Clostridiaceae at 0.25 and 0.50 days of age (38% and 50%, respectively) to a high abundance of Streptococcaceae at 1, 2, and 3 days of age (29%, 23%, and 18%, respectively). Later, at days 5, 10, and 20 of age, Lactobacillaceae were found to be predominant (45-50%), with significant increases in the genera *Prevotella* and *Lactobacillus* after weaning compared to suckling piglets, which may have been the

result of a change in diet from nursing to weaning (Frese et al., 2015; Guevarra et al., 2018).

Under modern commercial livestock management, weaning is likely one of the most disruptive stages, with increased stress from the abrupt separation from the dam, and the transition from milk to a solid-based feed. These can lead to unfavorable changes in intestinal mucosa and gut physiology, reduced metabolic activity, malabsorption of nutrients, and increased susceptibility to pathogens. Together, these conditions can contribute to unfavorable changes in gut microbial composition (Alain et al., 2014).

3.4 Eubiosis vs dysbiosis

The gut microbiota has direct interactions with host cells, and these associations are essential in maintaining mucosal immune function, epithelial barrier integrity, motility, and nutrient absorption (Krüger et al., 2014). Under normal conditions, the symbiotic relationships between the host and its gut microbiota contribute to intestinal health, thus directly benefiting animal productivity. When in this state, termed ‘eubiosis’, the quantitative and qualitative harmonic balance of gut microbial communities with their host results in a healthy metabolic and immunologic cooperation that benefit both the animal and its symbionts (Stecher et al., 2013).

In contrast, ‘dysbiosis’ refers to any disturbance in the normal microbiota of the GIT, i.e. quantitative and qualitative changes in its composition, that affects its normal metabolic activities (Stecher et al., 2013; Krüger et al., 2014). Abrupt changes in diet composition, immune deficiency as well as exposure to heavy metals, toxic substances,

bacterial toxins or antibiotics are conditions that can result in dysbiosis (Macpherson and Harris, 2004; Neish, 2009). Production stages such as weaning can disrupt gut microbial ecosystems, increasing susceptibility to dysbiosis until a stable microbiota has been re-established (Lalles et al., 2007). In the weaned pig gut, for instance, dysbiosis can be observed when a reduction in abundance of *Lactobacillus* species that are associated with the mucosa takes place, which can lead to proliferation of pathogenic bacteria followed by enterocyte invasion if levels of beneficial mucosal species are not restored (Lu and Walker, 2001; Konstantinov et al., 2006).

A number of strategies have been developed, investigated or proposed towards maintaining or restoring eubiosis in ruminants and non-ruminants. The administration of probiotic bacteria is being widely used as a means to promote balanced gut microbial communities and to prevent colonization by pathogenic bacteria (Gagliardi et al., 2018). An alternative is the use of prebiotics, which consists of compounds that can be metabolized by beneficial symbionts, but not by their host, in order to promote their growth (Gagliardi et al., 2018). Combinations of probiotics and prebiotics into products known as synbiotics, can also be administered. Other strategies include digesta / fecal transplantation, which is very effective but has the disadvantage of lacking a consistent formulation, as well as phage therapy, which is still under development.

4. Manipulating the gut microbiome

Traditionally, genetics, diet, environmental conditions, health and management have been the main areas of focus for improving animal production. The importance of the gut microbiota has been increasingly recognized for its contribution to animal

performance, notably because of its roles in nutrient utilization, feed efficiency, and immune response. Since microbiome function is dependent on the composition of its microbial communities, differences in microbial species and their respective abundance are likely to affect host performance.

A number of strategies have been developed that either directly or indirectly manipulate the gut microbiome, including antimicrobials (antibiotics, essential oils), probiotics and prebiotics (Hook et al., 2009; Ericsson and Franklin, 2015; Scott et al., 2015; Weimer, 2015). In many cases, further improvements of products or the development of new technologies are limited by gaps in our understanding of the mechanisms that dictate how gut microbiomes become established, how they recover from disruptions, and how they interact with their host (Clemmons et al., 2019).

4.1 Antibiotics

The administration of antibiotics at sub-therapeutic doses through feed has been a common practice for decades, as this management practice has positive effects on animal health and productivity, with a net reduction in the cost of production. For instance, pigs fed antibiotics were found to require 10 to 15% less feed to achieve a desired growth target. As feed accounts for more than 50% of the cost of production in livestock, the addition of antibiotics is thus economically beneficial (Chattopadhyay, 2014). Antibiotics improved the growth rate by an average of 16.4%, with improved feed utilization efficiency by 6.9% for young pigs (7-15 kg); in heavier pigs (17-49 kg), growth rates were increased by 10.6% and feed efficiency by 4.5%. When assessed over the entire growing to finishing period (24-89 kg), the growth rate was improved by 4.2 % and feed

efficiency by 2.2% (Cromwell, 2002). In addition to these beneficial effects on growth and feed efficiency, morbidity and mortality rates are reduced in livestock herds fed antibiotic at subtherapeutic levels, even under conditions of elevated stress and high risk of disease.

However, the prophylactic use of antibiotics in animal feed has raised public awareness and health concerns over the risk of selecting for pathogenic bacteria with cross resistance and multiple antibiotic resistance (Cromwell, 2002). During the past decade, the number of deaths caused by resistant strains exceeded the combined number of deaths caused by influenza, HIV, and traffic accident according to WHO (Yap, 2013). As a result, the practice of feeding sub-therapeutic doses of antibiotics for growth promotion has been banned by the EU in 2006 and by the FDA in the USA in 2017. Consequently, finding effective alternatives has become a high priority for the livestock industry. Some of the most actively researched alternatives include probiotics, prebiotics, acidifiers, as well as neutraceuticals such as copper and zinc (Thacker, 2013), with interest pursued in other potential alternatives such as antimicrobial peptides, essential oils, clay minerals, egg yolk antibodies, eucalyptus oil-medium chain fatty acids, and recombinant enzymes (Thacker, 2013). However, most of these potential alternatives have proven inconsistent, and have rarely shown an efficiency equal to that of antibiotics. Thus, if these products are to replace feed antibiotics, then further research is needed to improve their efficacy.

4.2 Essential oils

Essential oils (EO) are aromatic volatile oil compounds that are naturally produced by a number of edible, medicinal, or herbal plants, in which they are stored in a number of different ways, such as in secretory cells, cavities, canals, epidemic cells or glandular trichomes (Bakkali et al., 2008; Benchaar et al., 2008). They can be extracted by either steam distillation or solvent extraction from a number of different plant tissues, such as leaves, buds, flowers, stem, seeds, roots, bark, twigs or fruits (Bakkali et al., 2008). The quality, quantity, and composition of EO can fluctuate among the different parts of the same plants (Dorman and Deans, 2000), and also as a function of plant maturity (Delaquis et al., 2002), growth conditions (Cosentino et al., 1999) or processing methods (Calsamiglia et al., 2007).

EO typically consist of a mixture of different bioactive compounds that can exhibit a number of different properties, such as antibacterial, antiviral, antifungal, analgesic, locally anesthetic, sedative, anti-inflammatory or spasmolytic (Bakkali et al., 2008). Terpenoids are typically the most common bioactive compounds in EO, consisting mainly of monoterpenes (C_{10}) and sesquiterpenes (C_{15}), although diterpenes (C_{20}) may also be present. In addition, a variety of low molecular weight aliphatic hydrocarbons, acids, alcohols, aldehydes, acyclic esters, lactones as well as a variety of N- and S-containing compounds, coumarins and homologs of phenylpropanoids can be present (Dorman and Deans, 2000), and may contribute to the bioactive properties of EO.

4.2.1 Mode of Action of EO

Because of their ability to inhibit or slow the growth of bacteria, there has been great interest in developing essential oils as alternatives to antibiotics. However, commercial blends of EO have shown inconsistent results in animal performance trials (Benchaar et al., 2008), with their respective modes of action still poorly characterized (Helander et al., 1998). As the mechanisms of action of EO depend on their active compounds and their respective chemical groups, these may vary based on their source, as EO typically consist of not only two or three major components that are present at high concentrations, but also other compounds that are present in trace amounts (Bakkali et al., 2008). Overall, the phenolic components of EO are thought to be mainly responsible for their antimicrobial activity (Cosentino et al., 1999). Another confounding factor in the investigation of EO is their potential for additive, antagonistic and synergistic effects among the different compounds within the same blend or formulation (Burt, 2004). For instance, the main constituents of oregano EO, thymol and carvarcrol, were found to exhibit higher antibacterial activity than either compound alone (Lambert et al., 2001).

As many EO compounds are hydrophobic, EO tend to interact with cell membranes and accumulate in the lipid bilayer of bacteria, which can result in increased fluidity and expansion, reduced membrane stability, and leakage of ions and cellular content across the cell membrane (Calsamiglia et al., 2007; Nazzaro et al., 2013). Increasing permeability can lead to reduced proton or ion motive force, decreasing ATP synthesis and cell growth rates in affected microorganisms (Lambert et al., 2001). This may explain why EO are slightly more active against gram positive than gram negative bacteria, as gram negative bacteria have a peptidoglycan layer that can prevent or limit the

penetration of EOs into cells. Generally, low oxygen levels, low pH, and low temperature can improve the action of EO.

4.2.2 Effects of EO on ruminal fermentation

Due to the antimicrobial activity of EO, a number of researchers have investigated their potential to modulate ruminal fermentation as a means of improving feed efficiency and nutrient utilization by ruminants (Benchaar et al., 2008). However, various studies have shown inconsistent results, possibly because a wide range of EO formulations, inclusion rates, and animal diets were used. One likely reason for this level of inconsistency may be the wide variety of active compounds and their respective chemical structures amongst EO formulations (Benchaar et al., 2008). More successful outcomes have resulted from the use of *in vitro* systems. For instance, the addition of thymol to rumen fluid *in vitro* resulted in the accumulation of amino acid nitrogen and reduction of ammonia nitrogen concentrations, which suggested that deamination may have been inhibited (Borchers, 1965). In an *in vitro* study carried out using rumen fluid from deer and sheep, there were no effects of EO on rumen microbial fermentation at low inclusion rates (4 to 8 mL/L of liquid), but there was reduced gas production during fermentation when higher levels (12 mL/L of liquid) were provided (Oh et al., 1967).

4.2.3 Antimicrobial activity of EO

Chao et al. (2000) found that almost all EOs tested in their study had an inhibitory effect on bacteria, yeast, molds, and viruses. For bacteria, Gram-negatives were found to be more resistant than Gram positives, with *Pseudomonas aeruginosa* identified as the

most resistant bacterial species tested. The same group also observed that cinnamon bark (*Cinnamomum zeylancium*) and tea tree oils (*Melaleuca alternifolia*) had an inhibitory effect against all organisms tested, while coriander oil (*Coriandrum sativum*) was found to be most effective against the Gram-positive bacteria tested. In a study by Si et al. (2006), most of the 66 EOs tested were found to be efficient against *S. typhimurium* DT104, *E. coli* O157:H7, and *E. coli* with K88 pili, while minimal inhibition was observed against *Lactobacillus* or *Bifidobacteria*. The efficacy of the EO against *E. coli O157:H7* was maintained even after mixing with swine cecal content. In addition to inhibitory effects on *S. typhimurium* and *E. coli* O157:H7, other *in vitro* studies have also demonstrated EO antibacterial activity against *Listeria monocytogens, Shigella dysenteria*, *Bacillus cereus* and *Staphylococcus aureus* (Burt, 2004). Amongst 52 plant oils and extracts that were evaluated for their antimicrobial activity against *Candida albicans, Enterococcus faecalis, E. coli, Klebsiella puemoniae, P. aerogenosa, Salmonella enterica, Serratia marcescens* and *S. aureus,* lemon grass, oregano, and bay were found to inhibit all microorganisms tested (Hammer et al., 1999). In pigs, many studies have shown that EO supplementation results in decreased *E. coli* and increased *Lactobacillus* in ileum, colon or feces (Zeng et al., 2015).

4.2.4 Effects of EO on Rumen Microorganisms

While only a limited number of studies have been performed on the effects of EO on rumen microorganisms, their results have overall shown that the growth of certain species can be modulated using these compounds. The effect of a commercial blend of EO containing thymol, eugenol, vanillin, and limonene on ruminal microorganisms and

their protein metabolism was investigated *in vitro* using rumen fluid from dairy cattle fed either grass, maize silage, or a concentrate diet (McIntosh et al., 2003). The study determined that the EO formulation tested reduced the rate of amino acid deamination, and inhibited the growth of most pure cultures of ruminal bacteria at concentrations lower than 100 ppm. *Streptoccus bovis* was found to be the most resistant species, whereas *Prevotella ruminicola*, *Clostridium sticklandii*, and *Peptostreptococcus anaerobious* were the most sensitive species. When adapted to the presence of EO, *Prevotella ruminicola* and *Prevotella bryantii* were able to grow in the presence of higher concentrations of EO, while *C. sticklandii* and *P. anaerobious* remained sensitive. Similarly, supplementing rumen fluid with EO for 24 hours resulted in an increase in *in vitro* dry matter digestibility, and *in vitro* neutral detergent fiber digestibility, with the abundance of *Selenomonas ruminantium*, and *Rumninococcus albus* also found to be higher in response to EO (Kim et al., 2019). Using a microarray approach, (Patra and Yu, 2015) determined the impact of EOs (origanum oil, garlic oil and peppermint oil) on the composition of ruminal bacterial communities, with 67 Operational Taxonomic Units (OTUs) showing significant differences in abundance across treatments. A wide range of predominant bacterial groups were affected by the EOs tested, including OTUs affiliated to *Syntrophococcus sucromutans*, *Succiniclasticum ruminis*, *Lachnobacterium bovis*, *Prevotella*, *Clostridium*, *Roseburis*, *Psedobutyrivibrio*, *Lachnospriraceae*, *Ruminococcaceae*, *Prevotellaceae*, *Bactriodales*, and *Clostridiales*.

4.2.5 Effects on non-ruminant production

While the use of EOs has increased in the swine and poultry industries in recent years, their effects remain inconsistent and poorly understood. As stated in earlier sections, conflicting results on performance or microbial composition may be because of the type of EO investigated, the concentration used, or differences in digestive physiology between swine and poultry. For instance, Franz et al. (2010) and Windisch et al. (2008) have reported that the average improvement in weight gain, feed intake, and feed conversion from using EOs were respectively 2.0%, 0.9% and 3.0% for piglets compared to 0.5%, -1.6%, and -2.6% for poultry. Reviewing studies carried out in piglets and broilers, Zeng et al. (2015) reported inhibitory effects of different EOs against pathogens such as *C. perfringens* and *E. coli* as well as proliferating effects on beneficial bacteria such as *Lactobacilli*. In contrast, Cross et al. (2007) as well as Muhl and Liebert (2007) reported no effects of EOs on the gut microbial composition of either pigs or broilers.

4.3 Peptides

4.3.1 Mode of Action

Antimicrobial peptides (AMPs) are typically short in length (12–60 amino acid), with an overall positive charge, and also include hydrophobic residues (Hou et al., 2017a). Bacterial bacteriocins kill cells from other species that may be competing for resources in the same ecological niche. In plants and animals, the role of AMPs is to protect against bacteria and fungi. In vertebrates, AMPs have been shown to have antimicrobial activity at high concentration, as well as immune modulating and inflammation controlling

properties, and can be isolated from body fluids as well as from epithelial tissues of the mouth, lungs and skin. In complex microbial ecosystems such as found in the gut of animals, AMPs can be used to suppress harmful microorganisms and stimulate the growth of beneficial microorganisms such as *Lactobacillus* and *Bifidobacterium* (Wang et al., 2016).

The AMP mechanisms of action can be divided into two major categories: membrane targeting and non-membrane targeting. Membrane targeting AMPs can be either receptor mediated, which includes mostly bacterial AMPs, or non-receptor mediated, which includes most vertebrate and non-vertebrate AMPs (Kumar et al., 2018). The combination of amino acids with a positive charge and hydrophobic residues favors interactions of AMPs with phospholipids of cell membranes, resulting in the accumulation of AMPs and their disruptive self-assembly at the surface of target bacteria. Three main mechanisms have been proposed to explain the peptide-mediated permeation that takes place at target membranes: the barrel-stave model, the carpet model and the toroidal-pore model. In the barrel stave model, the peptides attached at the surface aggregate to form a bundle with a central lumen that can penetrate the hydrophobic core of the membrane. In the carpet model, AMPs bind to the heads of phospholipids on the surface of the cell membrane, then function like a detergent by disrupting the bilayer curvature. In the toroidal-pore model, peptide helices aggregate then insert themselves perpendicularly into the lipid bilayer (Wang et al., 2016; Kumar et al., 2018). Another proposed mode of action of AMPs, which is similar to that of penicillin, includes inhibition of cell-wall synthesis through interaction with precursor molecules that are required for this cellular process. For non-membrane targeting AMPs, mechanisms

include inhibition of protein or nucleic acid synthesis, as well as disruption of enzymatic activity. In this case, the mode of action depends mostly on the organisms by which they are synthesized. For instance, cecropins are insect AMPs that have strong inhibitory effects against bacteria such as *E. coli* and *P. aeruginosa*, by breaking the integrity of bacterial membranes (Silvestro et al., 2000).

4.3.2 Bioactive Peptides

Proteins are nitrogenous macromolecules composed of one or more chains of amino acids that are linked by peptide bonds. They can perform a range of different functions, including acting as enzymes, antibodies, structural components of body tissues or reserves of nutrients. Proteins represent the commercial product sold by most livestock industries, in the form of milk, meat, egg, or wool (Hou et al., 2017b). Sufficient intake of dietary protein is thus essential for all animal species to achieve optimal growth, production performance, and efficient use of dietary energy. Dietary proteins are hydrolyzed into free amino acids as well as di- and tri-peptides by the action of host proteases and oligopeptidases expressed in the small intestines, which are then absorbed by the intestinal epithelium (Hou et al., 2017b).

Dietary proteins fed to livestock can come from a number of different sources, including forages, grains, legumes, animal meal, as well as various by-products (Martínez-Alvarez et al., 2015). These different protein sources each have advantages and disadvantages. For instance, soybean meal represents one of the major protein sources for animal production, but it contains trypsin inhibitor and allergenic proteins, such as gylcinin and β-conglycinin, which can reduce its digestibility and affect animal health,

particularly in weaned animals, if the product is not processed appropriately (Martínez-Alvarez et al., 2015).

One approach to improve the digestibility or availability of dietary amino acids has been the development of feed ingredients generated from the microbial, enzymatic or chemical hydrolysis of animal and plant protein waste products. These peptide blends have shown several benefits as feed ingredients: they contain minimal levels of anti-nutritional factors, they are highly soluble over a wide range of pH values, and they tend to have a favorable amino acid profile (Dieterich et al., 2014; Martínez-Alvarez et al., 2015; Hou et al., 2017b). Additional reported benefits for animal performance include enhanced palatability (Martínez-Alvarez et al., 2015), increased intestinal absorption (Wong et al., 2008), as well as increased availability of poorly soluble amino acids such as cysteine and glutamine by providing them in the form of small peptides. For instance, dietary intake of a whey protein hydrolysate resulted in higher growth rates, higher nitrogen retention, and higher glutamine stores compared to a control diet with glutamine and arginine provided as free amino acids (Boza et al., 2000).

4.4 Enzymes as supplements in animal feed

The animal digestive system is not fully efficient at digesting and absorbing all nutrients available in the feed that is consumed. For instance, pigs and chickens are unable to digest approximately one fourth of the fed that they ingest. Potential explanations for this inefficiency include the presence of feed compounds that hinder the digestive process or the absence / low expression of the enzymes needed to release certain nutrients from the feed (Ojha et al., 2019).

Supplementation of feed with enzymes can then help by enhancing the nutritive value of certain feed ingredient by increasing the effectiveness of their digestion (Ojha et al., 2019). Enzymes that are capable of hydrolyzing crude fat, starch, proteins, and phytates not only increase the efficiency of feed utilization, but also prevent irritation of the intestinal mucosal layer by undigested feed ingredients, which can be detrimental to gut health (Ravindran, 2013). In addition to improved nutrient digestibility, enzymes can reduce the availability of nutrients that are preferred by pathogenic bacteria, lower digesta viscosity, and enhance nutrient absorption (Campbell and Bedford, 1992). Finally, by reducing the levels of undigested substrates and anti-nutritional factors, as well as by releasing prebiotics such as oligosaccharides from dietary non soluble polysaccharides (Kiarie et al., 2013; Bedford, 2018), supplemented enzymes can potentially modulate the composition of gut microbial communities (Kiarie et al., 2013).

4.4.1 Proteases

As a means to counter the increasing cost of protein sources that can be used for feeding livestock without affecting animal performance (Vieira et al., 2016), the use of exogenous proteases has become an attractive solution. In contrast to crystalline amino acids which provide an alternative to crude protein, exogenous proteases aim to complement the animal digestive system by hydrolyzing certain type of proteins that are resistant to host enzymes, thereby increasing the availability of amino acids that are provided in the feed (Mc Alpine et al., 2012). Increasing the digestibility and availability of dietary amino acids is particularly important in weaned animals, in which reduced growth and animal performance is typically observed, which may be an indication of

inadequacies in the levels or activity of host proteases (Le Huerou-Luron et al., 1993; Noy and Sklan, 1995; Hedemann and Jensen, 2004; Qaisrani et al., 2014; Lee et al., 2018). Interestingly, supplementation of exogenous proteases to160 finishing pigs on a low protein diet was reported to improve growth performance and increase ATTD of CP, while decreasing fecal ammonia emissions (Lei et al., 2017). Using 144 pigs (18-45 kg), Chen et al., (2017) observed improved AID of CP, as well as an increased villus height:crypt depth ratio, when exogenous proteases were used as additives to sorghum-based diet. Tactacan et al., (2016) demonstrated improved growth rate and nutrient digestibility, as well as reduced fecal NH_3 emission, on 50 nursery pig whose diet was supplemented with a commercial protease formulation. Similar positive effects of dietary supplementation with proteases on growth performance, protein digestibility, nutrient transport efficiency, and health status on 21-day-old nursery pig were reported by Zuo et al. (2015). In contrast, Caine et al. (1997) reported no effect of protease treatment of soybean meal on ileal digestibilites of CP and AA in an experiment carried out on 16 newly weaned pigs fitted with a modified post valve T-cecum cannula

While exogenous proteases can be supplemented on their own, they can also be used in combination with other enzymes towards improving digestibility of other feed ingredients (Omogbenigun et al., 2004; Cowieson and Adeola, 2005; Mc Alpine et al., 2012). In this context, Recharla et al (2019) investigated the potential of a multi-enzyme formulation (xylanase, α-amylase, β-glucanase, and protease) on a wide range of feed ingredients (corn meal, wheat meal, soybean meal, fish meal, oriental herbal extract, Italian rye grass, and peanut hull). They found no effect of enzymes on apparent nutrient digestibility and growth performance of the 36 pigs used for the experiment. However,

changes in gut bacterial communities were observed, with higher abundance of *Treponema* and *Barnesiella* and lower abundance of Prevotella, *Butyricicoccus*, *Ruminococcus* and *Succinivibrio*.

