INVESTIGATION OF HEALTH PROMOTING EFFECTS OF MANITOBA-GROWN RED OSIER DOGWOOD AS AN ALTERNATIVE TO ANTIBIOTICS IN NURSERY PIG PRODUCTION

by

Rachita Maniyar

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Animal Science

University of Manitoba

Winnipeg

Abstract

The objective of this study was to investigate the health-promoting effects of red osier dogwood extract as an alternative to antibiotics, in weaned piglets challenged with enterotoxigenic *Escherichia coli*. Twenty-eight weaned piglets (9.15±0.95 kg BW), confirmed to be genetically susceptible to ETEC, were individually assigned to one of four dietary treatments in a completely randomized design. The experimental diets were, negative control (NC), corn-wheat soybean meal diet with no additives; positive control (PC), NC plus antibiotics; ROD1, NC plus 0.1% ROD extract; ROD2, NC plus 0.2% ROD extract. Piglets were orally challenged on d 7 with ETEC F4. Feed disappearance, body weight, fecal score, and rectal temperature were recorded, and blood samples were taken before and after the challenge. On d 14, the piglets were euthanized to collect intestinal tissue samples for histomorphology, Ussing chamber analysis, and mRNA gene expression. Colon digesta was collected for analyzing microbial DNA. Data were analyzed with the MIXED procedure of SAS using individual piglet as the experimental unit. There were no differences ($P > 0.10$) in histomorphology and intestinal permeability. Piglets fed the NC diet tended ($P < 0.10$) to have a higher average daily gain post-inoculation than those fed ROD1 or ROD2. The fecal score of piglets fed the PC diet tended to ($P < 0.10$) or was significantly lower ($P <0.05$) than for piglets fed ROD1 or ROD2 on 0 and 2 days post-inoculation (dpi), respectively. On 0 dpi, piglets fed the ROD1 diet had significantly higher ($P < 0.05$) body temperature than those fed PC or ROD2. The anti-inflammatory cytokine IL-10 tended ($P < 0.10$) to be higher in the blood of ROD2 piglets as compared to the NC group, pre-inoculation. The IL-10 was significantly higher ($P < 0.05$) in the blood of ROD2 group than in the NC group and tended ($P < 0.10$) to be higher in the ROD2 piglets than in the PC or ROD1 group, post-inoculation. In the post-inoculation phase, the antioxidative enzyme superoxide dismutase showed a lower trend (*P*

< 0.10) in the ROD2 group as compared to the PC group. Similarly, malondialdehyde levels tended ($P < 0.10$) to be lower in the ROD2 group than in the ROD1 group. The mRNA gene expression of the catalase enzyme in the jejunum was significantly higher ($P < 0.05$) in the ROD1 group than in the NC group. The gene expression of IL-10 tended ($P < 0.10$) to be lower in ROD1 than in NC piglets. In the case of inflammatory cytokine IL-6, the PC group had significantly lower ($P < 0.05$) and ROD2 had a lower tendency ($P < 0.10$) of gene expression than the NC group. With regards to microbial DNA, *Bifidobacterium* was significantly lower ($P < 0.05$) in ROD2 and tended ($P < 0.10$) to be lower in ROD1 and PC as compared to NC piglets. Also, *E. coli* F4 tended ($P < 0.10$) to be lower in the PC group as compared to the NC group. In conclusion, red osier dogwood extract supplementation might have some health-promoting effects on gut health and oxidative status of ETEC challenged weaned piglets but is not comparable to antibiotic growth promoters.

Dedication

I would like to dedicate this work to my parents, Sangita and Manmohan, my husband, Sunny and to all the animals that contribute towards research in the field of Animal Science.

Acknowledgment

First and foremost, my sincere thanks and gratitude goes to my supervisor, Dr. C. M. Nyachoti for giving me this opportunity and guiding me throughout my research. I truly appreciate his support in the ever-changing conditions during the pandemic. I am also thankful to Dr. Chengbo Yang and Dr. Karmin O for being my committee members. The comments and suggestions given by my advisory committee on the key aspects of my research were very valuable and encouraging.

My gratitude goes to Robert Scales, Chief Executive Officer of Red Dog Enterprises Ltd. for developing and providing the red osier dogwood extract, without which the research would not have been possible.

A sincere thanks to all my group members Bonjin Koo, Dr. Debora Holanda, Atanas Karamanov, Jinyoung Lee, Jichen Song, Olumide Adeshakin, and Xiaoxiao Zhang for their significant contributions to this research and many cherished memories in Winnipeg. I am very grateful to Robert and Freddie from T. K. Cheung Centre for Animal Science Research for their immense help during the *E. coli* trial. I would like to appreciate all the members of Dr. Yang's lab, especially laboratory technician Fernando Esposito for his assistance as a friend and a colleague. I also appreciate all the staff members of the Animal Science Department at the University of Manitoba for all the administrative help.

Finally, I am eternally grateful to my amazing parents and parents-in-law for their unending encouragement and love. Many thanks to my brother, Akshay for always looking out for me. Special thanks to my pillar of strength, my husband, Sunny for giving me the love and warmth needed during my thesis completion.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ADFI	Average daily feed intake
ADG	Average daily gain
AGP	Antibiotic growth promoter
AMP	Antimicrobial peptide
BW	Body weight
CD	Crypt depth
CFU	Colony-forming unit
CP	Crude protein
Ct	Threshold cycle
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
ETEC	Enterotoxigenic *Escherichia coli*
FITC	Fluorescein isothiocyanate
GAE	Gallic acid equivalent
GF	Gain: Feed ratio
GIT	Gastrointestinal tract
GPX	Glutathione peroxidase

Ig	Immunoglobulin
Ig	Immunoglobulins
IL	Interleukin
KRB	Krebs-Ringer bicarbonate
MCFA	Medium chain fatty acid
MDA	Malondialdehyde
ME	Metabolizable energy
NC	Negative control
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffer saline
PC	Positive control
PWD	Post weaning diarrhea
RDE	Red osier dogwood extract
RNA	Ribonucleic acid
ROD	Red osier dogwood
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT-qPCR	Reverse transcription- quantitative polymerase chain reaction

SCFA	Short chain fatty acids
SOD	Superoxide dismutase
TAC	Total anthocyanin content
TBARS	Thiobarbituric acid reactive substances
TEER	Trans-epithelial electrical resistance
TNF-α	Tumor necrosis factor-alpha
TP	Total protein
TPC	Total polyphenol content
TSB	Tryptic soy broth
VFA	Volatile fatty acid
VH	Villous height

1 General Introduction

Weaning is a process of separation of animals from their mothers. It happens in all mammalian species in nature and is a gradual process. However, with the farming of domestic animals for meat production, the livestock industry conducts the process of weaning abruptly and at a young age of the animal. If not properly managed, this can cause a serious impact on the health of animals during their lifetime. It is of particular importance in pigs because they are weaned at an age of 3 to 4 weeks and many organ systems including the gut are still in development and not fully matured. If the pig gets sick after weaning, it also causes production losses to the farmer. In the period during weaning, a cascade of events occurs in the body of piglets and the environment around them. The piglets stay with their sows during the nursery phase for approximately 3 weeks and in 24 hours they have a change in the environment, that is the separation from sow and littermates, new pen mates, human handling, transportation, and most importantly shift in their diet from energy-rich, highly palatable sow milk to mostly plant-based, less digestible dry feed (Pluske et al., 1997; Lallès et al., 2007).

Some pigs do not consume feed for prolonged periods (up to 48 hours) post-weaning due to the tremendous stress, leading to post-weaning diarrhea (PWD) (Brooks et al., 2001). The PWD is a multifactorial disorder and is characterized by the proliferation of *Escherichia coli* in the intestine of piglets (Pluske et al., 2003). Around weaning, the digestive system of the piglet is still developing and therefore constantly changing. It has been reported that stomach secretions (HCl, pepsin, and lipase) and motility are reduced in early-weaned pigs compared to sow-reared pigs (Snoeck et al., 2004). The acidic environment is necessary for the stomach for protein breakdown and killing pathogenic bacteria. However, with higher stomach and intestinal pH, the weaned

piglets are more prone to diarrhea (Heo et al., 2013). Villus atrophy and crypt elongation along with a transient decrease in brush border enzyme secretion (lactase, maltase, amino peptidase) is observed in piglets, post-weaning (Hampson, 1986; Lallès and David, 2011).

Antibiotics are added to the pig's diet as a supplement for growth promotion. These antimicrobial compounds are added at subtherapeutic doses which aid in protection against pathogens and enhance growth performance. Two main reasons for antibiotics to play a great role as growth promoters are, their direct effect on the gut microbes and thinning of the gut wall (Frankel et al., 1994). Antibiotics modify the gut microbiota in such a way that there is a reduced competition for nutrients between host and pathogens, and decreased production of toxic microbial metabolites that defer growth in piglets (Anderson et al., 1999). Antibiotics also help indirectly in thinning the villi which facilitate more nutrient absorption (Frankel et al., 1994). Unfortunately, there are growing concerns regarding antibiotic usage in the livestock industry. Using antibiotics for other than therapeutic purposes can accelerate antimicrobial resistance in microbes and contaminate the food chain due to antibiotic residue in meat consumed by humans (Dibner and Richards, 2005). This was first brought to attention in the report of the Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine (Swann et al., 1969). The first European country to ban antibiotics as growth promoters was Sweden in 1986. Soon many countries like the United Kingdom, Denmark, United States, etc. followed and either banned or restricted antibiotic use in food-producing animals (Dibner and Richards, 2005). With the ban, extensive research started in several countries to find a natural alternative to antibiotics.

The research developed some exemplary alternatives like probiotics, prebiotics, synbiotics, feed enzymes, phytogenics, organic acids, nucleotides, antimicrobial peptides, seaweed extract, vaccines, and so on. These additives not only have a growth promotional effect on piglets but also

provide various nutritional and non-nutritional benefits. The most popular and researched feed additives are presented in detail in the literature review. Many antibiotic alternatives, when used in combination have a synergistic effect on gut health in piglets. However, there are still many challenges like digestibility, absorption, bioavailability, side effects, optimal concentration in the feed, and palatability of these feed additives. Therefore, it is important to find the solutions to all these problems before antibiotics are prohibited completely for animal growth promotion.

Plants and their extracts have been used in traditional medicine in every part of the world. There are recognized health benefits of common herbs and spices which are still used as a remedy for different illnesses in humans. When plant extracts were added to swine diets, similar results were obtained. Phytogenics, also known as plant extracts, not just have antimicrobial properties but also immunomodulatory, antiviral, antioxidative, and anti-inflammatory properties (Xiong et al., 2019). In experiments involving *E. coli* challenged weaned piglets, oleoresin supplements made from pepper, turmeric, and ginger extracts, increased the gene expression of tight junction proteins, improving the intestinal barrier function of the piglets (Liu et al., 2014b). Another study found that allicin present in garlic extract helped in reducing chemokine production in intestinal epithelial cells and leukocyte placement in the inflamed tissue *in vitro* (Lang et al., 2004). Phytogenics that are used as herbs like fennel, oregano, thyme, basil, cinnamon, mint, etc. are known to have antioxidative properties (Slamenova et al., 2008; Frankič et al., 2010a; Amorati et al., 2013; Wu et al., 2018a).

Red osier dogwood (ROD) was the plant of choice for this research as it is commonly found in Canada and northern parts of the United States, and it has been used by Indigenous people as traditional medicine. Moreover, there has been *in vitro* research done on dogwood extract which showed the antioxidative effect of the plant extract (Yang et al., 2019). When pigs were fed diet

having 4% raw dogwood plant, they had lower serum and ileal malondialdehyde and higher serum superoxide dismutase levels than the group of piglets fed control diet (corn-wheat-soybean meal) (Koo et al., 2018). There were no *in vivo* studies in pigs fed polyphenol extract made from dogwood plant. Hence, this study would be pivotal research in bringing red osier dogwood extract (RDE) closer to commercial use as an alternative to antibiotic growth promoters (AGP).

2 Literature review

2.1 Gut health

Gut health is a broad term that includes various physiological and functional features of an animal's gastrointestinal tract (GIT). The GIT is responsible for the first physiological step of bringing nutrients to the body's cells and plays a crucial role in regulating the development of young piglets (Guilloteau et al., 2010). A healthy gut can be defined as 'the absence or prevention or avoidance of disease so that the animal is able to perform its physiological functions in order to withstand exogenous and endogenous stressors' (Kogut and Arsenault 2016). The basis for this definition needs a GIT framework that includes, effective digestion and absorption of feed, effective structure and function of intestinal epithelium for barrier function, the interaction of the host with the intestinal microbiome, and a powerful immune status in absence of GIT illness (Bischoff, 2011; Celi et al., 2017). The general metabolism, growth performance, and physiology of pigs are highly influenced by GIT function in all stages of growth and development. The physiological changes in the swine digestive tract are a result of a complex interacting system formed by nutrition, the barrier function of the intestinal mucosa, and the intestinal microbiome (Pluske et al., 2018). Growth and maturation of intestinal epithelial cells, the establishment of gut barrier function by gut mucosa, gut microbiome for microbial fermentation of feed components, morphology and structure of intestine constitute GIT physiology and are essential aspects of gut health in piglets. To understand these elements of the digestive system it is useful to know the foundation of the digestive tract and its maturation.

2.2 Developmental physiology of the digestive tract in swine

In the pre- and post-natal period, GIT function and growth increases dynamically so that the young pig is prepared for its future development and ultimately survival. Incidents in this period are crucial because they can affect the pig's whole life by altering feed efficiency and/or health resilience (Williams, 2003; Morales J., 2006). There are three main developmental phases of the digestive system namely, the prenatal phase (mainly structural formation of organs), neonatal phase (milk and colostrum influence on the piglets' GIT physiology) and lastly the post-weaning phase (associated with significant changes and adaptation to solid feed and external stress factors) (Zabielski et al., 2008).

In the last stage of gestation, during the prenatal period, there is a sudden weight gain in the fetus, which is attributed to the growth of the immature digestive system (McPherson et al., 2004). During this period, the fetus ingests amniotic fluid while in utero and this regulates many gastric enzyme activities and GIT growth before birth. According to Sangild et al. (2002), when the esophagus of fetal piglets was ligated to prevent swallowing of amniotic fluid, body weight (BW), intestinal weight, aminopeptidase A activity, and glucose absorption were significantly lowered.

The early postnatal growth of GIT is seen in several studies, to be disproportionately faster than the rest of the body. During the first three days following birth, the stomach experiences profound functional maturation, with its gastric acid secretion capacity (expressed as a function of gastric tissue mass) increasing roughly threefold (Xu and Cranwell, 1990). Postnatally, the GIT of newborn piglets modifies quickly for the shift from parenteral to enteral nutrition. This adaptation is vital for piglets because their intestine readily absorbs macromolecules like immunoglobulins (Ig) to gain passive immunity from the sow's colostrum in the first 24 to 36 h after birth.

Furthermore, colostrum supplies essential nutrients in the form of lactose, fatty acids, and milk protein, and at least 68.5 kcal/kg BW of metabolizable energy is needed during the first 24 hours of life because the piglets' energy reserves, especially fat, are insufficient at birth (Le Dividich et al., 1994). With inadequate energy intake, the newborn piglets are unable to maintain body heat, which is linked to increased mortality and morbidity (Theil et al., 2014).

In the weaning period, the digestive system is still not fully matured and several changes in the gut microbiome, intestinal tissue structure and immune system are going on. During the 2 weeks post-weaning, changes happening in the intestinal physiology include a transient net increase in ion transport and glucose absorption, and a decrease in jejunal electrical resistance in piglets fasting for 2 days post-weaning (Boudry et al., 2004). Other than the basic digestive processes like prehension, digestion, active or passive absorption, and excretion, the GIT also regulates vitally important activities for normal physiological functioning and homeostasis in the body. In natural conditions, the weaning transition is a slow and gradual process that is achieved by the pig at approximately 20 weeks of age (Jensen and Stangel, 1992). However, in commercial farms, the pig undergoes a transition that includes an abrupt change in diet, transportation, handling by humans, new pen mates, and separation from their sow and siblings at a much younger age of 3 to 4 weeks. This drastic change can have short-term effects on pigs like diarrhea and loss of appetite and long-term effects like poor growth performance and additional days to market weight. This section briefly describes the three stages in the development of swine GIT. But it is not in the scope of this review to elucidate each stage in detail, therefore the focus in the subsequent chapters is on the weaning transition and post-weaning phase in piglets.

2.3 Changes during weaning

Many times, weaning is considered as only a shift in the diet and surroundings of the piglet. However, there is an intricate connection between the nutrition of the piglet, its gut barrier function or immunity, and its gut microbes i.e., microbiome, as shown in figure 2.1. These three domains maintain a delicate balance in the GIT and play major role in shaping the pig's physiology, health, and well-being. This balance is disrupted during weaning, causing a series of changes in the GIT. The following subsections summarize the changes in these three elements and their effects on gut health during weaning.

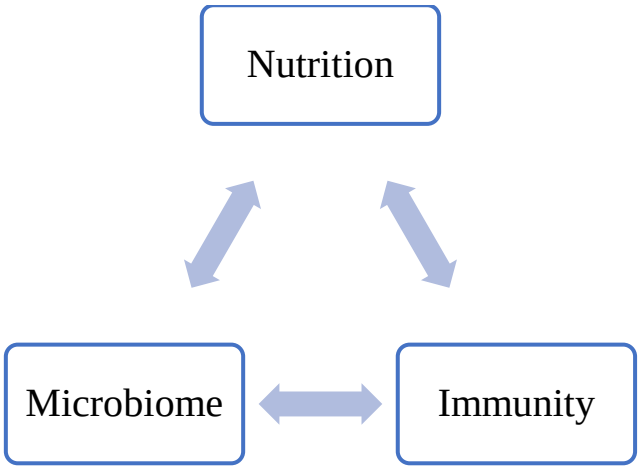

Figure 2.1 Mutual interaction between nutrition, microbiome, and gut immunity that maintains the structure and function of the GIT.

Adapted from Niewold, (2006).

2.3.1 Nutrition

As discussed earlier, weaning involves nutritional difficulty along with social and environmental disturbances. This results in decreased feed intake, sub-optimal growth, behavioral abnormalities, and gastrointestinal issues (Colson et al., 2006; Gresse et al., 2017). Multiple research projects have shown that a decrease in the voluntary feed intake critically affects future intestinal

development, growth, and disease susceptibility as well as villus height and maturation of gut lymphoid tissue (Pluske et al., 1997; Spreeuwenberg et al., 2001; Spreeuwenberg et al., 2003).

Ideally for optimal feed intake, creep feeding in nursing piglets should start at an age of 2 weeks or less and piglets should be provided with *ad libitum* feed in an easily accessible feeder space, along with the addition of feed supplements (Jayaraman and Nyachoti, 2017). In a study by Lee and Kim (2018), piglets fed creep feed at 7 days of age had more BW post-weaning than those which did not have creep feed or were given creep feed later than 7 days of age. According to Carstensen et al. (2005), voluntary consumption of creep feed by nursing piglets, just before weaning, significantly reduced the occurrence of colibacillosis post-weaning. Creep feeding is a husbandry practice that improves growth performance in piglets, acclimatizes the digestive system to dry feed, and promotes voluntary feed intake post-weaning.

Almost 50% of weaned piglets adapt to the dietary changes 24 h post-weaning, however, 10% do not eat the dry feed for up to 48 h (Brooks et al., 2001). According to a review written by Sève (2000), the metabolizable energy (ME) intake of weaned piglets is lowered by 30 to 40 percent compared to pre-weaning milk intake, and it takes roughly 2 weeks post-weaning to reach the required intake level of ME consumption. This shows that decreased feed intake directly affects growth performance. Piglets lose approximately 100 to 250 g of BW on the first day after weaning, irrespective of their age at weaning and it takes them at least 4 days to recover (Sève, 2000). Conversely, for piglets gaining more than 227 g/day in the first week after weaning, the days to the market were cut by 6 to 10 compared to piglets who gained less than 150 g/day (Kats et al., 1992). This suggests that growth and development during the first week after weaning influence the final number of days to market. Therefore, timely feed consumption in weaned piglets is of

utmost importance for a healthy gut, maximum growth and development, and consequently more profit for the producer.

2.3.2 Microbiome

Sow milk consumption alters the microbiome, resulting in a milk-oriented microflora from the birth of the piglet, which is the beginning of microbial colonization (Isaacson and Kim, 2012; Frese et al., 2015). After weaning of pigs, at the age of 3 to 4 weeks, solid feed constitutes their major source of energy and other nutrients (Gresse et al., 2017). As a result of the weaning process, the microbial population shifts in favor of microbial pathogens (Li et al., 2018). Post-weaning diarrhea is mainly caused by changes in gut microbiota composition due to weaning shock. After weaning, there is a decrease in bacteria like *Alloprevotella* and *Oscillospira,* and an increase in *Campylobacterales, Campylobacteraceae*, and *Campylobacter* in the gut of piglets (Li et al., 2018). A previous metagenomic study of the fecal microbiome found that diarrhea was related to an increased proportion in the number of *Prevotella, Sutterella, Campylobacter*, and *Fusobacteriaceae* (Yang et al., 2017). Succinate and acetate, which can be produced by *Alloprevotella*, may improve the intestinal barrier and show anti-inflammatory properties (Downes et al., 2013). Some species of *Oscillospira* generate butyrate, which is effective in treating inflammatory diseases (Gophna et al., 2017).

Dietary changes from basic to complex food sources during weaning, which affect the small intestine's absorption capacity and ultimately impact growth and feed efficiency, are key contributors driving rapid alterations in the microbiota in piglets during weaning (Upadhaya and Kim, 2021). Short-chain fatty acids (SCFAs) are produced by *Prevotella* species by breaking down structural polysaccharides using enzymes such as β-glucanase, mannanase, and xylanase, which

have the ability to degrade plant cell walls (Ivarsson et al., 2014). *Lactobacilli* break down the carbohydrates in the large intestine, where they are transformed by fermentation into SCFAs, which are then used by the pigs as a source of energy (Gänzle and Follador, 2012). The microbial population of the pig's gut is vast and dynamic, and it is not feasible to observe the changes constantly. Therefore, these are only some of the significant changes in microbial composition based on the functional requirements of the pig for adaptation to the weaning transition (Upadhaya and Kim, 2021).

2.3.3 Immunity

Weaning transition leads to histomorphological and functional changes in the gut, which includes villous atrophy and increased crypt depth, reduced nutrient absorption, and enzyme secretion, to name a few (Wijtten et al., 2011; Pluske et al., 2018). The intestinal barrier comprises a mucosal layer formed by extracellular and cellular components, which consists of epithelial cells (enterocytes, Goblet cells, Paneth cells, and enteric lymphocytes) and intercellular junctional complexes (tight junctions, adherens junctions, gap junctions, and desmosomes) (Salvo-Romero et al., 2015). This barrier acts as the first line of defense and protects the pig against the unknown substances that enter the digestive lumen. When the gut barrier breaks down, intestinal permeability increases, enabling pathogenic agents contained within the lumen to leak through the epithelium, making it simple to enter the sub-epithelial tissue. This leakage may lead to inflammation, diarrhea, malabsorption, and systemic illness, all of which can have a negative impact on the animal's health and development (Madara, 1990; Nabuurs et al., 1993).

It was reported that the intestinal permeability and secretory activity in the mid-jejunum and colon of pigs weaned at 19 days of age was higher than that of unweaned pigs in a study by Moeser et

al. (2007). A similar finding was noted in the study by Boudry et al. (2004), who observed a transitory drop in transepithelial electrical resistance with the help of the Ussing chamber in the jejunum of piglets weaned at 21 days of age. The effects of early weaning on the GI barrier, the immune system, and the neurological system may remain throughout maturity (Medland et al., 2016). Two days after weaning, pig intestinal T lymphocytes (CD4+ and CD8+) increase dramatically, amplifying the mRNA expression of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukins (IL), such as IL-6 and IL-8 in the mid-jejunum (Pié et al., 2004). Secretory IgA is an antibody that particularly protects the mucosal linings of the body, including intestinal mucosa. Therefore, it plays a very important role in the intestinal barrier function. Ushida et al. (2008), observed that weaning 25 d old piglets, resulted in a lower secretory IgA in feces for 5 consecutive days. It should be noted that, during the age of weaning, the immune system is undergoing modifications for maturation and these changes are aggravated by external stimuli during the weaning transition.

Many changes in intestinal physiology occur during the two weeks after weaning, some of which are transient, while others are long-term. Examples of short-term alterations include increased net ion transport and glucose absorption capacity in the jejunum, and decreased jejunal electric resistance in piglets fasted for two days after weaning (Lallès et al., 2007). The authors also observed that 2 weeks post-weaning glucose absorption and permeability to macromolecules in jejunum decreased, while the ileal transmucosal electric resistance increased and then stabilized. This shows that weaning induces temporary albeit acute changes caused by post-weaning fasting but with the resumption of voluntary feed intake, intestinal maturation resumes.

The alterations in nutrition, microbiome, and immunity have some serious effects on newly weaned piglets, making weaning the most crucial stage of pig production. The most concerning consequence is PWD. Most of the nutrition and management strategies in piglets are aimed at preventing PWD.

2.4 Post-weaning diarrhea

Post-weaning diarrhea is defined as an intestinal illness caused by multiple stressors, and signals include watery stools, poor growth, high morbidity, and high death rates (Wellock et al., 2008; Wu et al., 2015). It is a significant gastrointestinal illness that can alter the future growth and development of piglets and if not managed in time, it can be fatal. Multiple factors (as discussed above) in combination with ETEC are involved in causing PWD. Piglets are most susceptible to coliform infections during the first 2 weeks post-weaning. While some pathogens directly affect the intestinal tract, others put piglets in a more vulnerable situation. An example given by Nakamine et al. (1998), is the infection of porcine reproductive and respiratory syndrome virus, which weakens the immune response of piglets, allowing ETEC (enterotoxigenic *Escherichia coli*) to spread in the systemic circulation, causing septicemia and sometimes, death. According to Laine et al. (2008), farms that provided restricted feeding to weaned piglets had more occurrences of PWD than other farms that did not. Similarly, other elements like hygiene, temperature control, herd size, and feed supplements or additives have an impact on PWD infections (Melin et al., 1997; Lofstedt et al., 2002). Although, it is not possible to review each factor in detail, certain essential aspects which have a major role in triggering or aggravating diarrhea after weaning are discussed in the next section. Figure 2.2 summarizes the situation during early weaning and the crucial factors associated with it.