4.5 Prebiotics in animal diets

4.5.1 Mannan Oligosaccharides

Mannan oligosaccharides (MOSs) are mannose oligomers that cannot be digested by the host, but they can contribute to host gut health by preventing the binding of pathogens to gut epithelial cells (White et al., 2002; Burkey et al., 2004; Castillo et al., 2008; Liu et al., 2008) or by being metabolized by beneficial gut microorganisms (Gibson and Roberfroid, 1995; White et al., 2002; Liu et al., 2008; Halas and Nochta, 2012). They can be found in certain feed ingredients, and are notably abundant in the cell wall of baker's yeast (*Saccharomyces cerevisiae*). As baker's yeast is widely utilized in the food industry, its derivatives have become a common source of MOS products in human and animal nutrition (Halas and Nochta, 2012). In the context of human health, MOS have been reported to modulate obesity and the gut microbiota in mice fed high-fat diets (Wang et al., 2018). MOS have also been reported to decrease the onset of atherosclerosis by lowering plasma cholesterol levels, which was also accompanied by an increase in cecal butyrate levels, fecal excretion of bile acids, and interactions with the murine gut microbiota (Hoving et al., 2018). In weaned pigs, MOS was reported to increase growth performance and nutrient digestibility, while decreasing diarrhea scores (Zhao et al., 2012).

5. Hypothesis and Research Objectives

In this context, the general hypothesis tested by the research presented in this doctoral dissertation was that supplementation of livestock diets with feed additives containing essential oils, peptides, proteases and/or MOS changes the composition of symbiotic bacterial communities in the gastrointestinal tract of pre-weaned or early weaned animals.

This hypothesis was tested by these three objectives:

- determine the effects of a commercial blend of EO on the ruminal bacterial communities of dairy calves (Chapter 2)
- determine the effects of a commercially formulated peptide-based product on the fecal bacterial communities of nursery pigs raised in a wean-to-finish swine facility on a commercial scale (Chapter 3)
- determine the effects of a commercially formulated product that combined peptides, exogenous proteases and mannan oligosaccharides on the fecal bacterial communities of nursery pigs in a wean-to-finish swine facility on a commercial scale (Chapter 4)

The results presented in this doctoral dissertation demonstrate that different formulations of essential oils, peptide, and combinations of peptides-protease-MOS can change or modulate the gut bacterial communities of young animals. While additional research will be required to elucidate the biological mechanisms involved and how these changes in gut bacterial composition can benefit animal health and performance, they provide further support that the gut microbiome of young animals can be modulated using different types of feed additives.

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Chapter 2:

Feeding essential oils to neonatal Holstein dairy calves results in increased ruminal Prevotellaceae abundance and propionate concentrations

Related Publication: Poudel, P., K. Froehlich, D. P. Casper, and B. St-Pierre. 2019. Feeding Essential Oils to Neonatal Holstein Dairy Calves Results in Increased Ruminal Prevotellaceae Abundance and Propionate Concentrations. Microorganisms 7(5)doi: 10.3390/microorganisms7050120

Abstract

Since antibiotic use in animal production has become a public health concern, great efforts are being dedicated to find effective and viable alternatives. While essential oils (EO) have become attractive candidates for use in the livestock industry, their mode of action and microbial targets in food animals remain largely uncharacterized. To gain further insight, we investigated the rumen environment of neonatal calves fed calf starter pellets and milk replacer supplemented with a commercial blend of EO. Propionate concentrations were not only found to be higher in EO fed calves compared to controls ($P < 0.05$), but ruminal bacterial communities also differed greatly. For instance, the abundance of Firmicutes was significantly lower in samples from EO fed calves than in controls, which appeared to be mostly due to lower Lachnospiraceae levels ($P < 0.05$). In contrast, Bacteriodetes were more abundant in EO fed calves compared to controls, which was primarily the result of higher Prevotellaceae ($P < 0.05$). Notably, two bacterial Operational Taxonomic Units (OTUs) were significantly more abundant in EO fed calves; SD_Bt-00966 was found to be a close relative of *Prevotella ruminicola* (97%), while SD_Bt-00978 likely corresponded to an uncharacterized species of Gammaproteobacteria. In addition, Pearson correlation and canonical correspondence analyses revealed potential associations between other ruminal bacterial OTUs and either SCFA parameters or metrics for calf growth. Together, these results support that EO supplementation in growing dairy calves can modulate rumen function through short chain fatty acid (SCFA) production and growth of specific rumen bacterial groups.

1. Introduction

Antibiotics have traditionally been used in dairy calf production to increase immunity as well as reduce stress and susceptibility to pathogens. They have been shown to improve rumen development, increase growth performance, reduce neonatal diarrhea, and decrease the risk of calf mortality (Gustafson and Bowen, 1997). However, due to the increased incidence of bacterial resistance and potential risks for food security, antibiotic use in animal production has become a concern, leading to stricter regulation for this practice in the livestock sector. Indeed, policies such as the European Union ban on the use of antibiotics and ionophores in animal production, as well as the phasing out of prophylactic treatments for food animals produced in the USA by the Food and Drug Administration (FDA), have created an urgent need for alternatives. To be viable, these not only have to promote animal welfare, but also optimize animal production while posing only minimal risks to human health and the environment (Allen et al., 2013; Cheng et al., 2014).

Since antibiotics act as selection agents that ultimately affect the composition of host microbiomes, a common strategy to identify effective alternatives has been to explore the potency of other types of antimicrobials, which have included essential oils (EO), a group of plant secondary metabolites that can be extracted by distillation. As a group, they are very diverse in chemical structure and biological effects, with terpenoids and phenylpropanoids representing the most commonly found types of EO (Patra and Saxena, 2010). Studies carried out in ruminants have provided evidence that EO could be used instead of antibiotics for improving animal productivity (Khiaosa-ard and Zebeli, 2013). While certain reports found no discernable effects of EO supplementation on

production or ruminal parameters (Meyer et al., 2009; Tager and Krause, 2011), perhaps as a result of dosage or nature of the active compounds, other studies were successful in uncovering positive responses. Their reported effects on rumen function include inhibition of deamination and methanogenesis, resulting in lower ammonia nitrogen and methane, respectively (McIntosh et al., 2003). EO can also reduce ruminal acetate levels, while maintaining total short chain fatty acid (SCFA) production through increased propionate and butyrate production in the rumen (Calsamiglia et al., 2007). Positive effects of EO supplementation for dairy calf performance have also been reported, such as increased starter feed intake and improved feed efficiency (Hill et al., 2007). The benefits of EO on the performance of young ruminants are of particular interest, as they may be the result of changes in the gut microbiome caused by their antimicrobial activities. Furthermore, since the composition of gut microbial communities in neonatal and young animals tends to fluctuate until it becomes stably established later on in life (Jami et al., 2013), it is more likely to be responsive to manipulation during these early growth stages.

Ultimately, the purpose of modulating early gut microbiome composition would be to provide long term benefits to the performance and health of adult animals (Malmuthuge and Guan, 2017). However, the impact of EO supplementation on the microbiome of young ruminants remains largely unexplored. To gain further insight, we took advantage of a companion study to investigate the rumen environment of dairy calves fed a commercial blend of EO (Froehlich et al., 2017). In this context, the main objective of the investigation presented in this report was to determine the effects of EO supplementation on ruminal bacterial communities. Comparative analyses of ruminal

SCFA profiles and bacterial community composition performed between the two dietary groups indicated that supplementation of a standard dairy calf diet (i.e. milk replacer and pelleted calf starter) with the EO product resulted in an increase in rumen propionate concentration that was associated with profound differences in bacterial composition, which included the enrichment of specific uncharacterized bacteria.

2. Materials and Methods

2.1 Sample collection

The analyses described in this report were performed on samples collected during a previously reported companion study (Froehlich et al., 2017), which was conducted at the South Dakota State University (SDSU) Animal Research Wing (ARW; Brookings, SD), with all procedures approved by the SDSU Institutional Animal Care and Use Committee before the start of the trial. As part of the original animal study, the effect of EO supplementation at three different doses (0.5X, 1.88g/feeding; 1.0X, 3.75g/feeding; 1.5X, 5.63g/feeding) on dairy calves was investigated (Froehlich et al., 2017). The commercial supplement, manufactured by Ralco, Inc. (Stay Strong for Dairy Calves; Marshall, MN), was a blend of EO (carvacrol, caryophyllene, p-cymene, cineole, terpinene, and thymol) that also included arabinogalactans, a type of hemicellulose known to enhance immune function (Dion et al., 2016). All calves were housed individually, fed milk replacer (24:20% crude protein: fat; as-fed basis), and had *ad libitum* access to water and pelleted calf starter (see Supplementary Table 1 for calf starter ingredient composition) during the trial. Milk replacer was offered by bucket feeding twice every day until d35, then reduced to once every day starting at d36 to

facilitate weaning at d42. From week 1 until week 6, calf starter intake increased from approximately 2% of total dry matter intake to approximately 70% (Froehlich et al., 2017).

For the purpose of the microbiome study presented in this report, rumen fluid was sampled from a subset of 10 of the animals fed milk replacer supplemented with EO (1.0X, 3.75g/feeding), and from 10 of the animals fed milk replacer without supplementation. While calves fed the 0.5X EO dose performed significantly better for body weight and other parameters compared to calves fed the other EO doses, the absence of differences in the gain: feed ratio across treatments indicated that higher performance was the result of higher feed intake rather than increased efficiency (Froehlich et al., 2017). In this context, samples from the animals fed the 1.0X EO dose were selected for the rumen microbiome study rather than samples from the 0.5X treatment group, as a higher dose was more likely to result in a detectable effect on ruminal bacterial communities. Rumen samples were collected one day after weaning (day 43) from each animal by stomach tubing, with rinsing of the sampling equipment with warm water between each collection. Separate samples were collected for microbiome and SCFA analysis, with the latter supplemented with 25% metaphosphoric acid (W/V) at a ratio of 4:1 before freezing. All samples were stored at -20^{0}C until analyzed.

2.2 SCFA analysis

Rumen samples mixed with metaphosphoric acid were thawed, then centrifuged to remove particulate (16,000 x g, 1 min). For each sample, 800 ml of supernatant were

mixed with 200 □l of an internal standard (2-ethyl butyric acid, 20mM). Following injection, SCFAs were separated by gas liquid chromatography (Trace 1310, Thermo Scientific, Bellefonte, PA) on a 0.25 mm i.d. × 30 m capillary column with 0.25-μm film thickness (Nukol^{TM}, Supelco Inc., Bellefonte, PA). The injector port temperature was 200 °C, with a split ratio of 100:1, and a column flow of He at a rate of 0.8 mL/min. After starting at 140 °C for a duration of 9.5 min, the oven temperature was increased at a rate of 20 °C/min until it reached 200 °C, at which point it was maintained for 1 min. Detection was completed using a flame-ionization detector with a temperature of 250 °C. Data was analyzed by the software Chromeleon 7.2 CDS, with SCFA concentrations measured based on peak height. For calibration, a mixture of standards (Supelco Volatile Free Fatty Acid Mix 46975, Supelco Inc., Bellefonte, PA) was first analyzed for identification of SCFAs peaks, with 2-ethylbutyric acid serving as an internal standard. External calibration was performed using three different SCFA concentration levels, each measured twice.

2.3 Microbial DNA isolation and PCR amplification

Microbial DNA was isolated from rumen samples using a repeated bead beating plus column method (Yu and Morrison, 2004). The V1-V3 region of the bacterial 16S rRNA gene was PCR-amplified using the 27F forward (Edwards et al., 1989) and 519R reverse (Lane et al., 1985) primer pair. PCR reactions were performed with the Phusion *Taq* DNA polymerase (Thermo Scientific, Waltham, MA, USA) under the following conditions: hot start (4 min, 98 °C), followed by 35 cycles of denaturation (10s, 98 °C), annealing (30s, 50 °C) and extension (30 s, 72 °C), then ending with a final extension

period (10 min, 72 °C). PCR products were separated by agarose gel electrophoresis, and amplicons of the expected size (~500bp) were excised for gel purification using the QiaexII Gel extraction kit (Qiagen, Hilden, Germany). For each sample, approximately 400 ng of amplified DNA were submitted to Molecular Research DNA (MRDNA, Shallowater, TX, USA) for sequencing with the Illumina MiSeq 2X300 platform to generate overlapping paired end reads.

2.4 Computational analysis of PCR generated 16S rRNA amplicon sequences

Unless specified, sequence data analysis was performed using custom written Perl scripts (available upon request). Raw bacterial 16S rRNA gene V1-V3 amplicon sequences were provided by Molecular Research DNA as assembled contigs from overlapping MiSeq 2x300 paired-end reads from the same flow cell clusters. Reads were then selected to meet the following criteria: presence of both intact 27F (forward) and 519R (reverse) primer nucleotide sequences, length between 400 and 580 nt, and a minimal quality threshold of no more than 1% of nucleotides with a Phred quality score lower than 15.

Following quality screens, sequence reads were aligned, then clustered into Operational Taxonomic Units (OTUs) at a genetic distance cutoff of 5% sequence dissimilarity (Opdahl et al., 2018). While 3% is the most commonly used clustering cutoff for 16S rRNA, it was originally recommended for full length sequences, and may not be suitable for the analysis of specific subregions since nucleotide sequence variability is not constant across the entire length of the 16S rRNA gene. In this context, if 3% is a commonly accepted clustering cutoff for V4 or V4–V5 regions, which are the

least variable of the hypervariable regions, then a higher cutoff should be used for the V1-V3 region, since V1 is the most variable region of the 16S rRNA gene.

OTUs were screened for DNA sequence artifacts using the following methods. Chimeric sequences were first identified with the chimera.uchime and chimera.slayer commands from the MOTHUR open source software package (Schloss et al., 2009). Secondly, the integrity of the 5' and 3' ends of OTUs was evaluated using a database alignment search-based approach; when compared to their closest match of equal or longer sequence length from the NCBI nt database, as determined by BLAST (Altschul et al., 1997), OTUs with more than five nucleotides missing from the 5' or 3' end of their respective alignments were discarded as artifacts. Single read OTUs were subjected to an additional screen, where only sequences that had a perfect or near perfect match to a sequence in the NCBI nt database were kept for analysis, i.e. that the alignment had to span the entire sequence of the OTU, and a maximum of 1% of dissimilar nucleotides was tolerated.

After removal of sequence chimeras and artifacts, taxonomic assignment of valid OTUs was determined using a combination of RDP Classifier (Wang et al., 2007) and BLAST (Altschul et al., 1997). The List of Prokaryotic Names with Standing in Nomenclature (LPSN - http://www.bacterio.net) was also consulted for information on valid species belonging to taxa of interest (Parte, 2014).

2.5 Computational analysis for alpha and beta diversity

Using custom Perl scripts, all datasets were randomly rarefied to 1800 reads, which were then used to create 'shared'-type formatted files. All subsequent steps were

performed using commands in MOTHUR [19]. Chao1, Shannon and Simpson indices, as well as observed OTUs and coverage, were determined from the shared files using summary.single. For Principal Coordinate Analysis (PCoA), Bray-Curtis distances were first determined using summary.shared, which were then used as input for the command pcoa. Principal Components 1 (PC1) and 2 (PC2), representing the highest levels of variation, were plotted using Microsoft Excel. Canonical Correspondence Analysis (CCA) was conducted in R (version 3.2.3) using the command cca from the vegan package (version 3.2.5), with outputs plotted using the command plot.

2.6 Statistical Analyses

An unpaired *t*-test was used to compare rumen SCFAs levels as well as the abundance of bacterial taxonomic groups, respectively, between samples from calves fed the EO supplemented diet and calves fed the control diet. The *t*-test was conducted using the online GraphPad Software (https://www.graphpad.com/quickcalcs/ttest1.cfm). Pearson correlation coefficients and associated *P* values were calculated using Microsoft Excel. Means of two groups were considered to be significantly different when $P \leq 0.05$, and a tendency towards statistical significance was indicated when $0.05 < P \leq 0.10$.

3. Results

3.1 Comparative analysis of ruminal SCFA between EO supplemented and non-supplemented diets

Ruminant animal performance is dependent on ruminal SCFA production. Amongst the SCFAs analyzed (Figure 1), propionate was found in higher concentration

($P < 0.05$) in the rumen of EO fed calves (40.25 mM ± 3.03 mM) compared to calves fed the control diet (31.06 mM ± 3.14 mM). While numerically greater in animals on the EO-supplemented diet, acetate, valerate and total SCFAs concentrations were not found to be statistically different between the two diets. A trend ($P = 0.072$) for differences in the acetate: propionate ratio was observed between the two treatments (EO: 1.24 ± 0.04; Control: 1.38 ± 0.06).

3.2 Effects of EO on the taxonomic composition of ruminal bacteria in growing calves

From the 20 samples analyzed, a total of 347,254 high quality sequence reads were generated, with an average of 16,376 ± 4,472 per sample. Taxonomic analysis identified six phyla across all samples, with Firmicutes, Bacteriodetes and Proteobacteria being the most highly represented (Table 1, Figure 2). The relative abundance of Firmicutes was significantly lower ($P < 0.05$) in EO fed calves (43.68% ± 6.92%) compared to controls (73.22% ± 6.79%), which appeared to be mostly due to lower Lachnospiraceae levels in EO fed samples ($P < 0.05$). In contrast, Bacteriodetes were more abundant in EO fed calves (44.63% ± 6.28%) compared to controls (13.45% ± 6.02%), which was primarily the result of higher Prevotellaceae (44.20% ± 6.27% vs 9.70% ± 5.94) ($P < 0.05$). Proteobacteria, mostly represented by unclassified Gammaproteobacteria, were also found to be significantly higher ($P < 0.05$) in samples from EO fed calves compared to control calves (3.49% ± 1.32% vs 0.17% ± 0.13% respectively).

3.3 Effects of EO on the ruminal bacterial community structure in growing calves

To gain further insights into the community level compositional differences between EO and Control ruminal environments, alpha and beta diversity analyses were conducted. Diversity of ruminal bacteria was not affected by treatment with EO under these conditions, since no statistical differences were observed for Chao1, Simpson and Shannon indices (Table 2). However, a beta diversity analysis using Principle Coordinate Analysis (PCoA) based on Operational Taxonomic Unit (OTU)-level Bray-Curtis dissimilarity (Figure 3) supported that the composition of EO and Control samples were different from each other, as their respective data points were not evenly distributed between clusters.

From a total of 4,154 OTUs that were identified in this study, 31 OTUs were designated as main OTUs, which were defined as having a mean relative abundance of at least 1% for at least one treatment (Table 3, Table 4 and Suppl. Table 2). As a group, main OTUs represented 68.9% and 67.0% of sequence reads in EO and control fed samples, respectively. Only four main OTUs (SD_Bt-00966, SD_Bt-00967, SD_Bt-00986, and SD_Bt-36860) were found to have a sequence identity of 95% or greater to their closest valid relative, indicating that at least 27 main OTUs likely corresponded to uncharacterized ruminal bacterial species.

Overall, main OTUs showed a phylogenetic distribution reflecting their respective treatment, with higher representation of Bacteriodetes-affiliated OTUs in EO fed calves and higher abundance of Firmicutes OTUs in control calves. Five of the Bacteriodetes affiliated OTUs were found in higher abundance in EO fed calves compared to control, and were affiliated to the genus *Prevotella* (Table 3). Of these, only SD_Bt-00966

showed significantly higher relative abundance in calves fed EO (19.51% ± 5.32%) compared to control (2.69 % ± 1.80%). Its closest known relative was identified as *Prevotella ruminicola* (97% sequence identity). Firmicutes included by far the highest number of main OTUs, but none showed statistical differences based on treatment (Table 4). OTU SD_Bt-00179 was observed in greater abundance in EO samples (13.6X), but these differences were not found to be statistically significant. OTUs SD_Bt-00125, SD_Bt-00732, SD_Bt-00975, SD_Bt-00980, SD_Bt-00983, SD_Bt-00998, and SD_Bt-36860 were found to be between 10 to 75.2X greater in control fed calves compared to EO calves. While these differences could help explain the higher abundance of Firmicutes in control-fed calves, they were not supported by statistical analysis. Most Proteobacteria were represented by a single OTU (SD_Bt-00978), which was higher ($P < 0.05$) in EO fed calves compared to controls (3.44% ± 1.30% vs 0.17% ± 0.13%). The most abundant Actinobacteria OTU (SD_Bt-00967) was numerically lower in EO fed calves, but by only a 2.5X difference with controls. Based on its high sequence identity to its closest valid relative, this OTU may have represented a strain of *Olsenella umbonata* (Table 2).

3.4 Identification of potential associations between main OTUs and ruminant performance parameters

To explore potential associations between dairy calf performance parameters and ruminal bacterial OTUs, two approaches were used. First, canonical correspondence analyses were performed using SCFAs levels and growth parameters as explanatory variables, respectively (Figure 4). Based on the length of the arrows for the SCFA biplot,

which is indicative of the respective strength of association of the explanatory variables, the acetate: propionate ratio, total SCFAs, as well as the respective levels of propionate, acetate, and iso-butyrate were found to display overall the strongest associations with OTUs. SD_Bt-00179 uniquely showed high correspondence to multiple SCFA attributes (total SCFAs, acetate, and propionate), while other OTUs appeared more strongly associated with individual SCFA conditions, such as observed for the acetate: propionate ratio (SD_Bt-00125, SD_Bt-00975, and SD_Bt-00009). While butyrate did not show as strong an influence as other SCFAs by this analysis, CCA indicated a strong association between butyrate and SD_Bt-00732. When calf growth performance parameters were used as explanatory variables, body length and heart girth showed the strongest correspondence with OTUs. SD_Bt-00009, SD_Bt-30048 and SD_Bt-00070 were found to be more strongly associated with body length, while SD_Bt-00977, SD_Bt-00732 and SD_Bt-00967 were more strongly associated with heart girth.

Based on Pearson correlation coefficient analysis (Supplementary Table 2), butyrate concentrations, which are critical to the development of ruminal papillae in growing calves, were strongly associated ($P < 0.05$) with OTUs SD_Bt-00995 (r = 0.733) and SD_Bt-00732 (r = 0.654), and showed a tendency for correlation with SD_Bt-00992 (r = 0.622, $P = 0.055$). Valerate levels were also strongly correlated ($P < 0.05$) with OTU SD_Bt-00995 (r = 0.635), and showed a tendency with SD_Bt-00732 (r = 0.592, $P = 0.072$). Finally for SCFA parameters, a tendency for correlation was found for three OTUs with the acetate: propionate ratio: SD_Bt-00009 (r = 0.596, $P = 0.069$), SD_Bt-00070 (r = 0.575, $P = 0.082$), and SD_Bt-00718 (r = 0.558, $P = 0.094$). Amongst the calf

growth parameter tested, the only statistically supported association by Pearson correlation was a tendency between SD_Bt-00978 and hip width (r = 0.557, *P* = 0.094).

4. Discussion

Development of the ruminal microbiome in neonatal ruminants is a complex and dynamic process involving microbial colonization and succession that ultimately culminates in the establishment of a stable microbial community that can support the host animal by producing SCFAs through fermentation of ingested feed (Malmuthuge and Guan, 2017). This stage provides a window of opportunity for manipulation, potentially allowing to increase the productivity and health of mature host animals through modulating the composition of their developing rumen microbiome (Yáñez-Ruiz et al., 2015; Meale et al., 2017). While solid feed has so far been found to be the main factor affecting rumen microbiome composition and community structure during pre-ruminal microbial colonization (Steele et al., 2016), there is growing interest in identifying compounds that could be used as feed additives to improve the rumen function of calves as they mature. Since they exhibit antimicrobial properties, and have shown potential as alternatives to antibiotics, EO have become attractive candidates to serve this purpose (Calsamiglia et al., 2007).

For instance, thymol and carvacrol have been found to act as potent antimicrobials against pathogens such as *Escherichia coli, Salmonella typhimurium*, *Staphylococcus aureus, S. epidermidis*, as well as *Listeria monocytogenes* (Cosentino et al., 1999; Benchaar et al., 2008). These compounds were also reported to exhibit antimicrobial activity against ruminal bacteria. Indeed, EO had previously been reported

to inhibit the growth of most pure cultures of rumen bacteria (McIntosh et al., 2003). *Clostridium sticklandii* and *Peptostreptococcus anaerobius* were found to be the most sensitive species, while *Streptococcous bovis* was the most resistant. Certain species, such as *P. ruminicola* and *P. bryantii*, could also adapt to grow in the presence of higher EO concentrations. Similarly, Patra and Yu (2012) (Patra and Yu, 2012) have found significant reductions in growth of *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *R. albus* in the presence of EO from clove, eucalyptus, garlic, oregano, or peppermint.

In light of the limited available information on the effect of EO on ruminal microbiomes, we took advantage of a dairy calf production trial to investigate the response of ruminal bacterial composition and SCFA levels to dietary supplementation with EO. All dairy calves sampled in this study were on a diet regimen to promote early rumen development with the use of calf starter pellets which provided a mix of the main substrates that rumen microorganisms would metabolize from a typical solid diet. Milk replacer was offered in buckets once to twice every day for calves to drink from, which not only promoted solid feed consumption, but also minimized formation of the esophageal groove which is induced by suckling. Thus, under this regimen, development of the rumen would have started well prior to weaning, so there was no sudden transition of the rumen from non-functional to functional status. As the main precursor for glucose synthesis, higher propionate levels are generally considered beneficial for ruminant production (Bergman, 1990). Since significantly greater concentrations of propionate were observed in the rumen of EO fed calves, inclusion of this blend in the diet of young ruminants appeared to create a ruminal environment that was favorable for animal

performance. For growing calves, butyrate is typically considered the more desirable SCFA, as it is involved in initiating the development of rumen papillae through stimulation of rumen epithelial metabolism (Baldwin and McLeod, 2000). While propionate may also be used to a lesser extent as a source of energy for rumen papillae development (Tamate et al., 1962), its main effect would more likely be to improve animal performance rather than promoting rumen development.

Ruminal SCFA concentrations and profiles are dependent on the respective composition of the diet and of the host's ruminal microbial communities (Bergman, 1990). Accordingly, differences in rumen microbial community composition were observed between EO fed calves and controls. Based on16S rRNA composition analysis, the two most likely candidates for this effect would have been OTUs SD_Bt-00966 and SD_Bt-00978, as their respective abundances were found to be significantly greater in the rumen of EO fed calves compared to control calves by a factor of 7.2X and 20.2X. It remains to be determined whether SD_Bt-00966 and / or SD_Bt-00978 are responsible for higher propionate levels in response to EO, but we anticipate that they would likely have the ability to express one or more metabolic pathways for its production (Reichardt et al., 2014; Gonzalez-Garcia et al., 2017). While SD_Bt-00978 was phylogenetically too distant from its closest relative to reliably infer function based on its 16S rRNA gene sequence (*Haemophilus influenzae*, 84% sequence identity), SD_Bt-00966 presented a close match to *P. ruminicola* (97% sequence identity). This bacterial species has been defined as a carbohydrate utilizer (Russell and Baldwin, 1979; Strobel, 1992) with the ability to tolerate low pH (Russell and Dombrowski, 1980). Interestingly, *P. ruminicola* was reported to be able to grow in the presence of elevated EO (McIntosh et al., 2003),

and many strains possess the ability to decarboxylate succinic acid to propionic acid (Dehority, 1966; Wallnofer and Baldwin, 1967). While it remains to be determined whether SD_Bt-00966 represented a strain of *P. ruminicola* or if it corresponded to an uncharacterized species of *Prevotella*, these properties make it an interesting candidate to pursue towards linking increases in ruminal propionate to the addition of EO in a diet.

The predominance of Firmicutes in the rumen of calves fed the control diet is consistent with a number of studies conducted with pre-weaned dairy calves (Malmuthuge et al., 2014; Dias et al., 2017), while other groups have reported combinations of Bacteriodetes, Firmicutes, and Proteobacteria (Li et al., 2012; Jami et al., 2013). Notably, Patra and Yu (2015) (Patra and Yu, 2015) have observed a lower abundance of Firmicutes combined with higher levels of *Prevotella* in response to phenolic EO extracted from oregano, which is consistent with our observations. The same report also indicated that the effects of EO on rumen bacterial communities were dependent on the chemical nature of the EO provided as supplement. Indeed, the type of EO used, the composition of their active components, as well as their dosage, may affect their ability to modulate performance or the rumen environment (Benchaar et al., 2007; Calsamiglia et al., 2007; Patra, 2011). For instance, a phenolic structure, as well as the presence of hydroxyl groups and their respective position in a compound, can affect the antimicrobial potency of certain EO (Dorman and Deans, 2000; Burt, 2004). While the specific modes of action of EO still remain to be determined, they are thought to be more effective in combinations, as different types of compounds may be more likely to affect microbial growth or survival through distinct mechanisms. For instance, additive effects of carvacrol and thymol against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

have previously been reported (Lambert et al., 2001). As Gram negative bacteria appear to be less susceptible to the antimicrobial properties of EO compared to Gram-positive, perhaps because of their cell wall structure (Dorman and Deans, 2000) (Burt, 2004) compounds such as p-cymene, which can induce swelling of bacterial cell walls, could act in synergy with other EO components by facilitating their uptake into target cells (Ultee et al., 2002). Conversely, we would anticipate that bacterial species able to thrive in the presence of EO would possess structural and/or enzymatic adaptations to counter EO antibacterial mechanisms. In the context of the current search for effective and sustainable alternatives to antibiotics, further investigations of EO-resistant bacteria could yield valuable insight into cellular mechanisms that could be targeted by future generations of antimicrobials.

In addition to EO, the commercial additive used in this study also included arabinogalactans, a type of hemicellulose primarily composed of galactose and arabinose that is intended to enhance immune function (Dion et al., 2016). Intriguingly, uncharacterized ruminal spirochete strains that preferably metabolize arabinogalactans over cellulose have been identified (Paster and Canale-Parola, 1982), indicating the existence of a niche for this substrate in the rumen. However, considering that butyrate was more prominent than propionate in human fecal cultures grown with arabinogalactans (Vince et al., 1990), these polysaccharides may not be responsible for the increase in ruminal propionate observed in this study. As the effects of arabinogalactans on the ruminal environment and its microbiome remain largely unexplored, future investigations will be necessary to determine if they impact development of the rumen.

5. Conclusion

In conclusion, the results of this study support a beneficial effect of EO on the SCFA profile of dairy calves that would be expected to promote increased performance later in life. This report also indicates that at least two ruminal bacterial species belonging to distinct phylogenetic lineages may be upregulated by feeding EO to young calves. Together, these results thus support that EO can effectively be used to modulate the ruminal environment and microbiome of young bovine animals towards potentially improving their nutrition, performance and health during the productive stages of their life.

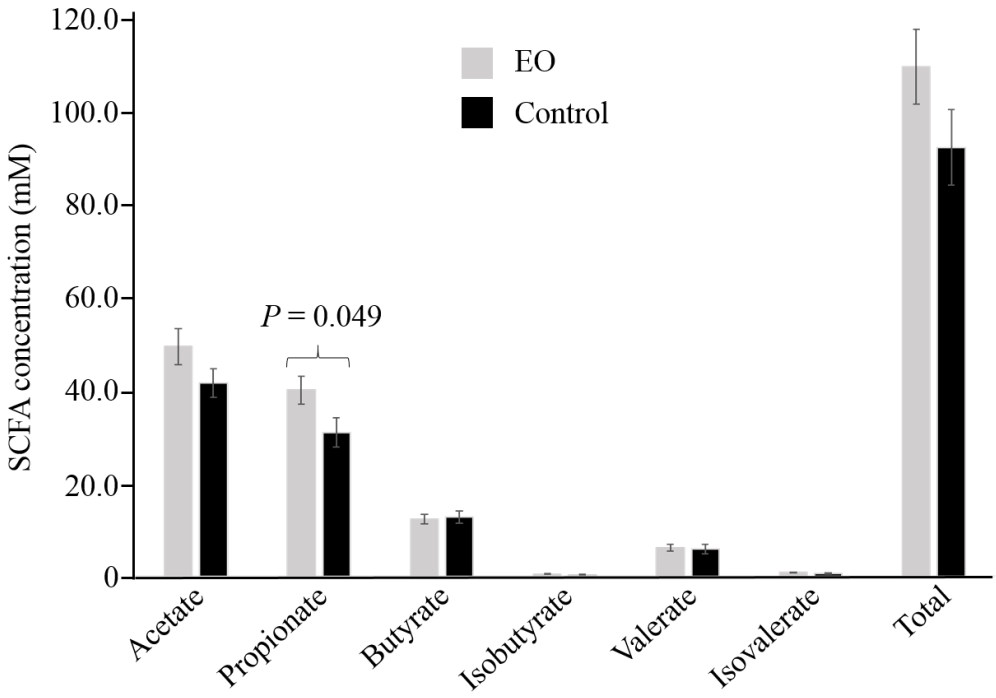


Figure 1. 1. SCFA profiles of rumen samples from EO-supplemented and Control diet-fed calves. Values shown represent the mean and standard error of the means for 10 samples per treatment.

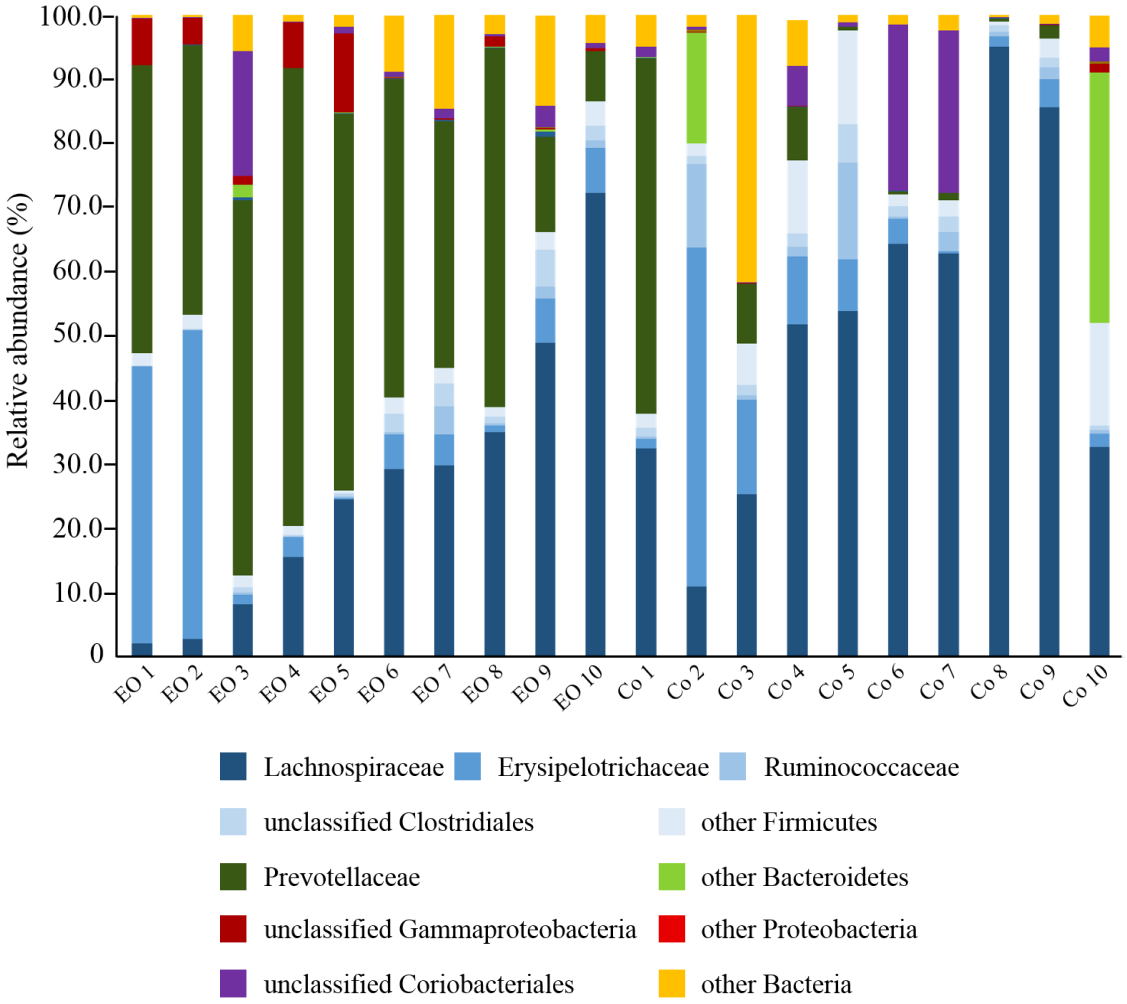


Figure 1.2. Family level taxonomic composition of rumen bacterial populations in EO fed calves and controls (Co). Families belonging to the same phylum are represented by different shades of the same color: Firmicutes (blue), Bacteriodetes (green), Proteobacteria (red), and Actinobacteria (purple).

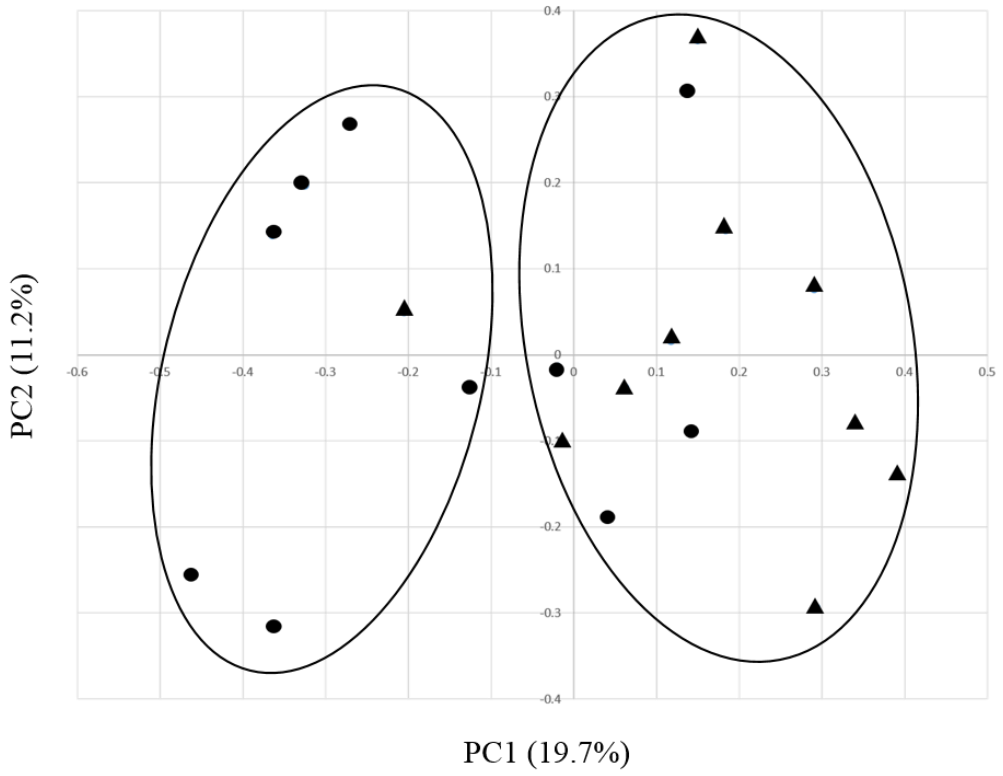
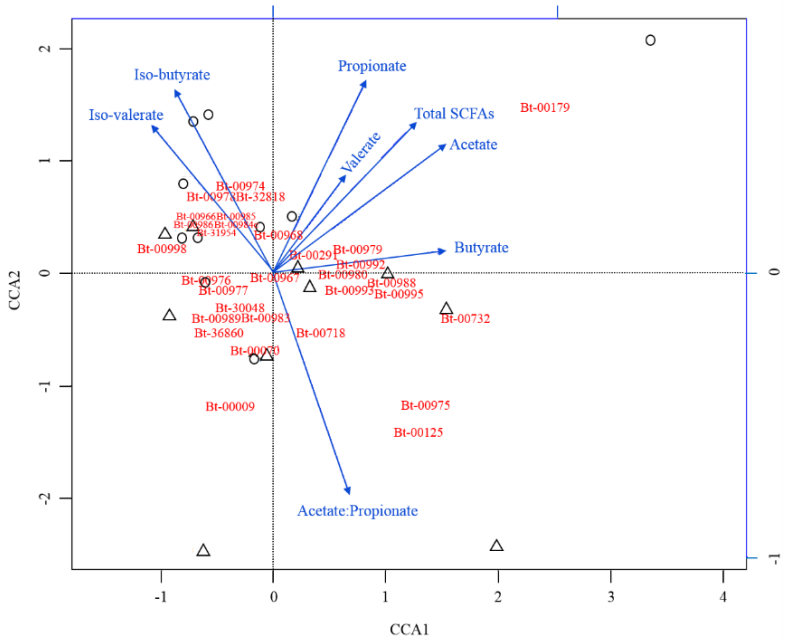


Figure 1.3. Comparison of rumen bacterial communities from EO-supplemented and Control diet-fed dairy calves using Principle Coordinate Analysis (PCoA). The x and y axes correspond to Principal Components 1 (PC1) and 2 (PC2), which explained the highest level of variation. EO and Control samples are represented by circles and triangles, respectively.

(A)



(B)

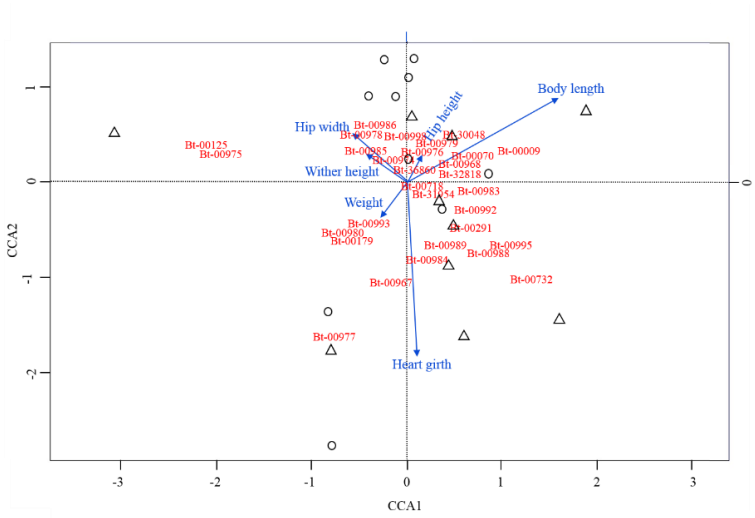


Figure 1.4. Canonical Correspondence Analysis (CCA) to uncover associations between main OTUs and SCFA parameters (A) or dairy calf performance attributes (B) as explanatory variables. The length of an arrow represents the relative influence of its corresponding explanatory variable on the distribution of the OTUs analyzed. EO and Control samples are represented by circles and triangles, respectively.

Table 1.1. Relative abundance (%) of main bacterial taxonomic groups in the rumen of dairy calves fed an EO-supplemented or Control diet. Values shown represent mean and standard error of the mean, respectively.

Taxonomic affiliation	EO	Control	*P*-value
Firmicutes	43.68 ± 6.92	73.22 ± 6.79	0.0069
Lachnospiraceae	26.87 ± 6.92	51.53 ± 8.44	0.0364
Erysipelotrichaceae	12.11 ± 5.65	9.99 ± 4.97	0.7812
Ruminococcaceae	0.91 ± 0.42	3.71 ± 1.74	0.1349
unclassified Clostridiales	1.72 ± 0.59	1.94 ± 0.48	0.7802
Other Firmicutes	2.07 ± 0.28	6.04 ± 1.82	0.0453
Bacteroidetes	44.63 ± 6.28	13.45 ± 6.02	0.0021
Prevotellaceae	44.20 ± 6.27	9.70 ± 5.94	0.0009
unclassified Bacteroidales	0.18 ± 0.07	0.06 ± 0.02	0.1359
Other Bacteroidetes	0.26 ± 0.20	5.64 ± 4.08	0.2048
Proteobacteria	3.51 ± 1.32	0.25 ± 0.17	0.0246
unclassified Gammaproteobacteria	3.49 ± 1.32	0.17 ± 0.13	0.0222
Other Proteobacteria	0.02 ± 0.01	0.08 ± 0.05	0.2111
Actinobacteria	2.77 ± 1.88	6.37 ± 3.28	0.3531
Coriobacteriales	2.75 ± 1.88	6.27 ± 3.28	0.3638
Other Bacteria	5.41 ± 1.70	6.72 ± 3.94	0.7643

Table 1.2. Alpha diversity indices and coverage from ruminal bacterial communities of dairy calves fed an EO-supplemented or Control diet. Values are presented as means and standard error of the mean, respectively.

Index	EO	Control	*P*-value
Chao1	484 ± 48	543 ± 80	0.5375
OTUs	206 ± 19	219 ± 25	0.6760
Shannon	3.18 ± 0.25	3.27 ± 0.24	0.8052
Simpson	0.16 ± 0.04	0.14 ± 0.03	0.7518
Coverage (%)	91.5 ± 0.8	90.7 ± 1.2	0.5723

Table 1.3. Relative abundance (%) of main Operational Taxonomic Units (OTUs) assigned to Bacteriodetes, Proteobacteria and Actinobacteria in rumen samples collected from dairy calves fed an EO-supplemented or Control diet. Values shown represent mean and standard error of the mean, respectively.