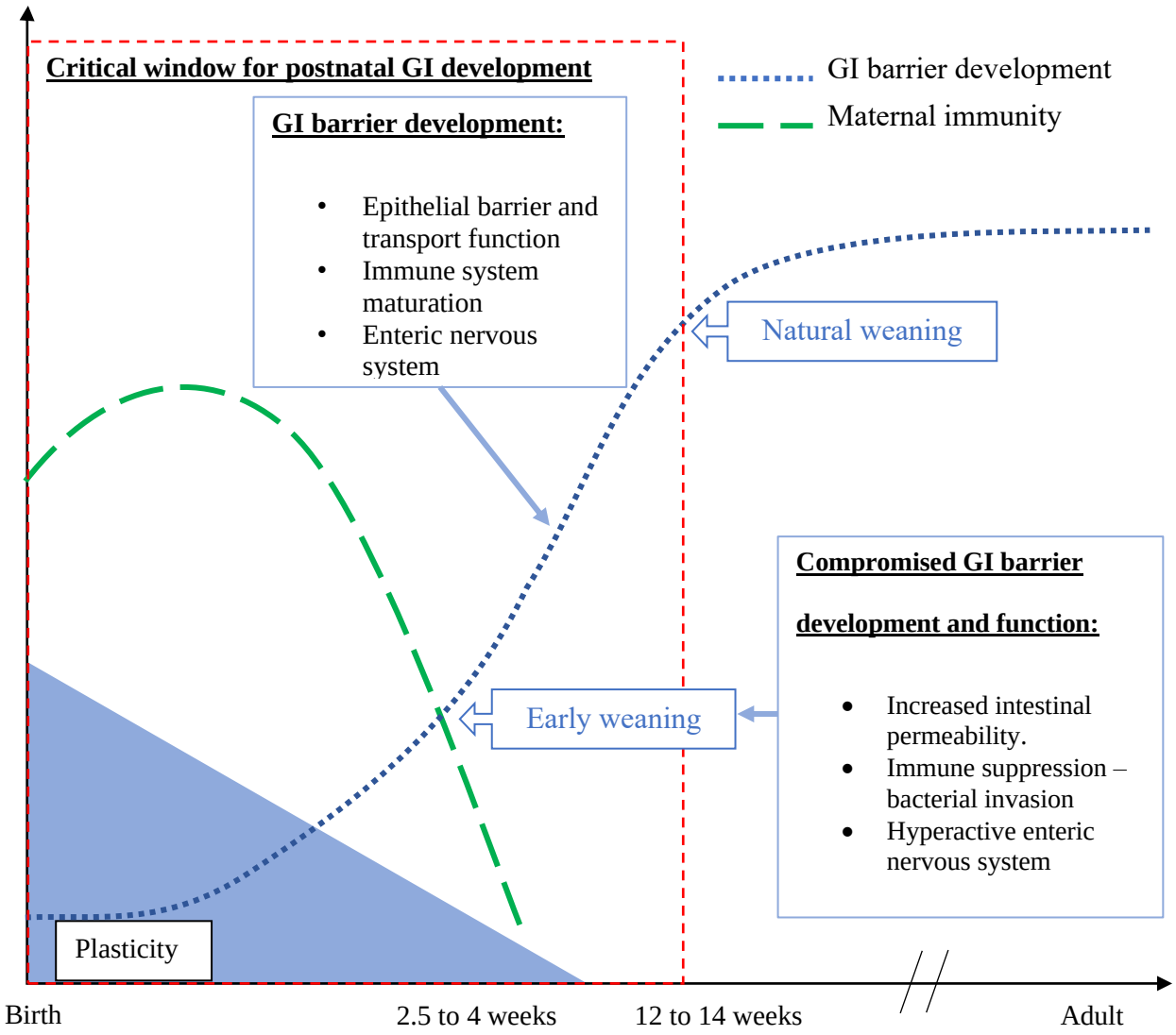

Figure 2.2 Summary of events during early weaning and their effects on gut health.

Adapted from Moeser et al. (2017).

2.4.1 Causes

Diarrhea occurring after weaning is a complex disorder and as mentioned earlier, there is not one particular cause of PWD but several conditions the pig is exposed to that lead to PWD. This section elucidates its causes and the mechanism behind the development of diarrhea in weaned piglets. Figure 2.3 summarizes some of the common causes that make piglets susceptible to PWD.

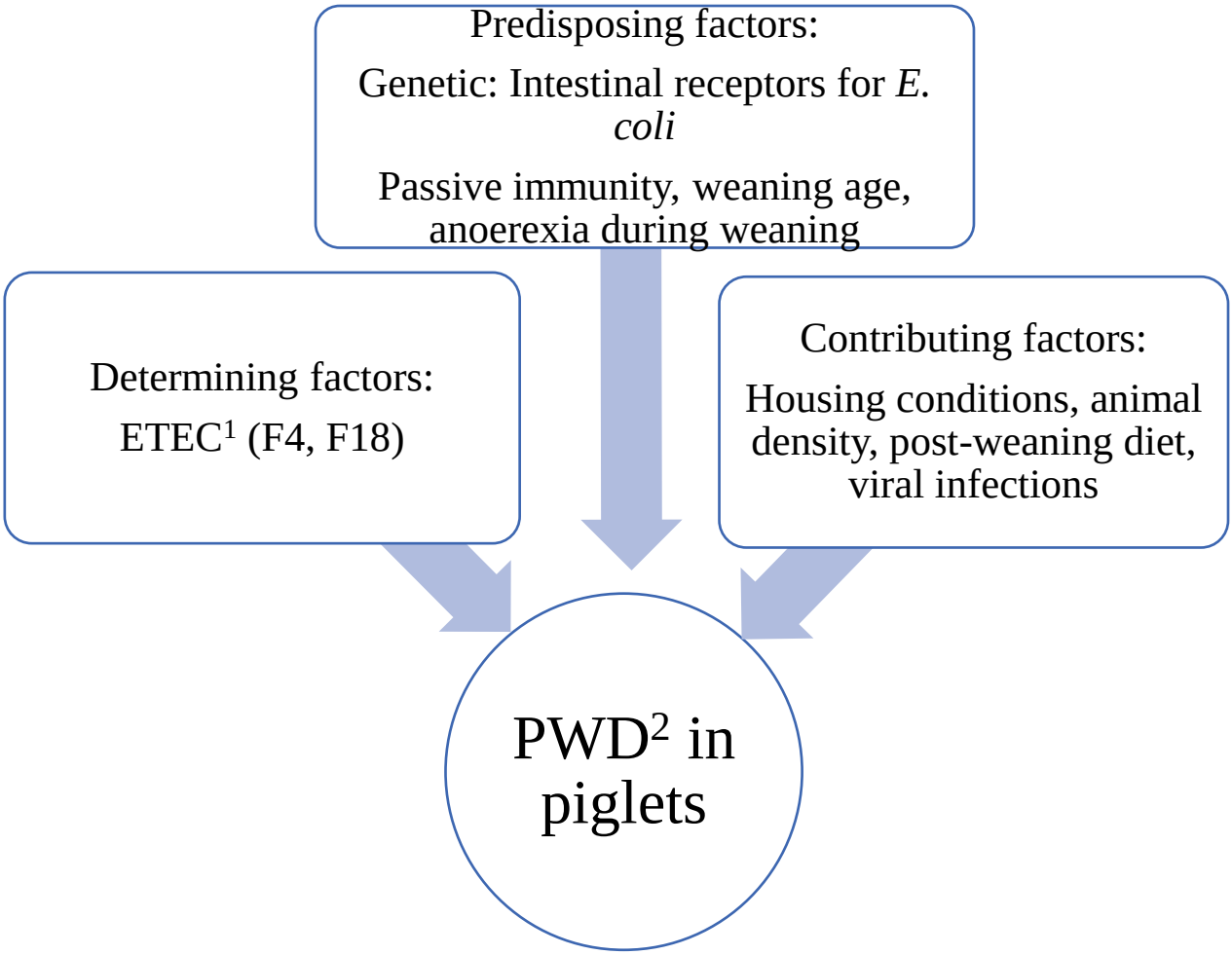

Figure 2.3 Schematic representation of the multifactorial causes of PWD.

Adapted from Rhouma et al. (2017a).
[1]Enterotoxigenic *Escherichia coli*
[2]Post-weaning diarrhea

2.4.1.1 Causative pathogen

Escherichia coli is a natural inhabitant of the gut, present in normal and sick pigs (Schierack et al., 2006). However, the fact remains that some *E. coli* serotypes with certain virulence genes (for instance, fimbrial and toxin genes) are linked to PWD in pigs and diarrhoeal illness in calves and humans (Nagy and Fekete, 2005). As some of these ETEC bacteria multiply in number, PWD is caused (Fairbrother et al., 2005; Nagy and Fekete, 2005). As the bacteria proliferate in the intestine, enterotoxins and adhesins are abundantly produced, both contributing the most to symptoms of

PWD. F4 and F18 are the two paramount adhesins in ETEC infections(Luppi et al., 2016). The occurrence and function of F4 and F18 specific receptors in the small intestine regulates the susceptibility of piglets to the infection (Delisle et al., 2012). *Escherichia coli* is the predominant causative agent of PWD, however, other pathogenic microbes contribute to the severity of this disease. Viruses like strains of rotavirus and sapovirus, which destroy the intestinal villi, and bacteria like *campylobacters* and *salmonella*, as well as protozoa like *Cryptosporidium*, promote diarrhea in this age group (Katsuda et al., 2006). It is also noted that plenty of β-hemolytic ETEC is excreted through feces during the occurrence of this illness (Schierack et al., 2006). The specific fimbriae (F4 or F18) present on ETEC adhere to the glycoprotein receptors of enterocytes in the small intestine. These receptors are located on the brush border of the villi. The precise method of the linkage between the fimbriae and receptors is not precisely known (van den Broeck et al., 2000).

2.4.1.2 High crude protein

According to researchers like Bikker et al. (2006) and Kluess et al. (2010), feeding a high-protein diet to piglets weaned at 3 to 4 weeks of age, may increase the risk of developing PWD. The mechanism of action behind that is the high level of crude protein (CP) in the diets of these early-weaned pigs, which might promote microbial fermentation of undigested protein and boost the growth of harmful bacteria in the intestine (Heo et al., 2015). This process produces an excess amount of branched-chain volatile fatty acids (VFA) and possibly toxic compounds like ammonia and amines, which can decrease the rate of development in piglets and increase the occurrence of diarrhea in pigs after they are weaned (Porter and Kenworthy, 1969; Rist et al., 2013; Rajoka et al., 2017). The abovementioned compounds are synthesized by bacteria such as *Bacteroides* spp., *Propionibacterium* spp., *Streptococcus,* and *Clostridium* species (Rist et al., 2014). For the most

part, these compounds are inflammatory and can damage barrier function (Hamer et al., 2012). The metabolites or bacterial toxins may then impede fluid reuptake and conceal small intestinal hypersecretion (Pieper et al., 2016). Hydrogen sulfide has a detrimental effect on gut health by damaging the mucus membrane and increasing the permeability of intestinal epithelium (Ijssennagger et al., 2015). Some studies have connected high-protein fermentation to a higher risk of developing cancer as well (Davis and Milner, 2009). Furthermore, there is an inhibition of the production of SCFAs due to a decrease in the quantity of Lactobacilli, which are prevalent before weaning (Jensen, 1998). When pigs are fed a high-protein diet, they not only have less expression of SCFAs, such as butyrate, which may allow the intestinal epithelium to recover more quickly, but also show a higher small intestine pH (Burrin et al., 2001; Htoo et al., 2007). The findings of Jeaurond et al. (2008) showed that dietary amounts of fermentable protein (eg. poultry meal) increased the abundance of *Clostridia* species in the large intestine, while pigs fed more fermentable carbohydrates (eg. sugar beet pulp) reduced the number. The changes occurring in the pig's digestive tract due to high crude protein are summarised in figure 2.4.

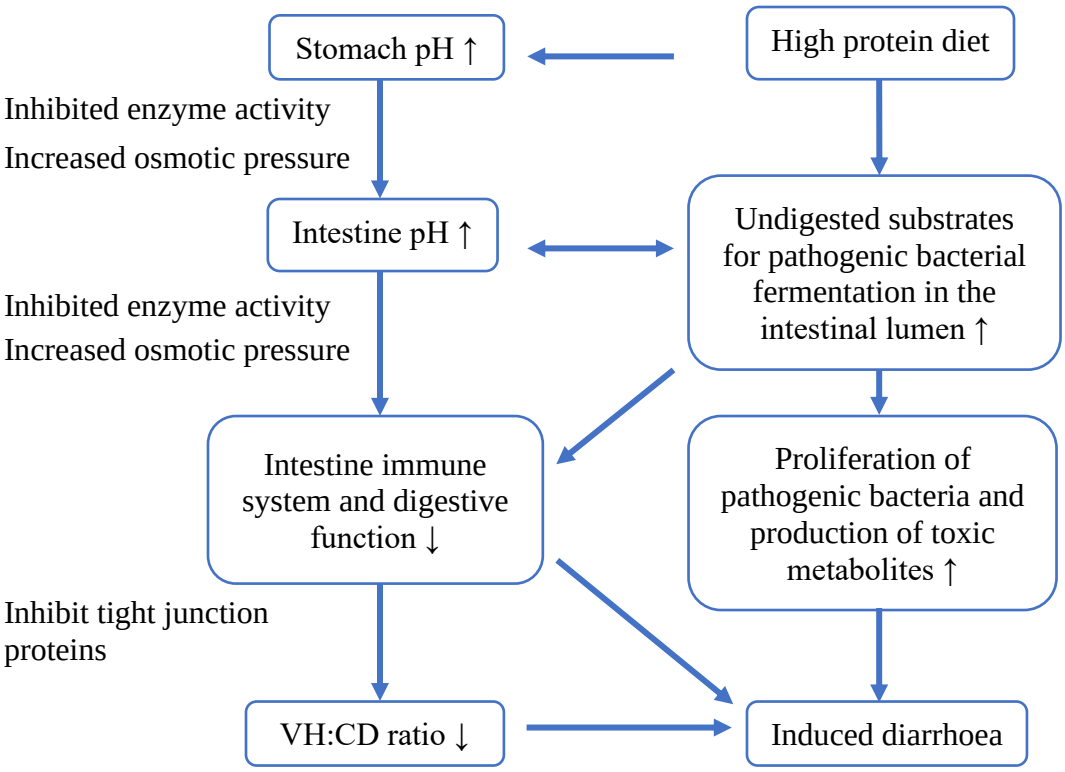

Figure 2.4 The possible mechanism of high protein diets induced post-weaning nutritional diarrhea.

Adapted from Gao et al. (2019).
↑ represent higher and ↓represent lower value, respectively.

2.4.1.3 Water and electrolyte imbalance

Almost 98 percent of the water in digesta along with electrolytes is absorbed by the small intestine and some amount is absorbed by the colon (Chowdhury and Lobo, 2011). Ions are absorbed into the body through ion exchangers present on the luminal surface of the small intestine (Hoque et al., 2014). While some ions are transported by an osmotic gradient, others need an energy-driven processes. The maintenance of electrolyte balance and absorption of water and electrolytes is a complicated procedure and is not yet completely understood. Calcium as limestone, phosphorus as calcium phosphate, sodium and chloride as salt, and sodium bicarbonate are regularly added to swine feeds, to meet their nutritional requirement as well as the gastrointestinal electrolyte balance (Liu et al., 2014a). Most of the pathogenic bacteria in the gut stimulate the secretion of fluid and

electrolytes into the lumen of the intestine with the help of the different toxins they produce (Viswanathan et al., 2008). Post-weaning electrolyte imbalances contribute substantially to PWD, produce major intestinal electrolyte instability, and adversely affect the development and growth performance of weaned piglets by significant salt and water depletion from their bodies (Patience et al., 1987; Guzmán-Pino et al., 2015). The possible explanation behind this is demonstrated in a flow diagram in Figure 2.5. To begin with, water and electrolytes are brought into the luminal space of the small intestine, employing the osmotic force provided by the unabsorbed solutes contained in the post-weaning diets (Buddington et al., 2012). Simultaneously, PWD causes villus atrophy and crypt enlargement, affecting the body's absorption and secretion processes negatively (Stenson, 2013). Finally, the unabsorbed dihydroxy bile acid and fatty acid promote active secretion (Hofmann et al., 2008). These organic acids then travel into the lipid phase of the plasma membrane (Kim et al., 2018) and lead to reduced intestinal absorption, increased intestinal output, and excess fecal water resulting in diarrhea (Ridlon et al., 2015).

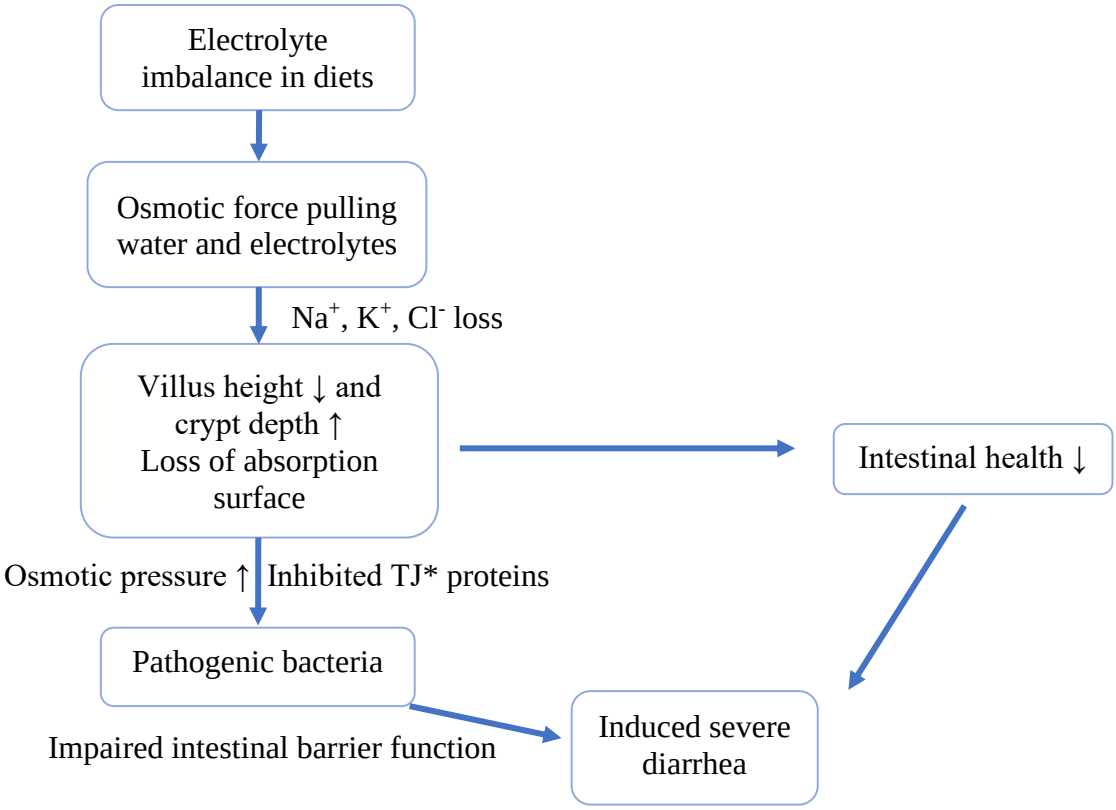

Figure 2.5 The possible mechanism of electrolyte imbalanced diets induced post-weaning nutritional diarrhea.

Adapted from Gao et al., (2019).
*Tight junction

2.4.2 Pathogenesis

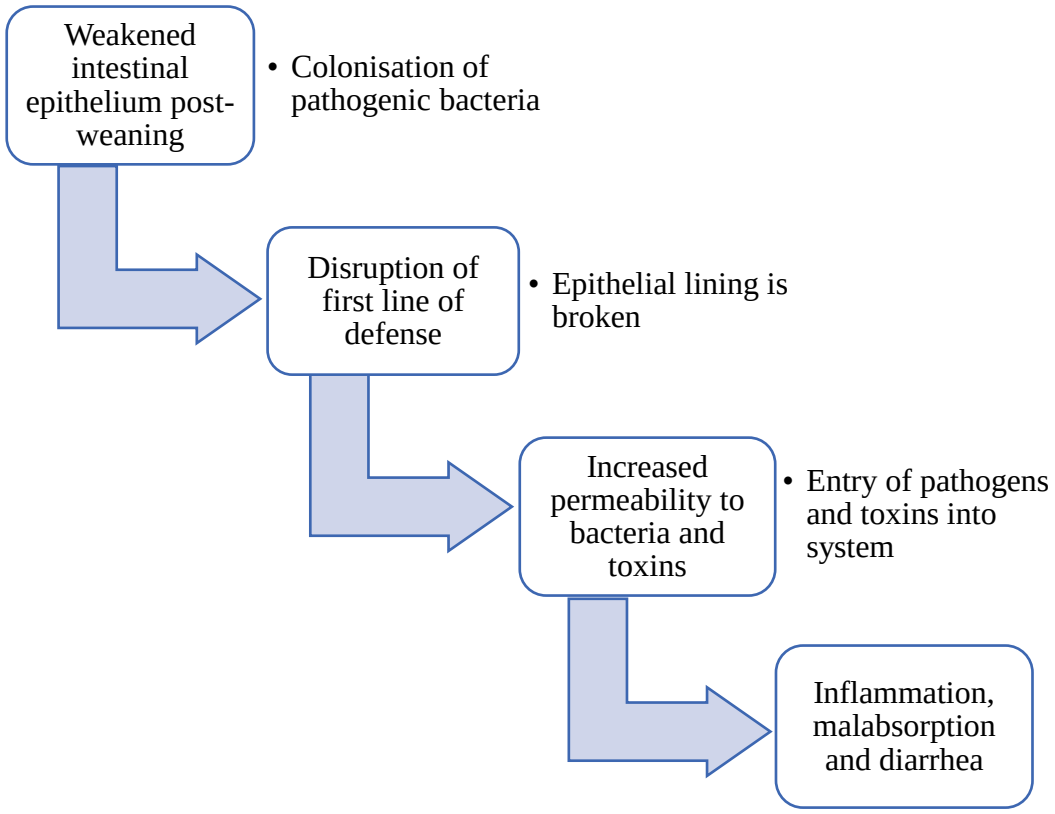

Figure 2.6 Pathogenesis of PWD.

Piglets can get exposed to ETEC in their surrounding through the mammary glands of their mother, the farrowing pen, or in the pens they are moved to for the nursery period (Gyles et al., 2010). Step by step effects and changes done by ETEC are shown in a simplified manner in Figure 2.6. Once the bacteria enter the GIT, they reach the small intestine and attach to the glycoprotein receptors of the epithelium with the support of fimbriae. Weaning reduces active immunity and damages gut integrity, providing favorable conditions for bacterial adhesion (Pluske et al., 2002). Soon after adhesion and colonization, the ETEC are empowered to release an enormous amount of enterotoxins in the lumen. The toxins produced are of 2 types, heat-labile which promotes sodium, chloride, and hydrogen carbonate ion secretion into the lumen; and heat-stable toxin, which lowers the absorption of liquid and salts into the body (Nagy and Fekete, 1999). Both toxins collectively

cause hypersecretion of water and electrolytes in the lumen, causing dysfunction of enterocytes and bringing excessive luminal contents in the colon, which are beyond its absorptive capacity (Heo et al., 2013). With the disproportionate volume of water and electrolytes, piglets suffer from dehydration, metabolic acidosis, malnutrition, osmotic diarrhea, and sometimes death (Zhang et al., 2006; Rhouma et al., 2016; Rhouma and Letellier, 2017; Roubos-van den Hil et al., 2017).

Other physiological processes that are happening in the piglet's body contribute to the symptoms of PWD. It has been observed that inflammatory responses in the intestines are triggered right after weaning and subside gradually. After weaning, McCracken et al. (1999) found that in two days, pigs given a soy or milk diet had a lower jejunal expression of major histocompatibility complex class 1 mRNA and higher CD4+ T cell counts in the jejunal villi, respectively. This leads to an increase in the pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (Pié et al., 2004). The reduced appetite post-weaning combined with its deleterious effects on intestinal morphology causes a regional inflammatory response in the intestine (McCracken et al., 1999).

As explained by Pluske et al. (2018), too much immune system excitation during weaning might have a detrimental effect on growth rate and feed efficiency. This physiological function has a high-energy requirement and pigs might suffer from excessive prostaglandin E2 production that can lead to pyrexia, cachexia, and poor performance. The effects of PWD vary based on the severity of the illness, but oxidative stress is a common feature of PWD. It is therefore very important to understand what it is and how it can be managed.

2.4.3 Oxidative stress

An imbalance between the production and elimination of free radicals such as reactive oxygen species (ROS) and reactive nitrogen species to an extent that there is an accumulation of ROS is

called oxidative stress. Free radicals are produced as a by-product of regular metabolic reactions occurring in the body. They are unstable and highly reactive enabling them to react with lipid, protein and DNA, therefore causing oxidative injury to the cells (Zhu et al., 2012). At the molecular level, they modify signalling pathways, mutate genetic material, change protein structures, and disrupt energy metabolism (Rahal et al., 2014). Weaning greatly escalates the production of free radicals and ROS (Rao, 2008; Vergauwen et al., 2015). This is caused by an increased expression of pro-inflammatory cytokines restructuring the tight junction proteins and decreasing their gene expression, ultimately increasing gut permeability (Hao et al., 2021). Nuclear factor-kappa B (NF-κB) is a protein complex that regulates the expression of genes causing inflammation and forms the link between ROS production and gut inflammation which is explained in figure 2.7. However, each animal has a natural mechanism to protect cells from oxidative stress. This ROS scavenging system is made up of various enzymes that act as antioxidants (Finkel, 2003; Minelli et al., 2009).

There are exogenous and endogenous antioxidants, which contribute in balancing prooxidant and antioxidant compounds in the body. The endogenous antioxidants comprise enzymes like superoxide dismutase (SOD), catalase and glutathione peroxidase. The SOD reduces oxidized species which are later on destroyed by other compounds like glutathione peroxidase and catalase (Yin, 2013). There are exogenous sources or antioxidants available as micronutrients in the diet like ascorbic acid (vitamin C), tocopherol (vitamin E), carotenoids, flavonoids, and anthocyanins. The PWD and its effects have created a field of special interest concerning feed additives supplemented during weaning. As the GIT undergoes rapid changes during the post-weaning transition, it is of utmost importance to manage and stabilize the growth and overall health of weaned piglets (Pluske, 2013). To achieve this, various growth promoters have been used in

commercial pig production. A thorough description is provided in the following sections about current and potential growth promoters available for swine.

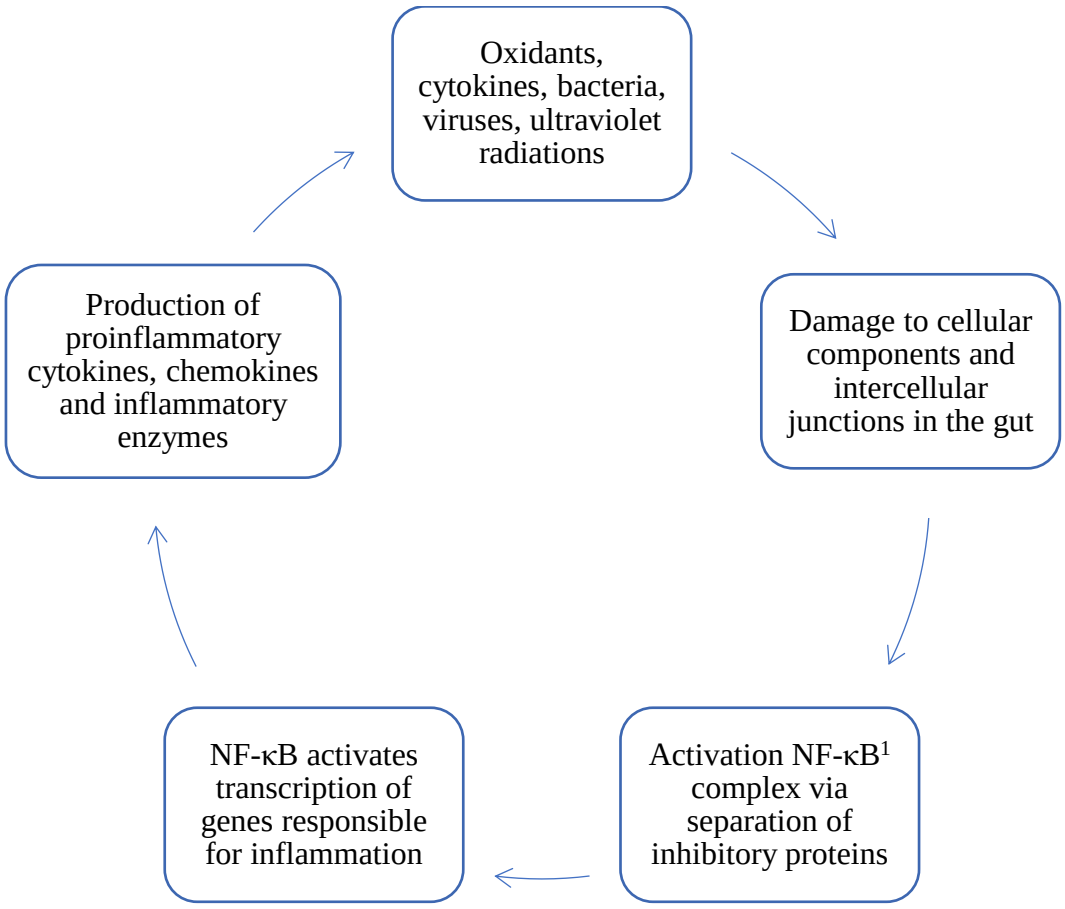

Figure 2.7 Link between inflammation and oxidative stress that leads to post-weaning diarrhea in piglets.