OTUs	EO	Control	*P*-value	Closest valid taxon (id%)
Bacteriodetes				
SD_Bt-00966[a]	19.51 ± 5.32	2.70 ± 1.80	0.008	*P. ruminicola* (97%)
SD_Bt-00976[a]	4.74 ± 1.32	8.01 ± 5.02	0.536	*P. ruminicola* (90%)
SD_Bt-00979[a]	2.35 ± 2.10	0.02 ± 0.01	0.281	*P. salivae* (89%)
SD_Bt-00985[a]	1.92 ± 1.02	0.18 ± 0.08	0.105	*P. salivae* (89%)
SD_Bt-00986[a]	0.91 ± 0.34	0.22 ± 0.13	0.080	*P. ruminicola* (95%)
SD_Bt-32818[a]	1.11 ± 0.79	0.08 ± 0.05	0.212	*P. multisaccharivorax* (93%)
Total	**30.55**	**11.22**		
Proteobacteria				
SD_Bt-00978[b]	3.44 ± 1.30	0.17 ± 0.13	0.022	*Haemophilus influenzae* (84%)
Actinobacteria				
SD_Bt-00967	1.96 ± 1.37	4.98 ± 2.69	0.331	*Olsenella umbonata* (99%)

Taxonomic affiliations: a. Prevotellaceae; b. unclassified Gammaproteobacteria.

Table 1.4. Relative abundance (%) of main Operational Taxonomic Units (OTUs) assigned to Firmicutes in rumen samples collected from dairy calves fed an EO-supplemented or Control diet. Values shown represent mean and standard error of the mean, respectively.

OTUs	EO	Control	*P*-value	Closest valid taxon (id%)
SD_Bt-00009[a]	4.15 ± 1.47	7.57 ± 5.85	0.577	*Butyrivibrio hungatei* (91%)
SD_Bt-00070[a]	1.74 ± 0.54	1.71 ± 0.97	0.982	*Clostridium aminophilum* (91%)
SD_Bt-00179[a]	3.54 ± 3.26	0.26 ± 0.17	0.329	*Lachnospira pectinoschiza* (89%)
SD_Bt-00291[a]	0.96 ± 0.55	2.60 ± 1.34	0.271	*Coprococcus catus* (90%)
SD_Bt-00718[a]	0.92 ± 0.34	1.41 ± 0.45	0.400	*Eisenbergiella tayi* (92%)
SD_Bt-00968[a]	0.84 ± 0.38	2.17 ± 1.46	0.389	*Butyrivibrio fibrisolvens* (90%)
SD_Bt-00977[a]	4.51 ± 3.06	0.64 ± 0.43	0.227	*Butyrivibrio fibrisolvens* (91%)
SD_Bt-00980[a]	0.52 ± 0.23	6.56 ± 4.38	0.185	*Butyrivibrio fibrisolvens* (89%)
SD_Bt-00983[a]	0.05 ± 0.03	3.76 ± 1.95	0.073	*Butyrivibrio fibrisolvens* (91%)
SD_Bt-00988[a]	0.48 ± 0.19	1.64 ± 0.85	0.198	*Lachnospira multipara* (91%)
SD_Bt-00993[a]	0.27 ± 0.15	1.00 ± 0.63	0.269	*Clostridium bolteae* (87%)
SD_Bt-00998[a]	0.15 ± 0.08	1.50 ± 1.39	0.347	*Clostridium lavalense* (90%)
SD_Bt-30048[a]	1.08 ± 0.46	1.40 ± 0.71	0.718	*Butyrivibrio fibrisolvens* (91%)
SD_Bt-31954[a]	0.50 ± 0.24	1.87 ± 0.94	0.176	*Butyrivibrio fibrisolvens* (90%)
SD_Bt-00974[b]	8.85 ± 5.45	1.14 ± 0.55	0.176	*Kandleria vitulina* (89%)
SD_Bt-00975[b]	0.48 ± 0.35	5.16 ± 5.00	0.363	*Catenibacterium mitsuokai* (88%)
SD_Bt-00989[b]	0.53 ± 0.29	1.71 ± 0.89	0.225	*Eubacterium cylindroides* (92%)
SD_Bt-00992[b]	0.62 ± 0.29	1.01 ± 0.50	0.509	*Solobacterium moorei* (91%)
SD_Bt-00125[c]	0.03 ± 0.01	1.25 ± 1.21	0.324	*Ruminococcus albus* (90%)
SD_Bt-00995[c]	0.63 ± 0.33	1.80 ± 1.29	0.390	*Ruminococcus albus* (86%)
SD_Bt-00732[d]	0.08 ± 0.05	1.11 ± 1.05	0.338	*Mogibacterium pumilum* (92%)
SD_Bt-00984[d]	1.25 ± 0.59	1.55 ± 0.98	0.797	*Syntrophococcus sucromutans* (91%)
SD_Bt-36860[e]	0.12 ± 0.06	1.72 ± 1.00	0.129	*Dialister succinatiphilus* (99%)
Total	**32.94**	**50.63**		

Taxonomic affiliations: a. Lachnospiraceae; b. Erysipelotrichaceae ; c. Ruminococcaceae;

d. Clostridia; e. Veillonellaceae.

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Chapter 3:

Dietary inclusion of a peptide-based feed additive can accelerate the maturation of the fecal bacterial microbiome in weaned pigs

Abstract

Weaning is one of the most critical transition stages of the swine production cycle, as the piglet gut physiology and microbiome need to rapidly adapt to changes in diet and environmental conditions. Based on their potential for producing a vast array of bioactive molecules, peptide formulations represent a largely untapped source of compounds that could be developed into feed additives to benefit animal health and nutrition. In this context, a commercial-scale nursery trial was performed to evaluate the impact of low inclusion of a peptide-based feed additive (Peptiva, Vitech Bio-Chem Corporation) on the performance and fecal microbiome of weaned pigs. While no significant differences in body weight, daily gain, daily feed intake nor gain:feed were observed between control and treatment animals ($P > 0.05$), an effect of Peptiva on the fecal bacterial composition of weaned pigs was observed. The first main observation was that the fecal bacterial profiles from pigs fed Control-Phase II and Control Phase III diets were found to be very distinct, suggesting that a transition or succession stage had occurred between the two phases. *Lactobacilli*, represented by four main OTUs (Ssd-00002, Ssd-00019, Ssd-00025, and Ssd-00053), were more abundant at the end of Phase II ($P < 0.05$), while *Streptococci*, mostly represented by OTUs Ssd-00039 and Ssd-00048, were in higher abundance at the end of Phase III ($P < 0.05$). Secondly, the fecal bacterial composition from pigs fed Peptiva Phase II diets showed similarities to both Control-Phase II and Control Phase III samples, while there was no difference in fecal bacterial composition between Control-Phase III and Peptiva Phase III samples. For instance, OTUs Ssd-00002, Ssd-00025 and Ssd-00053 were in lower abundance in Peptiva Phase II samples compared to Control Phase II ($P < 0.05$), but no significant difference was observed in

the abundance of these three OTUs when comparing Peptiva Phase II to Control Phase III ($P > 0.05$). Together, these results suggest that Peptiva can modulate the composition of the swine microbiome during a specific window of the nursery stage, potentially by accelerating its maturation.

1. Introduction

Weaning is one of the most critical transition stages of the swine production cycle, as decreased feed intake and poor performance from sudden changes in diet and environment can result in severe economic losses (Hötzel et al., 2011; Campbell et al., 2013). While a number of physiological conditions contribute to the performance and health challenges that commonly occur during the nursery phase, gastrointestinal dysfunction is generally involved. Typically, a combination of prolonged intestinal inflammation, immature immune system and transitioning gut microbial communities result in a compromised gut epithelial lining, decreased nutrient digestibility, and increased susceptibility to pathogen infection (Pluske et al., 1997; Pluske et al., 2002; Lee and Mazmanian, 2010; Kim et al., 2012; Brestoff and Artis, 2013; Campbell et al., 2013; Heo et al., 2013; Fouhse et al., 2016; Gresse et al., 2017; Moeser et al., 2017). Together, these conditions can lead to a higher incidence of diarrhea, resulting in higher weaned pig morbidity and mortality.

Conventional approaches to reduce the impact of weaning on nursery pig health and performance have typically combined antibiotic use to reduce the pathogen load with inclusion of high-quality protein ingredients to facilitate digestion and absorption (Maxwell et al., 2001). However, implementation of stricter regulations on the prophylactic use of medically important antimicrobials, as well as higher costs of traditionally used protein sources such as fish meal, have created a need for effective substitutes and the development of innovative strategies. For instance, products such as essential oils and antimicrobial peptides are becoming more widely used as alternative antimicrobials, while modified plant ingredients with reduced levels of anti-nutritional

factors (e.g. enzymatically or microbially modified soybean meal) are being included as lower cost protein-rich sources in dietary formulations (Franz et al., 2010; Koepke et al., 2017; Sinn et al., 2017). In addition to these substitutes, feed additives are also developed to target other functions, such as enhancing the immune response of weaned pigs (e.g. immunoglobulin or omega-3 fatty acids), stimulating digestive functions (e.g. butyrate, glutamate, threonine or cysteine), or promoting the establishment of beneficial gut microorganisms (probiotics, prebiotics) (de Lange et al., 2010; Berrocoso et al., 2012; Yuan et al., 2017).

Amongst the various products available, peptides have the unique potential to be used as multipurpose feed additives. Indeed, they are cost effective means of providing amino acids, as they are more stable, soluble, and can be absorbed at a faster rate than free amino acids (Webb et al., 1992; Lindemann et al., 2000; Hou et al., 2017). In addition, certain types of peptides can control various physiological functions by acting as either antimicrobials, antioxidants, immuno-modulators or signaling molecules (Bhat et al., 2015; Hou et al., 2017; Nasri, 2017). In the case of bioactive peptides supplemented in feed, they may act on either host cells and / or on the host's microbiome (Xiong et al., 2014; Xiao et al., 2015; Wang et al., 2016). As an example of peptide signaling to host cells, exorphins have been shown to modulate gastrointestinal motility, secretions, and endocrine metabolism once they have been released by digestion and absorbed by the gut epithelium (Froetschel, 1996). Conversely, modulation of gut microbiome composition by certain antimicrobial peptides has also been reported. For instance, colicins and cecropin AD can help control the proliferation of *Escherichia coli* strains that can cause post weaning diarrhea in swine (Stahl et al., 2004; Wu et al., 2012;

Wang et al., 2016). Antimicrobial peptides can also have positive effects on performance. Indeed, feeding a combination of lactoferrin, cecropin, defensin and plectasin resulted in higher average daily gain and final body weight compared to supplemented diets (Tang et al., 2012). Similarly, apparent total tract digestibility of either dry matter or crude protein was found to be higher with dietary supplementation of the antimicrobial peptide-P5 (Yoon et al., 2012).

Considering the importance of beneficial gut microbial communities for animal health and nutrition, manipulating the gut microbiome using peptides would represent an additional tool towards improving resistance to pathogens, optimizing the use of alternative feed ingredients or providing other benefits to the host animal. Typically, bioactive peptides remain inactive until they are released from their parent protein as a result of chemical, enzymatic, or microbial hydrolysis (Korhonen, 2009). Since their functional characteristics would depend on their length as well as their amino acid composition and sequence (Hou et al., 2017; Nasri, 2017), there likely exists a wide range of potential bioactive peptides that have yet to be identified or characterized. Indeed, the search for novel bioactive peptides is still ongoing even for highly investigated sources such as milk (Zanutto-Elgui et al., 2019). Thus, a reasonable expectation would then be that many peptide formulations would contain bioactive peptides that can perform functions other than simply supplying dietary amino acids. However, as the effects of peptide feed additives on the gut microbiome of food animals remain largely unexplored, additional insight is required to develop further improvements in this field.

In this context, the aim of the study presented in this report was to determine the effect of a commercially-formulated peptide additive, Peptiva, on the performance and

fecal bacterial communities of weaned pigs raised in a commercial wean-to-finish swine facility. This product has been previously reported as an acceptable protein supplement in nursery diets (Zhao et al., 2008), but had not been tested at low inclusion levels. In the current study, Peptiva supplementation did not result in improved weight gains or feed efficiency of weaned pigs under the conditions tested, but it was found to affect the fecal microbiome composition of animals during the first few weeks after weaning.

2. Materials and Methods

2.1 Animal performance trial and sample collection

The animal trial was conducted at the South Dakota State University (SDSU) Off-Site Wean-to-Finish Barn, with all procedures approved by the SDSU Institutional Animal Care and Use Committee before the start of the study (Protocol 17-035A). This swine facility is managed as a commercial-scale livestock barn to conduct nutritional and animal health research that can benefit producers in this sector. Weaned pigs (21 d of age, 5.6 ± 1.2 kg) were randomly allocated to 45 pens (24 pigs/pen), with each pen randomly assigned to one of three experimental diets: control diet (CON; formulated to meet the NRC (2012) nutrient requirements), Peptiva (PEP; control diet supplemented with Peptiva), and PEP with reduced amino acid content (PEP10; dietary amino acid content at 90% of NRC (2012) recommendations). All other dietary nutrients met or exceeded NRC (2012) recommendations for weaned pigs. Experimental diets were fed according to a standard nursery phase feeding program (Supplementary Table 1): Phase I (d0-d7), Phase II (d8-d21), and Phase III (d22 – 42). Peptiva is a commercial product manufactured by Vitech Bio-Chem Corporation (Glendale, CA, USA) which consists of fish peptides,

porcine digests and microbial peptides. In both PEP and PEP10 diets, Peptiva was included at 1%, 0.5%, and 0.3%, during Phases I, II, and III, respectively. The swine facility was separated into eight blocks based on pen location within the barn, and each treatment was equally represented in each block. Use of antibiotics for treatment of scours or poor health were administered on an individual pig basis using injectable antibiotics. No mass antibiotic treatment via feed or water medicator was used during the course of the trial.

Body weights of the animals were measured by pen at the start of the trial, then on a weekly basis until the end of Phase III. Individual pig weights were determined at the beginning of the trial, at the end of Phase II and at the end of Phase III. Samples for microbiome analysis were collected at the end of Phase II and at the end of Phase III from ten animals fed the CON diets and ten individuals fed the PEP diet. More specifically, two representative individuals from each of five representative pens were selected for fecal sample collection for each diet. Pen weight was used to identify representative pens for each dietary treatment, and individual weight was used to identify representative animals from each selected pen. Fecal samples were collected by rectal palpation, then stored frozen (-20 °C) until microbial genomic DNA extraction was performed.

At the conclusion of the trial, pens were randomly allotted to a separate grow finish trial, and the animals were marketed after achieving 130 kg body weight.

2.2 Microbial DNA isolation and PCR amplification of the 16S rRNA gene

Microbial genomic DNA was isolated from fecal samples using the repeated bead beating plus column method, as previously described [35]. The V1-V3 region of the bacterial 16S rRNA gene was PCR-amplified using the 27F forward (Edwards et al., 1989) and 519R reverse (Lane et al., 1985) primer pair. PCR reactions were performed with the Phusion Taq DNA polymerase (Thermo Scientific, Waltham, MA, USA) under the following conditions: hot start (4 min, 98 °C), followed by 35 cycles of denaturation (10 s, 98 °C), annealing (30 s, 50 °C) and extension (30 s, 72 °C), then ending with a final extension period (10 min, 72 °C). PCR products were separated by agarose gel electrophoresis, and amplicons of the expected size (~500bp) were excised for gel purification using the QiaexII Gel extraction kit (Qiagen, Hilden, Germany). For each sample, approximately 400 ng of amplified DNA were submitted to Molecular Research DNA (MRDNA, Shallowater, TX, USA) for sequencing with the Illumina MiSeq (2X300) platform to generate overlapping paired end reads.

2.3 Computational analysis of PCR generated 16S rRNA amplicon sequences

Unless specified, sequence data analysis was performed using custom written Perl scripts (available upon request). Raw bacterial 16S rRNA gene V1-V3 amplicon sequences were provided by Molecular Research DNA as assembled contigs from overlapping MiSeq (2X300) paired-end reads from the same flow cell clusters. Reads were then selected to meet the following criteria: presence of both intact 27F (forward) and 519R (reverse) primer nucleotide sequences, length between 400 and 580 nt, and a

minimal quality threshold of no more than 1% of nucleotides with a Phred quality score lower than 15.

Following quality screens, sequence reads were aligned, then clustered into Operational Taxonomic Units (OTUs) at a genetic distance cutoff of 5% sequence dissimilarity (Opdahl et al., 2018). While 3% is the most commonly used clustering cutoff for 16S rRNA, it was originally recommended for full length sequences, and may not be suitable for the analysis of specific subregions since nucleotide sequence variability is not constant across the entire length of the 16S rRNA gene. In this context, if 3% is a commonly accepted clustering cutoff for V4 or V4–V5 regions, which are the least variable of the hypervariable regions, then a higher cutoff should be used for the V1-V3 region, since V1 is the most variable region of the 16S rRNA gene. OTUs were screened for DNA sequence artifacts using the following methods. Chimeric sequences were first identified with the chimera.uchime and chimera.slayer commands from the MOTHUR open source software package (Schloss et al., 2009). Secondly, the integrity of the 5’ and 3’ ends of OTUs was evaluated using a database alignment search-based approach; when compared to their closest match of equal or longer sequence length from the NCBI nt database, as determined by BLAST (Altschul et al., 1997), OTUs with more than five nucleotides missing from the 5’ or 3’ end of their respective alignments were discarded as artifacts. Single read OTUs were subjected to an additional screen, where only sequences that had a perfect or near perfect match to a sequence in the NCBI nt database were kept for analysis, i.e. that the alignment had to span the entire sequence of the OTU, and a maximum of 1% of dissimilar nucleotides was tolerated.

After removal of sequence chimeras and artifacts, taxonomic assignment of valid OTUs was determined using a combination of RDP Classifier (Wang et al., 2007) and BLAST (Altschul et al., 1997). The List of Prokaryotic Names with Standing in Nomenclature (LPSN - http://www.bacterio.net) was also consulted for information on valid species belonging to taxa of interest (Parte, 2014).

2.4 Statistical analyses

Analysis of performance data was performed using the PROC MIXED procedure of SAS (Version 9.4; SAS Inst. Inc., Cary, NC) with pen as the experimental unit and pen nested within block as the random variable. Dietary treatment was considered the fixed effect. Data were *a priori* tested for normal distribution and homogeneity of variances. Initial body weight was used as covariate for analysis of weekly body weight. Differences between treatment means were tested using Tukey's adjusted means test where a significant main effect was observed, and data are presented as lsmeans +/- standard error of the mean. A Chi-squared test was used to evaluate the distribution of total pigs removed by treatment.

Using R (Version R-3.2.3), ANOVA (command aov) and post hoc Tukey Honest Significant Difference (command TukeyHSD) analyses were performed to compare the abundance of bacterial taxonomic groups and OTUs between different groups, respectively. Means were considered to be significantly different when $P \leq 0.05$, and a tendency towards statistical significance was indicated when $0.05 < P \leq 0.10$.

3. Results

3.1 Effect of low inclusion of Peptiva on swine performance during the nursery phase

To test the ability of Peptiva to improve the availability of dietary amino acids in swine nursery phase diets, animals fed a Peptiva-supplemented diet that included only 90% of the recommended amino acids requirements for nursery phase diets (PEP10) were compared to animals fed the control diet (CON). After the first 3 weeks, no effect of diet on body weight was observed ($P > 0.05$; Table 1). Starting at week 4, however, pigs fed the CON diet tended to be heavier than PEP10-fed pigs ($P = 0.07$), with CON-fed pigs continuing to be heavier than PEP10-fed pigs through to week 6 ($P < 0.05$). While there was no difference in average daily feed intake during Phases I and II across dietary treatments, an effect of diet on daily feed intake during Phase III was observed, where CON-fed pigs had greater daily intake than PEP-fed pigs ($P < 0.05$). No effect of dietary treatment on average daily weight gain or gain: feed was observed. While there were 3% fewer pigs removed from the PEP10 group compared to the CON group for the entire trial period (6 wks), a statistical difference in net pig removal rate by diet was not detected. No significant differences were noted in pen weight variation amongst treatment groups.

3.2 Effect of diet composition and Peptiva supplementation on the fecal bacterial profile of weaned pigs

To investigate the potential of Peptiva as a modulator of gut microbiome composition in weaned pigs, a comparative analysis using fecal bacterial communities as

a proxy was performed on samples collected at the end of Phase II and at the end of Phase III. The average number of high-quality, non-chimeric reads for 16S rRNA gene sequences across the four sample sets (CON II, CON III, PEP II and PEP III) ranged from 14972 ± 2792 to 26020 ± 3191 (Supplementary Table 2), with numerical differences amongst means not found to be significant ($P = 0.16$). Firmicutes was the most highly represented phylum, with sample set averages ranging from 77.4% to 85.3% (Table 2). While these variations in abundance at the phylum level were not found to be significant, the differences in representation for three families belonging to Firmicutes were supported by ANOVA. *Lactobacillaceae* were more abundant ($P < 0.05$) in CON II samples (44.8%) than in samples from pigs fed the PEP II, CON III or PEP III diets (13.0% – 16.0%). In contrast, *Streptococcaceae* were in lower abundance in CON II compared to CON III, and *Erysipelotrichaceae* were found at higher levels in PEP II samples compared to CON III or PEP III ($P < 0.05$). Other well represented families belonging to Firmicutes included *Lachnospiraceae* (5.9% - 13.2%) and *Clostridiaceae1* (5.9% - 18.9%), but the observed differences in abundance were not supported by ANOVA. The second most abundant phylum was Bacteriodetes, with *Prevotellaceae* identified as its most highly represented family (11.8% - 16.0); variation across datasets was not found to be significant for either of these taxonomic groups.

3.3 Comparative analysis of fecal bacterial composition by alpha and beta diversity

Community level compositional differences amongst fecal bacterial communities from CON II, CON III, PEP II and PEP III sample sets were further assessed using alpha and beta diversity analyses. A combined total of 8429 OTUs were identified across all

samples analyzed (Supplementary Table 3). No statistical difference was observed amongst means of the four dietary treatments for either observed OTUs, Ace, Chao1, Shannon or Simpson indices ($P > 0.05$; Table 3). However, principal coordinate analysis (PCoA) based on Bray-Curtis OTU composition dissimilarity revealed that samples could be clustered into three different groups according to their fecal bacterial community composition (Figure 1). Furthermore, uneven distribution of samples from different sets amongst the three clusters of the PCoA plot suggested that distinct OTU profiles could be associated with the fecal environments of particular sets of samples.

3.4 Identification of weaned pig OTUs responding to distinct dietary treatments

As the comparative taxonomic composition analysis and PCoA both indicated differences in bacterial composition amongst sample sets, the individual profiles of major OTUs were further investigated. A total of 23 OTUs that were found to have a mean relative abundance of at least 1% in at least one sample set were designated as major OTUs. Of these most abundant OTUs, at least seven were likely to correspond to uncharacterized species, as they each showed less than 95% sequence identity to their respective closest valid taxon. Thirteen major OTUs, all affiliated to Firmicutes, were found to vary across sample types ($P < 0.05$) (Table 4). Pair-wise differences between specific samples for nine of these varying OTUs were further revealed by the post-hoc Tukey honest significant difference test (Figure 2). Notably, the respective abundances of OTUs Ssd-00002, Ssd-00025 and Ssd-00053 were found to be significantly different in CON II compared to PEPII, CON III and PEP III sample sets ($P < 0.05$). OTUs Ssd-00019, Ssd-00048 and Ssd-00106 showed a slightly different profile, with their

respective abundances being significantly different between CON II and either CON III or PEP III ($P < 0.05$), while no significant difference was found between PEPII and either CON II, CON III or PEP III. Also, while Ssd-00140 was found at similar levels in CON II and PEP II, its abundance in these sample sets was significantly lower than in CON III and PEP III ($P < 0.05$).

3.5 Associations between main OTUs and dietary treatments

A correspondence analysis was conducted to further explore potential associations between main OTUs and dietary treatments (Figure 3). All CON II samples clustered together with OTUs Ssd-00002, Ssd-00019, Ssd-00025, Ssd-00053 and Ssd-000106. CON III and PEP III samples were clustered into two groups, with the major group being closely associated with OTUs SSd-00048, OTUs SSd-00061 and OTUs SSd-00140, while the minor group was closely associated with OTU Ssd-00001. PEP II samples showed a very distinct distribution pattern, as half of the samples clustered with the CON II group, while the remaining samples were associated with the CON III - PEP III major cluster.

4. Discussion

Products manufactured by hydrolysis of conventional protein ingredients have the potential to include bioactive peptides that can provide other functions or benefits in addition to supplying dietary amino acids. In this study, a commercial peptide-based additive, Peptiva, was tested as a possible source of bioactive molecules using two methods. First, its ability to compensate for reduced inclusion of dietary amino acid

levels in weaned pig diets, by increasing the digestibility or the efficiency of use of protein ingredients, was assessed. In the context of a commercial swine production system as used in this study, there was no difference in performance during Phases I and II post-weaning, but PEP 10-fed pigs were found to weigh significantly less than CON-fed pigs by the end of Phase III. These results would indicate that, at least in the first 6 weeks post-weaning, Peptiva supplementation at low inclusion levels was not sufficient to compensate for a 10% reduction in dietary amino acid levels.

The second potential activity of the Peptiva product investigated in this study was the ability to change or modulate the composition of the gut microbiome in weaned pigs. Since the composition of gut microbial communities has been associated with the health status and performance of individual hosts (Richards et al., 2005; Bäckhed et al., 2015; Gresse et al., 2017; Kim and Isaacson, 2017), compounds that can change gut symbiont profiles have the potential to be developed as tools to improve critical livestock production parameters (Han et al., 2018). To this end, fecal bacterial communities were used as a proxy for gut microbiome composition analysis in weaned pigs, from which two main observations were made: evidence of bacterial succession between Phase II and Phase III in control-fed animals, and a stage-specific effect in Pep-fed pigs.

4.1 Bacterial succession from Lactobacillaceae in Phase II to Streptococcaceae in Phase III

A comparison of the samples collected from control-fed pigs between Phase II and Phase III diets was suggestive of microbial succession, as major changes in taxonomic profiles and OTU composition were observed. For instance, members of the

Lactobacillaceae family were found to be more abundant at the end of Phase II compared to the end of Phase III, which included four main OTUs (Ssd-0002, Ssd-00019, Ssd-00025, and Ssd-00053). In young animals, *Lactobacilli* have been reported to prevent adhesion of pathogens to the gut mucosa, inhibit growth of pathogens through production of lactate, and / or stimulate colonization of beneficial bacteria (Fouhse et al., 2016; Huang et al., 2004; Yang et al., 2015; Valeriano et al., 2017). Because of these types of activities, *Lactobacillus* species are considered beneficial to the gastrointestinal tract of animals and are typically included in probiotic formulations. For instance, a probiotic formulation containing *L. gasseri, L. reuteri, L. acidophilus* and *L. fermentum* was reported to result in fewer incidences of diarrhea in weaned pigs and to lower *E. coli* counts after a pathogen challenge (Huang et al., 2004), while weaned pigs supplemented with *L. reuteri* were found to have higher average daily gain, longer ileal villi, as well as increased expression of the tight junction protein zonula occludens -1 (Yi et al., 2018). *Lactobacilli* have also been reported to have antimicrobial activity, as observed with *L. reuteri* which can inhibit the growth and mucosal adherence of enterotoxigenic *E. coli* (Wang et al., 2018), and *L. gasseri* which is known to produce a bacteriocin (Ritter et al., 2009). In the current study, three of the four most abundant *Lactobacillus*-affiliated OTUs were found to be closely related to *L. reuteri* or *L. gasseri.*

At the end of Phase III, members of the *Streptococcaceae* family became the most predominant bacterial group of the fecal microbiome in weaned pigs, while the abundance of *Lactobacillus*-affiliated bacteria was greatly reduced. Since the sequence identity to their respective closest *Streptococcus* relatives ranged between 90% and 96%, main OTUs Ssd-00039, Ssd-00048, Ssd-00061 and Ssd-00140 most likely corresponded

to uncharacterized species of this genus. While the biological activities of *Streptococci* in the gut have not been as extensively studied as for *Lactobacilli,* members of this genus are also known to be lactate producers and to express bacteriocin, and thus could be involved in protection against pathogen proliferation in weaned pigs (Georgalaki et al., 2002).

Of the factors that may be responsible for these observed changes in bacterial composition in pigs fed control diets, differences in diet formulation between Phase II and Phase III offer a reasonable explanation. Notably, three ingredients (dried whey, fish meal and zinc oxide) were included in Phase II diets, but not in Phase III diets (Supplementary Table 1). As its primary use is to prevent diarrhea, zinc oxide represents a likely candidate modulator of gut microbiome composition (Hojberg et al., 2005; Shen et al., 2014; Starke et al., 2014; Xia et al., 2017). However, its target bacterial groups in gut environments remain to be further investigated, as exemplified by two conflicting studies, one observing a decrease in *Lactobacilli* as a result of dietary inclusion of zinc oxide (Hojberg et al., 2005), while the other reported no effect (Li et al., 2001). Similarly, further investigations will be required to determine the effects of dried whey and fish meal, both used as high-quality protein ingredients, on the gut microbiome of weaned pigs.

4.2 Stage-specific effect of Peptiva on the microbiome of weaned pigs

The second main observation from the comparative analysis of fecal bacterial communities performed in this study was that the profiles of PEP II samples appeared to be intermediate between CON II and CON III profiles. This was well illustrated by

correspondence analysis, where PEP II samples appeared to be divided into two groups, with certain samples more similar to CON II profiles while others were more similar to CON III profiles. At the OTU level, the respective abundances of Ssd-00002, Ssd-00025, Ssd-00053 in PEP II were found to be statistically different from CON II, but not from CON III. In contrast, no difference in abundance was found for Ssd-000140 between PEP II and CON II samples, which were however both significantly lower than those observed in the CON III samples. Other OTUs, such as Ssd-00019, Ssd-00048, and Ssd-00106, were found to be statistically different between CON II and CON III, while no significant pair-wise difference was found between either CON II and PEP II or between CON III and PEP II. Finally, no major differences in fecal bacterial profiles were observed between CON III and PEP III samples, indicating that both sets of fecal bacterial communities had reached similar compositional profiles. While additional research will be required to further elucidate the mechanisms responsible for these effects, the results presented in this study would suggest that Peptiva can promote maturation of swine fecal bacterial communities during a specific period of the nursery phase.

5. Conclusions

In the context of the current understanding of gut microbiome development, early events that impact bacterial composition can have long term effects that persist in adults. For food animal production, this would suggest that development of practices or diet formulations that can establish more resistant, resilient and efficient gut microbiomes in neonates would provide lasting benefits into the growing and finishing stages. Based on their potential for producing a vast array of bioactive molecules, peptide formulations

represent a largely untapped source of compounds that could be further developed into feed additives to benefit animal health and nutrition.

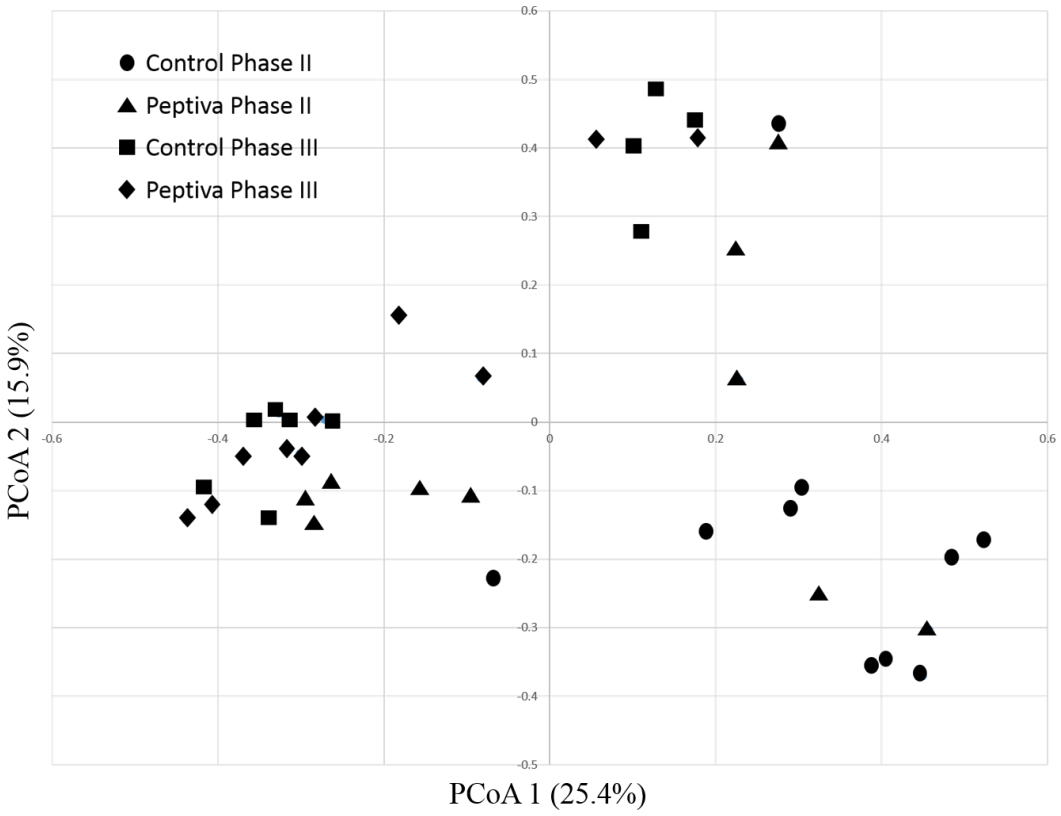
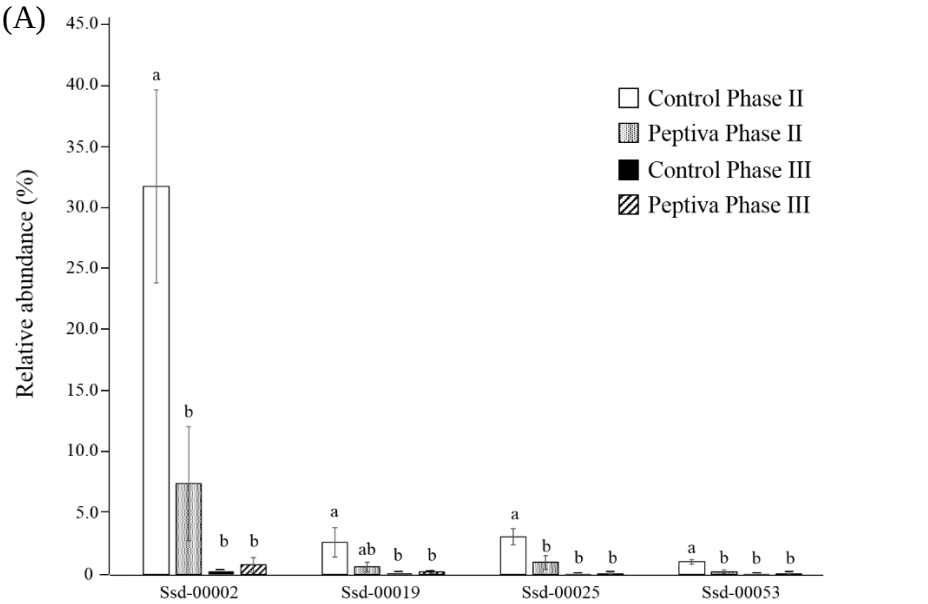


Figure 2.1. Comparison of fecal bacterial communities from weaned pigs under two different diets at two different time points. Principle Coordinate Analysis (PCoA) was performed using OTU composition-based Bray-Curtis dissimilarity. The x and y axes correspond to Principal Components 1 (PC1) and 2 (PC2), which explained the highest level of variation.

(A)



(B)

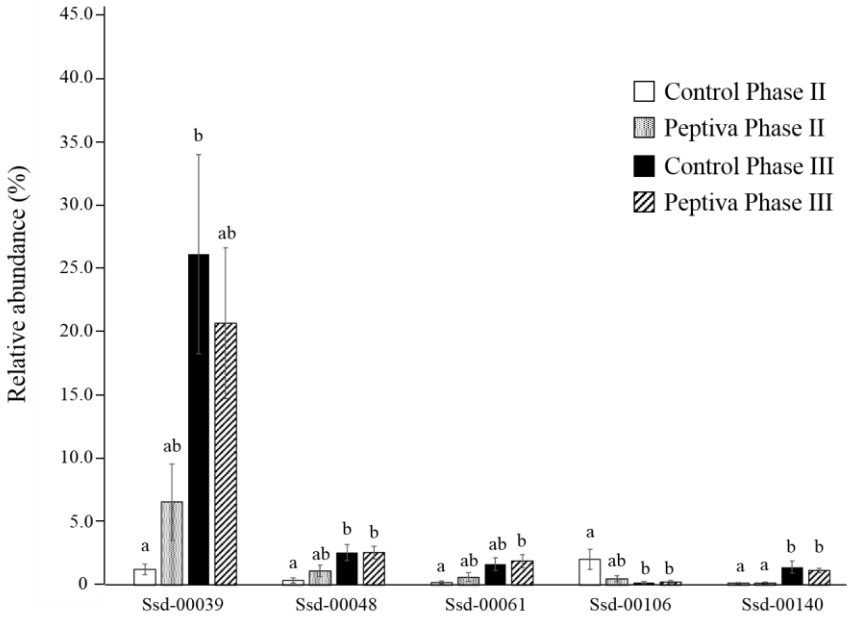


Figure 2.2. Main bacterial OTUs whose respective abundance was found to vary significantly amongst groups based on the post-hoc Tukey honest significant difference test ($P < 0.05$). OTUs affiliated to the genus *Lactobacillus* are shown in panel (A) while OTUs affiliated to the genera *Streptococcus* or *Roseburia* are shown in panel (B). For each OTU, means with different superscripts were significantly different as determined by the Tukey honest significant difference test.

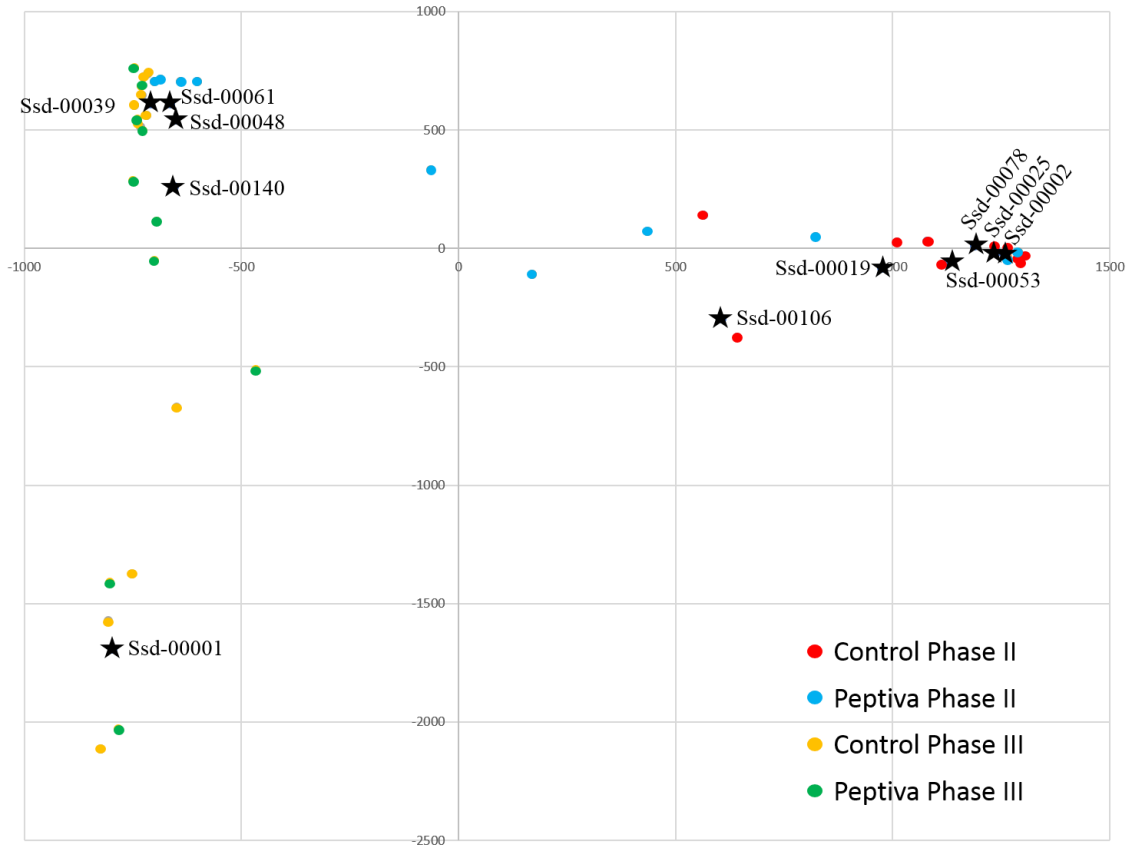


Figure 2.3. Correspondence analysis (CA) between sample type (circle) and main OTUs (star).

Table 2.1. Growth performance of weaned pigs fed diets containing Peptiva formulated at 100 or 90% of amino acid requirements (NRC (2012)).

	Control	PEP	PEP-10	SEM	P-value
Body weight, kg					
d0	5.9	5.8	5.7	0.1	0.602
d6	6.2	6.2	6.2	0.2	0.948
d13	7.8	7.7	7.6	0.1	0.267
d20	10.4	10.2	9.9	0.3	0.396
d27	12.6[a]	12.4[ab]	12.1[b]	0.2	0.067
d34	16.2[a]	15.8[ab]	15.2[b]	0.2	0.011
d41	20.8[a]	20.5[ab]	19.4[b]	0.3	0.008
Average daily gain, kg/d					
d0 – d7	0.063	0.054	0.062	0.024	0.958
d8 – d21	0.256	0.253	0.234	0.014	0.476
d22 – d42	0.471	0.469	0.440	0.019	0.415
Average daily feed intake, kg/d					
d0 – d7	0.108	0.096	0.097	0.014	0.797
d8 – d21	0.309	0.300	0.294	0.022	0.892
d22 – d42	0.659[a]	0.614[b]	0.633[ab]	0.012	0.034
Gain:feed, kg:kg					
d0 – d7	0.54	0.52	0.60	0.16	0.928
d8 – d21	0.81	0.83	0.76	0.05	0.528
d22 – d42	0.71	0.78	0.70	0.03	0.180
Pigs removed, #/pen	2.4	1.9	2.0	0.42	0.700
Total removed, #	36	35	26		
Total started, #	360	383	360		
Removal, %	10	9.1	7.2		
Pen coefficient of variation					
d0	0.230	0.199	0.226	0.011	0.083
d21	0.248	0.247	0.249	0.021	0.999
d42	0.240	0.242	0.269	0.021	0.545

Means with different superscripts are significantly different as determined by the Tukey honest significant difference test

Table 2.2. Mean relative abundance (%) and standard error of the mean for the main bacterial taxonomic groups in representative fecal samples from four dietary treatments

Taxonomic affiliation	Con PII	Pep PII	Con PIII	Pep PIII
Firmicutes	81.3 ± 6.1	77.4 ± 7.7	81.7 ± 6.6	85.3 ± 6.8
Lactobacillaceae#	44.8^{a} ± 9.0	13.4^{b} ± 6.8	13.0^{b} ± 5.1	16.0^{b} ± 5.9
Lachnospiraceae	13.2 ± 2.9	12.7 ± 2.4	7.8 ± 2.4	5.9 ± 1.3
Erysipelotrichaceae#	1.5^{ab} ± 0.5	3.7^{b} ± 1.5	0.7^{a} ± 0.2	0.7^{a} ± 0.2
Ruminococcaceae	5.4 ± 1.6	6.1 ± 2.0	2.2 ± 0.5	2.3 ± 0.7
Clostridiaceae1	5.9 ± 3.3	17.3 ± 6.2	11.8 ± 6.6	18.9 ± 6.0
Peptostreptococcaceae	1.2 ± 0.4	4.2 ± 2.5	1.1 ± 0.3	2.5 ± 1.0
Streptococcaceae#	2.1^{a} ± 0.7	9.0^{ab} ±4.3	32.2^{c} ± 9.2	25.6^{bc} ± 6.6
Veillonellaceae	0.5 ± 0.2	1.9 ± 1.3	3.4 ± 1.2	3.1 ± 0.9
unclassified Clostridiales	3.1 ± 1.0	4.2 ± 0.7	2.2 ± 0.4	2.8 ± 0.7
Other Firmicutes	3.6 ± 0.8	4.8 ± 1.1	7.4 ± 1.5	7.4 ± 1.1
Bacteroidetes	16.3 ± 5.9	20.4 ± 7.1	17.4 ± 6.5	13.5 ± 6.8
Prevotellaceae	14.0 ± 6.1	13.1 ± 6.4	16.0 ± 6.4	11.8 ± 6.8
Porphyromonadaceae	1.4 ± 0.6	4.5 ± 2.2	0.5 ± 0.2	1.1 ± 0.8
Other Bacteroidetes	0.8 ± 0.2	2.7 ± 1.8	0.8 ± 0.3	0.6 ± 0.3
Other Phyla	1.5 ± 1.0	1.2 ± 0.7	0.3 ± 0.09	0.5 ± 0.3
Unclassified Bacteria	0.9 ± 0.2	1.0 ± 0.3	0.6 ± 0.1	0.7 ± 0.2

#Taxa showing a significant difference ($P<0.05$) amongst means of different treatment groups.