Adapted from Gessner et al. (2017).
[1]NF-κB - Nuclear factor-kappa B

2.5 Antibiotics as growth promoters

Since animal agriculture has become increasingly industrialized, antibiotics have been rapidly integrated into the animal production system, where they serve as both preventative and therapeutic agents, in addition to being a cost-effective economic instrument (Begemann et al.,

2018). As years went by and animal farming became intensive and demanding, the distinctions between these applications became increasingly hazy. To expedite animal growth, notably in monogastric animals like pigs and poultry, shorten production cycles, and enhance space usage through a higher 'density' of herds, group treatment with antibiotics in feed or water became a standard (Nathan and Cars, 2014). Basically, antibiotics paved the way for the intensification of animal-based food production (Thoms, 2012). Antibiotics and high levels of trace elements (copper and zinc) have been highly successful in decreasing the economic burden of post-weaning diarrhea in the first two weeks following weaning. Enhanced therapeutic doses of copper (up to 170 mg/kg feed as a feed additive) and zinc (up to 2,500 mg/kg feed of zinc oxide as a prescribed veterinary medication) exceeding more than 20-fold the dietary needs have also been used routinely as prophylaxis for diarrhea in the diet formulation of piglets (López-Gálvez et al., 2021a).

As explained by Vervaeke et al. (1979); Nugon-Baudon et al. (1985); Dierick et al. (1986) and Dierick et al. (2002), antibiotics and heavy metals mainly work in the following ways,

1. They reduce the microbial load significantly in the proximal GIT and shift the microbial population towards the beneficial microbes.
2. They lower the net energy requirement by making more glucose available for the host. This happens because there is decreased microbial breakdown of glucose to SCFA.
3. They limit the microbial decarboxylation of amino acids to amines (cadaverine, histamine, and tyramine) and minimize the deamination to ammonia in the small intestine, which is where nutrients are actively absorbed in the animal.

4. They lead to an increase in the ileal digestibility coefficients of nutrients by moving the enzymatic digestion of major nutrients to more proximal locations in the gut.

However, in the United Kingdom the government was soon obliged to create the Swann Committee when veterinarians in the country warned about episodes of antibiotic resistance on animal farms that also harmed humans and were highlighted in the media. According to the Swann report of 1969, antibiotics used for growth promotion and similar objectives are harmful to both human and animal health because they cause bacteria to become resistant to them (Medicine and Swann, 1969; Wise, 2007; Kahn, 2016; Kirchhelle, 2018). Similarly, compounds with heavy metals also came with a lot of harmful aftermath like accumulation in soil, causing environmental pollution of soil and water; might influence the development of resistant bacteria (Panel, 2014; Panel, 2016), and may influence the production of genes that affect the immunological response of pigs (Debski, 2016). Other countries also had similar observations and started adopting measures to curb these effects.

An explanation for the beneficial connection between AGP usage and the growth performance of piglets, according to Cardinal et al. (2021), is a decreased intestinal inflammatory response. For example, Mazutti et al. (2016) proved that animals who got colistin or tylosin at subtherapeutic doses throughout the nursery, gained up to 1.74 kg more weight at the end of the nursery phase. A similar observation was noted by Helm et al. (2019) that the average daily gain of pigs treated with subtherapeutic doses of chlor-tetracycline, rose by 0.110 kg/day. Both authors accredit the increased weight to the lower costs of immunological stimulation caused by AGPs' impact on gut microbiota. Microbial dysbiosis, characterized by an overpopulation of possible pathogens such as *Escherichia* and *Clostridium*, as well as the decrease in healthy bacteria such as *Bacteroides*,

Bifidobacterium, and *Lactobacillus*, is a long-term consequence of using AGP. As an added downside, the AGPs decreased the microbiome diversity, which resulted in detrimental effects on the overall health of piglets (Correa-Fiz et al., 2019; Guevarra et al., 2019).

In several countries, processed pork products like ham, salami and sausages come from the commercial pig farms that are still highly dependent on antibiotics for growth promotion. Waluszewski et al. (2021) write that there might be a relationship between the use of AGP and difficulties in treating secondary bacterial infections caused by viruses like the coronavirus in humans.

It is based on a 2010 estimate that in the United States, approximately 63,151 tonnes of antibiotics are used in animal agriculture, and this will rise by 67% by 2030 (van Boeckel et al., 2015). Almost 71 percent of the United State's yearly antibiotic usage is attributed to the livestock sector. In terms of its contribution to the spread of antibiotic-resistant bacteria in animals and people, these activities ultimately pose a serious public health hazard. The use of antibiotics in animal feed for production purposes is prohibited from 2017 in the United States as stated in GFI #213, published by the United States Food and Drug Administration (US Department of Health and Human Services and Medicine, 2013).

2.6 Alternatives to antibiotics

2.6.1 Prebiotics

Prebiotics are additives that act as a substrate for utilization by the host microbes, for the benefit of the health of the host (Gibson et al., 2017). According to the definition above, an ideal prebiotic should be resistant to stomach acids and digestive enzymes, so that the microbes in the large

intestine can utilize it for their metabolism. Certain non-digestible carbohydrates commonly used as prebiotics are lactulose, mannan oligosaccharides, galactomannan oligosaccharides, inulin, fructooligosaccharides, isomaltooligosaccharides, chitooligosaccharides, resistant starch, dietary fiber, and lactose (López-Gálvez et al., 2021b). The majority of prebiotics are produced or extracted from polysaccharides found in plants and algae (Wu et al., 2017). A chemical treatment that hydrolyzes polysaccharides (example, isomalto-oligosaccharides from starch) or enzyme-catalyzed synthesis from disaccharides can also yield prebiotics (van den Broek et al., 2008).

Prebiotics influence gut health primarily through promoting the growth of helpful gut microbes and boosting the production of SCFA (lactate, propionate, acetate, butyrate). Lactose is significant for weaned piglets because it goes to the large intestine and gets fermented by *lactobacillus* yielding lactate. Lactate lowers the pH and hence, is detrimental to pathogens. Lactose also boosts the production of SCFA, which are an important source of energy for colonocytes (López-Gálvez et al., 2021b). From previous research it is evident that prebiotics can alter the gut microbiota and benefit the host however, there have been inconsistent results in some experiments, and we still need to explore their interaction with other feed components, and their influence on the immune system and other organ systems of the body.

2.6.2 Probiotics

Probiotics, also known as direct-fed microbials, are fed to animals as live microbes for increasing their growth performance and overall health. There are three groups commonly used in livestock, *Bifidobacterium*, Lactobacilli, and *Eubacteria* (NRC, 2012). Probiotics composed of *Bifidobacterium* are spore-forming, making them capable of surviving high temperatures and low

pH (Ferrari et al., 2014). Extracellular fiber degrading enzymes are efficiently produced by these bacteria, enhancing nutrient digestibility and uptake (Schreier, 2014). Lactic acid-producing bacteria do not form spores hence, their preservation during feed processing is critical (de Lange et al., 2010). They are abundantly found in the GIT of young pigs aiding in lactic acid production and lowering the pH, suppressing pathogens in the gut (Richards et al., 2005; Li et al., 2003). However, the concentration of these bacteria decreases once the pigs are weaned, therefore, supplementation of *Lactobacillus* is needed for improving their health (Niers et al., 2005). It can be concluded from the research data available, that probiotics certainly have a positive effect on pigs. *Lactobacillus* provides better gut health and management after weaning while *Bifidobacterium* aids in nutrient digestibility and energy bioavailability, contributing to weight gain for grower-finisher pigs. Most bacteria belonging to these three genera are beneficial for intestinal health and help in the prevention of GIT diseases (van der Aar et al., 2017). They produce more lactic and acetic acid in the colon, thereby lowering pH and amplifying fermentation and SCFA. These aid in reducing the pathogenic bacterial population (Smiricky-Tjardes et al., 2003). Not just that, they might also help in breaking down the complex structures of non-starch polysaccharides into smaller compounds digestible by the pigs, making energy sources more available (Jaworski et al., 2017). Also, they may contribute to stabilizing the piglets' immune system by inhibiting the production of pro-inflammatory cytokines by the intestinal cells, adding the unused energy towards growth (Cho et al., 2011).

There are not many conclusive experiments on yeast, however, it has many potential health benefits. Based on a study done by Bontempo et al. (2006), weaned piglets supplemented with live dietary yeast showed higher growth performance and improved intestinal health than piglets fed a control diet without any additives. Moreover, weaned piglets, when fed a combination of bovine

casein hydrolysate and yeast β-glucan, had growth parameters, fecal scores, and inhibition of the NF-κB pathway similar to piglets supplemented with zinc oxide (Mukhopadhya et al., 2019). More trials with antibiotic comparison groups and standardization against random variables like pig breed, age, management practices, probiotic quantity and durability, timespan for supplementation, etc. are needed to replace antibiotics.

2.6.3 Postbiotics and synbiotics

Postbiotics are compounds that are produced from microbial fermentation. They can include a variety of carbohydrates, amino acids, and proteins. Synbiotics are a combination of probiotics and prebiotics, supplemented together to observe their synergistic effects on livestock (Laird et al., 2021). Both postbiotics and synbiotics have recently emerged and gained attention due to their positive effect on gut health and growth performance. Postbiotics of *Saccharomyces cerevisiae* increased biodiversity in the intestine and reduced ammonia and *E. coli* in treatment pigs compared to the control group (Kiarie et al., 2011). Similarly, supplements containing fermentation products of *L. acidophilus* increased the gain to feed ratio of weaned piglets (Nordeste et al., 2017). Some synbiotics have shown increased growth performance in pigs as compared to probiotics alone (Wang et al., 2019). However, there are a wide array of mixtures possible with prebiotics and probiotics, and every combination has to be tested *in vitro* and *in vivo* before drawing any conclusions.

2.6.4 Organic acids

Organic acids are carboxylic acids having a particular carboxyl structure in each molecule. One kind of organic acid is a fatty acid, comprising an aliphatic chain and forming the main energy source in animal diets (Kil et al., 2011). They are classified based on the number of carbon atoms,

as SCFA (1-5 carbon) and medium-chain fatty acids (MCFA; 6-12 carbon). The principal mechanisms behind the productive outcome on the gut microbiome, architecture, and digestibility due to organic acids are as follows: reduced stomach pH promoting protein digestion; boosting small intestinal function and enzyme output; antimicrobial activity; increased gastric retention, and aiding in mucosal barrier function (Dierick et al., 2002).

Commonly used organic acids in the animal industry are formic, fumaric, lactic, and citric acid (Suiryanrayna and Ramana, 2015). Formic acid, citric acid, and benzoic acid have been shown to increase growth performance when fed to weaned pigs (Diao et al., 2016; Luise et al., 2017). Eckel et al. (1992) reported increased apparent total tract digestibility of protein when pigs were supplemented with formic acid and its salts but Gabert and Sauer (1995) noted a lower ileal amino acid digestibility in pigs. Moreover, the digestibility of calcium and phosphorus in growing pigs (Bühler et al., 2010; Xu et al., 2018) and crude protein in weaning pigs (Halas et al., 2010) was enhanced when they were supplemented with benzoic acid. Zentek et al. (2013) integrated organic acids with MCFA and reported increased anti-microbial activity, growth, and digestibility in piglets as compared to feeding them individually. This was proven in another study where *Lactobacillus* count increased and pro-inflammatory cytokines were less expressed in pigs (Kuang et al., 2015). Similarly, Long et al. (2018) mentioned a reduction in hydroxyl radicals in the blood of weaned piglets after feeding mixed organic acids. This also illustrates the antioxidative power of organic acids and MCFA in combination. Research shows that organic acids are compelling feed additives and might completely replace AGP in future.

2.6.5 Nucleotides

Nucleotides are mainly known as monomers of nucleic acids i.e., DNA and RNA which form the genetic material of each organism. They are a macromolecule comprising a nitrogenous base, either purine or pyrimidine, attached to a pentose sugar and a phosphate group. Pyrimidine is a six-atom single aromatic ring seen in compounds such as uridine, cytosine and thymine, and purine are made up of a five and six atom dual aromatic ring and includes compounds like hypoxanthine, adenine, and guanine (Rudolph, 1994).

Besides forming nucleic acids, nucleotides assist in energy metabolism, oxidation and reduction reactions, carrying intermediates in physiological reactions, development of the immune system, gut barrier function, and microbiome (Liu et al., 2018). The demand for nucleotides increases during immunological stress because they are fundamental in both cellular and humoral immune responses. Domeneghini et al. (2004) and Šperanda et al. (2008) observed that macrophages and lymphocytes were upscaled in the small intestinal epithelium of piglets when fed with nucleotides. Similar studies were made where nucleotides protected DNA in blood lymphocytes (Salobir et al., 2005), reduced TNF-α and IL-6 in serum of ETEC challenged piglets (Hung, 2015), and increased serum IgA (Lee et al., 2007; Sauer et al., 2012b; Sauer et al., 2012a). Nucleosides are nitrogenous compounds similar to nucleotides, except that they do not have a phosphate group attached to them. Diets not including nucleotides were fed to 2 groups of piglets, one as control and the other group as nucleoside supplemented. The group with nucleoside supplementation expressed a higher concentration of healthy bacteria and a lower concentration of *Clostridium perfringens* as compared to the control group (Mateo and Stein, 2004). After adding nucleotides (Weaver and Kim, 2014) or nucleotide-rich yeast extract (Waititu et al., 2016), the pigs' growth parameters were enhanced. On the contrary, many other studies did not note such an observation (di Giancamillo

et al., 2003; Martinez-Puig et al., 2007; Moore et al., 2011). Changes in the type, quantity, and duration of the feeding of nucleotides can show such variabilities across different experiments. In the future, nucleotides could be a probable candidate for enhancing the adaptive ability and growth performance of piglets after weaning.

2.6.6 Antimicrobial peptides

Antimicrobial peptides (AMP) are a varied group of polypeptide molecules commonly found in every living organism. They can be obtained from various sources from prokaryotes to mammals or can be synthesized chemically. The AMP can fight against a broad spectrum of gram-positive and gram-negative bacteria, fungi, viruses, and parasites (Hancock, 2001). They are small, positively charged compounds with a hydrophobic and hydrophilic region facilitating a trouble-free entry into the bacterial cell (Zasloff, 2002). Examples of AMP are lactoferrins and defensins. Wang et al. (2006), reported that augmentation with recombinant lactoferrin improved intestinal architecture and performance parameters in weaned piglets. Similarly, several types of defensins have up-regulated the expression of mucin and tight junction proteins resulting in fortified mucosal barrier function (Robinson et al., 2015). The two ways in which AMP are supplemented to the animals are, as exogenous AMP either synthesized or obtained from another organism, or as ingredients that prompt AMP production inside the body (Robinson et al., 2018). It is important to note that AMPs are proteins and might be broken down in the upper GIT, while most of the pathogenic organisms reside in the lower intestinal tract (Xiong et al., 2019). While there are not many consistent results obtained yet, AMPs are a very promising alternative to antibiotics.

2.6.7 Feed enzymes

Feed enzymes are added to the diet, largely to facilitate the digestion of particular feed ingredients so that their bioavailability is increased. There are a variety of enzymes used commercially, like phytases, lysozymes, carbohydrases, and proteases. Each enzyme works differently and has been shown to benefit gut health. A study compared a multi-enzyme additive (protease, α-amylase, xylanase, β-mannanase, glucose oxidase, acid cellulose, and galactosidase) averse to an antibiotic supplemented group and both showed similar growth performance as well as a significantly higher digestibility than the antibiotic group (Han et al., 2017). Other researchers exhibited optimistic results on oxidative stress (Duarte et al., 2017), gut morphology, and microbiome (Chen et al., 2017). Similarly, lysozymes supplied through drinking water reduced the amplification of ETEC as antibiotic supplementation (Nyachoti et al., 2012). Feed enzymes are a very promising AGP replacement, and they aid in digestion which is a bonus for a healthy gut. More research in the future can bring us closer to a multi-enzyme product that would not only act as an AGP alternative but also have various other benefits for nutrient digestibility and intestinal health.

2.6.8 Seaweed extract

Marine seaweeds have been used for decades for their numerous health uses as an antioxidant, anticancer, anticoagulant, and anti-inflammatory. They are considered indispensable by the pharmaceutical industry. With regards to the feed industry, products of brown marine algae such as alginates, fucoidans, laminarins, and phlorotannin have been evaluated as probable AGP replacements (Michiels et al., 2012). Laminarin (300mg/kg of feed) and zinc oxide have similar effects on growth performance and gain to feed ratio when supplemented to weaned piglets as well as better nutrient digestibility and gut morphology compared to the negative control group without any supplements (Dillon et al., 2010; McDonnell et al., 2010). Similar results were obtained by

Heim et al. (2014) when fucoidans were supplemented at 240 mg/kg of feed. However, laminarin and fucoidans cannot be combined as they displayed incongruity by cancelling each other's usefulness, as demonstrated by Walsh et al. (2013). Also, it was noted that at higher doses of laminarin, the production of pro-inflammatory cytokines was promoted having deleterious effects on the pigs (O'Doherty et al., 2017). The research data obtained leads to the positive use of seaweed extracts in the future, but there are still some ambiguities that need to be resolved like the effective duration and dose of the supplement, bioavailability, and interactions with other compounds. Although commercial seaweed products are available in some regions, they are still not popular worldwide.

2.6.9 Vaccines

Vaccines have been a great tool in the eradication or prevention of many infectious and deadly diseases. But it is not easy to make them, as each pathogen acts differently and the body's responses also vary. In the case of bacteria, there are factors like strains, type of toxin produced, specific antigen, immune response, route of administration and more to be considered before making a commercial vaccine. In the last decade, vaccine studies exploring possibilities of subunit, encapsulated, and parenteral vaccines have been done for ETEC-led PWD but only a live oral vaccine has been made available until now (Melkebeek et al., 2013; Hedegaard and Heegaard, 2016). Countless vaccines target a fimbrial antigen but the antibodies generated are only useful for a particular strain and do not provide cross-protection (Fairbrother et al., 2017; Rhouma et al., 2017). Enterotoxin-based vaccine research also showed no cross-immunity because of high variability in nature and quantity of toxins amongst serotypes (H. Wang et al., 2020). Vaccination can be provided in two ways, by immunizing sows and conferring piglets with passive immunity or immunizing weaned piglets for stimulating active immunity. Immunizing sows will only protect

piglets during nursing. Once they are weaned, the immunity will slowly subside. On the other hand, scheduling administration is fundamental in piglet immunization. Vaccine effectiveness of Coliprotec F4 (Prevtec Microbia GmbH, Germany) comprising an $F4^+$ tame *E. coli* strain was tested by Fairbrother et al. (2017) while administering ETEC challenge strain on days 3, 7 and 21 post-vaccination on piglets weaned at 18 days of age and vaccinated one day post-weaning. They observed that diarrhea, and ETEC colonization and shedding were significantly higher in pigs challenged on day 3 as compared to those challenged on day 7 or 21. This shows that vaccine administration should be done at a precise time to gain full protection against PWD caused by ETEC.

There have been limitations in transporting live vaccines globally due to biosecurity reasons but the progress achieved until now is remarkable and can pave the way towards antibiotic-free production. Having said that, it is still vital to find antibiotic alternatives until sufficient data and proof for vaccination is available.

2.6.10 Phage therapy

Phage therapy indicates supplementing viral particles called bacteriophages targeting a specific strain of bacteria in the gut. The virus then carries out a lytic cycle wherein, it enters the host (pathogenic bacteria), takes control over the biochemical processes, and starts replicating inside the cell until it is exhausted resulting in the breakdown of the bacterial cell wall (Cha et al., 2012a). The virus then infects other bacteria of the same strain and repeats the cycle. It is noteworthy that the high specificity of the target bacteria allows us to control the bacteria to be killed without disturbing the remaining gut microbiome. Several trials have shown that phage therapy can mitigate the symptoms induced by ETEC infection in weaned piglets as well as abating the risk of

spreading infection from one farm to another by decreasing the fecal load of target bacteria (Jamalludeen et al., 2009; Cha et al., 2012b; Lee et al., 2017).

In some situations, however, the limited target range of phages may not be beneficial because it restricts manufacturing a universal product for all farms as the strain of bacteria on one farm is most probably not similar to another. Additionally, there is only limited research on various phages, no detailed data is available about phages particularly useful for livestock and most of them are not commercially available yet.

2.6.11 Plant extracts

Plant extracts, also known as phytogenics, are chemical compounds produced by plants as secondary metabolites with various important functions. They provide protection to plants from ultraviolet radiation, pathogens, oxidative stress, and harsh climatic conditions. They are derived from natural sources with minimum alteration and stand as one of the significant alternatives for antibiotics. Humans and animals cannot synthesize most of the phytogenic compounds and therefore need an exogenous source of polyphenols from plants. The four most extensively studied properties of phytogenics are broad-spectrum antimicrobial, potential anti-viral, anti-oxidative and immunomodulation, because of which they are becoming popular among researchers (Liu et al., 2012; Liu et al., 2013). There are five methods for the extraction of polyphenols including (1) solvent extraction; (2) pressurized liquid extraction; (3) ultrasonic-assisted extraction; (4) microwave-assisted extraction and (5) supercritical extraction (Rasouli et al., 2017). Supplementing plant extracts demonstrated strengthened intestinal health and immune function, further leading to better growth performance and resistance to diarrhea in weaned piglets. Curcumin, turmeric oleoresin, and capsicum oleoresin are phytogenics that might be responsible

for an increase in gene expression of tight junction and cell-cell junction proteins and a decrease in gene expression related to immune stimulation due to antigens, in piglets after inoculation with ETEC (Wang et al., 2012; Liu et al., 2014c). Chemokine production in intestinal epithelial cells reduced after garlic extract supplementation, indicating inhibition in the count of circulating leucocytes in areas of inflammation (Lang et al., 2004). In many studies done *in vivo* and *in vitro*, extracts derived from common herbs like ginger, thyme, fennel, pepper, oregano, cinnamon, basil, garlic, mint, and clove have shown strong free radical scavenging properties (Frankič et al., 2010b; Amorati et al., 2013; Wu et al., 2018b). Phenolic acids have been described as the active components in the extracts that aid in the protection of proteins and lipids from oxidative damage of peroxyl radicals (Djeridane et al., 2006; Lobo et al., 2010). Positive effects on the immune system, like increased lymphocyte proliferation rate, phagocytic rate and IgA, IgM levels in the blood of weaned piglets, were reported after administering encapsulated essential oilscontaining thymol and cinnamaldehyde at 50, 100, or 150 g/ton of feed (Li et al., 2012). A mixture of oregano, anise, and citrus peel (40 mg/kg diet) added to the piglet diet enhanced the anti-inflammatory effect by reducing the gene expression of NF-κB and TNF-α (Kroismayr et al., 2008). The antibacterial, antifungal, antiviral, and anti-coccidial properties are said to be present in some well-known phytogenics like carvacrol, eugenol, thymol, capsaicin, cineole, and so on (Windisch et al., 2008).

Plant extracts are without a doubt beneficial for both humans and animals and can be obtained without harming the environment or presenting any adverse effects on human or animal life. But like any other additive, there are still many unresolved issues with phytogenics. They are less bioavailable because of poor absorption, high metabolism, and rapid excretion. Since the pathogens are in the lower GIT, it is important that phytogenics are delivered to that location after ingestion. To ensure that, various encapsulation methods have been implemented. Less data is

available on the interactions of polyphenols with each other and with different feed components. More polyphenols that are present in nature are yet to be explored. We still do not have availability of pure plant extracts from different parts of the world. The content of polyphenols in plant extracts varies tremendously as it depends on harvesting time, storage conditions, extraction processes, part of plant taken, etc. With time and research, there will be an answer to all the above disparities.

2.7 Red osier dogwood

Cornus sericea or commonly known as red osier dogwood is a plant naturally found everywhere in Canada and some northern states in the United States. It thrives in moist or swampy soil in open woodlands and has little white flowers in Summer and early Autumn, as well as white berries in the Fall. Through various studies done on *Cornus* species, it is shown that its fruits are rich in flavonoids like rutin, quercetin, and kaempferol (Seeram et al., 2002; Vareed et al., 2006; Pawlowska et al., 2010). In the past, many tribes of Native Americans used the plant as a remedy for weakness or to cure sores, stomach pain, and skin diseases (Smith, 1929). However, with leaves and barks, there is only a limited amount of information available about their polyphenolic contents.

There is some research with piglets showing the beneficial effects of polyphenols obtained from ROD. Jayaraman et al. (2018) and Koo et al. (2018) found that supplementing weaned pig diets with 4 % powdered ROD reduced oxidative stress and enhanced growth performance. In the study by Yang et al. (2019), RDE increased trans-epithelial electrical resistance (TEER) and decreased permeability in Caco-2 intestinal cell culture. The RDE protected the cell culture from hydrogen peroxide H_2O_2-induced oxidative damage, *in vitro*. In addition to directly scavenging free radicals and boosting the cellular antioxidant system, RDE increases cell activity via restoring tight

junction proteins and increasing their expression (Jiang et al., 2019). Similarly, it has been shown that rumen protein degradation may be reduced, and protein efficiency increased by ROD feeding in finishing beef heifers (Gomaa et al., 2018). Mogire et al. (2021) found that ROD had no influence on growth performance in broiler chickens, but it did enhance gastrointestinal function and had no negative impact on meat quality.

According to Isaak et al. (2013), the total phenolic content found after methanolic extraction of air-dried ROD leaves, stems, and bark is much higher than its counterpart in the olive plant (figure 2.8). Phenolics are more acidic and stable than regular alcohols because of the dispersed pi electrons. ROS and other free radicals feed on these extra electrons or hydrogen atoms and are neutralized. These sturdy hydroxyl compounds also work as singlet oxygen destroyers, metal chelators, and enzyme inhibitors (Bravo, 1998). Hence, the phenolic content of a sample is mostly responsible for its antioxidant action. Oxygen radical absorbance capacity (ORAC) assay is an accurate measurement of antioxidant activity, as it assesses the sample's capacity to prevent fluorescein from being oxidized by a peroxyl radical generator. The ORAC analysis by Isaak et al. (2013), showed values substantially higher for ROD than other antioxidant-rich plants like basil leaves, parsley leaves, and tea leaves (figure 2.9). Another group of compounds that are soluble in water and gives a red, purple, or blue pigment to plants, fruits, and flowers and are a crucial component of seed dissemination and pollination are called anthocyanins. Anthocyanins were also analyzed from stem, leaves, and bark of ROD, and in the spring, the sample had a good amount of anthocyanin and in the winter, it was exceptionally high (figure 2.10).

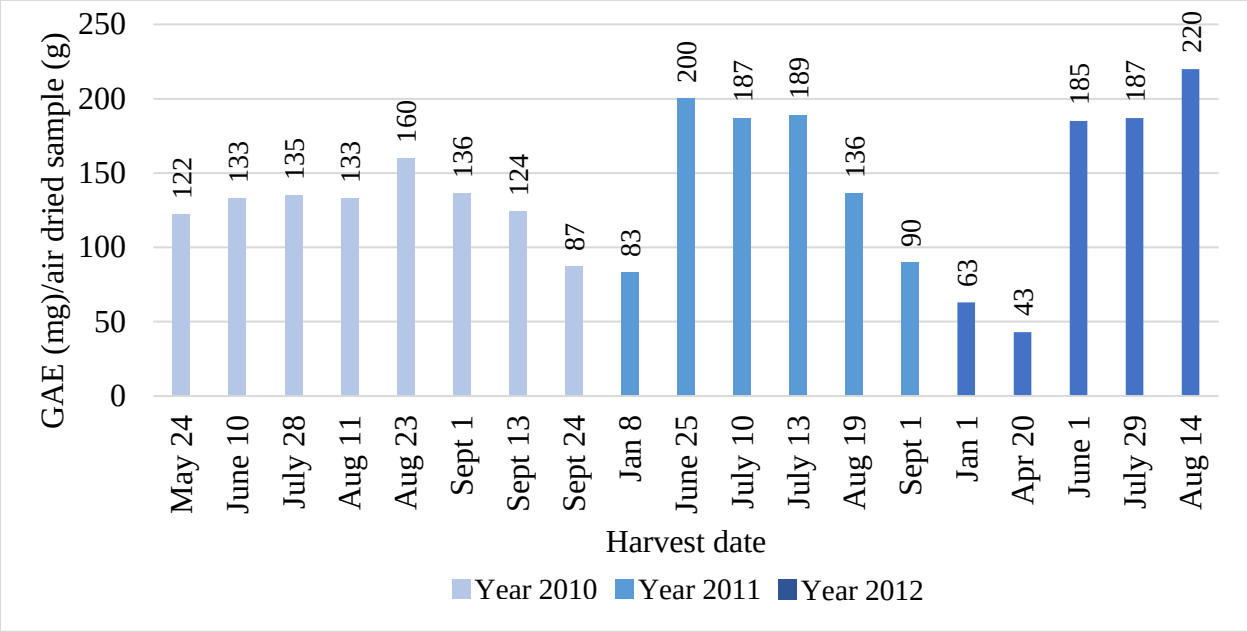

Figure 2.8 Total phenolic content in plant material harvested from Cornus sericea at various intervals over a 3-yr period.

Values are the mean of triplicate analysis of samples (mean ± SD, n=3).
Adapted from Isaak et al. (2013).
GAE – Gallic acid equivalent

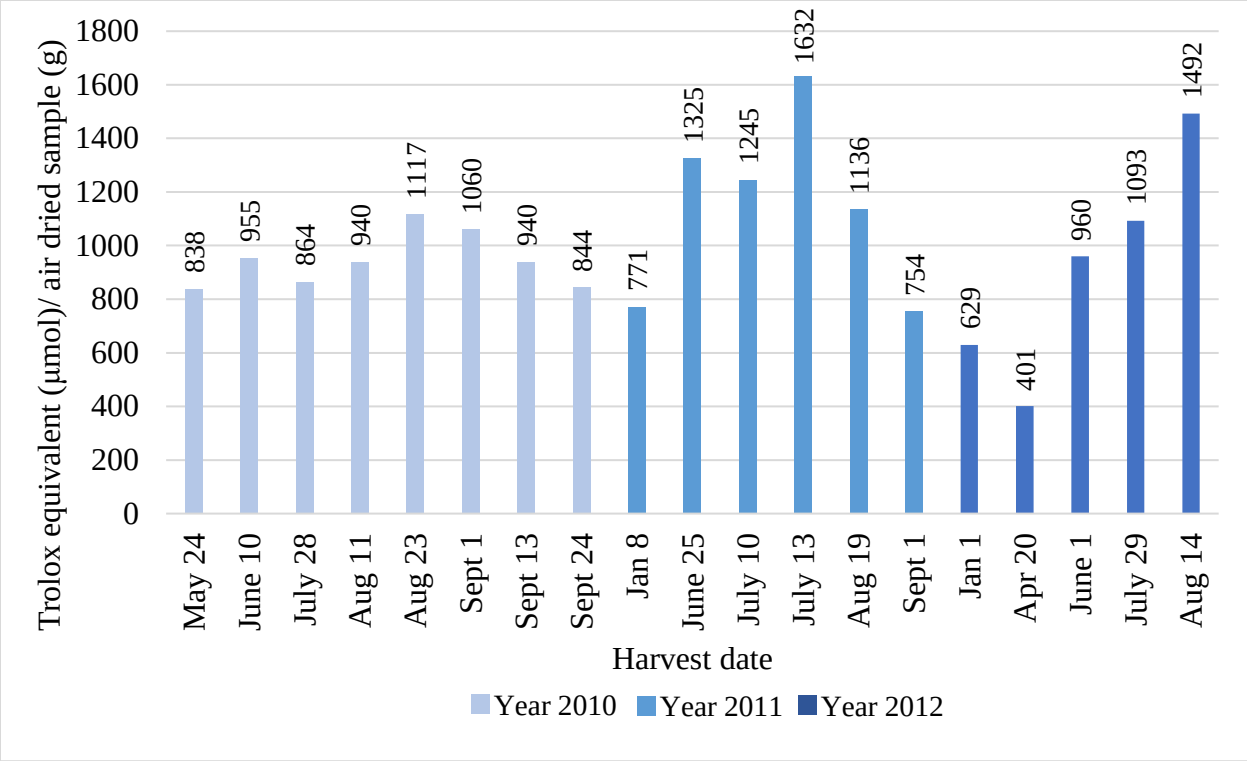

Figure 2.9 Oxygen radical absorbance capacity in plant material harvested from Cornus sericea at various intervals over a 3-yr period.

Values are the mean of quadruplicate analysis of samples (mean ± SD, n=4).
Adapted from Isaak et al. (2013).

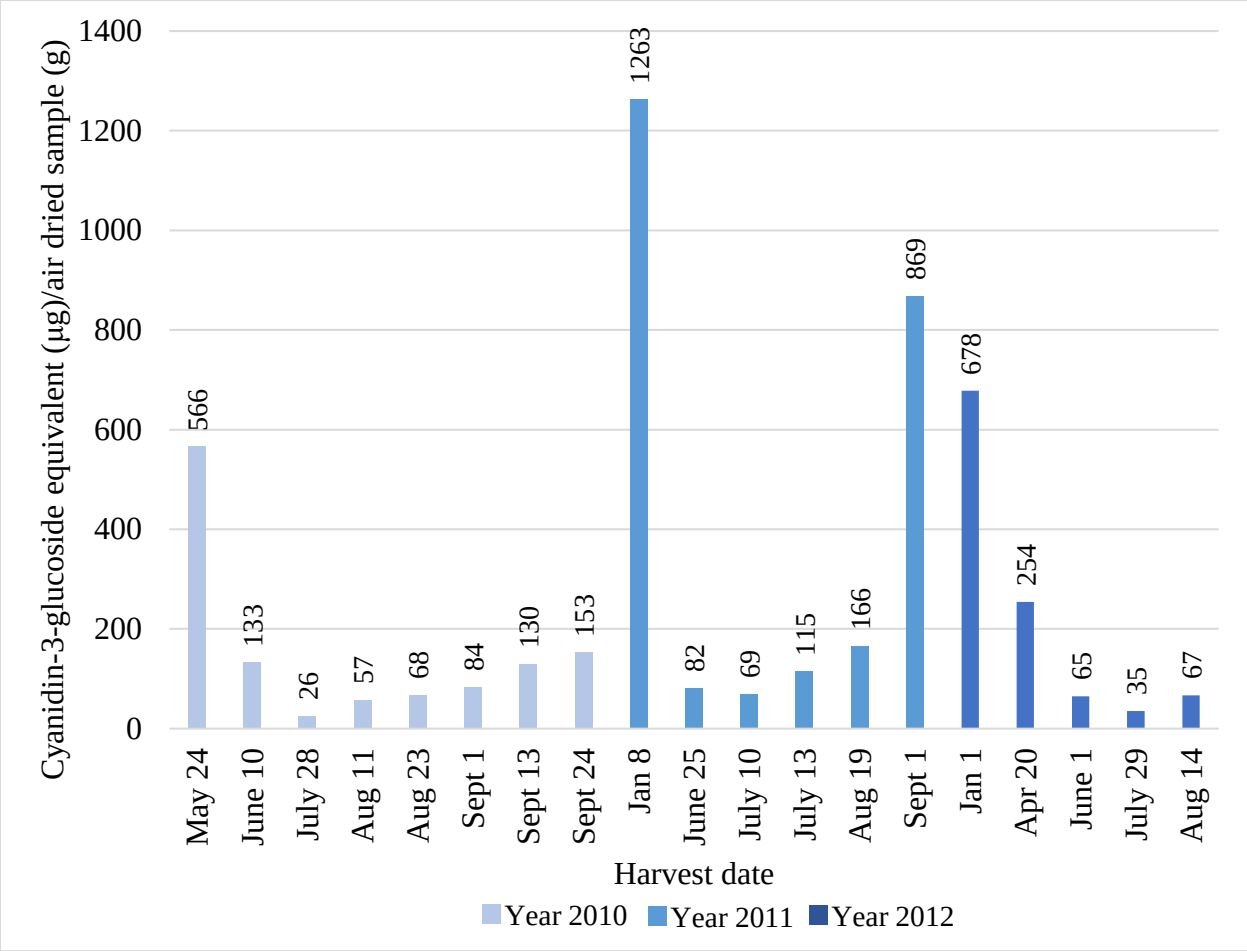

Figure 2.10 Total anthocyanin content in plant material harvested from Cornus sericea at various intervals over a 3-yr period.

Values are the mean of quadruplicate analysis of samples (mean ± SD, n=4).
Adapted from Isaak et al. (2013).

Based on the analysis of Apea-Bah et al. (2020), five phenolic compounds were identified in samples collected from ROD bark and leaves. These include two phenolic acids namely, glucogallic acid and ellagic acid; and three flavonoids namely, rutin, quercetin, and quercetin 3-*O*-malonylglucoside.

It can be concluded, based on the research data that there is a huge scope for plants containing polyphenols like ROD, to replace antibiotics. This will pave the way for safer meat consumption for humans and healthier life for livestock. In the next years, more animal-based experiments

should be conducted to know the actual impact of antibiotic alternatives on animal health and meat production. The plant-derived products can be a breakthrough for antibiotic replacement in the pork industry.

3 Hypothesis and Objectives

3.1 Hypothesis

Dietary supplementation of red osier dogwood extract will improve antioxidant defense system and gut health indices of weaned piglets challenged with *E. coli*.

3.2 Objectives

The experiment was designed to address the following specific objectives:

1. To evaluate the effects of dogwood extract on gut and systemic health.
2. To compare the effects of dogwood extract with antibiotic growth promoters using contrasts.
3. To investigate a dose-response associated with different levels of dogwood extract.

4 Materials and Methods

All experimental procedures were reviewed and approved (animal care approval number AC11414) by the University of Manitoba Animal Care Committee (Winnipeg, Manitoba) and pigs were cared for according to the guidelines of the Canadian Council on Animal Care (CCAC, 2009).

4.1 Red osier dogwood extract

The RDE was supplied by Robert Scales, chief executive officer of Red Dog Enterprises Ltd. (Swan River, Manitoba, Canada). This company with the help of the Food Development Centre (Portage La Prairie, Manitoba, Canada) prepared the RDE. The ROD plant was harvested from July 1 to 11 in 2018 from Swan River (Manitoba, Canada). The plant was grown in an insulated land free from chemical contamination. Stem, branches, and leaves were collected and cut into smaller pieces, dried with an air dryer, and stored in food-grade bags until they were sent for extraction.

The ROD extraction was performed in the following steps in the given order, water steeping (at 98 ± 2^{o}C), solid separation, clarification, concentration, and, lastly, spray drying. The moisture content, water activity, total aerobic count, total phenolic content, and ORAC values were measured after preparing the extract.

Before mixing the RDE with the basal diet, a phenolic composition analysis was done at the Department of Food and Human Nutritional Sciences, University of Manitoba. Under this investigation, phenolic compounds were extracted, total phenolic content (TPC), total anthocyanin content (TAC), and phenolic composition of the extract were obtained. The compounds identified in the dogwood extract were gallic acid, rutin, ellagic acid, and quercetin (Table 4.1). Anthocyanins were not present in any of the samples. Since the commercial feed contains some

cereals and legumes, they most likely have some constituent phenolic compounds. It was, therefore, useful to determine the TPC of both the NC and treatment diets to enable a good comparison between them.

However, the TPC of treatment diets was also calculated, due to the small amount of individual polyphenol in the mixed diets, it was not feasible to detect the peaks of phenolic compounds with the mass spectrometer.

Table 4.1 Total phenolic content and phenolic composition of red dogwood extract and experimental diets.

Sample[1]	TPC[2]	Gallic acid	Rutin	Ellagic acid	Quercetin
RDE	263.11	47.86	9.86	8.84	0.25
NC	0.40	-	-	-	-
ROD1	0.43	-	-	-	-
ROD2	0.45	-	-	-	-

[1]RDE, Red osier dogwood extract; NC, Negative Control, corn, wheat, soybean meal diet; ROD1, NC plus 0.1% RDE; ROD2, NC plus 0.2% RDE.

[2] TPC – total phenolic content, expressed as mg GAE (gallic acid equivalent)/g sample, dry weight basis; concentration of all phenolic compounds (gallic acid, rutin, ellagic acid, quercetin) are expressed as mg GAE/g sample, dry weight basis.

Values are mean ± standard deviation of 3 replicates.

4.2 Gene screening of piglets

Based on the publication by Jensen et al. (2006), only piglets genetically susceptible to ETEC F4 were selected for the experiment. A specific receptor in the pigs' intestine determines if the ETEC F4 bacterium can attach to the digestive tract or not. This is controlled by an autosomal dominant

Mendelian trait with 2 alleles, S: adhesion and dominant; R: non-adhesion and recessive (Gibbons et al., 1977). Tissue samples were collected on day 3 post-farrowing when tail docking was being done of the newborn piglets and later stored at -20°C. The DNA extraction was done using the method described by Truett et al. (2000). It was done in 2 steps, first, lysis was performed for DNA extraction using 25 mM hot sodium hydroxide and 0.2 mM EDTA (Ethylenediamine tetraacetic acid) solution, followed by neutralization with 40 mM Tris-HCl.

The extracted DNA was used to amplify the MUC4 gene using PCR by adding DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 2 mmol. L^{-1} $MgCl_2$, 200 µmol. L^{-1} of each dNTP, 400 µmol. L^{-1} of each primer and 2 µl of DNA made the volume of 25µl. Thermocycling was done 35 times with each cycle having an initial denaturation at 95°C for 3 minutes followed by 95°C for 30 s, annealing at 65°C, and extension at 72°C in T100 thermal cycler (Bio-Rad Life Science Inc., Hercules, California, USA). The amplified MUC4 gene having the size of 367 bp was digested using FastDigest Xbal (Thermo Fisher Scientific) at 37°C for 5 minutes following the kit's instructions. An agar gel electrophoresis was conducted on the final product to separate the DNA fragments based on their size. The 2% agar gel was submerged in Tris-borate-EDTA buffer, and the samples were stained with SYBR green (Invitrogen, Thermo Fisher Scientific, Frederick, Maryland, USA). The electrophoresis was run at 80 V and 160 mA for 40 minutes. ChemiDoc MP imaging system version 3.0.1 (Bio-Rad Life Science Inc., Hercules, California, USA) was used to visualize the results. Only the susceptible allele was broken down into 151 bp and 216 bp DNA fragments as seen in Figure 4.1. Those piglets with susceptible alleles were chosen for the experiment.

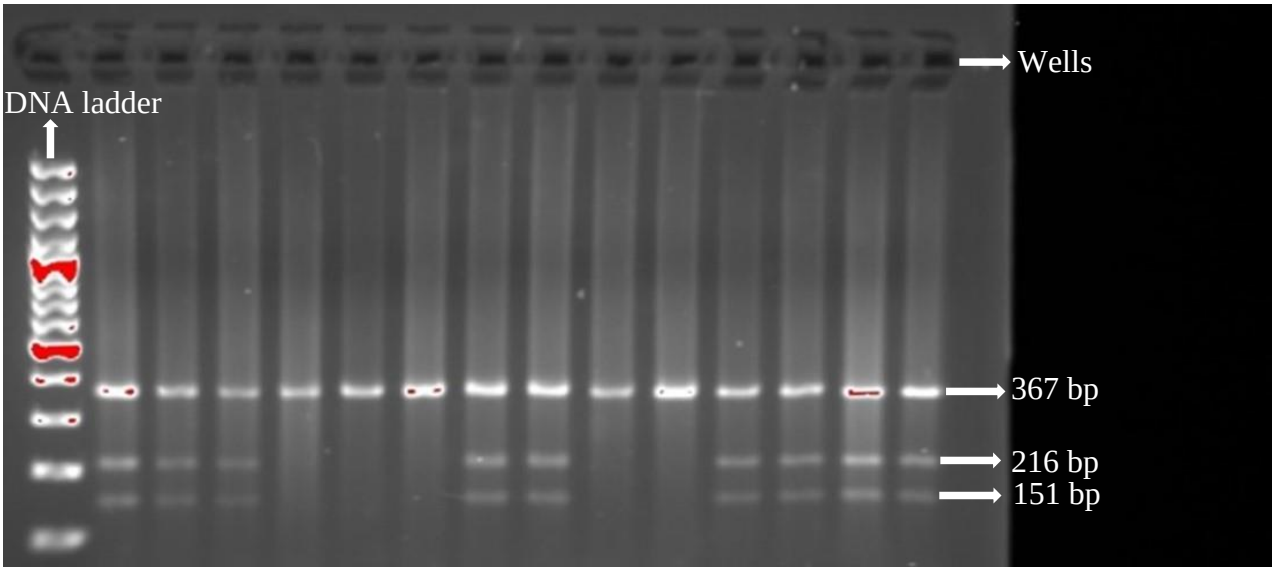

Figure 4.1 Visualisation of the DNA fragments after electrophoresis.
The samples were put in wells before starting electrophoresis. The luminescent lines show the DNA fragments based on their weights (in bp). For reference, a DNA ladder is put in the first well. It contains DNA of different bp and indicates the proper performance of electrophoresis. Those samples which were broken down into 3 fragments (2 additional lines on the image in Figure 4.1) contained the gene with R and S allele and were susceptible to ETEC.

4.3 Preparation of ETEC F4 inoculation

The ETEC F4 inoculum was prepared in Dr. Song Liu's lab in Department of Biosystems Engineering, University of Manitoba. The tryptic soy broth (TSB) powder was dissolved in double-distilled water and autoclaved with the intent to provide a growth medium for *E. coli*. One bead of ETEC F4 was taken from the frozen stock and added to 10 mL autoclaved TSB. An additional tube with just TSB was used. Both the tubes were loosely capped (to let oxygen in) and placed on a shaker with the speed of 150 rpm to incubate at 37°C for 16 to 18 hours. After incubating for one night, the tube with TSB was tested for contaminations.

Approximately, 100 to 200 μL of the bacterial culture was added to fresh TSB and placed on a shaker for another 2 hours until it reached the log phase. Using a small sample, the bacterial OD value was measured, and the same amount of fresh TSB was taken as blank. The wavelength in the UV spectrophotometer was set to 600 nm and the result was within the normal range of 0.3 to 0.5. The amount of ETEC F4 was calculated in CFU/mL and diluted with phosphate buffer saline (PBS) till the target concentration of 5 X 10^6 CFU/mL was achieved. The culture was stored in a box with ice packs before usage.

4.4 Animals and housing

A total of 28 and 2 spare female piglets (TN70 X Tempo) susceptible to ETEC F4, weaned at 21 d of age with an average BW of 9.03±0.35 kg were acquired from the Glenlea Swine Research Unit at the University of Manitoba. Only female piglets were selected to eliminate any sex-based effects on immunity, growth performance, or antioxidant status. These piglets were individually housed in a Containment Level – 2 room at the T. K Cheung Centre for Animal Science Research, University of Manitoba. Room temperature was regulated and maintained at 29±1°C in the pre-inoculation phase and reduced by 1.5°C in the post-inoculation phase. Each piglet was provided with an enrichment toy, *ad-libitum* feed, and water.

4.5 Experimental design and dietary treatments

A completely randomized design was implemented with 4 dietary treatments and each treatment constituting 7 replicates. A corn wheat soybean meal basal diet was prepared to meet or exceed the NRC (2012) requirements. RDE and antibiotic were added to the basal diet according to the treatment groups. The four dietary treatments were negative control (NC) with no additives; positive control (PC) with known additive, antibiotic avilamycin (Surmax 100® Elanco Inc.);

ROD1 with testing additive, 0.1% RDE and ROD2 with testing additive, 0.2% RDE. The dietary composition on an as-fed basis is shown in Table 4.2.

Table 4.2 Ingredient and calculated nutrient composition of experimental diets (%, as-fed basis).

Treatment*	NC	PC	ROD1	ROD2
Ingredient, %				
Corn	41.87	41.87	41.77	41.67
Wheat	15.00	15.00	15.00	15.00
Soybean meal	27.50	27.50	27.50	27.50
Fish meal	3.00	3.00	3.00	3.00
Dried whey	3.50	3.50	3.50	3.50
Vegetable oils	3.50	3.50	3.50	3.50
Limestone	1.20	1.20	1.20	1.20
Monocalcium phosphate	1.50	1.50	1.50	1.50
Salt	0.80	0.80	0.80	0.80
Vit-Min premix[1]	1.00	1.00	1.00	1.00
Lysine-HCl	0.55	0.55	0.55	0.55
DL-Methionine	0.20	0.20	0.20	0.20
L-Threonine	0.18	0.18	0.18	0.18
L-Tryptophan	0.10	0.10	0.10	0.10
L-Valine	0.10	0.10	0.10	0.10
RDE	-	-	0.10	0.20
Antibiotic	-	0.1	-	-
Calculated Nutrient, %				
ME[2], kcal/kg	3,413	3,413	3,410	3,407
NE[3], kcal/kg	2,552	2,552	2,549	2,546
Crude protein, %	22.00	22.00	22.00	22.00
Ether extracts, %	5.90	5.90	5.90	5.90
Linoleic acid, %	2.90	2.90	2.90	2.90
NDF[4], %	8.10	8.10	8.10	8.10
ADF[5], %	3.40	3.40	3.40	3.40
NSP[6], %	11.20	11.20	11.20	11.20
Calcium, %	1.00	1.00	1.00	1.00
Total phosphorus, %	0.70	0.70	0.70	0.70
STTD[7] P, %	0.50	0.50	0.50	0.50
SID[8] Lysine, %	1.40	1.40	1.40	1.40

*NC, negative control, corn, wheat, soybean meal diet; PC, positive control, NC plus antibiotics; ROD1, NC plus 0.1% RDE; ROD2, NC plus 0.2% RDE; RDE, Red osier dogwood extract. Vitamin and mineral premix[1], metabolizable energy[2], net energy[3], neutral detergent fibre[4], acid detergent fibre[5], non-starch polysaccharides[6], standard total tract digestibility[7], standard ileal digestibility[8]. Diets were formulated to meet or exceed the daily nutrient recommendation for nursery pigs weighing 7 to 11 kg (NRC, 2012).

4.6 Sample collection

In the 14 d experiment, there was a 7 d adaptation period for the piglets and on d 7, they were challenged with ETEC after which they were under observation for 7 d. BW and feed disappearance along with feed wastage were measured every week to calculate growth performance parameters like average daily gain (ADG), average daily feed intake (ADFI), and gain: feed ratio (GF). Blood was collected in a BD Vacutainer® PST^{TM} lithium heparin tube (Becton, Dickinson, and Company, NJ, USA) before and 48 hours after the inoculation. On the d 7, 5 mL of the ETEC inoculum was orally administered in each pig using a polyethylene syringe. Body temperature was recorded pre-inoculation, 3 hpi (hours post-inoculation), 24 hpi, and 48 hpi by measuring the rectal temperature. The fecal score was noted before inoculation and every 8 hpi for the first 72 hpi and every 12 hours, afterward. The scale for fecal consistency score was 0 = normal; 1 = soft feces; 2 = mild diarrhoea; 3 = severe diarrhoea (Marquardt et al., 1999). The fecal scores were noted by a person not knowing the treatment groups, to have unbiased result. The health status of piglets was checked every 8 hpi for 48 hours and any concerns or observations were conveyed to the veterinarian in charge. On d 14, intestinal tissue samples were collected from the piglets after euthanasia. Pigs were first sedated using a combination of drugs, xylazine at the rate of 4 mg/kg of BW (Xylamax®) and acepromazine at the rate of 1.1 mg/kg of BW (Acevet 25®), and then euthanized with a captive bolt gun. As soon as the pigs were euthanized, they were transported to a clean room for sampling. With a surgical blade, the small intestine was promptly exteriorized by making an incision along the midline of the lower abdomen. The ileocecal junction was tracked to locate the mid-jejunum approximately 2 m proximally to the junction. A tissue sample of 10 cm length was taken from mid-jejunum and cleaned with PBS to remove any digesta and then temporarily stored in 50 mL tubes containing Krebs-Ringer bicarbonate (KRB) buffer

(Sigma Aldrich Co LLC, Oakville, Ontario, Canada) to maintain the osmotic balance and pH range at the physiological level and prevent calcium precipitation. These sample tubes were transferred immediately on ice to the laboratory for TEER and FITC (fluorescein isothiocyanate) dextran flux with the help of the Ussing chamber. Another section of mid-jejunum tissue of 2 cm was cleaned with PBS and fixed in 10% formalin for histomorphological evaluation. Similarly, a 10 cm long tissue sample from mid-jejunum was cleaned, stored in aluminium foil coated with RNase*ZAP*™ (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) to decontaminate the surface and protect samples from RNases and frozen in liquid nitrogen before transferring to a -80°C freezer for further use in gene expression analysis. Lastly, the colon was located approximately 40 cm away from the ileocecal junction and digesta samples were collected from the lumen for microbial DNA analysis. The digesta was collected in sterile sampling bags, snap-frozen using liquid nitrogen and later moved to a -80°C freezer.

4.7 Histomorphology

The histomorphology samples were processed and stained by the pathology laboratory in the Department of Human Anatomy and Cell Science, University of Manitoba. They were embedded in paraffin blocks, sliced into 5 µm thick sections, and stained with Alcian blue/periodic acid Schiff stain. The slides were observed under an Axio Scope A1 microscope (Carl Zeiss Micro-Imaging GmbH, Göttingen, Germany) with a color camera. The images captured from the microscope were used for measuring villus height (VH) and crypt depth (CD) with the help of Infinity Analyze programme (version 6.5.4; Lumenera Corporation, Ottawa, Ontario, Canada). A minimum of fifty intact villi and crypts were selected from one tissue cross-section per animal and VH:CD ratio was calculated from the measurements.