Means with different superscripts are significantly different as determined by the Tukey honest significant difference test

Table 2.3. Alpha diversity indices and coverage from four dietary treatments. Values are presented as means and standard error of the mean, respectively.

Index	CON II	CON III	PEP II	PEP III	*P*-value
OTUs	383 ± 45	343 ± 32.5	400 ± 48	318 ± 33	0.471
Ace	1395 ± 184	1145 ± 159	1397 ± 187	1110 ± 171	0.510
Chao1	909 ± 121	790 ± 112	920 ± 112	703 ± 86	0.448
Shannon	3.41 ± 0.33	3.12 ± 0.23	3.72 ± 0.27	3.16 ± 0.21	0.357
Simpson	0.197 ± 0.05	0.222 ± 0.03	0.121 ± 0.03	0.189 ± 0.03	0.274
Coverage (%)	92.3 ± 0.93	93.3 ± 0.73	92.1 ± 1.0	93.8 ± 0.77	0.462

Table 2.4. Mean relative abundance (%) and standard error of the mean for the most abundant OTUs in representative fecal samples from four dietary treatments.

OTUs	Con PII	Con PIII	Pep PII	Pep PIII	Closest valid taxon (id%)
Firmicutes					
Ssd-00001[a#]	0.1 ± 0.02	10.4 ± 4.9	0.07 ±0.01	12.0 ± 5.0	*L. amylovorus* (99%)
Ssd-00002[a#]	31.9 ± 7.9	0.3 ± 0.08	7.5 ± 4.7	0.8 ± 0.6	*L. gasseri* (99%)
Ssd-00008[a]	0.06 ± 0.04	0.05 ± 0.03	1.2 ± 1.1	0.2 ± 0.2	*L. mucosae* (99%)
Ssd-00019[a#]	2.6 ± 1.2	0.1 ± 002	0.6 ± 0.4	0.2 ± 0.1	*L. reuteri* (99%)
Ssd-00025[a#]	3.1 ± 0.7	0.02 ± 0.01	1.0 ± 0.6	0.05 ± 0.03	*L. taiwanensis* (95%)
Ssd-00053[a#]	1.0 ± 0.2	0.02 ± 0.01	0.2 ± 0.1	0.06 ± 0.03	*L. reuteri* (95%)
Ssd-00078[a#]	1.5 ± 0.3	0.01 ± 0.01	1.3 ± 0.8	0.03 ± 0.02	*L. taiwanensis* (88.1%)
Ssd-00013[b#]	0.1 ± 0.08	0.01 ± 0.01	1.0 ± 0.5	0.01 ± 0.01	*S. ventriculi* (98%)
Ssd-00092[b]	0.2 ± 0.1	0.8 ± 0.4	0.3 ± 0.1	1.0 ± 0.4	*C. paraputrificum* (89%)
Ssd-00238[b]	0.6 ± 0.4	0.5 ± 0.1	1.0 ± 0.3	0.8 ± 0.2	*C. saccharo.* (93%)
Ssd-00134[b]	4.3 ± 2.6	9.1 ± 5.6	13.7 ± 5.5	14.4 ± 4.8	*C. saccharo.* (97%)
Ssd-00014[c]	0.7 ± 0.2	0.6 ± 0.2	3.1 ± 2.1	1.6 ± 0.7	*T. mayombei* (97%)
Ssd-00039[d#]	1.3 ± 0.4	26.2 ± 7.9	6.6 ± 3.0	20.7 ± 6.0	*St. macedonicus* (95%)
Ssd-00048[d#]	0.4 ± 0.2	2.6 ± 0.6	1.1 ± 0.5	2.6 ± 0.5	*St. alactolyticus* (96%)
Ssd-00061[d#]	0.2 ± 0.1	1.7 ± 0.5	0.6 ± 0.4	1.9 ± 0.5	*St. alactolyticus* (90%)
Ssd-00140[d#]	0.2 ± 0.06	1.4 ± 0.5	0.2 ± 0.08	1.2 ± 0.2	*St. salivarius* (91%)
Ssd-00071[e]	0.1 ± 0.06	1.2 ± 0.6	0.08 ±0.04	1.1 ± 0.7	*M. indica* (98%)
Ssd-00188[f]	0.5 ± 0.2	1.6 ± 0.9	2.5 ± 1.4	0.6 ± 0.3	*E. rectale* (99%)
Ssd-00106[g#]	2.0 ± 0.8	0.2 ± 0.09	0.5 ± 0.2	0.3 ± 0.1	*R. faecis* (98%)
Ssd-00123[h#]	0.2 ± 0.08	0.08 ± 0.04	1.3 ± 0.7	0.03 ± 0.01	*Ca. mitsuokai* (97%)
Bacteriodetes					
Ssd-00003[i]	7.0 ± 4.1	9.6 ± 4.4	6.3 ± 3.9	6.5 ± 5.2	*P. copri* (98%)
Ssd-00502[i]	1.4 ± 1.4	0.02 ± 0.01	0.01 ±0.01	0.01 ± 0.01	*Ma. massiliensis* (84%)
Ssd-00366[j]	1.1 ± 0.5	0.4 ± 0.1	4.0 ± 2.1	0.8 ± 0.7	*Pa. distasonis* (84%)

OTUs showing a significant difference ($P<0.05$) amongst means of different treatment groups.

Taxonomic affiliations: a. *Lactobacillaceae*, b. *Clostridiaceae*, c. *Peptostreptococcaceae*, d. *Streptococcaceae*, e. *Veillonellaceae*, f. *Eubacteriaceae*, g. *Lachnospiraceae*, h. *Erysipelotrichidae*, i. *Prevotellaceae*, j. *Porphyromonadaceae*,

Abbreviations: *Ca.* : *Catenibacterium* ; *C.*: *Clostridium ; E.: Eubacterium; L.*: *Lactobacillus* ; *Ma.*: *Massiliprevotella* ; *M.: Megasphaera* ; *Pa.*: *Parabacteroides* ; *P.*: Prevotella; *R.*: *Roseburia ; saccharo.*: *saccharoperbutylacetonicum ; S.*: *Sarcina*; *St.*: *Streptococcus* ; *T.*: *Terrisporobacter*.

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Chapter 4:

Effects of inclusion of peptide based commercial products, MOS and protease in weaned pigs' diets on growth performance and fecal microbial composition

Abstract

Different commercial feed additives have been designed and used successfully as antibiotic alternatives to prevent digestive disorders and lower animal performance during postweaning period of pigs. This study was aimed to investigate the impact of peptide-based product along with mannose oligosaccharides (MOS) and protease on the performance and fecal microbiome of weaning pigs. Approximately 1,125 weaned pigs were randomly assigned to one of four experimental diets: Control, PeptivaM, PeptivaM with protease, and PeptivaM with reduced amino acid content. Experimental diets were fed as a standard nursery phase feeding program: Phase II (d8-d21) and Phase III (d22 – 49). No statistically significant effect of experimental diets on body weight was observed in all phases. While, the pigs fed PeptivaMp showed higher ($p < 0.05$) average daily gain in phase II compared to others. Significant effect of PeptivaM on gain:feed was observed at phase II in comparison to control. While, there was reduced ($P < 0.05$) feed efficiency in PeptivaMp 10 compared to control showing no effect of it to compensate the reduced amino acid level in the diet. Bacterial profiles determined by analysis of high throughput sequencing data generated from PCR-amplified DNA targeting the V1-V3 region of the 16S rRNA gene determined the effect of phases and experimental diets. The bacterial profiles of control phase II and control phase III fecal samples were found to be very distinct, suggesting that a transition or succession stage had occurred between the two phases. *Lactobacillus,* represented by two OTUs, Ssd-00001 and Ssd-00123 were most abundant ($P < 0.05$) in phase III, while *Ruminococcus*, represented by one OTU was highly abundant ($P < 0.05$) in phase II. The taxonomic and OTU composition were

affected by the treatments. For instance, *Streptococcus*, represented by one OTU Ssd-00039 was most abundant in PeptivaMp phase III, while *Lactobacillus* represented by another OTU Ssd-00123 was most abundant in PeptivaMp phase II which is similar to control phase II. Together, these results showed Peptiva along with MOS and protease can modulate the swine gut microbiome during nursery period.

1. Introduction

The management and feeding strategies to stimulate gut development and maintaining gut heath are very important aspects during weaning transition of young pig which have direct influence to improve feed efficiency, pig health and growth to market, hence profitability. These strategies at this stage should be aimed to improve productivity around the weaning time and minimize the use of antibiotics and expensive feed ingredients (de Lange et al., 2010). After weaning, the young piglets face series of abrupt changes such as removal from the sow, moving to a new environment and mixing with unfamiliar animals (Campbell et al., 2013; Pluske, 2016). Most importantly, the abrupt change of diet from highly digestible and palatable liquid milk from their sows to less digestible and palatable plant based dry feed is the most limiting factor that causes reduction in feed intake following the week after weaning (van Beers-Schreurs et al., 1998; Dong and Pluske, 2007; Campbell et al., 2013) which, in turn has negative effects on gut function and increased susceptibility to enteric pathogens and other disorders along with lower immune protection (Pluske et al., 1997; Madec et al., 1998; Dong and Pluske, 2007). Along with low feed intake, the weaned piglets experience physiological changes in enzymes activities and absorption and secretion in the gut. Weaning induces both acute and long lasting structural and functional changes in the small intestine including shortening of villi and increase in crypt depth (Pluske et al., 1997; Boudry et al., 2004; Campbell et al., 2013). There is reduced brush border digestive enzyme activities after weaning (Pluske et al., 1997). Similarly, there is significant reduction in pancreatic secretions for trypsin, chymotrypsin, and amylase activity (Hedemann and Jensen, 2004; Lallès et al., 2004). This disruption in structure and function of small

intestine impact the digestive, absorptive, and secretary capacity along with intestinal barrier function at this young age and may contribute to post-weaning diarrhea leading to high morbidity and mortality rates, slow growth rate, and poor feed conversion with huge economic loss for the industry.

To promote early growth and muscle deposition, the diets for weaned piglets usually have high levels of protein. However, due to impaired digestion, absorption and enzymatic activities of small intestine due to weaning stress, the high quantity of dietary nutrients like proteins may accumulate in the gut and promote microbial fermentation that causes dysbiosis leading to proliferation of pathogenic bacteria (Htoo et al., 2007; Tactacan et al., 2016). Additionally, the bacterial fermentation of undigested protein produces volatile fatty acids (VFAs) and other substances like ammonia and amines that can induce diarrhea and reduced growth (Porter and Kenworthy, 1969; Dong et al., 1996; Gaskins, 2000) in weaning piglets and also the excess ammonia excretion (Nahm, 2003; Tactacan et al., 2016).

Soybean meal is generally considered as a primary source of protein for swine diet but due to presence of anti-nutritional factors and lower methionine and lysine content compared with animal source protein (Friedman and Brandon, 2001; Jo et al., 2012). Soybean anti-nutritional factors include trypsin inhibitors, lectins of which only trypsin inhibitors are influenced by heat. A bigger issue of soybean meal is in young pigs is the main soy proteins, conglycinin and B-conglycinin which are not inactivated by heat which can lead to poor digestibility and adverse nutritional effects (Friedman and Brandon, 2001). Moreover, overheating may negatively affect the value of proteins and

the availability of some amino acids lysine and arginine (Choe et al., 2017). As an alternative, the dietary supplementation of single or multiple enzyme preparations such as α-amylase, β-mannanase, xylanase, phytase, cellulose, and protease to the diets of pigs and poultry is very common due to its beneficial effects (de Souza et al., 2007; Cowieson and Ravindran, 2008; Yoon et al., 2010; Jo et al., 2012). Proteases supplementation can degrade protein anti-nutritional factors in the feed (Rooke et al., 1998; Guggenbuhl et al., 2012) and can improve its the energy value as they can help to degrade starch bound proteins thus increasing starch digestibility (Wang et al., 2008). Protease supplementation has shown improved feed efficiency, protein utilization, nutrient digestibility, growth performance, and lower manure odor emission in grower-finisher pigs fed different basal diets (Guggenbuhl et al., 2012; O'Shea et al., 2014; Upadhaya et al., 2016; Choe et al., 2017). Moreover, there was improved growth rate, nutrient digestibility, improved intestinal development, enzymes activities of stomach pepsin, pancreatic amylase and trypsin, and reduced fecal NH_3 emission in feces in weaned piglets (Guggenbuhl et al., 2012; Zuo et al., 2015; Tactacan et al., 2016). On contrary, in another study, protease treatment of soybean meal had no effect on ileal digestibilities of CP and AAs in newly weaned piglets (Caine et al., 1997).

Antibiotics were used to reduce diarrhea incidences and to promote growth for young piglets, however, due to increased antibiotics resistance concerns, resulted in major restrictions in antibiotic use in food animal production in European Union and USA, stimulating investigations into effective alternate feed additives. In swine industry, wide researches have been done on the alternatives including probiotics, prebiotics, enzymes, acidifiers, plant extracts, and minerals such as copper and zinc and majority of

these compounds have inconsistent results and are rarely equal to antibiotics (Thacker, 2013). Among the various commercial products available, peptides are the unique alternatives as they have multiple benefits. These peptides have been demonstrated to have broad spectrum antibiotic effects against bacteria, mycobacteria, viruses, and fungi (Reddy et al., 2004). The transport of AA in the form of peptides in soy, egg white or milk protein hydrolysate was demonstrated to be faster route of uptake than free amino acids into the portal blood after duodenal infusion in rat and pigs (Rerat et al., 1988; Kodera et al., 2006). Two or three AAs can be transported into the cell by the transporter PepT1 for the same energy expenditure required to transport a single free AA which seems more energy efficient than free AAs absorption (Webb et al., 1992; Daniel, 2004). There are other dietary large peptides absorbed which have been shown to have biologic activity to modulate neural, endocrine, immune, anti-microbial, enhancing mineral absorption and availability, and antioxidant functions which is largely dependent on the source and their processing methods (Zaloga and Siddiqui, 2004; Bhat et al., 2015; Hou et al., 2017; Nasri, 2017). Dietary supplementation of various antimicrobial peptides in pigs has been reported to have positive effects on performance, nutrient digestibility, intestinal morphology, immune function, intestinal microbiota (Tang et al., 2009; Yoon et al., 2012; Xiao et al., 2015). There was enhancement of growth performance, improved nutrient digestibility, and reduction of incidence of post-weaning diarrhea in weaned piglets fed with various antimicrobial peptides individually or in mixture such as AMP-A3, AMP-A5, colicin A1, cecropin AD, cipB-lactoferricin-lactoferrampin, defensing, and plectasin (Cutler et al., 2007; Tang et al., 2012; Wu et al., 2012; Yoon et al., 2013; Yoon et al., 2014). Mannose Oligosaccharides (MOS) are complex sugars derived most

commonly from the cell wall of yeasts *Saccharomyces cerevisiae* (White et al., 2002). MOS has been reported as a viable alternative to antibiotics and potent growth promotor used in diets of pigs (A.F. and M.J, 2000; Rozeboom et al., 2005). Most of the previous studies demonstrated that addition of MOS to the diets increased ADG, feed efficiency, and higher weaning weights (P.R. et al., 2001; Davis et al., 2002).

There are no studies carried out to determine the effects of antimicrobial peptides, MOS, and protease in the feed of weaned piglets on animal performance and gut microbiome. The hypothesis of this study was that there will be increased performance and modulation effect on fecal bacterial communities by the supplementation of peptides, MOS, and protease in the diet of weaned piglets. Therefore, in this context, the aims of present study were to evaluate the effects of commercial based peptide product along with MOS and protease on the performance and fecal bacterial communities in nursery piglets.

2.Materials and Methods

2.1 Animals and diets

The animal trial was conducted at the South Dakota State University (SDSU) On-Site Wean-to-Finish Barn, with all procedures approved by the SDSU Institutional Animal Care and Use Committee before the start of the study. Approximately 1,125 weaned pigs (~ 7 kg; 21 d of age; blocked by weight) were randomly divided into 44 pens with each pen randomly assigned to one of four experimental diets (Table). The experimental diets used were Control (CON; formulated to meet nutrient requirement of

NRC 2012 requirement without peptiva and protease), PeptivaM (PEP M; control diet supplemented with PeptivaM product); PeptivaM with protease (PEP M PRO; control diet supplemented with PeptivaM product and protease); and PeptivaM with reduced amino acid content (PEP10; dietary amino acid content at 90% of NRC-2012 recommendations; otherwise met recommended requirements for all other nutrients; supplemented with Peptiva). Experimental diets were fed as a standard nursery phase feeding program (Supplementary Table): Phase II (d8-d21) and Phase III (d22 – 36). In all experimental diets, Peptiva was included at 0.3%. All pigs were received a common Phase I starter diet for 5 – 7d. Phase I and II diets contained Mecadox at 25g/ton. When necessary, water antibiotics were used to provide additional control for health-related issues. PeptivaM is a commercial product manufactured by Vitech Bio-Chem Corporation (Glendale, CA, USA) which consists of fish peptides, porcine digests and microbial peptides in combination with mannan-oligosaccharide (MOS).

2.2 Growth performance and health assessment

Piglets were randomly assigned to pens at weaning based on gate cut procedure. Treatments were randomized to pens based on mean pen weight to achieve ≤10% CV in pen weight between pens within treatment. Pens of pigs were weight at entry and at the end of each Phase. The swine facility was equipped with a Feed Logic system for feeding which was also used to monitor feed dispensed and disappearance for each pen. Diarrhea assessment was performed by pen from d0 – 10 (pen diarrhea score, incidence and duration). Veterinary treatments (reason, treatment, duration) and removals were recorded on a pen and individual pig basis for the duration of the trial. At the end of

Phase I and II, 2 representative pigs/pen was selected, based on growth performance, for blood sampling. Collected serum was analyzed for IgA as an indirect marker of intestinal inflammation.

2.3 Gut bacterial composition analysis

A comparative analysis of gut bacterial composition was performed between pigs fed the CON, PEP M and PEP PRO at phases II and III on 10 animal on each treatment. Selection of individuals for gut bacterial composition analyses was based on the performance. Collection of fecal samples was done at the start of Phase II (pre-treatment) and at the end of Phase II and III (post-treatment). Fecal samples were collected by rectal palpation, then stored frozen (-20 °C) until microbial genomic DNA extraction was performed.

2.4 Microbial DNA isolation and PCR amplification of the 16S rRNA gene

Microbial genomic DNA was isolated from fecal samples using the repeated bead beating plus column method, as previously described (Yu and Morrison, 2004). The V1-V3 region of the bacterial 16S rRNA gene was PCR-amplified using the 27F forward (Edwards et al., 1989) and 519R reverse lane (Lane et al., 1985) primer pair. PCR reactions were performed with the Phusion Taq DNA polymerase (Thermo Scientific, Waltham, MA, USA) under the following conditions: hot start (4 min, 98 °C), followed by 35 cycles of denaturation (10 S, 98 °C), annealing (30 S, 50 °C) and extension (30 S, 72 °C), then ending with a final extension period (10 min, 72 °C). PCR products were separated by agarose gel electrophoresis, and amplicons of the expected size (~500bp)

were excised for gel purification using the QiaexII Gel extraction kit (Qiagen, Hilden, Germany). For each sample, approximately 400 ng of amplified DNA were submitted to Molecular Research DNA (MRDNA, Shallowater, TX, USA) for sequencing with the Illumina MiSeq (2X300) platform to generate overlapping paired end reads.

2.5 Computational analysis of PCR generated 16S rRNA amplicon sequences

Unless specified, sequence data analysis was performed using custom written Perl scripts (available upon request). Raw bacterial 16S rRNA gene V1-V3 amplicon sequences were provided by Molecular Research DNA as assembled contigs from overlapping MiSeq (2x300) paired-end reads from the same flow cell clusters. Reads were then selected to meet the following criteria: presence of both intact 27F (forward) and 519R (reverse) primer nucleotide sequences, length between 400 and 580 nt, and a minimal quality threshold of no more than 1% of nucleotides with a Phred quality score lower than 15.

Following quality screens, sequence reads were aligned, then clustered into Operational Taxonomic Units (OTUs) at a genetic distance cutoff of 5% sequence dissimilarity (Opdahl et al., 2018). OTUs were screened for DNA sequence artifacts using the following methods. Chimeric sequences were first identified with the chimera.uchime and chimera.slayer commands from the MOTHUR open source software package (Schloss et al., 2009). Secondly, the integrity of the 5’ and 3’ ends of OTUs was evaluated using a database alignment search-based approach; when compared to their closest match of equal or longer sequence length from the NCBI nt database, as determined by BLAST (Altschul et al., 1997), OTUs with more than five nucleotides

missing from the 5’ or 3’ end of their respective alignments were discarded as artifacts. Single read OTUs were subjected to an additional screen, where only sequences that had a perfect or near perfect match to a sequence in the NCBI nt database were kept for analysis, i.e. that the alignment had to span the entire sequence of the OTU, and a maximum of 1% of dissimilar nucleotides was tolerated.

After removal of sequence chimeras and artifacts, taxonomic assignment of valid OTUs was determined using a combination of RDP Classifier (Wang et al., 2007) and BLAST (Altschul et al., 1997). The List of Prokaryotic Names with Standing in Nomenclature (LPSN - http://www.bacterio.net) was also consulted for information on valid species belonging to taxa of interest (Parte, 2014).

2.6 Statistical analyses

Growth performance was analyzed using the PROC MIXED procedure of SAS (Version 9.4; SAS Inst. Inc., Cary, NC), with pen as the experimental unit and pen as the random variable. The contrast statement was used for pre-planned comparisons. Chi-squared analysis was used to evaluate health assessment data. Differences between treatment means were tested using Tukey’s adjusted means test where a significant interaction was observed.

Using R (Version R-3.2.3), ANOVA (command aov) and post hoc Tukey Honest Significant Difference (command TukeyHSD) analyses were performed to compare the abundance of bacterial taxonomic groups and OTUs between different groups, respectively and for alpha diversity indices. Means were considered to be significantly

different when $P \leq 0.05$, and a tendency towards statistical significance was indicated when $0.05 < P \leq 0.10$.