4.8 RNA isolation and RT-qPCR (Reverse transcription-quantitative polymerase chain reaction)

The intestinal tissue samples from mid-jejunum stored in the -80^{o} C freezer were ground using a mortar and pestle. The ground sample was weighed (approximately 80 mg) and placed in a microcentrifuge tube for extraction using TRIzol® Plus RNA purification kit (Invitrogen, Thermo Fisher Scientific Inc.) and tissue homogenizer. TRIzol® reagent lysed the cell and the silica cartridge purified RNA after centrifugation. The quality and quantity of RNA in each sample was tested using a Nanodrop^{TM} 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc. Ottawa, Ontario, Canada). The value of OD260:OD280 was in the range of 1.9 to 2.1 indicating highly pure nucleic acid with minimum contamination. The samples were then run through agar gel electrophoresis to ensure the presence of RNA in the samples. All samples were diluted to a standard concentration of 200 ng/µL. Using the High-capacity cDNA reverse transcription kit (Applied Biosystems®, Thermo Fisher Scientific Inc.; Woolston, Warrington, UK) RNA was converted into cDNA. Instructions given in its manual were followed. The cDNA obtained was diluted to a standard concentration of 20 ng/µL before performing qPCR. In order to perform qPCR, a mixture of 5µL iTAP^{TM} Universal SYBR® Green Supermix, 0.5µL forward primer (10 pmol/µL concentration), 0.5 µL reverse primer (10 pmol/µL concentration), 2µL nuclease-free water, and 2µL cDNA sample was added to the wells of Hard-Shell PCR plates (Bio-Rad Laboratories Inc., USA). The plate was sealed using Microseal® 'B' seal (Bio-Rad Laboratories Inc., UK) and centrifuged at 3,000 rpm (revolutions per minute) to spin down the contents. The RT-PCR was run on CFX Connect ^{TM} Real-Time PCR Detection System (Bio-Rad Laboratories Inc., USA). The thermal cycle for each plate was: denaturation at 95°C for 5 minutes followed by 35 cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 30 seconds, and ending with a melt curve program from 65°C to 95°C. The mRNA Ct (cycle threshold) of each target gene was

normalized using a housekeeping gene and relative mRNA Ct was determined using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001). All primers were developed using the primer designing tool, Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and manufactured by IDT (Integrated DNA Technology Inc., Coralville, IA, USA). The genes along with their accession ID and forward and reverse primer sequence are shown in Table 4.3. Each sample for each gene was run in duplicate and every gene had a set of the negative control.

Table 4.3 Gene, primer sequences and accession ID used for gene expression of inflammatory cytokines, tight junction proteins, antioxidants, and housekeeping genes.

Gene[1]	primer sequence[2]	Accession ID
IL-6	F-AAGGTGATGCCACCTCAGAC	M86722
	R-TCTGCCAGTACCTCCTTGCT	
IL-10	F-CATCCACTTCCCAACCAGCC	NM_214041
	R-CTCCCCATCACTCTCTGCCTTC	
TNFA	F-ATGGATGGGTGGATGAGAAA	X54001
	R-TGGAAACTGTTGGGGAGAAG	
CLDN1	F-CTGTGGATGTCCTGCGTGT	NM_001244539.1
	R-GGTTGCTTGCAAAGTGGTGTT	
OCLN	F-GAGAGAGTGGACAGCCCCAT	NM_001163647
	R-TGCTGCTGTAATGAGGCTGC	
ZO1	F-GATCCTGACCCGGTGTCTGA	XM_021098856
	R-TTGGTGGGTTTGGTGGGTTG	
MUC2	F-CCAGGTCGAGTACATCCTGC	XM_021082584.1
	R-GTGCTGACCATGGCCCC	
SOD	F-GTACCAGTGCAGGTCCTCAC	NM_001190422
	R-TTTGCCAGCAGTCACATTGC	
CAT	F-ACACAGGCACATGAACGGAT	NM_214301
	R-GTCCCGGATGCCATAGTCAG	

GSH-Px	F-TGTGGTTTACGGATTCTGG	NM_214407.1
	R-CCTTGGGCTGGACTTTCA	
ACTB	F-GGATGCAGAAGGAGATCACG	XM_021086047.1
	R-ATCTGCTGGAAGGTGGACAG	
HPRT	F-GGACTTGAATCATGTTTGTG	NM_001032376
	R-CAGATGTTTCCAAACTCAAC	

[1]IL-6; Interleukin-6; IL-10, Interleukin-10; TNFA, tumour necrosis factor-alpha; CLDN-1, Claudin-1; OCLN, Occludin; ZO-1, Zona occludens-1; MUC-2, Mucin-2; SOD, Superoxide dismutase; CAT, Catalase; GPx, Glutathione peroxidase; ACTB, Beta-actin; HPRT, Hypoxanthine-guanine phosphoribosyl transferase. [2]F, forward; R, reverse.

4.9 Relative microbial abundance in colon digesta using qPCR

The colonic digesta samples were thawed on ice after removing from -80°C and approximately 250 mg sample was used for DNA extraction using QIAamp® PowerFecal® Pro DNA Kit (QIAGEN, Hilden, Germany) and following the manufacturer's instructions. The concentration and sample purity were tested using Nanodrop™ 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc.). All samples had an absorbance ratio of approximately 1.8 (OD230:OD260). Based on the concentration in each sample, they were diluted to a standard level of 20 ng/μL. The bacterial DNA analysed were, *Escherichia coli* F4, *Bifidobacterium*, Lactobacilli, and *Clostridium*. For qPCR, the procedure described in section 4.8 was followed. The bacteria with its primer name and sequence are mentioned in Table 4.4. Each sample for each bacterium was run in duplicate and every bacterium had a set of the negative control.

Table 4.4 Bacteria, primer name and primer sequences used for identification of bacterial genomic DNA.

Target bacteria	Primer name[2]	Primer sequence
Eubacteria	F: F338	ACTCCTACGGGAGGCAG
	R: R518	GTATTACCGCGGCTGCTG

Escherichia coli F4	F: faeGF	CACTGGCAATTGCTGCATCT
	R: faeGR	ACCACCGATATCGACCGAAC
Bifidobacterium	F: BifF	TCGCGTCYGGTGTGAAAG
	R: BifR	CCACATCCAGCRTCCAC
Lactobacilli subgroup[1]	F: LactF	AGCAGTAGGGAATCTTCCA
	R: LactR	CACCGCTACACATGGAG
Clostridium perfringens	F: CPerf165F	CGCATAACGTTGAAAGATGG
	R: CPerf269R	CCTTGGTAGGCCGTTACCC

[1]*Lactobacillus* and *Lactococcus* [2]F, forward; R, reverse.

4.10 Blood plasma antioxidant status

After collection, blood was centrifuged at 3,000 rpm for 10 minutes and plasma was removed and stored at -80°C. For knowing the plasma antioxidant status, superoxide dismutase activity, lipid peroxidation, and TAC were measured. Serial dilution was performed before assaying to measure precise sample values in the curve fitting model. The SOD assay was performed using a kit from Cayman Chemical, MI, USA, and as per the manufacturer's manual. The final absorbance was measured at 450 nm using a Synergy HTX multi-mode microplate reader (BioTek Instruments Inc., Vermont, USA). The SOD activity of the sample was calculated by plotting a linearized standard curve with final SOD activity as its function. The lipid peroxidation results in the formation of malondialdehyde (MDA) which can be measured as Thiobarbituric Acid Reactive Substances (TBARS) (Ohkawa et al., 1979). As per the instructions in the TBARS assay kit (R&D Systems, Inc. Minneapolis, MN, USA) the samples first had an acid treatment followed by a reaction between MDA and Thiobarbituric Acid in the presence of heat and acid. The absorbance of the colored end product was read at OD 532 nm in the same microplate reader used for SOD and the TBARS concentration was calculated by plotting a standard curve. The TAC was measured using an antioxidant assay kit from Cayman Chemical, MI, USA. The assay is based on the

capacity of antioxidants in the sample to inhibit the oxidation of ABTS® (2,2'-Azino-di- [3-ethylbenzthiazoline sulphonate]) to ABTS®$^{+}$ by metmyoglobin. This depicts if there is more antioxidant, less absorbance of ABTS® is seen in the microplate reader mentioned above at OD 750 nm. The TAC was calculated as the function of absorbance after obtaining a linear regression equation from a standard curve.

4.11 Blood plasma cytokines and total protein

Blood plasma cytokines were measured by performing an enzyme-linked immunosorbent assay (ELISA) and total protein (TP) was evaluated using Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, IL, USA). The reagents for ELISA were provided by RayBio® Porcine TNF-alpha ELISA kit, RayBio® Porcine IL-6 ELISA kit (RayBiotech, Inc. GA, USA), and Invitrogen™ Swine IL-10 ELISA Kit (Bender MedSystems GmbH, Thermo Fisher Scientific Inc. Vienna, Austria). For highly specific and sensitive results, solid-phase sandwich ELISA was performed. The microtiter plates were coated with primary antibodies. A cytokine-specific biotin conjugate was used as a secondary antibody in each assay and horseradish peroxidase streptavidin as its enzyme and tetramethylbenzidine as the substrate chromogen. At each step, the wells were washed four times with wash buffer and after substrate addition, the plate was incubated in absence of direct light. The absorbance of individual plates was measured at OD 450 nm in the Synergy HTX multi-mode microplate reader. A standard curve of optical density with standard concentration as its function was plotted and used for calculating sample concentration. For total protein, a bicinchoninic acid-based colorimetric detection and quantification assay was performed and plate absorbance was measured at OD 562 nm. A color response curve was plotted with bovine serum albumin as the standard and sample values were derived from the linear regression equation.

Each plasma sample for individual assays was run in duplicate with a set of blanks in every analysis.

4.12 Intestinal permeability

The mid-jejunal tissue samples stored in KRB buffer were promptly transported to the laboratory for TEER measurement in Ussing chamber (VCC-MC8; Physiologic Instruments Inc., San Diego, CA). The set up included pairs of current (Ag wire) and voltage (Ag/AgCl pellet) electrodes placed in 3% agar bridges and filled in KRB buffer without glucose. The mucosal and serosal chambers were constantly supplied with 5% carbon dioxide and %% oxygen gas. Glucose (10 mmol/L) enriched 5ml of KBR buffer was added to the serosal chamber. A temperature of 37°C was maintained in the chambers with the help of water jacketed reservoir. In the Ussing chambers the tissue was placed using tissue slider with a 1 cm^2 opening after the serosal and longitudinal muscle layers were gently removed. After placing the tissue, 10 minutes were required for the tissue to equilibrate, followed by measurements for 10 minutes. The mucosal side was treated with 0.1 mg/ml of FITC-D4 (Sigma Aldrich Co.) and a sample of 1 ml was taken from the serosal side, afterwards. These samples were later used for calculating FITC flux. The fluorescence was measured at 485 nm and 528 nm at the excitation and emission wavelengths, respectively in a Bio-Tek PowerWave HT Microplate Scanning Spectrophotometer (Bio-Tek Instruments Inc.) The FITC-D4 concentration was calculated from a standard curve prepared from known dilutions.

4.13 Statistical analyses

All data were analysed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). An individual pig was considered the experimental unit and each treatment had 7 experimental units. Experimental diets (NC; PC; ROD1; ROD2) were considered as fixed variables in a completely randomized design. Data were considered significantly different at $P <$

0.05 and tended to be different at $P < 0.10$. Orthogonal contrasts were used to compare NC and PC, determine the response to increasing levels of RDE and compare RDE supplementation to NC and PC treatment groups. To calculate treatment means and compare their differences the LSMEANS statement with the option of Tukey-adjusted PDIFF was used. Each table in the result shows the least square mean for each treatment group with a pooled standard error of means alongside the level of significance of each contrast. All the comparisons in mRNA gene expression and microbial DNA results were made with the NC group.

5 Results

5.1 Growth performance

In the trial, piglets in all the treatment groups had similar ($P > 0.05$) BW and other growth parameters like ADG, ADFI, and GF throughout the experiment. Although, the ADG of the NC group tended to be higher ($P < 0.10$) than RDE treatment groups (ROD1 & ROD2) (Table 5.1).

5.2 Fecal score and body temperature

There were no significant differences ($P > 0.05$) in the fecal score between NC, ROD1, and ROD2 groups. However, the diarrhea incidence in the PC group compared to the RDE treatment groups tended to be lower ($P < 0.10$) 3 hours after inoculation and was significantly reduced ($P < 0.05$) on 3 dpi as seen in Figure 5.1.

The body temperature data is presented in Figure 5.2. The differences in body temperature before and after inoculation were non-significant ($P > 0.05$) except two observations made at 3 hpi which showed that PC and ROD2 group piglets had significantly lower ($P < 0.05$) body temperature than the ROD1 group.

5.3 Intestinal permeability and histomorphology

With the help of the Ussing chamber and microplate absorbance reader, the TEER and flux of FITC were measured for intestinal tissue. Although numerically TEER of ROD1 was higher than other treatment groups there were no significant differences ($P > 0.05$) observed. Similarly, treatment groups did not show any significant differences ($P > 0.05$) in FITC values (Table 5.3). For histomorphological evaluation VH, CD, and VH:CD ratio data is indicated in Table 5.2. There were no significant differences ($P > 0.05$) among treatment groups in any of these response criteria.

5.4 Plasma cytokines and antioxidants

The plasma variables were measured before and after the inoculation. The three cytokines (IL-6, IL-10 and TNF-α) did not show any significant differences ($P > 0.05$) in the predetermined contrasts (Table 5.4). However, in a comparison between ROD2 and NC group, IL-10 tended to be higher ($P < 0.10$) in the ROD2 group than in NC during the pre-inoculation phase. It remained significantly higher ($P < 0.05$) in ROD2 as compared to NC in the post-inoculation phase. Trends ($P < 0.10$) for an increase in IL-10 were observed in ROD2 supplemented piglets as compared to PC or ROD1 groups post-inoculation.

The data for plasma antioxidants and total protein (TP) is summarized in Table 5.5. The values of SOD, MDA, TAC, and TP were similar ($P > 0.05$) in all the treatment groups before inoculation. This situation tended to change post-inoculation with SOD trending higher ($P < 0.10$) in PC than in the ROD1 and ROD2 group and MDA trending lower ($P < 0.10$) in ROD2 as compared to ROD1.

5.5 mRNA gene expression and microbial DNA

Gene expression for inflammatory genes like *IL-6*, *IL-10*, and *TNF-α*; antioxidant genes like *SOD*, *GPx*, and *CAT*; barrier function genes like *ZO-1*, *OCLN*, *CLDN-1*, and *MUC-2* from mid-jejunal tissue is illustrated in Figure 5.4. Except for *IL-10*, *IL-6*, and *CAT*, other genes did not have a significant difference ($P > 0.05$) among treatments. There was a significantly higher ($P < 0.05$) expression of *CAT* in the ROD1 group. Similarly, *IL-10* showed a tendency to be lower ($P < 0.10$) in ROD1 than NC. The expression of *IL-6* was notably lower ($P < 0.05$) in PC and trended to be lower ($P < 0.10$) in ROD2 compared to NC.

Bacterial DNA for *Bifidobacterium, Clostridium, Lactobacillus,* and *Escherichia coli* F4 was quantified relatively to total eubacteria population for each treatment and displayed in Figure 5.3. Piglets in group ROD2 had a significantly lower ($P < 0.05$) amount of *Bifidobacterium* in their colon as compared to NC. Following a similar trend were ROD1 and PC treatment groups. The only group to show a tendency of lower ($P < 0.10$) *E. coli* F4 than NC piglets is the antibiotic supplemented group, PC.

Table 5.1 Effect of antibiotic and RDE supplementation on growth performance of piglets challenged with Escherichia coli F4.

Item[3]	Treatment[1,5]				SEM[2]	*P*-value[4]			
	NC	PC	ROD1	ROD2		1	2	3	4
BW, kg									
initial	9.05	9.03	9.00	9.06	0.35	0.982	0.900	0.969	0.990
pre-inoculation	10.36	10.24	10.59	10.35	0.56	0.874	0.749	0.862	0.723
post-inoculation	13.56	12.87	13.30	12.53	0.77	0.500	0.468	0.474	0.958
ADG, g/day									
pre-inoculation	163	151	198	144	37	0.802	0.294	0.849	0.634
post-inoculation	433	354	357	293	52	0.255	0.375	**0.083**	0.636
overall	322	274	307	237	39	0.352	0.196	0.274	0.968
ADFI, g/day									
pre-inoculation	252	239	293	236	31	0.744	0.187	0.739	0.483
post-inoculation	509	449	455	424	43	0.296	0.603	0.175	0.858
overall	392	348	389	320	39	0.397	0.207	0.410	0.888
GF, g/g									
pre-inoculation	0.61	0.60	0.65	0.54	0.08	0.924	0.317	0.923	0.990
post-inoculation	0.90	0.84	0.85	0.72	0.09	0.629	0.304	0.298	0.617
overall	0.81	0.79	0.79	0.71	0.07	0.820	0.353	0.422	0.583

[1]NC, negative control, corn, wheat soybean meal diet; PC, positive control, NC plus antibiotics; ROD1, NC plus 0.1% RDE; ROD2, NC plus 0.2% RDE.

[2]SEM, Standard error of the mean.

[3]BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; GF, gain: feed ratio.

[4]*P*- value, level of significance; 1: NC *vs.* PC; 2: ROD1 *vs.* ROD2; 3: NC *vs.* ROD1 + ROD2; 4: PC *vs.* ROD1 + ROD2.

[5]Each value represents the mean of 7 replicates except for ROD2 with 6 replicates due to the elimination of 1 piglet in the pre-inoculation phase.

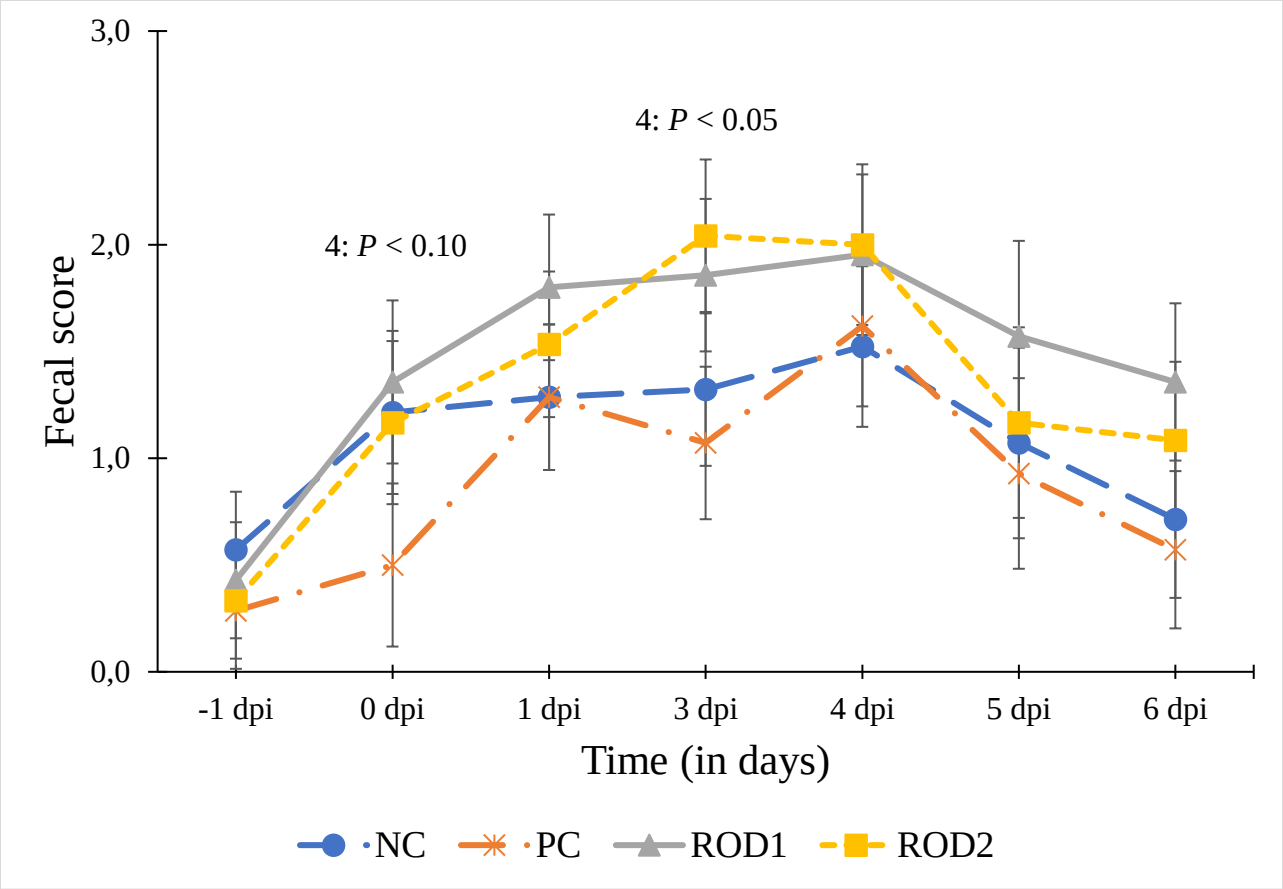

Figure 5.1 Effect of antibiotic and RDE supplementation on the fecal score of piglets challenged with *Escherichia coli* F4.
NC, negative control, corn, wheat soybean meal diet; PC, positive control, NC plus antibiotics; ROD1, NC plus 0.1% RDE; ROD2, NC plus 0.2% RDE; dpi, days post-inoculation.
4: PC *vs.* ROD1 + ROD2.
P: Level of significance (*P* – value)
Fecal score = 0, normal feces; 1, soft feces; 2, mild diarrhea; and 3, severe diarrhea.
Each value represents the mean of 7 replicates except for ROD2 which represents the mean of 6 replicates due to the elimination of 1 piglet in the pre-inoculation phase.

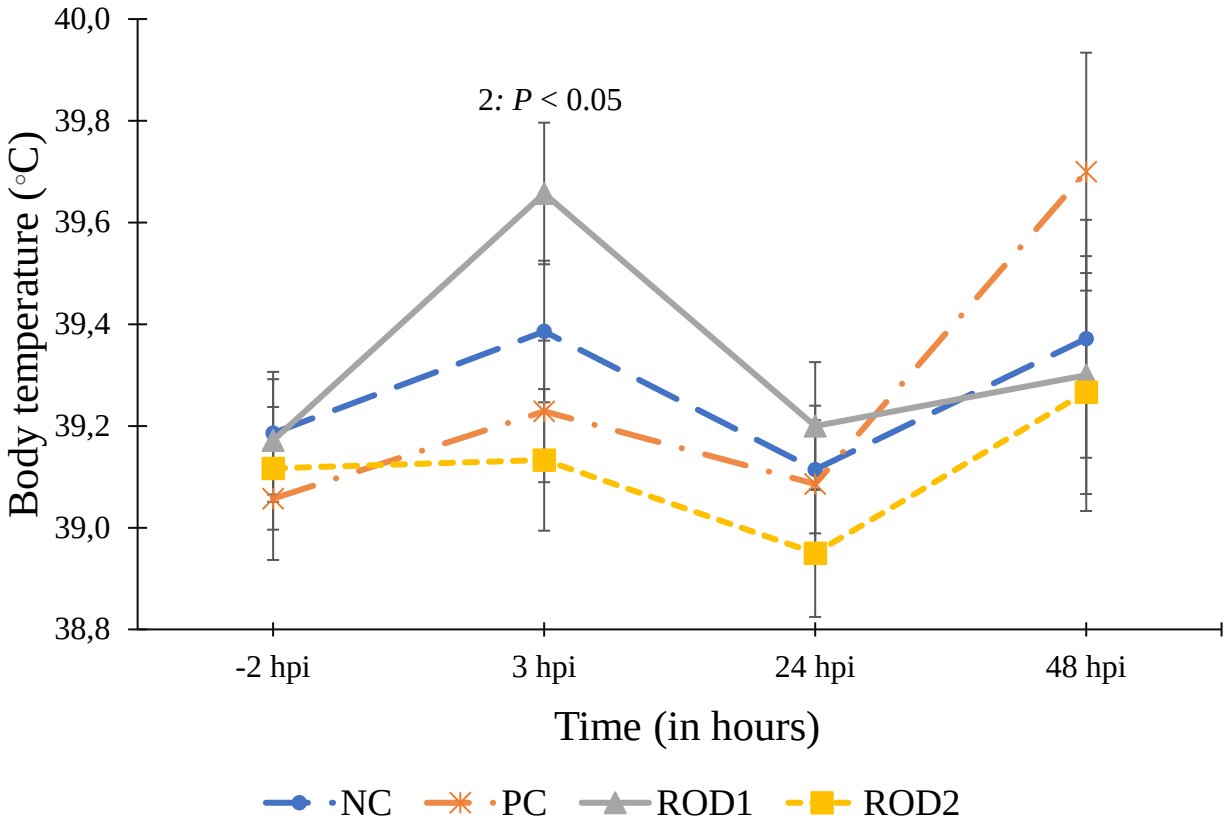

Figure 5.2 Effect of antibiotic and RDE supplementation on body temperature of piglets challenged with *Escherichia coli* F4.
NC, negative control, corn, wheat soybean meal diet; PC, positive control, NC plus antibiotics; ROD1, NC plus 0.1% RDE; ROD2, NC plus 0.2% RDE; hpi, hours post-inoculation.
2: ROD1 *vs.* ROD2
P: Level of significance (*P*-value)
Each value represents the mean of 7 replicates except for ROD2 which represents the mean of 6 replicates due to the elimination of 1 piglet in the pre-inoculation phase.

Table 5.2 Effect of antibiotic and RDE supplementation on intestinal morphology of piglets challenged with *Escherichia coli* F4.

Item[3]	Treatment[1,5]				SEM[2]	*P*-value[4]			
	NC	PC	ROD1	ROD2		1	2	3	4
VH, μm	376	330	350	388	34	0.319	0.430	0.862	0.335
CD, μm	271	270	256	270	15	0.974	0.499	0.637	0.663
VH:CD, μm/ μm	1.60	1.40	1.61	1.73	0.18	0.430	0.622	0.797	0.251

[1]NC, negative control, corn, wheat soybean meal diet; PC, positive control, NC plus antibiotics; ROD1, NC plus 0.1% RDE; ROD2, NC plus 0.2% RDE.
[2]SEM, Standard error of the mean.
[3]VH, Villous height; CD, Crypt depth.
[4]*P*- value, level of significance; 1: NC *vs.* PC; 2: ROD1 *vs.* ROD2; 3: NC *vs.* ROD1 + ROD2; 4: PC *vs.* ROD1 + ROD2.
[5]Each value represents the mean of 7 replicates except for ROD2 which represents the mean of 6 replicates due to the elimination of 1 piglet in the pre-inoculation phase.

Table 5.3 Effect of antibiotic and RDE supplementation on intestinal permeability of piglets challenged with *Escherichia coli* F4.

Item[3]	Treatment[1,5]				SEM[2]	*P*-value[4]			
	NC	PC	ROD1	ROD2		1	2	3	4
TEER, Ω·cm^2	58.0	56.0	62.7	56.2	12.9	0.930	0.716	0.920	0.841
FITC flux, μg/cm^2·hr·mL	55.0	91.0	70.3	79.1	18.5	0.157	0.732	0.374	0.454

[1]NC, negative control, corn, wheat soybean meal diet; PC, positive control, NC plus antibiotics; ROD1, NC plus 0.1% RDE; ROD2, NC plus 0.2% RDE.
[2]SEM, Standard error of the mean.
[3]TEER, Trans-epithelial electrical resistance; FITC, fluorescein isothiocyanate.
[4]*P*- value, level of significance; 1: NC *vs.* PC; 2: ROD1 *vs.* ROD2; 3: NC *vs.* ROD1 + ROD2; 4: PC *vs.* ROD1 + ROD2.
[5]Each value represents the mean of 7 replicates except for ROD2 which represents the mean of 6 replicates due to the elimination of 1 piglet in the pre-inoculation phase.

Table 5.4 Effect of antibiotic and RDE supplementation on plasma level of inflammatory cytokines of piglets challenged with *Escherichia coli* F4.