3. Results

3.1 Effects of experimental diets on production performance of nursery pigs

The effect of PeptivaM and protease and reduced amino acid supplementation (90% of the recommended amino acids requirement) on Peptiva-supplemented diet were evaluated for nursery pigs from weaning to day 49. No statistically significant effect of three experimental diets (ANOVA, *P > 0.05;* Table 1) on body weight was observed on all phases. However, at day 35, i.e. at phase III, the pigs fed PeptivaM and PeptivaM tended (ANOVA, *P < 0.10*) to have higher body weight than pigs fed control or PeptivaMp 10 with PeptivaM intermediate. While the pigs fed PeptivaMp showed higher (*p < 0.05*) average daily gain from day 15 to day 35 compared to control and PeptivaMp 10 and in other phases there was no effects of treatments on average daily gain. Similarly, at day 36 to day 49, i.e. phase III, the pigs fed PeptivaMp 10 were observed to have higher average daily feed intake than pigs fed control diet, whereas, other dietary treatments were observed to have no effects. Significant effect of PeptivaM on gain:feed was observed from day 15 to day 35 i.e. phase II in comparison to control. In contrast, significant effect of control diet on feed efficiency (gain:feed) was observed from day 36 to day 49, i.e. phase III in comparison to other dietary treatments. Whereas, from weaning to day 35, i.e. till the end of phase II, significant effect of PeptivaM on gain:feed was observed while from weaning to day 49, i.e. phase III significant effect of PeptivaM and Control on feed efficiency was observed demonstrating similar effect of both dietary

treatments. While, it was reduced ($P < 0.05$) in PeptivaMp 10 compared to Control during the final common diet phase and overall. There was no difference in body weight, gain, or feed intake between PeptivaMp 10 and Control diets throughout the trial.

3.2 Effects of PeptivaM and Protease supplementation on fecal bacterial profile of nursery pigs

A comparative taxonomic analysis was carried out to evaluate the modulation effects of supplementation of PetivaM and the enzyme protease in the diet of nursery pigs on the fecal samples collected at the end of phase II and phase III. Of the six identified phyla across all samples, Firmicutes was the most highly represented one ranging from 71.40% to 77.89% followed by Bacteroidetes ranging from 7.06% to 13.64% (Table 2). The variations in abundance at the phylum level were not found to be statistically different among the samples. At the family level, *Lactobacillaceae* were the most abundant family ($P < 0.05$) and they were significantly higher in CON III samples (21.23%) and PEPM III (20.72%) than in samples from the piglets fed CON II, PEPM P II, PEPM P III, and PEPM II (3.08% - 14.97%). In contrast, other families *Lachnospiraceae, Ruminococcaceae, Acidaminococcaceae,* and *Eubacteriaceae* were in lower abundance in CON III, PEPM P II, PEPM P III, and PEPM II but were in significantly higher abundance ($P < 0.05$) in CON II (19.64%, 18.68%, 3.38% and 1.42% respectively). Whereas, *Streptococcaceae* were found at higher level ($P < 0.05$) in PEPM P III samples (18.88%) compared to other samples. Under the phyla Bacteriodetes, *Porphyromonadaceae* were significantly higher ($P < 0.05$) in PEPM II

samples (3.04%) than others. Other unclassified bacteria represented significantly higher ($P < 0.05$; 17.24%) in CON III samples compared to other remaining samples.

3.3 Comparative analysis of fecal bacterial composition by alpha and beta diversity

Alpha and beta diversity analyses were conducted to assess community level compositional differences amongst fecal bacterial communities from CON II, CON III, PEPM II, PEPM III, PEPM P II, and PEPM P III sample sets. There was no statistical difference identified across all the samples analyzed for either observed OTUs, Ace, Chao1, Shannon or Simpson indices (ANOVA P > 0.05; Table 4). The 32 main OTUs identified, with a mean relative abundance of at least 1% in at least one sample set, were plotted in principal coordinate analysis PCoA plot based on Bray-Curtis OTU composition dissimilarity (Figure 1). The plot indicated clear differences in bacterial OTUs composition amongst the sample sets at different phases. The samples from phase II, either from control or treatments were clustered together (cluster 1), whereas, the samples from phase III were clustered in another group (cluster 2). There were few other samples from phase II from both control and treatment were on grouped on another clusters. The uneven distribution of samples from different sets amongst the three clusters of the PCoA plot suggested that distinct OTU profiles could be associated with the fecal environments of particular sets of samples.

3.4 Identification of OTUs responding to Peptiva, MOS, and Protease dietary treatments

From a total of 4,332 OTUs identified in the study, 32 OTUs were designated as major OTUs that were found to have a mean relative abundance of at least 1% in at least one sample set. The main OTUs represented 44.38% to 49.07% of the sequence reads in different samples in which the proportion of Firmicutes related OTUs was the highest ranging from 38.30% to 45.67%. Of the total main OTUs, 19 OTUs were found to have a sequence identity of 95% or greater to their closest relative, indicating remaining 12 OTUs likely correspond to uncharacterized fecal bacterial species of nursery piglets. Fourteen main OTUs corresponding to Firmicutes and one main OTU corresponding to Actinobacteria were found to be statistically different across the samples (ANOVA, $P<0.05;$ Table 3). While further doing pairwise differences between specific samples for those 15 OTUs by using post-hoc Tukey honest test didn't show significant difference for 5 OTUs affiliated to Firmicutes. Among all the main OTUs identified the relative abundance of OTU SD_Ssd-00039 was observed to be the highest (17.19%; $P< 0.05$) in the diet supplemented with PeptivaM and protease at phase III and another OTU, SD_Ssd-00188 was also found to be in higher abundance in the same sample set. Notably, the abundance of OTUs SD_Ssd-00001 was found to different in PeptivaM phase III samples and control phase II samples. Similarly, OTUs SD_Ssd-00014, and SD_Ssd-00304 were observed to be significantly different in PeptivaM phase III samples compared to other samples. The abundances of other three OTUs, SD_Ssd-00123, SD_Ssd-00705, and SD_Ssd-00840 were found to be different in PeptivaM and protease

samples at phase II than others. While the abundances of SD_Ssd-00014 and SD_Ssd-01254 were observed to be higher in control phase I and phase II respectively.

4. Discussion

4.1 Growth Performance

The peptide product Peptiva in combination with mannan oligosaccharides (MOS) and enzyme protease used in this study was hypothesized to have a synergistic effect on nursery pig's performance. Previous studies on dietary bioactive peptides demonstrated beneficial effects on animal performance due to high content of short peptides and free amino acids which are palatable and more readily absorbed than intact protein (Gilbert et al., 2008). Our results demonstrated that the pigs on PeptivaMp 10 diet had lower body weight and feed efficiency than the control and other treatment groups although there was there was significantly higher ADF during d36 to d49. This indicated that Peptiva supplementation at lower amino acids level was not able to compensate the required dietary amino acids such as lysine, methionine, threonine, and tryptophan. In contrast, although there was no effect on BW, ADG, and ADF, PeptivaM demonstrated improved feed efficiency during d15 to d35, d0 to d35 and d0 to d49. Several studies have shown improved ADG, ADFI, digestibility of DM and CP, feed efficiency by dietary supplementation of several types of antimicrobial peptides including lactoferrin, cecropin, defensin, plectasin, and AMP-P3, P5 in nursery diets (Tang et al., 2012; Yoon et al., 2012; Yoon et al., 2013; Yoon et al., 2014; Xiao et al., 2015). For MOS, there is inconsistent results in nursery pigs as some studies (White et al., 2002; van der Peet-

Schwering et al., 2007) reported no benefits, whereas, others (Davis et al., 2002; Castillo et al., 2008; LeMieux et al., 2010) demonstrated improved growth and feed efficiency. While several publications reported improved growth rate of piglets by supplementing the sow diet with dietary MOS in the last 2-3 weeks of gestation and during lactation (Halas and Nochta, 2012). Our results showed increased ADG during d15 and d35 of dietary supplementation of protease supplemented diet on pig performance but not on BW and feed efficiency, while other studies demonstrated increased growth performance (Rooke et al., 1998; Zuo et al., 2015), increased protein digestibility, nutrient transport efficiency (Zuo et al., 2015), apparent ileal digestibility (AID) (Guggenbuhl et al., 2012) after addition of protease in weaned piglets diets. MOS used in this study consists of these CHO and protein compounds that it could similarly elicit and effect on pig growth through competitive exclusion of pathogens via competition for common bacterial binding sites, hence limiting pathogen colonization and reducing enteric infection. MOS affect this change as they consist of carbohydrates and proteins in their cell wall in the form of chained and branched structures of glucose, mannose and N-acetylglucosamine (Ballou, 1970) which can act as high affinity ligands, offering a competitive binding site for the bacteria (Ofek et al., 1977; Spring et al., 2000). The pathogens move through the intestine with MOS, without colonization that could attach the lumen of the intestine and cause enteric infection (Spring et al., 2000).Further studies are required to elucidate the combined influence of these feed additives in piglet's production performance.

4.2 Microbial succession

The composition of the microbial community and its functional capacity during weaning transition of pig production play very importance roles to establish and maintain a beneficial gut microbiota. This is very crucial in early ages as the early gut colonizers are fundamental in the establishment of stable microbial community affecting the health and growth performance of pigs later in life (Guevarra et al., 2018; Guevarra et al., 2019). The antibiotic alternative products like peptides and MOS in combination with protease evaluated in our study demonstrated modulation effects on gut microbiota between phase II and phase III and clear microbial succession of phase II and phase III microbiome of nursery pigs. The members of *Lactobacillaceae* family was significantly higher in Control phase III than in Control phase II which included four major OTUs (Ssd-00001, Ssd-00123, Ssd-00019, and Ssd-00706). Similar result was also demonstrated by the study of Kim et al. (Kim et al., 2015) where Lactobacilli was highly abundant with 11% of the total bacterial population in 10 week old pigs, whereas, it was only 3.2% in 22 week old pigs. Our study showed significantly higher abundance of OTU *Lactobacillus amylovorus* in control phase III samples than control phase II which is in contrast to the finding of Pieper et al. (Pieper et al., 2008) where they reported higher abundance of *L. amylovorus* and *L. sobrious* from day 1 to day 11 in the gut of piglets. Lactobacillus are predominant bacterial community of porcine GIT colonizing soon after birth which play an important role to influence intestinal physiology, regulate the immune system, and balance the intestinal ecology of the host (Naito et al., 1995; Judith M. Bateup, 1998; Valeriano et al., 2017) although the bacterial succession occurs throughout the pig's lifetime (Tannock et al., 1990). Specifically, in our study, there was high abundant of

OTU, *L. amylovorus* in control phase III in compared to control phase II. *L. amylovorus* has probiotic properties having antimicrobial activity against enteric pathogens producing large quantities of lactic acid (Nakamura, 1981; Kant et al., 2011).

Similarly, the OTU, *L. reuteri* was numerically higher in Control phase II samples which has also been reported to have probiotics effects. They have been found to interact with host cells for the protection of epithelial cells and have capacity to colonize, adhere to intestinal mucin (Miyoshi et al., 2006; Yu et al., 2007; Li et al., 2008; Hou et al., 2014), and can produce antimicrobial substances such as lactic acid, hydrogen peroxide, reuterin, reutericyclin to inhibit the growth of enteric pathogens (Morita et al., 2008; Martinez et al., 2009; Hou et al., 2015). In pigs, the administration of probiotics *L. reuteri* have been reported to have beneficial effects on performance, prevention of diarrhea, stress relief, gut microbiota modulation, and immunomodulation (Hou et al., 2015). Whereas, the members of *Lachnospiraceae* and Ruminococcaceae was lower in Control phase III than Control phase II samples. For instance, in our study, *Faecalibacterium prausnitzii,* an OTU under the family Ruminococcacae was found to be significantly higher in Control phase II. Lower *Fa. prausnitzii* has been reported to be associated with risk of Inflammatory Bowel Disease (IBD), ulcerative colitis, and Crohn disease in human (Miquel et al., 2014). *Fa. Prausnitzii* is a producer of butyrate (Barcenilla et al., 2000) which is an important energy source for colonic epithelial cells and this OTU has potential to be used as a livestock probiotics (Foditsch et al., 2015).

4.3 Effects of Experimental diets on OTUs composition

For the experimental groups fed with PeptivaM P at the phase III the members of Streptococcaceae family became the most predominant bacterial group of fecal microbiomes, which included only one OTU SD_Ssd-0039 which has 99% sequence identity. While the members of Lactobacillaceae family was greatly reduced in this group. As described by Farrow et al. (Farrow et al., 1984), *St. alactolyticus* has been isolated from the intestine of pigs and feces of chicken. Robinson et al. (Robinson et al., 1988) reported *St. intestinalis* to be the predominant commensal member of the pig colonic microbiota and later by Vandamme et al. (Vandamme et al., 1999) suggested *St. intestinalis* to be junior synonym of *St. alactolyticus* and pigs were considered to be a host of *St. alactolyticus*. As *St. alactolyticus* are lactic acid bacteria, they have been reported to have several beneficial effects on the host (Salminen and Deighton, 1992). They have been reported to suppress the growth of intestinal pathogens (Hudault et al., 1997; Pascual et al., 1999) and to enhance the immune functions in human and mice (Gill et al., 2000; Vitini et al., 2000).

Composition of feed ingredients are one of major factors that affect the fecal bacterial composition. In this study the amount of two ingredients (limestone and monocalcium phosphate) were used in phase III but not in phase II diet. The increase in dietary calcium increased pH of gizzard (Walk et al., 2012) and digesta (Ptak et al., 2015) and this changes in digesta pH may result in shifts of microbiota profiles and their activity. Similarly, there was significant change of energy source feed ingredients corn and PGF oat blend as there was no amount of oat blend phase III diets which might have also affect the changes in the fecal OTUs composition. Further research will be needed

to elucidate the mechanisms of action of those feed ingredients on fecal bacterial composition of nursery pigs.

Table 3. 1. Growth performance of weaned pigs fed diets containing Peptiva, MOS, and protease formulated at 100 or 90% of NRC (2012) AA requirements for weaned pigs[1].

	A Control	B PeptivaMp	C PeptivaMp-10	D PeptivaM	SEM	P-value[2]	Contrast
BW, kg							
d0	6.9	6.9	7.0	6.8	0.11	0.453	
d14	12.4	12.4	12.4	12.4	0.18	0.791	0.791
d35	23.8	24.4	23.7	24.2	0.23	0.139	0.074
d49	35.1	35.4	34.6	35.0	0.27	0.196	0.776
ADG, kg/d							
d014	0.355	0.357	0.360	0.359	0.012	0.985	0.795
d1535	0.534^{b}	0.568^{a}	0.536^{b}	$0.558^{a,b}$	0.010	0.017	0.009
d3649	0.851	0.833	0.823	0.829	0.017	0.404	0.191
d035	0.443	0.461	0.447	0.458	0.007	0.204	0.057
d049	0.563	0.574	0.558	0.569	0.006	0.259	0.248
ADF, kg/d							
d014	0.349	0.371	0.383	0.342	0.013	0.133	0.624
d1535	0.875	0.842	0.817	0.809	0.028	0.296	0.134
d3649	1.303^{x}	$1.419^{x,y}$	1.437^{y}	1.429^{y}	0.044	0.050	0.012
d035	0.611	0.606	0.600	0.575	0.016	0.364	0.273
d049	0.847	0.882	0.883	0.863	0.011	0.089	0.072
g:f, kg:kg							
d014	1.009	0.962	0.921	1.100	0.050	0.082	0.713
d1535	0.603^{a}	$0.674^{a,b}$	$0.657^{a,b}$	0.707^{b}	0.028	0.028	0.005
d3649	0.652^{a}	0.591^{b}	0.579^{b}	0.583^{b}	0.016	0.007	0.002
d035	0.726^{a}	$0.765^{a,b}$	$0.745^{a,b}$	0.819^{b}	0.020	0.009	0.008
d049	0.666^{a}	$0.653^{a,b}$	0.633^{b}	0.662^{a}	0.007	0.007	0.312

[1]Experimental diets were fed from 7 – 42 d post-weaning (d0 – 35) followed by a common diet for 14 d.

[2]Within a row, means without common superscripts ^{a,b} differ $P < 0.05$ and ^{x,y} differ $P < 0.10$.

[3]Contrast between 'untreated' (Control) and 'treated' (PeptivaMP and PeptivaM).

Table 3.2. Mean relative abundance (%) of main bacterial taxonomic groups in representative fecal samples

Taxonomy	CON II[1]	CON III[2]	PEPMP II[3]	PEPMP III[4]	PEPM II[5]	PEP M III[6]	P-value
Firmicutes	72.64	71.40	71.67	77.55	74.90	77.89	0.399
Streptococcaceae[#]	0.34[b]	4.99[b]	1.32[b]	18.88[a]	2.02[b]	10.03[ab]	2.411e-06
Lactobacillaceae[#]	3.08[b]	21.23[a]	4.32[b]	14.97[ab]	8.71[ab]	20.72[a]	0.00029
Erysipelotrichaceae#	14.53[ab]	2.65[c]	20.41[a]	3.29[c]	18.36[a]	6.70b[c]	3.317e-07
Lachnospiraceae[#]	19.64[a]	9.17[b]	10.52[b]	13.43[ab]	16.41[ab]	13.13[ab]	0.016
Clostridiaceae 1	2.21	4.40	2.33	1.85	1.71	4.07	0.251
Peptostreptococcaceae[#]	0.20[b]	2.60[a]	0.92[ab]	1.78[ab]	0.28[b]	2.64[a]	0.00094
Ruminococcaceae[#]	18.68[a]	11.59[abc]	16.33[ab]	9.72[bc]	14.14[abc]	8.39[c]	0.0018
Acidaminococcaceae[#]	3.38[a]	0.09[b]	1.35[ab]	0.11[b]	1.56[ab]	0.27[b]	0.016
Clostridiales_Incertae Sedis XIII[#]	2.12a[b]	0.89[b]	4.16[a]	0.98[b]	3.57[ab]	1.25[ab]	0.005
unclassified Clostridiales	3.82	5.31	4.67	4.36	4.17	4.36	0.872
Eubacteriaceae[#]	1.42[a]	0.12[b]	0.40[b]	0.07b	0.55[b]	0.12b	9.746e-05
Other Firmicutes	3.22[b]	8.37[a]	4.95[b]	8.10[a]	3.43[b]	6.22[ab]	3.461e-06
Bacteroidetes	13.64	8.90	14.35	7.06	10.74	7.44	0.181
Prevotellaceae	9.27	5.70	9.88	5.43	5.78	5.30	0.398
Porphyromonadaceae[#]	1.47[ab]	1.17[ab]	1.74[ab]	0.58[b]	3.04[a]	0.53[b]	0.028
Other Bacteriodetes	2.9	2.03	2.73	1.05	1.92	1.61	0.285
Unclassified Bacteria[#]	8.16[ab]	17.24[a]	9.05[ab]	13.40[ab]	8.04[b]	12.27[ab]	0.029
Other Phyla	5.56	2.46	4.93	1.99	6.32	2.40	0.043

[1]basal diet phase I, [2]basal diet phase II, [3]basal diet plus Peptiva with mannan-oligosaccharide & & protease phase II, [4]basal diet plus Peptiva with mannan-oligosaccharide & protease phase III, [5]basal diet plus Peptiva & mannan-oligosaccharide phase II, [6]basal diet plus Peptiva & mannan-oligosaccharide phase III; [#]Taxa showing a statistically significant difference ($P<0.05$) amongst means of different treatment groups; Means with different superscripts are significantly different as determined by the Tukey honest significant difference test

Table 3.3. Mean relative abundance (%) of most abundant OTUs in representative fecal samples from six dietary treatments.

OTUs	CON II	CON III	PEM PII	PEM PIII	PEP M PII	PEP M PIII	*P-Value*	Closest valid taxon (id%)
Firmicutes								
SD_Ssd-00039#	0.27[b]	4.46[b]	1.12[b]	17.19[a]	1.72[b]	8.95[ab]	< 0.05	*St. alactolyticus* (99%)
SD_Ssd-00001#	0.32[b]	10.57[a]	1.92[b]	6.06[ab]	2.03[b]	11.02[a]	< 0.05	*L. amylovorus* (99%)
SD_Ssd-00123#	0.11[b]	7.23[ab]	9.32[a]	0.23[b]	7.45[ab]	2.83[ab]	0.005	*L. vitulina* (87%)
SD_Ssd-00019	2.11	5.43	1.48	3.97	5.25	5.73	0.23	*L. reuteri* (99%)
SD_Ssd-00706	0.48	1.31	0.56	1.05	0.29	1.05	0.05	*L. paracasei* (81%)
SD_Ssd-00064	0.51	8.431	2.02	1.01	3.73	1.54	0.05	*B. luti* (97%)
SD_Ssd-00308	1.49	0.547	4.12	0.98	4.35	1.41	0.009	*Ho. biformi* (97%)
SD_Ssd-00134	1.61	3.06	1.60	1.13	1.10	2.57	0.36	*Cl. saccharoperbutylacetonicum* (97%)
SD_Ssd-00224	1.23	0.63	0.96	0.51	0.94	0.47	0.12	*Cl. nexile* (95%)
SD_Ssd-01077	0.35	2.92	0.56	1.42	1.08	1.17	0.24	*Ery. Rhusiopathiae* (85%)
SD_Ssd-00002	0.03	2.86	0.08	2.61	0.12	1.40	0.05	*L. johnsonii* (99%)
SD_Ssd-00014#	0.07[b]	2.24[a]	0.70[ab]	1.45[ab]	0.20[b]	2.26[a]	0.0008	*T. glycolicus* (97%)
SD_Ssd-00892#	0.71[ab]	0.42[b]	1.66[ab]	0.35[b]	2.30[a]	0.44[b]	0.012	*So. moorei* (89%)
SD_Ssd-00409	1.01	1.37	0.83	1.12	0.96	0.57	0.68	*Dys. welbionis* (91%)
SD_Ssd-00188#	0.13[ab]	0.57[ab]	0.04[b]	2.25[a]	0.77[ab]	1.27[ab]	0.03	*Eu. rectale* (99%)
SD_Ssd-00993	1.33	0.33	1.67	0.20	1.61	0.25	0.14	*Fa. cylindroides* (88%)
SD_Ssd-00416	2.67	0.06	0.82	0.08	1.28	0.08	0.07	*Pha. succinatutens* (95%)
SD_Ssd-01079	0.62	0.78	1.25	0.27	1.72	0.04	0.18	*Mah. australiensis* (84%)
SD_Ssd-00304#	0.22[b]	0.88[ab]	0.49[ab]	0.95[ab]	0.49[ab]	1.42[a]	0.022	*Clo. bacterium* (90%)
SD_Ssd-01078	0.73	1.10	0.76	0.86	0.69	0.37	0.87	*So. moorei* (84%)

SD_Ssd-01254#	2.32[a]	0.07[b]	0.288[ab]	0.09[b]	0.96[ab]	0.03[b]	0.03	*Fa. prausnitzii* (99%)
SD_Ssd-01081	1.34	0.60	0.46	0.75	0.11	0.36	0.73	Breznakia pachnodae (81%)
SD_Ssd-00705#	0.23[b]	0.62[ab]	1.63[a]	0.20[b]	0.61[ab]	0.23[b]	0.02	*Clo. bacterium* (86%)
SD_Ssd-01244	2.05	0.00	0.77	0.00	0.26	0.00	0.26	*R. bromii* (92%)
SD_Ssd-01080	0.31	0.19	1.59	0.21	0.53	0.13	0.12	*Ihubacter massiliensis* (92%)
SD_Ssd-00928#	1.14	0.12	0.36	0.08	0.60	0.07	< 0.05	*R. gnavus* (96%)
SD_Ssd-01246	0.64	0.00	1.23	0.00	0.49	0.00	0.13	Sharpea azabuensis (97%)
Total	39.06	41.78	38.30	45.03	41.64	45.67		
Bacteriodetes								
SD_Ssd-00003	3.74	2.07	3.13	2.47	1.57	2.36	0.8077	*P. copri* (98%)
SD_Ssd-00021	1.04	0.10	1.33	0.05	0.75	0.52	0.2142	*P. copri* (95%)
SD_Ssd-00815	0.50	0.30	0.61	0.21	1.28	0.05	0.3041	*Par. distasonis* (92%)
Total	5.28	2.47	5.07	2.74	3.60	2.93		
Actinobacteria								
SD_Ssd-00840#	1.20[ab]	0.12[b]	1.83[a]	0.23[b]	1.69[ab]	0.47[ab]	0.004	*Co. aerofaciens* (98%)
SD_Ssd-00416	2.67	0.06	0.82	0.08	1.28	0.08	0.0679	*Pha. succinatutens (95%)*
Total	3.87	0.18	2.65	0.31	2.97	0.55		

OTUs showing a statistically significant difference ($P<0.05$) amongst means of different treatment groups.

Abbreviations: *St.: Streptococcus; L.: Lactobacillus; B.: Blautia; Ho.: Holdemanella; Ery. Erysipelothrix; T.: Terrisporobacter; So.: Solobacterium; Eu.: Eubacterium; Clo.: Clostridiales; Fa.: Faecalibacterium; R.: Ruminococcus; D.: Dorea; Cl.: Clostridium; Ma.: Mageeibacillus; P.: Prevotella; Co.: Collinsella; Pha.: Phascolarctobacterium, Dys.: Dysosmobacter, Fae.: Faecalitalea, Pha.: Phascolarctobacterium, Mah.: Mahella, So.: Solobacterium, Ihu.: Ihubacter, R.: Ruminococcus, Br.: Breznakia, Par.: Parabacteroides*

Table 3.4. Alpha diversity indices and coverage from three dietary treatments at phase I and II. Values are presented as means.

Index	CON II	CON III	PEPM II	PEPM III	PEPM P II	PEPM P III	*P*-value
OTUs	559.00	762.57	660.12	671.66	660.12	705.30	0.129
Ace	822.25	992.95	970.68	916.35	990.43	926.77	0.494
Chao1	762.15	999.21	890.23	910.31	905.91	923.62	0.325
Shannon	4.16	4.68	4.37	4.39	4.44	4.42	0.401
Simpson	0.054	0.035	0.042	0.046	0.038	0.059	0.425
Coverage (%)	98.88	98.60	98.69	98.70	98.67	98.70	0.512

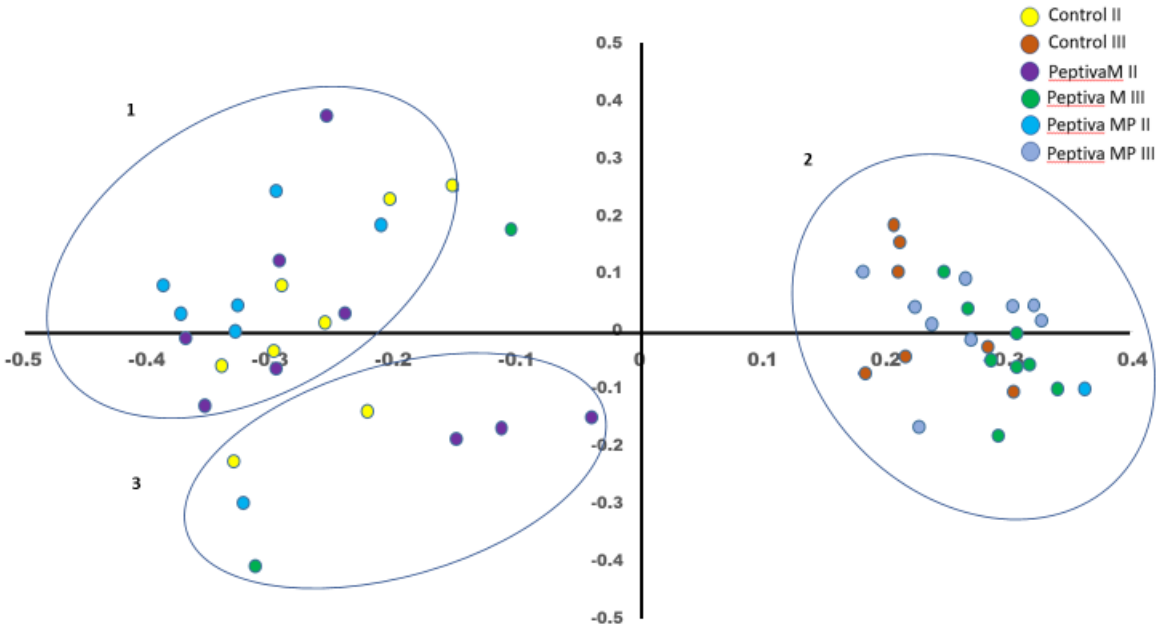

Figure 3.1. PCoA plot of main OTUs of control and treatment samples at phase II and phase III.

Table 3.5. Diet formulations

	Phase 2				Phase 3			
Item[1]	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Corn	662.5	651.5	672.2	652.2	953.10	942.1	948.10	942.8
Soybean Meal	420.0	420.0	410.0	420.0	525.0	525.0	525.0	525.0
Soybean or Corn Oil	40.0	40.0	37.0	40.0	40.0	40.0	42.0	40.0
DDGS	150.0	150.0	150.0	150.0	200.0	200.0	200.0	200.0
Lysine HCl	11.50	11.50	7.90	11.50	10.00	10.00	6.20	10.00
L-Threonine	4.50	4.50	2.80	4.50	3.70	3.70	1.80	3.70
DL-Methionine	2.10	2.10	0.40	2.10	2.40	2.40	0.70	2.40
Limestone					12.0	12.0	12.0	12.0
Monocalcium phosphate					5.10	5.10	5.10	5.10
L-Tryptophan	1.40	1.40	0.70	1.40	0.70	0.70	0.10	0.70
TBCC								
Salt	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
PGF Oat Blend	200.0	200.0	200.0	200.0				
Mecadox	25 g/ton in each Phase 2 diet							
Peptiva		9.90	9.90	9.90		9.90	9.90	9.90
blended protease&MOS		1.10	1.10			1.10	1.10	
MOS				0.44				0.44
Total	2000.0	2000.0	2000.0	2000.0	2000.0	2000.0	2000.0	2000.0

[1]Abbreviations: DDGS, dried distillers grains with solubles; PGF GMOS, Pipestone Grow-finish XX; VTM, vitamin/mineral trace mix; TBCC, tribasic copper chloride.

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Chapter 5:

Perspective and Future Outlook

5.1 Perspective and Future Outlook

The gut microbiota contributes to host functions such as energy harvesting capacity, production of SCFAs, production of vitamins, microbial protein synthesis, immunity and resistance against pathogens, which ultimately benefit the health, well-being and performance of the host (Kim and Isaacson, 2015; Stokes, 2017; Yang et al., 2017; Li et al., 2019). The microbiota also contributes to the development and cellular differentiation of host gut tissues, including the intestinal epithelium, the mucosal layer, as well as lymphoid structures and other immune cells (Sharma et al., 1995; Mebius, 2003; Smith and Garrett, 2011). It is then not surprising that studies have also found that the composition of the gut microbiota is associated with performance traits such as feed efficiency in both ruminant and no-ruminants (Shabat et al., 2016; Vigors et al., 2016; Li and Guan, 2017; Yang et al., 2017). Therefore, targeting the gut microbiota to modulate its function has the potential to improve animal productivity and wellbeing, which would greatly benefit the livestock industry.

Weaning would be a stage of production particularly well suited for modulation of microbiota composition and activity, as it represents a critical stage in microbiome transition, when young animals are particularly vulnerable to disease. Indeed, the abrupt change of diet from highly palatable and digestible milk to dry feed composed of plant-based ingredients is likely one of the main disrupting factors affecting the microbiota at this stage. In addition, a combination of stressors, including separation from the dam, and changes in physical and social environments, also contribute to an increased risk of dysbiosis and higher susceptibility to post weaning diarrhea (Campbell et al., 2013; Windeyer et al., 2014) leading to higher morbidity and mortality rates.

Traditionally, antibiotics were used prophylactically to prevent pathogen proliferation, and to minimize the impact of gut microbial instability during weaning. However, this practice is not without unexpected effects, as it has been associated with a negative impact on animal performance, due at least in part to gut microbial dysbiosis (Malmuthuge et al., 2015; Malmuthuge and Guan, 2017). With the implementation of stricter regulations on the use of antibiotics in order to reduce the risk of further spreading antibiotic resistance to human and animal pathogens, there has been a pressing need for the development of viable alternatives and innovative strategies to replace antibiotics without compromising animal health and production. This opportunity has resulted in the development of commercial feed additives, some of which are based on EO or antimicrobial peptide formulations. However, the use of these products has so far yielded mostly inconsistent outcomes, so they have yet to prove themselves as true substitutes to antibiotics.

It has been proposed that individuals of the same host species share sets of common microbial groups designated as ‘core’ microbiomes (Turnbaugh et al., 2007). According to this model, while the respective abundance of each microbial core group can vary between animals, depending on individual differences in diet, feeding behavior, genotype, physiological and immunological status, ambient environment, pathobiology as well as stress level (Turnbaugh et al., 2007), these shared microbial groups would define the gut microbiota of an animal species. In mammals, the phyla Firmicutes, Bacteriodetes and Proteobacteria have been consistently identified as components of the gut microbiome regardless of diet or environment, and may thus represent core bacterial groups. Ideally, core groups should be defined at the species or OTU level, as these

would represent more specific targets for modulation than higher phylogenetic ranks which may include a wider range of metabolic functions.

In this context, the research described in this dissertation aimed to provide insight on the dynamics of gut bacterial composition in weaned calves and pigs in response to commercial feed additives with different formulations. Based on the findings from these projects, this chapter aims to describe potential follow up research that could be of value to the scientific community and to the livestock industry.

5.2 Implication and Future Direction

5.2.1 Focus on uncultured bacterial species

The conversion of plant fiber material into SCFAs that occurs in the gut is only possible because of the metabolic activity of microbial communities. Indeed, host genomes do not encode enzymes that can efficiently hydrolyze plant structural polysaccharides and efficiently metabolize sugars other than glucose, fructose and galactose (Kobayashi, 2006). In mammalian herbivores, this metabolic activity is the main contributor of energy for the animal. For ruminants in particular, research efforts have mainly focused on the rumen to gain a deeper understanding of the physiology of this gut compartment and the ecology of its microbial communities. The ultimate goal of these efforts is to maximize animal production through optimization of digestion and fermentation of the diet by providing suitable micro-habitat conditions for rumen microorganisms (Kobayashi, 2006). In non-ruminants, gut microbial communities contribute more to maintaining gut homeostasis than to the nutrition of their host. In combination with a balanced nutrition, a functional immune system and a structurally

sound mucosae, maintaining a stable microbiome comprised primarily of beneficial microorganisms would then ensure ‘gut health’ (Pluske et al., 2018).

The development and recent advancements in DNA-based, culture-independent techniques have revealed that most gut microbial species remain unknown or have yet to be characterized. In the rumen, for instance, it has been estimated that only 10% or less of rumen bacteria have been cultured. The complexity of gut microbial communities and the dynamic functional interactions amongst microbial species remain a great hurdle towards gaining further insight. Despite these challenges, future efforts need to continue on characterizing unknown species and their metabolic potential. As technological advances continue for culture-independent techniques, such 16S rRNA-based taxonomic profiling, metagenomics, transcriptomics, proteomics, and metabolomics, the identification of uncharacterized microorganisms will become more efficient, and so will synergies with traditional culturing methods and associations with animal production parameters.

5.2.2 Investigating the microbiome in different segments of the gut

The gastrointestinal tract includes a wide range of different habitats to support microbial life, both longitudinally, i.e. from the proximal to the distal end, and radially, i.e. from the lumen to the epithelial surface of the host, resulting in a diverse set of micro-environments with the potential to support an equally diverse range of microbial populations (Zhao et al., 2015). Zhao et al. (Zhao et al., 2015), for instance, reported distinct microbial communities between the small intestine and the colon, which can be

attributed to differences in micro-environmental conditions. The small intestine is mainly responsible for enzymatic digestion of feed and absorption (Zhao et al., 2015), has higher oxygen level, and has a faster transit time for feed when compared to the colon (Kelly et al., 2017). The abundance of soluble carbohydrates for bacteria is higher in the small intestine (Zoetendal et al., 2012), whereas the main substrates available for microbial growth in the colon are complex carbohydrates such as non-starch polysaccharides that are resistant to digestion in the small intestine (Zhao et al., 2015). In accordance with these observations, Zhao et al. (2015) found that the bacterial profile in feces was quite different from bacterial profiles found in the small intestine. For instance, Firmicutes were found to be the main phylum in fecal samples (>90%), a proxy for the colon, whereas Proteobacteria were found to be the most abundant group (>70%) in the small intestine.

Similarly, the lumen habitat is distinct from the mucosal layer, since the lumen has a lower oxygen content than the mucosae, resulting in distinct micro-environments based on the ability to grow in the presence of oxygen (Albenberg et al., 2014). In addition, the mucosae represents an abundant source of mucin glycoproteins (McGuckin et al., 2011), acting as a source of nutrients for mucosal bacteria while blocking potential enteric pathogens from reaching the epithelial cell layer. Luminal bacterial populations were found to be different and more diverse from those associated with the mucosal layers in different segments of the gut (Kelly et al., 2017). For instance, an OTU assigned to *Helicobacter* was highly abundant in the caecal mucosae (18%), whereas it was less than 0.1% in the caecal lumen. Similarly, an OTU most closely related to *Prevotella*

copri, was only found at 6% within the caecal mucosal layer compared to 17% in the lumen.

In the context of our experiments performed using weaned pigs, fecal samples were used as a proxy for gut bacterial communities. However, based on the differences in conditions between the different regions of the gut, it would be expected that fecal microbial profiles may not be representative of microbial communities from other gut compartments. Thus, before deciding on a specific strategy to implement based on fecal bacterial communities, it would be wise to determine the microbial profile of all segments of the gut, and include both luminal and mucosal samples as part of future investigations.

5.2.3 Assessing the metabolic potential of OTUs using metagenomics

Determining the composition, diversity and function of microbial groups is key in studying microbial communities. The limited ability of traditional culture-dependent techniques to provide a comprehensive picture of complex microbial communities can be complemented by the use of DNA-based approaches using Next Generation Sequencing technologies. High throughput sequencing of amplicons from target genes (e.g. 16S rRNA) and shotgun metagenomics can provide in-depth taxonomic and functional compositional profiles of microbial communities. The two approaches are complementary; while amplicon-based target gene analyses are restricted by primer sequence specificity to particular microbial groups and provide taxonomic profiling with typically limited functional insights, metagenomic analyses provide information on metabolic potential from data that is generated in a non-targeted fashion from genomic DNA extracted from an environment of interest (Janda and Abbott, 2007).

When the goal is to determine the bacterial composition of a microbiome in a given sample, i.e. determining the phylogenic and taxonomic profile of communities consisting of cultured and uncultured bacterial species, sequencing of the 16S rRNA gene offers a number of advantages. This gene is expected to be present in all bacteria, and has an ideal structure for this type of analysis, with alternating conserved and variable regions; the conserved regions can be used to design primers for PCR amplification while the variable regions between them can be used for taxonomic profiling (Janda and Abbott, 2007). Limitations with this approach is the variation in copy numbers between different species which can skew representation of bacterial groups (Escobar-Zepeda et al., 2018), that sequencing of different variable regions of the 16S rRNA gene from the same sample can potentially give different results, that primers may introduce biases, and that PCR may generate artifacts that can be difficult to distinguish from low abundance 16SrRNA.

Metagenomics data is generated from the direct sequencing of genomic DNA from an environmental sample without the need for PCR amplification other than when library preparation takes place. Since sequence data is generated from genomic DNA molecules belonging to microbial species sampled from a given sample, its main strength is in allowing metabolic profiling through gene annotation for the sampled microbial community (Sedlar et al., 2016). While metagenomics data can be used for taxonomic assignment, its main disadvantage is the current lack of microbial genomic data for most environments that are investigated; for most sequence reads generated, it is more than likely that there will be no corresponding nucleotide match in public databases. So far, the compromise has been to use predicted amino acid sequences not just for gene

annotation, but also for taxonomic profiling. However, genome sequence-based definition of species needs to be established at the nucleotide level rather than the amino acid level. Indeed, because the genetic code is degenerate, high conservation of an amino acid sequence because of selection for biochemical function could confound high amino acid conservation due to phylogeny. As 61 codons are used to encode 20 amino acid in the coding sequence of genes, only two amino acids (methionine and tryptophan) are encoded by only one codon, with other amino acids (leucine, arginine and serine) are encoded by six different codons. Consequently, two identical amino acid sequences, which would appear to belong to the same species, could have very different genomic DNA sequences and actually belong to two different species.

From the results of the EO trial presented in chapter 2, the mean relative abundance of OTU SD_Bt-00966, whose closest relative was *Prevotella ruminicola,* amongst 10 calves that were fed an EO-supplemented diet was 19.5%, which was 7.2 time higher than the abundance of this OTU in the rumen of calves fed the non-supplemented (control) diet. If a metagenomics approach had been used instead of 16S rRNA, we would predict that, on average, approximately 20% of sequence reads generated from EO-fed calves' samples would belong to OTU SD_Bt-00966. We would also predict that taxonomic assignments based on the amino acid-coding sequences translated from the metagenomics data would reveal affiliation of sequence reads from the genome of OTU SD_Bt-00966 to *Prevotella* species. However, it is unclear whether these affiliation predictions would be accurate.

5.2.4 Design of probiotics to modulate gut microbial profiles

Probiotics are live microbial feed supplements that are designed to provide beneficial effects to animal growth, production performance and and/or immune responses by improving the intestinal microbial balance (Krehbiel et al., 2003; Isolauri et al., 2004; Patel et al., 2015). In feedlot cattle and dairy cows, probiotics supplementation can decrease the incidence of acidosis, and can also improve the immune response in stressed calves (Krehbiel et al., 2003). However, several investigations have shown that it is very difficult for probiotic microorganisms to establish themselves in environments as complex and as dynamic as the gut of animals, thus requiring the need to feed probiotics daily for sustained effects (Jensen, 1998). Other challenges with probiotics are inconsistent results, which may be due to the narrow selection of bacterial strains that have been developed into commercial probiotics. Most frequently used strains are affiliated to *Lactobacillus*, *Bifidobacteria*, *Enterococcus*, *Bacillus* or *S. cerevisiea*. Strong arguments can be made that there is no solid ecological and scientific basis for the narrow choice of bacterial species currently used to design probiotics (Jensen, 1998), as the main reason for their popularity as probiotics is because they are easy to culture (Lee, 1985).

It would seem like a more effective strategy would be to use complex mixtures of bacteria that are native gut dwellers as probiotics rather than just one bacterial strain. In this context, future work could involve culturing and further genomic characterization of the main OTUs identified as a result of this dissertation. We would predict that probiotics developed from prominent gut OTUs would be more effective to improve animal performance. The use of such probiotics would be of highest values at stages when the gut microbiota is in transition, such as after birth or weaning, after transportation, to treat

a metabolic condition such as acidosis, or after treatment with antibiotics (Jensen, 1998). For probiotics to be viable and effective, considerations should be given to their potential for stable establishment in the gut, specificity to host animal, and their genetic stability. Furthermore, proper and optimal use of probiotics would benefit from a deeper knowledge of their mechanisms of action at the cellular and biochemical levels.

5.2.5 Design of encapsulated feed additive products for specific intestinal segments

In non-ruminants, digestion of dietary protein is primarily performed by gastric and pancreatic proteases through hydrolysis into peptides and free amino acids. Large peptides are further hydrolyzed by the action of peptidases present at the brush border of enterocytes, while oligopeptides can be absorbed intact into the enterocytes through peptide transporters. Inside enterocytes, peptides can be further hydrolyzed into amino acids or enter the blood system (Miner-Williams et al., 2014).

In the trial described in Chapter 3, no significant effect of Peptiva was observed on performance, but an effect on fecal bacterial populations was observed at Phase II, but not at Phase III. Based on the intestinal digestion and absorption mechanisms described in the previous paragraph, it is possible that by the time the pigs under study had reached Phase III, their peptide hydrolysis effectiveness had improved to a point where dietary Peptiva peptides were digested and absorbed by the host before they could reach the large intestine. It is also possible that Peptiva peptides are most effective on bacterial populations of the small intestine, which were not investigated in the study described in Chapter 3. In this context, future follow up experiments could include the design of

encapsulated bioactive peptides, such as Peptiva, for targeted release in the large intestine. Based on the fecal bacterial composition studies performed, this is the location where Peptiva would be predicted to modulate microbial composition and abundance. Encapsulation would protect the feed additive from being hydrolyzed or metabolized before it reaches their targeted site.

5.2.6 Work on interaction of gut microbiota and host

In addition to digestion and absorption of nutrients, the gut has other important functions including immunity as well as acting as a selective barrier against harmful antigens, toxins, and pathogens (Lallès et al., 2004; Omonijo et al., 2018). Maintaining the integrity of the intestinal epithelium is important to its function as a barrier against pathogens and toxins present in the lumen. An important function of the intestinal immune system is to minimize the exposure of host tissues to bacteria and to decrease pathogenic bacteria proliferation. However, while the gut immune system is likely an important contributor to controlling gut microbiota composition (Hooper et al., 2012), it has to balance its activities between effectively controlling colonization by pathogens and showing tolerance to antigens derived from commensal bacteria and compounds from feeds (Pitman and Blumberg, 2000).

However, the relationship between the host and its intestinal microbiota is symbiotic. It is well known that the gut microbiota impacts physiological, developmental, nutritional and immunological processes of the host, with an overall impact on host health and performance (Richards et al., 2005). Typically, one of the challenges in

investigating host-symbiont relationships is the variability in the composition and diversity of intestinal microbiota in mammals, which can affect the reproducibility and significance of experimental results obtained from animal trials (Fiebiger et al., 2016). One strategy to overcome this type of challenge would be to use germ-free and gnotobiotic animal models. Therefore, in a similar fashion to the experiment carried out to investigate the host response to a simplified microbiota consisting of *Lactobacillus johnsonii*, *Bifidobacterium longum*, and *Escherichia coli* in gnotobiotic mice (Denou et al., 2009), it would be of interest to design an experiment using a gnotobiotic animal model to investigate host-microbial symbiont interactions in the context of the OTUs described in this dissertation.

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