Item[3]	Treatment[1,5]				SEM[2]	*P*-value[4]			
	NC	PC	ROD1	ROD2		1	2	3	4
IL-6, pg/ml									
pre-inoculation	518.64	875.85	559.13	614.14	371.22	0.207	0.851	0.781	0.245
post-inoculation	903.84	1894.06	1141.33	1088.28	886.26	0.151	0.940	0.722	0.197
IL-10, pg/ml									
pre-inoculation	38.83	52.77	44.90	77.56	13.31	0.466	0.109	0.188	0.614
post-inoculation	33.60	40.67	37.07	79.41	14.86	0.740	**0.065**	0.195	0.351
TNF-α, pg/ml									
pre-inoculation	562.92	127.26	63.40	565.93	219.90	0.920	0.392	0.511	0.442
post-inoculation	27.48	22.98	73.49	33.31	31.28	0.175	0.134	0.373	0.499

[1]NC, negative control, corn, wheat soybean meal diet; PC, positive control, NC plus antibiotics; ROD1, NC plus 0.1% RDE; ROD2, NC plus 0.2% RDE.

[2]SEM, Standard error of the mean.

[3]IL-6, Interleukin-6; IL-10, Interleukin-10; TNF-α, Tumour necrosis factor-alpha.

[4]*P*- value, level of significance; 1: NC *vs.* PC; 2: ROD1 *vs.* ROD2; 3: NC *vs.* ROD1 + ROD2; 4: PC *vs.* ROD1 + ROD2.

[5]Each value represents the mean of 7 replicates except for ROD2 which represents the mean of 6 replicates due to the elimination of 1 piglet in the pre-inoculation phase.

Table 5.5 Effect of antibiotic and RDE supplementation on plasma antioxidant status of piglets challenged with *Escherichia coli* F4.

Item[3]	Treatment[1,5]				SEM[2]	*P*-value[4]			
	NC	PC	ROD1	ROD2		1	2	3	4
SOD, *U/ml									
pre-inoculation	0.78	0.51	0.53	0.65	0.11	0.113	0.481	0.191	0.600
post-inoculation	0.84	0.99	0.72	0.58	0.16	0.512	0.555	0.328	**0.092**
MDA, µM/ml									
pre-inoculation	0.87	0.92	0.84	0.67	0.13	0.813	0.370	0.464	0.320
post-inoculation	1.12	0.83	1.21	0.77	0.16	0.219	**0.081**	0.531	0.431

TAC, mM/ml									
pre-inoculation	0.69	0.73	0.72	0.72	0.03	0.307	0.939	0.366	0.792
post-inoculation	0.75	0.72	0.71	0.78	0.03	0.301	0.112	0.743	0.391
TP, µg/ml									
pre-inoculation	39031	26294	27809	20458	7992	0.271	0.824	0.329	0.777
post-inoculation	23079	34043	13755	13847	9687	0.432	0.995	0.448	0.106

[1]NC, negative control, corn, wheat soybean meal diet; PC, positive control, NC plus antibiotics; ROD1, NC plus 0.1% RDE; ROD2, NC plus 0.2% RDE.

[2]SEM, Standard error of the mean.

[3]SOD, Superoxide dismutase; MDA, malondialdehyde; TAC, total antioxidant capacity; TP, total protein.

*U/ml, one unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

[4]*P*- value, level of significance; 1: NC *vs.* PC; 2: ROD1 *vs.* ROD2; 3: NC *vs.* ROD1 + ROD2; 4: PC *vs.* ROD1 + ROD2.

[5]Each value represents the mean of 7 replicates except for ROD2 which represents the mean of 6 replicates due to the elimination of 1 piglet in the pre-inoculation phase.

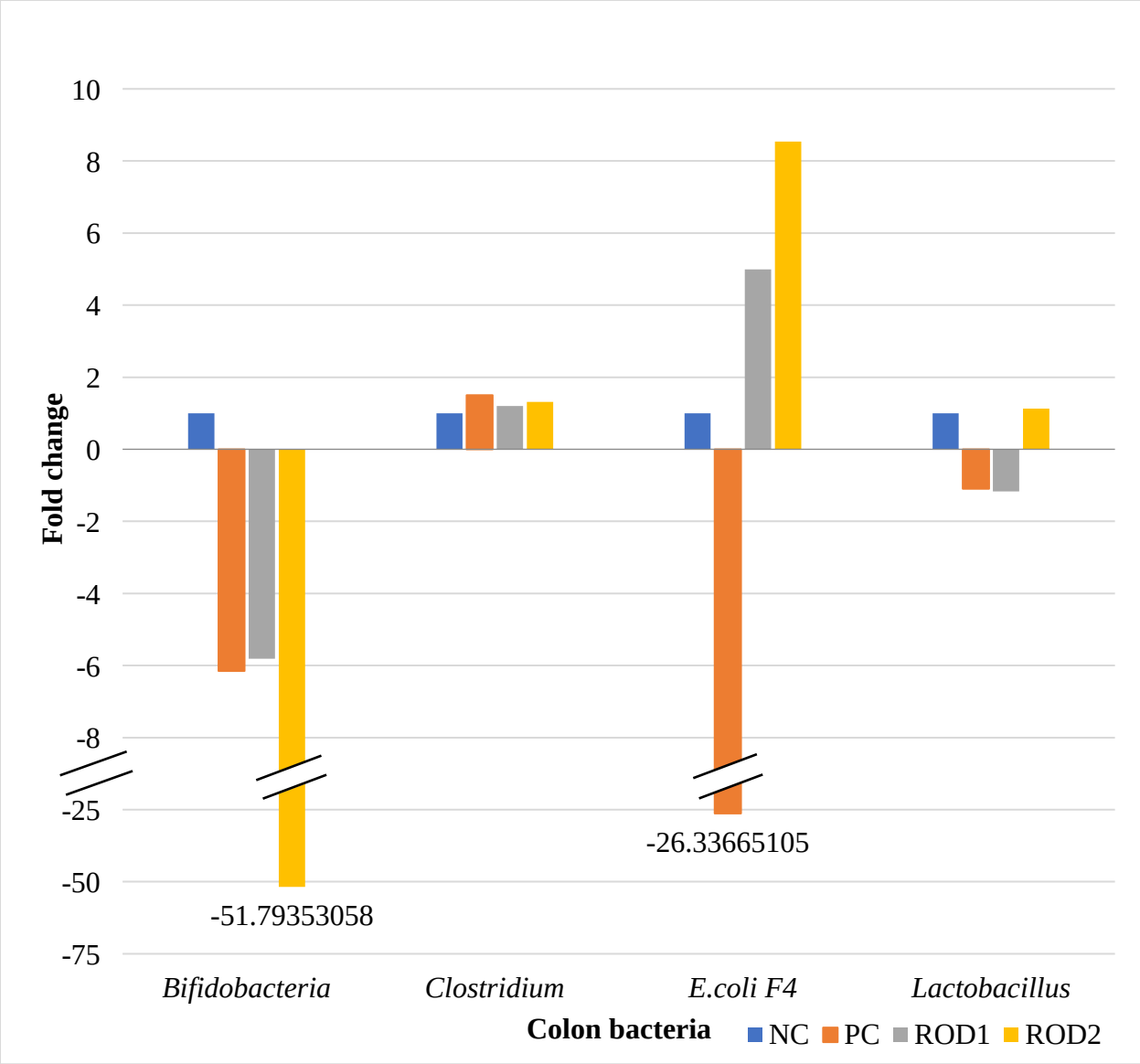

Figure 5.3 Effect of antibiotic and RDE supplementation on microbial DNA in the colon of piglets challenged with *Escherichia coli* F4.
NC, negative control, corn, wheat soybean meal diet; PC, positive control, NC plus antibiotics; ROD1, NC plus 0.1% RDE; ROD2, NC plus 0.2% RDE.
SEM, Standard error of the mean.
P- value, level of significance; 1: NC *vs.* PC; 2: NC *vs.* ROD1; 3: NC *vs.* ROD2.
Each value represents the mean of 7 replicates except for ROD2 which represents the mean of 6 replicates due to the elimination of 1 piglet in the pre-inoculation phase.

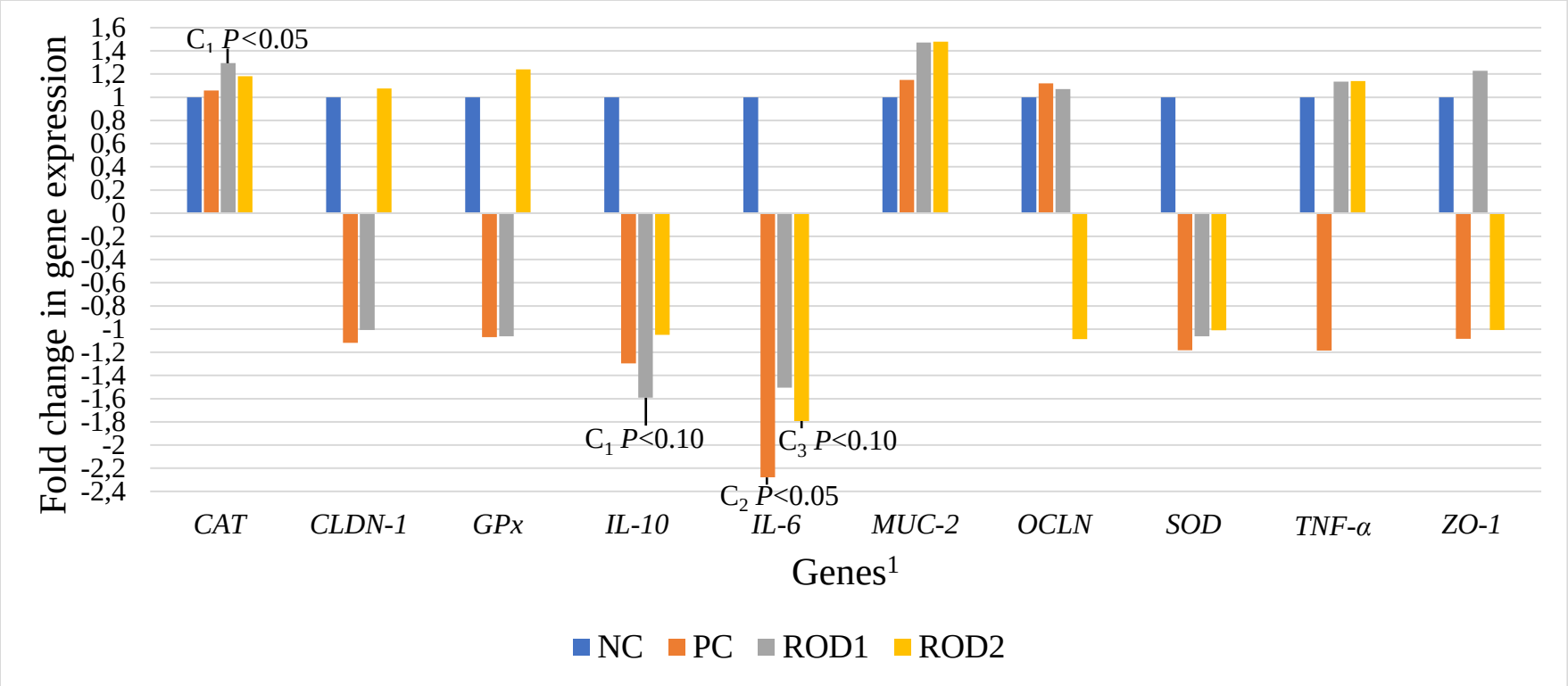

Figure 5.4 Effect of antibiotic and RDE supplementation on mRNA gene expression in mid-jejunum of piglets challenged with *Escherichia coli* F4.

NC, negative control, corn, wheat soybean meal diet; PC, positive control, NC plus antibiotics; ROD1, NC plus 0.1% RDE; ROD2, NC plus 0.2% RDE.

C_1: contrast 1: NC *vs.* ROD1; C_2: contrast 2: NC *vs.* PC; C_3: Contrast 3: NC *vs.* ROD2.

P: Level of significance (*P*-value)

Each value represents the mean of 7 replicates except for ROD2 which represents the mean of 6 replicates due to the elimination of 1 piglet in the pre-inoculation phase.

[1]*CAT, Catalase; CLDN-1, Claudin-1; GPx, Glutathione peroxidase; IL-10, Interleukin-10; IL-6; Interleukin-6; MUC-2, Mucin-2; OCLN, Occludin; SOD, Superoxide dismutase; TNF-α, tumour necrosis factor-alpha; ZO-1, Zona occludens-1.*

6 Discussion

Pigs are social creatures and any disruption in their environment can cause tremendous stress in their lives. Due to early and artificial weaning in most commercial swine facilities, the piglets undergo many changes that affects their growth and might make them severely anorexic affecting the rest of their productive time. This very delicate phase of a pig's life makes it very critical to provide proper nourishment and care to weaned piglets to minimize any economic losses to farmers or illnesses to pigs. Researchers have studied the post-weaning phase and established many therapeutic, natural, and managerial ways to reduce stress and promote gut health and growth performance in weaned piglets. However, there is still more to expand the knowledge about weaning and its complications.

In research experiments, pigs are provided with all the necessities and recreational articles to let them exhibit natural behavior. But most importantly, in the research setup, there are a smaller number of pigs, and they are kept in a controlled environment where each pig may be housed an individual pen, as in the current study. This affects their stress level as there is no social hierarchy or fights as well as ensuring hygienic uniform feed and water supply to each piglet. Due to these conditions, translating research findings to commercial farms are difficult because there are many factors that are not accounted for in a "research controlled environment" (Cornelison et al., 2018). Thus, such factors may influence the outcomes observed in "research pigs" towards an unexpected direction in "commercial pigs".

In the current ETEC challenge study, there were no significant differences in the BW, ADFI, or GF among the four treatment groups of the experiment. However, there was a trend in the NC group after the challenge that showed higher ADG as compared to RDE treatment groups (ROD1

and ROD2). Similar observations were noted in other polyphenol-rich supplements like dietary grape pomace (Wang et al., 2020), gallic acid (Cai et al., 2020), apple polyphenols (Xu et al., 2019), and various other plant extracts like *Boswellia serrata*, *Uncaria tomentosa*, *Tanacetum parthenium* and *Asteraceae, Verbenaceae* and *Lamiaceae* (Corino et al., 2021) where weaned piglets supplemented with phytogenics did not show any significant difference in GP in comparison to the non-supplemented group. Moreover, Bruins et al. (2011) found that black tea extract can diminish the growth of *E. coli* challenged weaned piglets as compared to the control group.

Out of all the swine pathogens, *E. coli* is the most prominent bacteria to exacerbate PWD in weaned piglets. Therefore, the ETEC challenge model is widely used in scientific experiments for the prevention of PWD. To find an alternative strategy in place of AGPs, several phytogenics and their combinations with or without the inclusion of other feed additives are being studied around the world. In this study, the fecal consistency of RDE treatment groups had a higher score than that of PC group, which was supplemented by an antibiotic. Positive control piglets already showed a tendency towards less diarrhea at 3 hpi and they significantly improved 3 dpi, with reference to ROD1 and ROD2. In an ETEC challenge study done by Caprarulo et al. (2022), the treatment group supplemented with phytogenic additive FRESTA® F (Delacon Biotechnik GmBH) (containing caraway oil, lemon oil, and dried herbs and spices) along with SCFA and MCFA showed the lowest diarrhea occurrence as compared to positive control with antibiotics, negative control, and phytogenic additive only treatments. This shows that although phytogenics alone might not significantly alleviate diarrhea, their synergistic effect with other feed additives can be remarkable.

When bacteria circulate through the blood it produces sepsis, resulting in increased production of inflammatory cytokines and chemokines. This inflammatory response throughout the body is responsible for elevated body temperature causing pyrexia. Many studies have had similar observations as a symptom of *E. coli* infection in piglets (Zhang et al., 2010; Lee et al., 2017). However, Spitzer et al. (2014) found that almost all the piglets, irrespective of the treatment, had hypothermia approximately 1 to 3 days after the ETEC challenge. A possible explanation for this could be when animals are anorexic due to a loss of appetite their body tries to conserve heat for internal organs by reducing body temperature (Romanovsky and Székely, 1998; Schieber and Ayres, 2016). As shown in Figure 4.2, ROD2 piglets had a stable body temperature throughout the experiment. However, PC and ROD1 piglets showed a significant jump in body temperature 3 hours after inoculation. According to the 'Swine Health Guide' of the American Association of Swine Veterinarians, the normal body temperature of weaned piglets ranges from 38.6^{o}C to 39.5^{o}C (American Association of Swine Veterinarians). ROD2 group piglets were below the upper-temperature limit along with the NC group. This might be because RDE has anti-inflammatory properties and can reduce inflammatory cytokine production in the body however, the reason behind NC group is not quite clear.

Longer villi and shorter crypt have been associated with better growth performance and intestinal health as villi aid in increased absorption of nutrients and short crypt indicates decreased cell regeneration thereby reducing energy expenditure (Iji et al., 2001; de Los Santos et al., 2007). This additional energy can be then used in BW gain and increase in muscle size. Dietary quercetin supplementation in piglets increased VH, VH:CD ratio, and decreased CD in the jejunum as compared to the non-supplemented group among weaned piglets (Xu et al., 2021). A similar observation was recorded when piglets challenged with *E. coli* were supplemented with red osier

dogwood (Koo et al., 2021). In other species like broiler chicken, longer villi, and shorter crypt than the control group was reported when supplemented with phytogenic blends (Oso et al., 2019). Another important feature of intestinal health is mucosal permeability. The intestinal mucosa protects the body by being the first line of defense against oral toxins while facilitating nutrient transport at the same time. To measure this selective permeability, the Ussing chamber is a useful tool. When RDE was evaluated *in vitro* it increased the TEER and decreased the FITC transport across the intestinal cells (Yang et al., 2019) suggesting an improved intestinal barrier function. Similar *in vitro* results were produced by Vergauwen et al. (2016) using quercetin and rosmarinic acid. However, in the current experiment, there were no significant changes due to dietary treatments in the intestinal permeability and histomorphology in piglets. It is difficult to conclude that NC, PC, ROD1, and ROD2 all act indifferently on the above intestinal parameters because in a large population unlike small sample size, differences in the values might have statistical significance. It is necessary to conduct large-scale animal studies in order to get definitive results.

Plasma cytokines are a systemic indicator of inflammation in the body. Cytokines and chemokines are produced by the body in response to external agents like stress, pathogens, and nutritional imbalance. They aid in protecting the body against toxic foreign substances but also utilize protein required for growth in weaned piglets. Tea polyphenols have been shown to reduce pro-inflammatory cytokines such as IL-1, TNF-α, and IFN-γ in blood after pigs were challenged with diquat, an herbicide inducing oxidative stress (Deng et al., 2010). This shows that phenolic compounds supplement the anti-oxidative system of the body and help in reducing inflammation. In the present study, IL-10 an anti-inflammatory cytokine is significantly higher in ROD2 than NC group and a tended to be higher than PC and ROD1 in the post-inoculation period. It is also showing a higher trend in ROD2 than NC in the pre-inoculation period. Upregulation of IL-10

during an inflammatory trigger can help in balancing the immune response and preventing any inflammatory injury to the pigs. There were no remarkable differences in TNF-α or IL-6 plasma levels among treatment groups, however, it is also important to understand the systemic antioxidant levels and the effect of each treatment on them.

A balance between the production and elimination of ROS is essential for prohibiting tissue damage due to oxidative stress. The antioxidative system in the body regulates this cycle during normal circumstances, however, when external factors lead to the overproduction of ROS the endogenous antioxidants fail to prevent the damage. For this reason, it is beneficial to supplement piglets with antioxidant-rich compounds during weaning. Early weaning has been associated with oxidative damage. A study done by Yin et al. (2014) shows high levels of plasma MDA, protein carbonyl, and 8-hydroxydeoxyguanosine indicating lipid, protein, and DNA damage, respectively when pigs were weaned at 14 days of age. Yin et al. (2014) also noted low levels of antioxidant enzymes including SOD, catalase, and glutathione peroxidase in the blood. The plasma levels of SOD were measured in the current study. Table 4.5 shows slightly higher levels of SOD in PC piglets than in ROD2 piglets. On the other hand, the MDA level in the ROD2 group post-inoculation was the lowest and was significantly lower than for the ROD1 group, indicating less lipid peroxidation in ROD2 piglets.

Since, jejunum is the region of the small intestine most affected by oxidative damage and inflammation (Campbell et al., 2013), a tissue sample from the mid-jejunum was analyzed for mRNA gene expression in the present study. In mid-jejunum, the mRNA gene expression of the antioxidative enzyme, *catalase* was significantly higher in the ROD1 group as compared to the NC group. Similarly, there was reduced expression of anti-inflammatory cytokine *IL-10* in the ROD1 group than in the NC group. Piglets receiving the PC diet showed less signs of jejunal

inflammation than those receiving the NC diet as the *IL-6* expression was significantly lower in them. There have been many studies in other species including broiler chickens showing an increase in gene expression of tight junction proteins in the small intestine after feeding carvacrol essential oil as a supplement (Christaki et al., 2020). Similarly, curcumin and resveratrol have been associated with lower gene expression of pro-inflammatory cytokines in the jejunum of weaned piglets (Gan et al., 2019). There is ample evidence based on research to prove that phytogenics work as antioxidants and anti-inflammatory agents for intestinal tissues. In the current study, we could observe such anti-inflammatory effect by reduced gene expression of *IL-6* in ROD2 group and increased gene expression of *catalase* in ROD1 group in comparison to NC. Similarly, antioxidative effect was seen when serum MDA levels were lower in pigs supplemented with higher level of RDE than in pigs supplemented with lower level of RDE.

In the case of bacterial DNA in the colon, the PC group presented the least value for *E. coli* F4. This is correlated to the antimicrobial effect of the antibiotic. Other results in the bacterial DNA analysis were inconclusive.

7 Summary

For pork producers, weaning of piglets has always been a crucial phase and in the past, there have been many advancements that have ameliorated the weaning transition. Antibiotics have been used for decades to keep mortality and morbidity low during this phase and enhance the weight gain and health of piglets. However, with the rise of concern regarding the use of AGPs among scientists, producers, and consumers, there has been a concorded effort to acquire alternatives for the growth promotion and wellbeing of young piglets. With increasing awareness people tend to choose meat options that are organic and antibiotic-free. This concern led to the development of various natural non-therapeutic alternatives having more than one benefit for the health like better gut permeability and histology, less gene expression of pro-inflammatory cytokines, strengthening antioxidant system, and defense against gut pathogens. Plant-derived polyphenols have shown antioxidative, anti-microbial, and anti-inflammatory properties *in vitro* as well as *in vivo* experimental settings. Red osier dogwood plant contains high levels of polyphenols having such properties like rutin, quercetin, ellagic acid, and gallic acid. In this study, it was hypothesized that dietary supplementation of RDE will improve antioxidant defense and gut health indices of weaned piglets challenged with *E. coli*. The objectives that were accomplished through this research were,

1. Evaluation of the effects of RDE on gut and systemic health by measuring growth parameters, intestinal permeability, structure, and systemic and local inflammatory as well as oxidative biomarkers.
2. Comparison of the effects of RDE with AGP using contrasts and analysis of variance.
3. Investigating if there is a dose-response associated with different levels of RDE.

It is possible to conclude from the data of this experiment that ROD might alleviate oxidative stress and PWD symptoms during weaning and have an anti-inflammatory effect on pigs. However, it is

still uncertain to conclude that it will be able to replace antibiotics used for growth promotion in the future. Further investigation can provide a more definitive conclusion. Some things to consider for future studies are written below.

8 Future studies

This study has helped to know the effects of ROD on gut health and growth performance of weaned piglets challenged with ETEC. However, polyphenols from plant extracts are novel to the livestock industry and their many aspects are yet to be explored.

Polyphenols are abundant in nature and hardly a fraction of them have been studied till now. Along with this, experiments of already known polyphenol containing plant extracts like RDE in combination with other feed additives like organic acids, nucleotides, probiotics, feed enzymes, etc. should be examined for their synergistic effects.

Better availability and standardization of plant extracts from different parts of the world should be done. The information on some phytogenics and their ingredients are still ambiguous, and their contents change with each harvest, making it difficult to calculate the optimum dose in each livestock species. Although there are few studies of RDE in pigs, chickens, and cattle, a standard dose has not yet been established. For this, further animal studies should be promoted.

9 References

Van der Aar, P. J., Molist, F. V., & Van Der Klis, J. D. (2017). The central role of intestinal health on the effect of feed additives on feed intake in swine and poultry. Animal feed science and technology 233, 64-75.

Amorati, R., M. C. Foti, and L. Valgimigli. 2013. Antioxidant activity of essential oils. Journal of Agricultural and Food Chemistry 61:10835–10847.

Anderson, D. B., V. J. McCracken, R. I. Aminovi, J. M. Simpson, R. I. Mackie, M. W. A. Verstegen, and H. R. Gaskins. 1999. Gut microbiology and growth-promoting antibiotics in swine. Pig News and Information 20:115–122.

Apea-Bah, F. B., D. Head, R. Scales, R. Bazylo, and T. Beta. 2020. Hydrothermal extraction, a promising method for concentrating phenolic antioxidants from red osier dogwood (*Cornus stolonifer*) leaves and stems. Heliyon 6: e05158

Begemann, S., E. Perkins, I. Van Hoyweghen, R. Christley, and F. Watkins. 2017. How political cultures produce different antibiotic policies in agriculture: A historical comparative case study between the United Kingdom and Sweden. Sociologia Ruralis 58:765–785.

Bikker, P., A. Dirkzwager, J. Fledderus, P. Trevisi, I. le Huërou-Luron, J. P. Lallès, and A. Awati. 2006. The effect of dietary protein and fermentable carbohydrates levels on growth performance and intestinal characteristics in newly weaned piglets. Journal of animal science 84:3337–3345.

Bischoff. 2011. “Gut health”: a new objective in medicine? BMC Medicine 9:1–14.

van Boeckel, T. P., C. Brower, M. Gilbert, B. T. Grenfell, S. A. Levin, T. P. Robinson, A. Teillant, and R. Laxminarayan. 2015. Global trends in antimicrobial use in food animals. Proceedings of the National Academy of Sciences 112:5649–5654.

Bontempo, V., A. di Giancamillo, G. Savoini, V. Dell'Orto, and C. Domeneghini. 2006. Live yeast dietary supplementation acts upon intestinal morpho-functional aspects and growth in weanling piglets. Animal Feed Science and Technology 129:224–236.

Boudry, G., V. Péron, I. le Huërou-Luron, J. P. Lallès, and B. Sève. 2004. Weaning induces both transient and long-lasting modifications of absorptive, secretory, and barrier properties of piglet intestine. Journal of Nutrition 134:2256–2262.

Bravo, L. 1998. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutrition Review. 56:317-333.

van den Broeck, W., E. Cox, B. Oudega, and B. M. Goddeeris. 2000. The F4 fimbrial antigen of *Escherichia coli* and its receptors. Veterinary Microbiology 71:223–244.

van den Broek, L. A. M., S. W. A. Hinz, G. Beldman, J. P. Vincken, and A. G. J. Voragen. 2008. Bifidobacterium carbohydrases-their role in breakdown and synthesis of (potential) prebiotics. Molecular Nutrition and Food Research 52:146–163.

Brooks, P. H., C. A. Moran, J. D. Beal, V. Demeckova, A. Campbell, M. A. Varley, and J. Wiseman. 2001. The weaner pig: Nutrition and management. 1st ed. CABI publishing, CAB International, Wallingford, Oxon, UK.

Bruins, M. J., M. A. M. Vente-Spreeuwenberg, C. H. Smits, and L. G. J. Frenken. 2010. Black tea reduces diarrhoea prevalence but decreases growth performance in enterotoxigenic *Escherichia*

coli-infected post-weaning piglets. Journal of Animal Physiology and Animal Nutrition 95:388–398.

Buddington, R. K., P. T. Sangild, B. Hance, E. Y. Huang, and D. D. Black. 2012. Prenatal gastrointestinal development in the pig and responses after preterm birth. Journal of Animal Science 90:290–298.

Bühler, K., A. Liesegang, B. Bucher, C. Wenk, and J. Broz. 2010. Influence of benzoic acid and phytase in low-phosphorus diets on bone characteristics in growing-finishing pigs. Journal of Animal Science 88:3363–3371.

Burrin, D. G., Y. Petersen, B. Stoll, and P. Sangild. 2001. Glucagon-like peptide 2 : a nutrient-responsive gut growth factor. The Journal of Nutrition 131:709–712.

Cai, L., Y. P. Li, Z. X. Wei, X. L. Li, and X. R. Jiang. 2020. Effects of dietary gallic acid on growth performance, diarrhea incidence, intestinal morphology, plasma antioxidant indices, and immune response in weaned piglets. Animal Feed Science and Technology 261:114391.

Campbell, J. M., J. D. Crenshaw, and J. Polo. 2013. The biological stress of early weaned piglets. Journal of Animal Science and Biotechnology 4:19.

Caprarulo, V., L. Turin, M. Hejna, S. Reggi, M. Dell'Anno, P. Riccaboni, P. Trevisi, D. Luise, A. Baldi, and L. Rossi. 2022. Protective Effect of Phytogenic based Additives in Enterotoxigenic *Escherichia Coli* Challenged Piglets. Research Square Platform LLC.

Cardinal, K. M., I. Andretta, M. K. da Silva, T. B. Stefanello, B. Schroeder, and A. M. L. Ribeiro. 2021. Estimation of productive losses caused by withdrawal of antibiotic growth promoter from pig diets – Meta-analysis. Scientia Agricola 78.

Carstensen, L., A. Ersboll, K. Jensen, and J. Nielsen. 2005. *Escherichia coli* post-weaning diarrhoea occurrence in piglets with monitored exposure to creep feed. Veterinary Microbiology 110:113–123.

Celi, P., A. J. Cowieson, F. Fru-Nji, R. E. Steinert, A.-M. Kluenter, and V. Verlhac. 2017. Gastrointestinal functionality in animal nutrition and health: new opportunities for sustainable animal production. Animal Feed Science and Technology 234:88–100.

Cha, S. B., A. N. Yoo, W. J. Lee, M. K. Shin, M. H. Jung, S. W. Shin, Y. W. Cho, and H. S. Yoo. 2012. Effect of bacteriophage in enterotoxigenic *Escherichia coli* (ETEC) infected pigs. Journal of Veterinary Medical Science 74:1037–1039.

Chen, H., I. Park, S. Zhang, and S. W. Kim. 2017. Effect of supplemental protease on growth performance, nutrient digestibility, and gut health in nursery pigs fed diets with corn or sorghum. Journal of Animal Science 95:195.

Cho, J. H., P. Y. Zhao, and I. H. Kim. 2011. Probiotics as a dietary additive for pigs: A review. Journal of Animal and Veterinary Advances 10:2127–2134.

Chowdhury, A. H., and D. N. Lobo. 2011. Fluids and gastrointestinal function. Current Opinion in Clinical Nutrition and Metabolic Care 14:469–476.

Christaki, E., I. Giannenas, E. Bonos, and P. Florou-Paneri. 2020. Innovative uses of aromatic plants as natural supplements in nutrition. In: Feed Additives. Academic press, Elsevier, San Diego, CA, USA. p. 19–34.

Colson, V., P. Orgeur, A. Foury, and P. Mormède. 2006. Consequences of weaning piglets at 21 and 28 days on growth, behaviour and hormonal responses. Applied Animal Behaviour Science. 98:70–88.

Corino, C., M. Prost, B. Pizzi, and R. Rossi. 2021. Dietary plant extracts improve the antioxidant reserves in weaned piglets. Antioxidants 10:702.

Cornelison, A. S., L. A. Karriker, N. H. Williams, B. J. Haberl, K. J. Stalder, L. L. Schulz, and J. F. Patience. 2018. Impact of health challenges on pig growth performance, carcass characteristics, and net returns under commercial conditions. Translational Animal Science 2:50–61.

Correa-Fiz, F., J. M. Gonçalves dos Santos, F. Illas, and V. Aragon. 2019. Antimicrobial removal on piglets promotes health and higher bacterial diversity in the nasal microbiota. Scientific Reports 9:6545.

Davis, C. D., and J. A. Milner. 2009. Gastrointestinal microflora, food components and colon cancer prevention. The Journal of Nutritional Biochemistry. 20:743–752.

Debski, B. 2016. Supplementation of pig's diet with zinc and copper as alternative to conventional antimicrobials. Polish Journal of Veterinary Sciences. 19.

Delisle, B., C. Calinescu, M. A. Mateescu, J. M. Fairbrother, and E. Nadeau. 2012. Oral immunization with F4 fimbriae and CpG formulated with carboxymethyl starch enhances F4-specific mucosal immune response and modulates Th1 and Th2 cytokines in weaned pigs. Journal of Pharmacy & Pharmaceutical Sciences 15:642.

Deng, Q., J. Xu, B. Yu, J. He, K. Zhang, X. Ding, and D. Chen. 2010. Effect of dietary tea polyphenols on growth performance and cell-mediated immune response of post-weaning piglets under oxidative stress. Archives of Animal Nutrition 64:12–21.

Diao, H., Z. Gao, B. Yu, P. Zheng, J. He, J. Yu, Z. Huang, D. Chen, and X. Mao. 2016. Effects of benzoic acid (VevoVitall®) on the performance and jejunal digestive physiology in young pigs. Journal of Animal Science and Biotechnology 7:32.

Dibner, J. J., and J. D. Richards. 2005. Antibiotic growth promoters in agriculture: history and mode of action. Poultry Science 84:634–643.

Dierick, N. A., J. A. Decuypere, K. Molly, E. van Beek, and E. Vanderbeke. 2002. The combined use of triacylglycerols (TAGs) containing medium chain fatty acids (MCFAs) and exogenous lipolytic enzymes as an alternative to nutritional antibiotics in piglet nutrition: II. *In vivo* release of MCFAs in gastric cannulated and slaughtered piglets by endogenous and exogenous lipases; effects on the luminal gut flora and growth performance. Livestock Production Science 76:1-16.

Dierick, N. A., I. J. Vervaeke, J. A. Decuypere, and H. K. Henderickx. 1986. Influence of the gut flora and of some growth-promoting feed additives on nitrogen metabolism in pigs. II. Studies *in vivo*. Livestock Production Science 14:177–193.

Dillon, S., T. Sweeney, S. Figat, J. J. Callan, and J. v. O'Doherty. 2010. The effects of lactose inclusion and seaweed extract on performance, nutrient digestibility and microbial populations in newly weaned piglets. Livestock Science 134:205–207.

le Dividich, J., P. Herpin, and R. M. Rosario-Ludovino. 1994. Utilization of colostral energy by the newborn pig. Journal of Animal Science 72:2082–2089.

Djeridane, A., M. Yousfi, B. Nadjemi, D. Boutassouna, P. Stocker, and N. Vidal. 2006. Antioxidant activity of some algerian medicinal plants extracts containing phenolic compounds. Food Chemistry 97:654–660.

Domeneghini, C., A. di Giancamillo, G. Savoini, R. Paratte, V. Bontempo, and V. Dell'Orto. 2004. Structural patterns of swine ileal mucosa following L-glutamine and nucleotide administration during the weaning period. A histochemical and histometrical study. Histology and Histopathology 19:49–58.

Downes, J., F. E. Dewhirst, A. C. R. Tanner, and W. G. Wade. 2013. Description of *Alloprevotella rava* gen. nov., sp. nov., isolated from the human oral cavity, and reclassification of *Prevotella tannerae* Moore et al. 1994 as *Alloprevotella tannerae* gen. nov., comb. nov. International Journal of Systematic and Evolutionary Microbiology 63:1214-1218.

Duarte, M. E., I. Park, W. Parnsen, F. X. Zhou, and S. W. Kim. 2017. Effects of combinational use of xylanase and protease on growth performance and gut health of newly weaned pigs. Journal of Animal Science 95:202–202.

Eckel, B., M. Kirchgessner, and F. X. Roth. 1992. Zum Einfluß von Ameisensäure auf tägliche Zunahmen, Futteraufnahme, Futterverwertung und Verdaulichkeit. Journal of Animal Physiology and Animal Nutrition 67:93–100.

Fairbrother, J. M., É. Nadeau, L. Bélanger, C. L. Tremblay, D. Tremblay, M. Brunelle, R. Wolf, K. Hellmann, and Á. Hidalgo. 2017. Immunogenicity and protective efficacy of a single-dose live non-pathogenic Escherichia coli oral vaccine against F4-positive enterotoxigenic *Escherichia coli* challenge in pigs. Vaccine 35:353–360.

Fairbrother, J. M., É. Nadeau, and C. L. Gyles. 2005. *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. Animal Health Research Reviews 6:17–39.

Ferrari, E., A. S. Jarnagin, and B. F. Schmidt. 2014. Commercial production of extracellular enzymes. In: Bacillus subtilis and Other Gram-Positive Bacteria. ASM Press, Washington, DC, USA. p. 917–937.

Finkel, T. 2003. Oxidant signals and oxidative stress. Current Opinion in Cell Biology 15:247–254.

Frankel, W. L., W. Zhang, A. Singh, D. M. Klurfeld, S. Don, T. Sakata, I. Modlin, and J. L. Rombeau. 1994. Mediation of the trophic effects of short-chain fatty acids on the rat jejunum and colon. Gastroenterology 106:375–380.

Frankič, T., A. Levart, and J. Salobir. 2010a. The effect of vitamin E and plant extract mixture composed of carvacrol, cinnamaldehyde and capsaicin on oxidative stress induced by high PUFA load in young pigs. Animal 4:572–578.

Frese, S. A., K. Parker, C. C. Calvert, and D. A. Mills. 2015. Diet shapes the gut microbiome of pigs during nursing and weaning. Microbiome 3:1–10.

Gabert, V. M., and W. C. Sauer. 1995. The effect of fumaric acid and sodium fumarate supplementation to diets for weanling pigs on amino acid digestibility and volatile fatty acid concentrations in ileal digesta. Animal Feed Science and Technology 53:243–254.

Gan, Z., W. Wei, Y. Li, J. Wu, Y. Zhao, L. Zhang, T. Wang, and X. Zhong. 2019. Curcumin and Resveratrol regulate intestinal bacteria and alleviate intestinal inflammation in weaned piglets. Molecules 24:1220.

Gänzle, M., and R. Follador. 2012. Metabolism of oligosaccharides and starch in lactobacilli: a review. Frontiers in Microbiology 3:340.

di Giancamillo, A., C. Domeneghini, R. Paratte, V. Dell'Orto, and V. Bontempo. 2003. Oral feeding with L-glutamine and nucleotides: impact on some GALT (gut associated lymphoid tissue) parameters and cell proliferation/death rates in weaning piglets. Italian Journal of Animal Science 2:364–366.

Gibbons, R. A., R. Sellwood, M. Burrows, and P. A. Hunter. 1977. Inheritance of resistance to neonatal *E. coli* diarrhoea in the pig: examination of the genetic system. Theoretical and Applied Genetics 51:65–70.

Gibson, G. R., R. Hutkins, M. E. Sanders, S. L. Prescott, R. A. Reimer, S. J. Salminen, K. Scott, C. Stanton, K. S. Swanson, P. D. Cani, K. Verbeke, and G. Reid. 2017. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nature Reviews Gastroenterology & Hepatology 14:491–502.

Gomaa, W. M. S., L. Y. Wei, G. M. Mosaad, H. Aamer, T. W. Alexander, and W. Z. Yang. 2018. In situ ruminal digestibility of red osier dogwood in finishing beef heifers. Canadian Journal of Animal Science 98:888–892.

Gophna, U., T. Konikoff, and H. B. Nielsen. 2017. Oscillospira and related bacteria–From metagenomic species to metabolic features. Environmental Microbiology 19:835–841.

Gresse, R., F. Chaucheyras-Durand, M. A. Fleury, T. van de Wiele, E. Forano, and S. Blanquet-Diot. 2017. Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. Trends in Microbiology 25:851–873.

Guevarra, R. B., J. H. Lee, S. H. Lee, M.-J. Seok, D. W. Kim, B. N. Kang, T. J. Johnson, R. E. Isaacson, and H. B. Kim. 2019. Piglet gut microbial shifts early in life: causes and effects. Journal of Animal Science and Biotechnology 10:1.

Guilloteau, P., R. Zabielski, H. M. Hammon, and C. C. Metges. 2010. Nutritional programming of gastrointestinal tract development. Is the pig a good model for man? Nutrition Research Reviews 23:4–22.

Guzmán-Pino, S. A., D. Solà-Oriol, R. Davin, E. G. Manzanilla, and J. F. Pérez. 2015. Influence of dietary electrolyte balance on feed preference and growth performance of post weaned piglets. Journal of Animal Science 93:2840–2848.

Gyles, C. L., J. F. Prescott, G. Songer, and C. O. Thoen. 2010. Pathogenesis of bacterial infections in animals 4th ed. Blackwell Publishing, Ames, Iowa, USA .

Halas, D., C. F. Hansen, D. J. Hampson, B. P. Mullan, J. C. Kim, R. H. Wilson, and J. R. Pluske. 2010. Dietary supplementation with benzoic acid improves apparent ileal digestibility of total nitrogen and increases villous height and caecal microbial diversity in weaner pigs. Animal Feed Science and Technology 160:137–147.

Hamer, H. M., V. de Preter, K. Windey, and K. Verbeke. 2012. Functional analysis of colonic bacterial metabolism: relevant to health? American Journal of Physiology-Gastrointestinal and Liver Physiology 302:G1–G9.

Hampson, D. J. 1986. Alterations in piglet small intestinal structure at weaning. Research in Veterinary Science 40:32–40.

Han, X. Y., F. Y. Yan, X. Z. Nie, W. Xia, S. Chen, X. Zhang, and L. Qian. 2017. Effect of replacing antibiotics using multi-enzyme preparations on production performance and antioxidant activity in piglets. Journal of Integrative Agriculture 16:640–647.

Hancock, R. E. 2001. Cationic peptides: effectors in innate immunity and novel antimicrobials. The Lancet Infectious Diseases 1:156–164.

Hao, Y., M. Xing, and X. Gu. 2021. Research Progress on Oxidative Stress and Its Nutritional Regulation Strategies in Pigs. Animals 11:1384.

Hedegaard, C. J., and P. M. H. Heegaard. 2016. Passive immunisation, an old idea revisited: Basic principles and application to modern animal production systems. Veterinary Immunology and Immunopathology 174:50–63.

Heim, G., A. M. Walsh, T. Sweeney, D. N. Doyle, C. J. O'Shea, M. T. Ryan, and J. V. O'Doherty. 2014. Effect of seaweed-derived laminarin and fucoidan and zinc oxide on gut morphology, nutrient transporters, nutrient digestibility, growth performance and selected microbial populations in weaned pigs. British Journal of Nutrition 111:1577–1585.

Helm, E. T., S. Curry, J. M. Trachsel, M. Schroyen, and N. K. Gabler. 2019. Evaluating nursery pig responses to in-feed sub-therapeutic antibiotics. PLOS ONE 14:e0216070.

Heo, J. M., J. C. Kim, J. Yoo, and J. R. Pluske. 2015. A between-experiment analysis of relationships linking dietary protein intake and post-weaning diarrhea in weanling pigs under conditions of

experimental infection with an enterotoxigenic strain of *Escherichia coli*. Animal Science Journal 86:286–293.

Heo, J. M., F. O. Opapeju, J. R. Pluske, J. C. Kim, D. J. Hampson, and C. M. Nyachoti. 2013. Gastrointestinal health and function in weaned pigs: A review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. Journal of Animal Physiology and Animal Nutrition 97:207–237.

Hofmann, A. F., V. Loening-Baucke, J. E. Lavine, L. R. Hagey, J. H. Steinbach, C. A. Packard, T. L. Griffin, and D. A. Chatfield. 2008. Altered bile acid metabolism in childhood functional constipation: inactivation of secretory bile acids by sulfation in a subset of patients. Journal of Pediatric Gastroenterology and Nutrition 47:598–606.

Hoque, K. M., S. Chakraborty, I. A. Sheikh, and O. M. Woodward. 2014. New advances in the pathophysiology of intestinal ion transport and barrier function in diarrhea and the impact on therapy. Expert Review of Anti-infective Therapy 10:687–699.

Htoo, J. K., B. A. Araiza, W. C. Sauer, M. Rademacher, Y. Zhang, M. Cervantes, and R. T. Zijlstra. 2007. Effect of dietary protein content on ileal amino acid digestibility, growth performance, and formation of microbial metabolites in ileal and cecal digesta of early-weaned pigs Journal of Animal Science 85:3303–3312.

Hung, I. F. 2015. The effect of dietary nucleotides in sow and nursery piglet diets on reproduction, growth, and immune response. Thesis and dissertations-Animal and Food Sciences 48

Iji, P. A., A. Saki, and D. R. Tivey. 2001. Body and intestinal growth of broiler chicks on a commercial starter diet. 1. Intestinal weight and mucosal development. British Poultry Science 42:505–513.

Ijssennagger, N., C. Belzer, G. J. Hooiveld, J. Dekker, S. W. C. van Mil, M. Müller, M. Kleerebezem, and R. van der Meer. 2015. Gut microbiota facilitates dietary heme-induced epithelial hyperproliferation by opening the mucus barrier in colon. Proceedings of the National Academy of Sciences 112:10038–10043.

Isaacson, R., and H. B. Kim. 2012. The intestinal microbiome of the pig. Animal Health Research Reviews 13:100–109.

Isaak, C. K., J. C. Petkau, O. Karmin, K. Ominski, J. C. Rodriguez-Lecompte, and Y. L. Siow. 2013. Seasonal variations in phenolic compounds and antioxidant capacity of Cornus stolonifera plant material: Applications in agriculture. Canadian Journal of Plant Science 93:725–734.

Ivarsson, E., S. Roos, H. Y. Liu, and J. E. Lindberg. 2014. Fermentable non-starch polysaccharides increases the abundance of *Bacteroides–Prevotella–Porphyromonas* in ileal microbial community of growing pigs. Animal 8:1777–1787.

Jamalludeen, N., R. P. Johnson, P. E. Shewen, and C. L. Gyles. 2009. Evaluation of bacteriophages for prevention and treatment of diarrhea due to experimental enterotoxigenic Escherichia coli O149 infection of pigs. Veterinary Microbiology 136:135–141.

Jaworski, N. W., A. Owusu-Asiedu, M. C. Walsh, J. C. McCann, J. J. Loor, and H. H. Stein. 2017. Effects of a 3 strain-based direct-fed microbial and dietary fiber concentration on growth performance and expression of genes related to absorption and metabolism of volatile fatty acids in weanling pigs. Journal of Animal Science 95:308–319.

Jayaraman, B., S. Amarakoon, and B. Koo. 2018. Effects of dietary supplementation with ground red-osier dogwood (Cornus stolonifera) on growth performance, blood profile, and ileal

histomorphology in weaned pigs challenged with Escherichia coli K88+. Journal of Animal Science 96:322.

Jayaraman, B., and C. M. Nyachoti. 2017. Husbandry practices and gut health outcomes in weaned piglets: A review. Animal Nutrition 3:205–211.

Jeaurond, E. A., M. Rademacher, J. R. Pluske, C. H. Zhu, and C. F. M. de Lange. 2008. Impact of feeding fermentable proteins and carbohydrates on growth performance, gut health and gastrointestinal function of newly weaned pigs. Canadian Journal of Animal Science 88:271–281.

Jensen, B. B. 1998. The impact of feed additives on the microbial ecology of the gut in young pigs. Journal of Animal and Feed Science 7:45–64.

Jensen, G. M., K. Frydendahl, O. Svendsen, C. B. Jørgensen, S. Cirera, M. Fredholm, J. P. Nielsen, and K. Møller. 2006. Experimental infection with *Escherichia coli* O149: F4ac in weaned piglets. Veterinary Microbiology 115:243–249.

Jensen, P., and G. Stangel. 1992. Behaviour of piglets during weaning in a seminatural enclosure. Applied Animal Behaviour Science 33:227–238.

Jiang, Q., H. Zhang, R. Yang, Q. Hui, Y. Chen, L. Mats, R. Tsao, and C. Yang. 2019. Red osier dogwood extract prevent inflammatory responses in Caco-2 cells and a Caco-2 BBe1/EA. hy926 cell co-culture model. Antioxidants 8:428.

Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine, and M. M. Swann. 1969. Report of the Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine. HM Stationery Office.

Kahn, L. H. 2016. One health and the politics of antimicrobial resistance. Johns Hopkins University Press, Baltimore, USA.

Kats, L. J., M. D. Tokach, R. D. Goodband, and J. L. Nelssen. 1992. Influence of weaning weight and growth during the first week postweaning on subsequent pig performance. In: Swine Day, Manhattan, Kansas, USA. p. 15-17.

Katsuda, K., M. Kohmoto, K. Kawashima, and H. Tsunemitsu. 2006. Frequency of enteropathogen detection in suckling and weaned pigs with diarrhea in Japan. Journal of Veterinary Diagnostic Investigation 18:350–354.

Kiarie, E., S. Bhandari, M. Scott, D. O. Krause, and C. M. Nyachoti. 2011. Growth performance and gastrointestinal microbial ecology responses of piglets receiving Saccharomyces cerevisiae fermentation products after an oral challenge with *Escherichia coli* (K88). Journal of Animal Science 89:1062–1078.

Kil, D. Y., W. B. Kwon, and B. G. Kim. 2011. Dietary acidifiers in weanling pig diets: a review. Revista Colombiana de Ciencias Pecuarias 24:231–247.

Kim, M., L. Friesen, J. Park, H. M. Kim, and C. H. Kim. 2018. Microbial metabolites, short-chain fatty acids, restrain tissue bacterial load, chronic inflammation, and associated cancer in the colon of mice. European Journal of Immunology 48:1235–1247.

Kirchhelle, C. 2018. Pharming animals: a global history of antibiotics in food production (1935–2017). Palgrave Communications 4:1–13.

Kluess, J., U. Schoenhusen, W. B. Souffrant, P. H. Jones, and B. G. Miller. 2010. Impact of diet composition on ileal digestibility and small intestinal morphology in early-weaned pigs fitted with a T-cannula. Animal 4:586–594.

Kogut, M. H., and R. J. Arsenault. 2016. Gut health: The new paradigm in food animal production. Frontiers in Veterinary Science 3:71.

Koo, B., S. B. Amarakoon, B. Jayaraman, Y. L. Siow, S. Prashar, Y. Shang, O. Karmin, and C. M. Nyachoti. 2021. Effects of dietary red-osier dogwood (*Cornus stolonifera*) on growth performance, blood profile, ileal morphology, and oxidative status in weaned pigs challenged with *Escherichia coli* K88 +. Canadian Journal of Animal Science 101:96–105.

Koo, B., S. Amarakoon, B. Jayaraman, Y. Siow, S. Prashar, Y. Shang and C. Nyachoti. 2018. Effects of dietary supplementation with ground red-osier dogwood (*Cornus stolonifera*) on oxidative status in weaned pigs challenged with *Escherichia coli* K88+. Journal of Animal Science 96:308.

Kroismayr, A., J. Sehm, M. W. Pfaffl, K. Schedle, C. Plitzner, and W. Windisch. 2008. Effects of avilamycin and essential oils on mRNA expression of apoptotic and inflammatory markers and gut morphology of piglets. Czech Journal of Animal Science 53:377–387.

Kuang, Y., Y. Wang, Y. Zhang, Y. Song, X. Zhang, Y. Lin, L. Che, S. Xu, D. Wu, B. Xue, and Z. Fang. 2015. Effects of dietary combinations of organic acids and medium chain fatty acids as a replacement of zinc oxide on growth, digestibility and immunity of weaned pigs. Animal Feed Science and Technology 208:145–157.

Laine, T. M., T. Lyytikäinen, M. Yliaho, and M. Anttila. 2008. Risk factors for post-weaning diarrhoea on piglet producing farms in Finland. Acta Veterinaria Scandinavica 50:21.

Laird, T. J., S. Abraham, D. Jordan, J. R. Pluske, D. J. Hampson, D. J. Trott, and M. O'Dea. 2021. Porcine enterotoxigenic *Escherichia coli*: Antimicrobial resistance and development of microbial-based alternative control strategies. Veterinary Microbiology 258:109117.

Lallès, J. P., P. Bosi, H. Smidt, and C. R. Stokes. 2007. Weaning—a challenge to gut physiologists. Livestock Science 108:82–93.

Lallès, J. P., and J. C. David. 2011. Fasting and refeeding modulate the expression of stress proteins along the gastrointestinal tract of weaned pigs. Journal of Animal Physiology and Animal Nutrition 95:478–488.

Lallès, J.-P., P. Bosi, H. Smidt, and C. R. Stokes. 2007. Nutritional management of gut health in pigs around weaning. Proceedings of the Nutrition Society 66:260–268.

Lang, A., M. Lahav, E. Sakhnini, I. Barshack, H. H. Fidder, B. Avidan, E. Bardan, R. Hershkoviz, S. Bar-Meir, and Y. Chowers. 2004. Allicin inhibits spontaneous and TNF-α induced secretion of proinflammatory cytokines and chemokines from intestinal epithelial cells. Clinical Nutrition 23:1199–1208.

de Lange, C. F. M., J. Pluske, J. Gong, and C. M. Nyachoti. 2010. Strategic use of feed ingredients and feed additives to stimulate gut health and development in young pigs. Livestock Science 134:124–134.

Lee, C. Y., S. J. Kim, B. C. Park, and J. H. Han. 2017. Effects of dietary supplementation of bacteriophages against enterotoxigenic *Escherichia coli* (ETEC) K88 on clinical symptoms of post-weaning pigs challenged with the ETEC pathogen. Journal of Animal Physiology and Animal Nutrition 101:88–95.

Lee, D. N., S. R. Liu, Y. T. Chen, R. C. Wang, S. Y. Lin, and C. F. Weng. 2007. Effects of diets supplemented with organic acids and nucleotides on growth, immune responses and digestive tract development in weaned pigs. Journal of Animal Physiology and Animal Nutrition 91:508–518.

Lee, S. I., and I. H. Kim. 2018. Creep feeding improves growth performance of suckling piglets. Revista Brasileira de Zootecnia 47.

Li, M., J. Gong, M. Cottrill, H. Yu, C. de Lange, J. Burton and E. Topp. 2003. Evaluation of QIAamp® DNA Stool Mini Kit for ecological studies of gut microbiota. Journal of Microbiological Methods 54:13-20.

Li, P., X. Piao, Y. Ru, X. Han, L. Xue, and H. Zhang. 2012. Effects of adding essential oil to the diet of weaned pigs on performance, nutrient utilization, immune response and intestinal health. Asian-Australasian Journal of Animal Sciences 25:1617–1626.

Li, Y., Y. Guo, Z. Wen, X. Jiang, X. Ma, and X. Han. 2018. Weaning stress perturbs gut microbiome and its metabolic profile in piglets. Scientific Reports 8:1–12.

Liu, Y., T. M. Che, M. Song, J. J. Lee, J. A. S. Almeida, D. Bravo, W. G. van Alstine, and J. E. Pettigrew. 2013. Dietary plant extracts improve immune responses and growth efficiency of pigs experimentally infected with porcine reproductive and respiratory syndrome virus. Journal of Animal Science 91:5668–5679.

Liu, Y., C. D. Espinosa, J. J. Abelilla, G. A. Casas, L. V. Lagos, S. A. Lee, W. B. Kwon, J. K. Mathai, D. M. D. L. Navarro, N. W. Jaworski, and H. H. Stein. 2018. Non-antibiotic feed additives in diets for pigs: A review. Animal Nutrition 4:113–125.

Liu, Y., Y. L. Ma, J. M. Zhao, M. Vazquez-Añón, and H. H. Stein. 2014a. Digestibility and retention of zinc, copper, manganese, iron, calcium, and phosphorus in pigs fed diets containing inorganic or organic minerals. Journal of Animal Science 92:3407–3415.

Liu, Y., M. Song, T. M. Che, D. Bravo, and J. E. Pettigrew. 2012. Anti-inflammatory effects of several plant extracts on porcine alveolar macrophages in vitro. Journal of Animal Science 90:2774–2783.

Liu, Y., M. Song, T. M. Che, J. J. Lee, D. Bravo, C. W. Maddox, and J. E. Pettigrew. 2014b. Dietary plant extracts modulate gene expression profiles in ileal mucosa of weaned pigs after an *Escherichia coli* infection. Journal of Animal Science 92:2050–2062.

Liu, Y., M. Song, T. M. Che, J. J. Lee, D. Bravo, C. W. Maddox, and J. E. Pettigrew. 2014c. Dietary plant extracts modulate gene expression profiles in ileal mucosa of weaned pigs after an Escherichia coli infection. Journal of Animal Science 92:2050–2062.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408.

Lobo, V., A. Patil, A. Phatak, and N. Chandra. 2010. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacognosy Reviews 4:118.

Lofstedt, M., N. Holmgren, and N. Lundeheim. 2002. Risk factors for postweaning diarrhoea in pigs. Svensk Veterinartidning 54:457–461.

Long, S. F., Y. T. Xu, L. Pan, Q. Q. Wang, C. L. Wang, J. Y. Wu, Y. Y. Wu, Y. M. Han, C. H. Yun, and X. S. Piao. 2018. Mixed organic acids as antibiotic substitutes improve performance, serum

immunity, intestinal morphology and microbiota for weaned piglets. Animal Feed Science and Technology 235:23–32.

López-Gálvez, G., M. López-Alonso, A. Pechova, B. Mayo, N. Dierick, and J. Gropp. 2021a. Alternatives to antibiotics and trace elements (copper and zinc) to improve gut health and zootechnical parameters in piglets: A review. Animal Feed Science and Technology 271:114727.

López-Gálvez, G., M. López-Alonso, A. Pechova, B. Mayo, N. Dierick, and J. Gropp. 2021b. Alternatives to antibiotics and trace elements (copper and zinc) to improve gut health and zootechnical parameters in piglets: A review. Animal Feed Science and Technology 271:114727.

de los Santos, F. S., A. M. Donoghue, M. B. Farnell, G. R. Huff, W. E. Huff, and D. J. Donoghue. 2007. Gastrointestinal maturation is accelerated in turkey poults supplemented with a mannan-oligosaccharide yeast extract (alphamune). Poultry Science 86:921–930.

Luise, D., V. Motta, C. Salvarani, M. Chiappelli, L. Fusco, M. Bertocchi, M. Mazzoni, G. Maiorano, L. N. Costa, J. van Milgen, P. Bosi, and P. Trevisi. 2017. Long-term administration of formic acid to weaners: Influence on intestinal microbiota, immunity parameters and growth performance. Animal Feed Science and Technology 232:160–168.

Luppi, A., M. Gibellini, T. Gin, F. Vangroenweghe, V. Vandenbroucke, R. Bauerfeind, P. Bonilauri, G. Labarque, and Á. Hidalgo. 2016. Prevalence of virulence factors in enterotoxigenic Escherichia coli isolated from pigs with post-weaning diarrhoea in Europe. Porcine Health Management 2:1–6.

Madara, J. L. 1990. Warner-Lambert/Parke-Davis Award lecture. Pathobiology of the intestinal epithelial barrier. The American Journal of Pathology 137:1273.

Marquardt, R. R., L. Z. Jin, J.-W. Kim, L. Fang, A. A. Frohlich, and S. K. Baidoo. 1999. Passive protective effect of egg-yolk antibodies against enterotoxigenic *Escherichia coli* K88+ infection in neonatal and early-weaned piglets. FEMS Immunology & Medical Microbiology 23:283–288.

Martinez-Puig, D., E. G. Manzanilla, J. Morales, E. Borda, J. F. Pérez, C. Piñeiro, and C. Chetrit. 2007. Dietary nucleotide supplementation reduces occurrence of diarrhoea in early weaned pigs. Livestock Science 108:276–279.

Mateo C.D. Stein H.H. 2004. Nucleotides and young animal health: can we enhance intestinal tract development and immune function? In: Nutritional Biotechnology in the Feed Industry. Proc. Alltech's 20th Annual Symposium. Department of Animal and Range Sciences, South Dakota State University, Brookings, SD, USA.

Mazutti, K., L. B. Costa, L. V. Nascimento, T. F. Filho, B. C. B. Beirão, P. C. M. Júnior, and A. Maiorka. 2016. Effect of colistin and tylosin used as feed additives on the performance, diarrhea incidence, and immune response of nursery pigs. Semina: Ciências Agrárias 37:1947–1962.

McCracken, B. A., M. E. Spurlock, M. A. Roos, F. A. Zuckermann, and H. R. Gaskins. 1999. Weaning anorexia may contribute to local inflammation in the piglet small intestine. The Journal of Nutrition 129:613–619.

McDonnell, P., S. Figat, and J. V. O'Doherty. 2010. The effect of dietary laminarin and fucoidan in the diet of the weanling piglet on performance, selected faecal microbial populations and volatile fatty acid concentrations. Animal 4:579–585.

McPherson, R. L., F. Ji, G. Wu, J. R. Blanton Jr, and S. W. Kim. 2004. Growth and compositional changes of fetal tissues in pigs. Journal of Animal Science 82:2534–2540.

Medland, J. E., C. S. Pohl, L. L. Edwards, S. Frandsen, K. Bagley, Y. Li, and A. J. Moeser. 2016. Early life adversity in piglets induces long-term upregulation of the enteric cholinergic nervous system and heightened, sex-specific secretomotor neuron responses. Neurogastroenterology & Motility 28:1317–1329.

Melin, L., M. Jensen-Waern, A. Johannisson, M. Ederoth, M. Katouli, and P. Wallgren. 1997. Development of selected faecal microfloras and of phagocytic and killing capacity of neutophils in young pigs. Veterinary Microbiology 54:287–300.

Melkebeek, V., B. M. Goddeeris, and E. Cox. 2013. ETEC vaccination in pigs. Veterinary Immunology and Immunopathology 152:37–42.

Michiels, J., E. Skrivanova, J. Missotten, A. Ovyn, J. Mrazek, S. de Smet, and N. Dierick. 2012. Intact brown seaweed (*Ascophyllum nodosum*) in diets of weaned piglets: effects on performance, gut bacteria and morphology and plasma oxidative status. Journal of Animal Physiology and Animal Nutrition 96:1101–1111.

Minelli, A., I. Bellezza, C. Conte, and Z. Culig. 2009. Oxidative stress-related aging: A role for prostate cancer? Biochimica et Biophysica Acta (BBA) - Reviews on Cancer 1795:83–91.

Moeser, A. J., C. S. Pohl, and M. Rajput. 2017. Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs. Animal Nutrition 3:313–321.

Moeser, A. J., K. A. Ryan, P. K. Nighot, and A. T. Blikslager. 2007. Gastrointestinal dysfunction induced by early weaning is attenuated by delayed weaning and mast cell blockade in pigs. American Journal of Physiology - Gastrointestinal and Liver Physiology 293.

Mogire, M. K., J. Choi, P. Lu, Chongwu Yang, S. Liu, D. Adewole, A. R. Rodas-González, and Chengbo Yang. 2021. Effecs of red-osier dogwood extracts on growth performance, intestinal digestive, and absorptive functions and meat quality of broiler chickens. Canadian Journal of Animal Science 101:687-703.

Moore, K. L., B. P. Mullan, J. R. Pluske, J. C. Kim, and D. N. D'Souza. 2011. The use of nucleotides, vitamins and functional amino acids to enhance the structure of the small intestine and circulating measures of immune function in the post-weaned piglet. Animal Feed Science and Technology 165:184–190.

Morales J., P. C. 2006. Progeny of primiparous sows: Lower health status and productive performance. Suis 32:16–24.

Mukhopadhya, A., J. v. O'Doherty, and T. Sweeney. 2019. A combination of yeast beta-glucan and milk hydrolysate is a suitable alternative to zinc oxide in the race to alleviate post-weaning diarrhoea in piglets. Scientific Reports 9:616.

Nabuurs, M. J. A., A. Hoogendoorn, E. J. van der Molen, and A. L. M. van Osta. 1993. Villus height and crypt depth in weaned and unweaned pigs, reared under various circumstances in the Netherlands. Research in Veterinary Science 55:78–84.

Nagy, B., and P. Z. Fekete. 1999. Enterotoxigenic *Escherichia coli* (ETEC) in farm animals. Veterinary Research 30:259–284.

Nagy, B., and P. Z. Fekete. 2005. Enterotoxigenic *Escherichia coli* in veterinary medicine. International Journal of Medical Microbiology 295:443–454.

Nakamine, M., Y. Kono, S. Abe, C. Hoshino, J. Shirai, and T. Ezaki. 1998. Dual infection with enterotoxigenic *Escherichia coli* and porcine reproductive and respiratory syndrome virus observed in weaning pigs that died suddenly. Journal of veterinary medical science 60:555–561.

Nathan, C., and O. Cars. 2014. Antibiotic resistance—problems, progress, and prospects. New England Journal of Medicine 371:1761–1763.

National Pork Board. 2013. Swine health recommendations: Exhibitors of all pigs going to exhibits or sales. Swine Health Guide.

Niers, L. E. M., H. M. Timmerman, G. T. Rijkers, G. M. van Bleek, N. O. P. van Uden, E. F. Knol, M. L. Kapsenberg, J. L. L. Kimpen, and M. O. Hoekstra. 2005. Identification of strong interleukin-10 inducing lactic acid bacteria which down-regulate T helper type 2 cytokines. Clinical and Experimental Allergy 35:1481–1489.

Niewold, T. A. 2006. Intestinal genomics for evaluation of alternatives for AGP, current situation and perspectives. Antimicrobial Growth Promoters: Where do we go from here 361–368.

Nordeste, R., A. Tessema, S. Sharma, Z. Kovač, C. Wang, R. Morales, and M. W. Griffiths. 2017. Molecules produced by probiotics prevent enteric colibacillosis in pigs. BMC Veterinary Research 13.

National Research Council. 2012. Nutrient Requirements of Swine. 11th revised edition. The National Academies Press, Washington D. C., USA.

Nugon-Baudon, L., O. Szylit, M. Chaigneau, N. Dierick, and P. Raibaud. 1985. *In vitro* and *in vivo* production of amines by a lactobacillus strain isolated from cock crop. Annales de l'Institut Pasteur/Microbiologie. 136:63–73.

Nyachoti, C. M., E. Kiarie, S. K. Bhandari, G. Zhang, and D. O. Krause. 2012. Weaned pig responses to *Escherichia coli* K88 oral challenge when receiving a lysozyme supplement. Journal of Animal Science 90:252–260.

O'Doherty, J. v., M. A. Bouwhuis, and T. Sweeney. 2017. Novel marine polysaccharides and maternal nutrition to stimulate gut health and performance in post-weaned pigs. Animal Production Science 57:2376.

Ohkawa, H., N. Ohishi, and K. Yagi. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry 95:351–358.

Oso, A. O., R. U. Suganthi, G. B. M. Reddy, P. K. Malik, G. Thirumalaisamy, V. B. Awachat, S. Selvaraju, A. Arangasamy, and R. Bhatta. 2019. Effect of dietary supplementation with phytogenic blend on growth performance, apparent ileal digestibility of nutrients, intestinal morphology, and cecal microflora of broiler chickens. Poultry Science 98:4755–4766.

EFSA Panel on Additives and Products or Substances used in Animal Feed. 2014. Scientific opinion on the potential reduction of the currently authorised maximum zinc content in complete feed. EFSA Journal 12:3668.

EFSA Panel on Additives and Products or Substances used in Animal Feed. 2016. Scientific opinion on the revision of the currently authorised maximum copper content in complete feed. EFSA Journal 14:4563.

Patience, J. F., R. E. Austic, and R. D. Boyd. 1987. Effect of dietary electrolyte balance on growth and acid-base status in swine. Journal of Animal Science 64:457–466.

Pawlowska, A. M., F. Camangi, A. Braca. 2010. Quali-quantitative analysis of flavonoids of *Cornus mas* L.(Cornaceae) fruits. Food chemistry 119:1257-1261.

Pié, S., J. P. Lallès, F. Blazy, J. Laffitte, B. Sève, and I. P. Oswald. 2004. Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. The Journal of Nutrition 134:641–647.

Pieper, R., C. V. Tudela, M. Taciak, J. Bindelle, J. F. Pérez, and J. Zentek. 2016. Health relevance of intestinal protein fermentation in young pigs. Animal Health Research Reviews 17:137–147.

Pluske, J., D. Hampson, and I. Williams. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livestock Production Science. 51:215–236.

Pluske, J. R. 2013. Feed-and feed additives-related aspects of gut health and development in weanling pigs. Journal of Animal Science and Biotechnology 4:1–7.

Pluske, J. R., J. le Dividich, and M. W. A. Verstegen. 2003. Weaning the pig, concepts and consequences. 1st edition. Wageningen Academic Publishers, The Netherlands.

Pluske, J. R., D. J. Hampson, and I. H. Williams. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: A review. Livestock Production Science 51:215–236.

Pluske, J. R., D. W. Pethick, D. E. Hopwood, and D. J. Hampson. 2002. Nutritional influences on some major enteric bacterial diseases of pig. Nutrition Research Reviews 15:333–371.

Pluske, J. R., D. L. Turpin, and J. C. Kim. 2018. Gastrointestinal tract (gut) health in the young pig. Animal Nutrition 4:187–196.

Porter, P., and R. Kenworthy. 1969. A study of intestinal and urinary amines in pigs in relation to weaning. Research in Veterinary Science 10:440–447.

Rahal, A., A. Kumar, V. Singh, B. Yadav, R. Tiwari, S. Chakraborty, and K. Dhama. 2014. Oxidative stress, prooxidants, and antioxidants: the interplay. BioMed Research International 1–19.

Rajoka, M. S. R., J. Shi, H. M. Mehwish, J. Zhu, Q. Li, D. Shao, Q. Huang, and H. Yang. 2017. Interaction between diet composition and gut microbiota and its impact on gastrointestinal tract health. Food Science and Human Wellness 6:121–130.

Rao, R. 2008. Oxidative stress-induced disruption of epithelial and endothelial tight junctions. Frontiers in Bioscience 13:7210–7226.

Rasouli, H., M. H. Farzaei, and R. Khodarahmi. 2017. Polyphenols and their benefits: A review. International Journal of Food Properties 20:1700–1741.

Rhouma, M., F. Beaudry, and A. Letellier. 2016. Resistance to colistin: what is the fate for this antibiotic in pig production? International Journal of Antimicrobial Agents 48:119–126.

Rhouma, M., J. M. Fairbrother, W. Thériault, F. Beaudry, N. Bergeron, S. Laurent-Lewandowski, and A. Letellier. 2017. The fecal presence of enterotoxin and F4 genes as an indicator of efficacy of treatment with colistin sulfate in pigs. BMC Microbiology 17:6.

Rhouma, M., and A. Letellier. 2017. Extended-spectrum beta-lactamases, carbapenemases and the mcr-1 gene: is there a historical link? International Journal of Antimicrobial Agents 49:269–271.

Richards, J. D., J. Gong, and C. F. M. de Lange. 2005. The gastrointestinal microbiota and its role in monogastric nutrition and health with an emphasis on pigs: Current understanding, possible modulations, and new technologies for ecological studies. Canadian Journal of Animal Science 85:421–435.

Ridlon, J. M., D.-J. Kang, P. B. Hylemon, and J. S. Bajaj. 2015. Gut microbiota, cirrhosis, and alcohol regulate bile acid metabolism in the gut. Digestive Diseases 33:338–345.

Rist, V. T. S., E. Weiss, M. Eklund, and R. Mosenthin. 2013. Impact of dietary protein on microbiota composition and activity in the gastrointestinal tract of piglets in relation to gut health: a review. Animal 7:1067–1078.

Rist, V. T. S., E. Weiss, N. Sauer, R. Mosenthin, and M. Eklund. 2014. Effect of dietary protein supply originating from soybean meal or casein on the intestinal microbiota of piglets. Anaerobe 25:72–79.

Robinson, K., Z. Deng, Y. Hou, and G. Zhang. 2015. Regulation of the intestinal barrier function by host defense peptides. Frontiers in Veterinary Science 2.

Robinson, K., X. Ma, Y. Liu, S. Qiao, Y. Hou, and G. Zhang. 2018. Dietary modulation of endogenous host defense peptide synthesis as an alternative approach to in-feed antibiotics. Animal Nutrition 4:160–169.

Romanovsky, A. A., and M. Székely. 1998. Fever and hypothermia: two adaptive thermoregulatory responses to systemic inflammation. Medical Hypotheses 50:219–226.

Roubos-van den Hil, P. J., R. Litjens, A.-K. Oudshoorn, J. W. Resink, and C. H. M. Smits. 2017. New perspectives to the enterotoxigenic E. coli F4 porcine infection model: Susceptibility genotypes in relation to performance, diarrhoea and bacterial shedding. Veterinary Microbiology 202:58–63.

Rudolph, F. B. 1994. The biochemistry and physiology of nucleotides. The Journal of Nutrition 124:124S-127S.

Salobir, J., V. Rezar, T. Pajk, and A. Levart. 2005. Effect of nucleotide supplementation on lymphocyte DNA damage induced by dietary oxidative stress in pigs. Animal Science 81:135–140.

Salvo-Romero, E., C. Alonso-Cotoner, C. Pardo-Camacho, M. Casado-Bedmar, and M. Vicario. 2015. The intestinal barrier function and its involvement in digestive disease. Revista Española de Enfermedades Digestiva 107:686–696.

Sangild, P. T., M. Schmidt, J. Elnif, C. R. Björnvad, B. R. Weström, and R. K. Buddington. 2002. Prenatal development of gastrointestinal function in the pig and the effects of fetal esophageal obstruction. Pediatric Research 52:416–424.

Sauer, N., M. Eklund, E. Bauer, M. G. Gänzle, C. J. Field, R. T. Zijlstra, and R. Mosenthin. 2012. The effects of pure nucleotides on performance, humoral immunity, gut structure and numbers of intestinal bacteria of newly weaned pigs. Journal of Animal Science 90:3126–3134.

Sauer, N., M. Eklund, S. Roth, F. Rink, D. Jezierny, E. Bauer, and R. Mosenthin. 2012. Short-term effect of dietary yeast nucleotide supplementation on small intestinal enzyme activities, bacterial populations and metabolites and ileal nutrient digestibilities in newly weaned pigs. Journal of Animal Physiology and Animal Nutrition 96:700–708.

Schieber, A. M. P., and J. S. Ayres. 2016. Thermoregulation as a disease tolerance defense strategy. Pathogens and Disease 74: 106.

Schierack, P., H. Steinrück, S. Kleta, and W. Vahjen. 2006. Virulence factor gene profiles of *Escherichia coli* isolates from clinically healthy pigs. Applied and environmental microbiology 72:6680–6686.

Schreier, H. J. 2014. Biosynthesis of Glutamine and Glutamate and the Assimilation of Ammonia. Bacillus subtilis and Other Gram-Positive Bacteria 281–298.

Seeram, N. P., R. Schutzki, A. Chandra, and M. G. Nair. 2002. Characterization, quantification, and bioactivities of anthocyanins in Cornus species. Journal of Agricultural and Food Chemistry 50:2519–2523.

Sève, B. 2000. Effects of underfeeding during the weaning period on growth, metabolism, and hormonal adjustments in the piglet. Domestic Animal Endocrinology 19:63–74.

Slamenova, D., E. Horvathova, L. Marsalkova, and L. Wsolova. 2008. Carvacrol given to rats in drinking water reduces the level of DNA lesions induced in freshly isolated hepatocytes and testicular cells by H_2O_2. Neoplasma 55:394.

Smiricky-Tjardes, M. R., C. M. Grieshop, E. A. Flickinger, L. L. Bauer, and G. C. Fahey. 2003. Dietary galactooligosaccharides affect ileal and total-tract nutrient digestibility, ileal and fecal bacterial concentrations, and ileal fermentative characteristics of growing pigs. Journal of Animal Science 81:2535–2545.

Smith, H. I. 1929. Materia medica of the Bella Coola and neighbouring tribes of British Columbia. In: Annual Report for 1927. F. A. Acland, printer to the King’s Most Excellent Majesty, Ottawa, Ontario, Canada.

Snoeck, V., N. Huyghebaert, E. Cox, A. Vermeire, J. Saunders, J. P. Remon, F. Verschooten, and B. M. Goddeeris. 2004. Gastrointestinal transit time of nondisintegrating radio-opaque pellets in suckling and recently weaned piglets. Journal of Controlled Release 94:143–153.

Šperanda, M., M. Didara, T. Šperanda, M. Domacinovic, H. Valpotic, J. Kovacevic, Z. Antunovic, and J. Novoselec. 2008. Hydrolyzed brewery yeast product like immunomodulator in weaned piglets. Archiva Zootechnica 11:52–60.

Spitzer, F., W. Vahjen, R. Pieper, B. Martinez-Vallespin, and J. Zentek. 2014. A standardised challenge model with an enterotoxigenic F4+ *Escherichia coli* strain in piglets assessing clinical traits and faecal shedding of fae and est-II toxin genes. Archives of Animal Nutrition 68:448–459.

Spreeuwenberg, M. A. M., J. M. A. J. Verdonk, H. R. Gaskins, and M. W. A. Verstegen. 2001. Small intestine epithelial barrier function is compromised in pigs with low feed intake at weaning. Journal of Nutrition 131:1520–1527.

Stenson, W. F. 2013. Postnatal growth in the intestine. Current Opinion in Gastroenterology 29:107–111.

Suiryanrayna, M. V. A. N., and J. v. Ramana. 2015. A review of the effects of dietary organic acids fed to swine. Journal of Animal Science and Biotechnology 6:1–11.

Theil, P. K., C. Lauridsen, and H. Quesnel. 2014. Neonatal piglet survival: Impact of sow nutrition around parturition on fetal glycogen deposition and production and composition of colostrum and transient milk. Animal 8:1021–1030.

Thoms, U. 2012. Between promise and threat. NTM Zeitschrift für Geschichte der Wissenschaften, Technik und Medizin 20:181–214.

Truett, G. E., P. Heeger, R. L. Mynatt, A. A. Truett, J. A. Walker, and M. L. Warman. 2000. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). Biotechniques 29:52–54.

Upadhaya, S.-D., and I.-H. Kim. 2021. The impact of weaning stress on gut health and the mechanistic aspects of several feed additives contributing to improved gut health function in weanling piglets—a review. Animals 11:2418.

United States Food and Drug Administration and Centre for Veterinary Medicine. 2013. CVM GFI# 213 New animal drugs and new animal drug combination products administered in or on medicated feed or drinking water of food-producing animals: recommendations for drug sponsors for voluntarily aligning product use conditions with GFI# 209 FDA Guidance Documents.

Ushida, K., C. Kameue, T. Tsukahara, K. Fukuta, and N. Nakanishi. 2008. Decreasing traits of fecal immunoglobulin A in neonatal and weaning piglets. Journal of Veterinary Medical Science 70:849–852.

Vareed, S., M. Reddy, R. E. Schutzki, M. G. Nair. 2006. Anthocyanins in *Cornus alternifolia, Cornus controversa, Cornus kousa* and *Cornus florida* fruits with health benefits. Life Sciences 78:777-784.

Vente-Spreeuwenberg, M. A. M., J. Verdonk, M. W. A. Verstegen, and A. C. Beynen. 2003. Villus height and gut development in weaned piglets receiving diets containing either glucose, lactose or starch. British Journal of Nutrition 90:907–913.

Vergauwen, H., S. Prims, J. Degroote, W. Wang, C. Casteleyn, S. Van Cruchten, S. De Smet, J. Michiels, and C. Van Ginneken. 2016. *In vitro* investigation of six antioxidants for pig diets. Antioxidants 5:41.

Vergauwen, H., B. Tambuyzer, K. Jennes, J. Degroote, W. Wang, S. De Smet, J. Michiels, and C. van Ginneken. 2015. Trolox and ascorbic acid reduce direct and indirect oxidative stress in the IPEC-J2 cells, an *in vitro* model for the porcine gastrointestinal tract. PLOS ONE. 10:e0120485.

Vervaeke, I. J., J. A. Decuypere, N. A. Dierick, and H. K. Henderick. 1979. Quantitative *in vitro* evaluation of the energy metabolism influenced by virginiamycin and spiramycin used as growth promoters in pig nutrition. Journal of Animal Science 49:846–856.

Viswanathan, V. K., K. Hodges, and G. Hecht. 2008. Enteric infection meets intestinal function: how bacterial pathogens cause diarrhoea. Nature Reviews Microbiology 7:110–119.

Waititu, S. M., J. M. Heo, R. Patterson, and C. M. Nyachoti. 2016. Dietary yeast-based nucleotides as an alternative to in-feed antibiotics in promoting growth performance and nutrient utilization in weaned pigs. Canadian Journal of Animal Science 96:289–293.

Walsh, A.M., T. Sweeney, C. J. O'Shea, D. N. Doyle, and J. V. O. 'Doherty. 2013. Effect of supplementing varying inclusion levels of laminarin and fucoidan on growth performance, digestibility of diet components, selected faecal microbial populations and volatile fatty acid concentrations in weaned pigs. Animal Feed Science and Technology 183:151–159.

Walsh, A. M., T. Sweeney, C. J. O'Shea, D. N. Doyle, and J. v. O'Doherty. 2013. Effect of dietary laminarin and fucoidan on selected microbiota, intestinal morphology and immune status of the newly weaned pig. British Journal of Nutrition 110:1630–1638.

Waluszewski, A., A. Cinti, and A. Perna. 2021. Antibiotics in pig meat production: restrictions as the odd case and overuse as normality? Experiences from Sweden and Italy Humanities and Social Sciences Communications 8:172.

Wang, H., R. Sanz Garcia, E. Cox, and B. Devriendt. 2020. Porcine enterotoxigenic *Escherichia coli* strains differ in their capacity to secrete enterotoxins through varying YghG Levels. Applied and Environmental Microbiology 86.

Wang, J., S. Wang, H. Liu, D. Zhang, Y. Wang, and H. Ji. 2019. Effects of oligosaccharides on the growth and stress tolerance of *Lactobacillus plantarum* ZLP001 *in vitro*, and the potential synbiotic effects of *L. plantarum* ZLP001 and fructo-oligosaccharide in post-weaning piglets. Journal of Animal Science 97:4588–4597.

Wang, R., H. Yu, H. Fang, Y. Jin, Y. Zhao, J. Shen, C. Zhou, R. Li, J. Wang, Y. Fu, and J. Zhang. 2020. Effects of dietary grape pomace on the intestinal microbiota and growth performance of weaned piglets. Archives of Animal Nutrition 74:296–308.

Wang, W., Zhu, Xie, Li, Xiao, Li, Liu, S. Wang, and D. Cui. 2012. Enhanced bioavailability and efficiency of curcumin for the treatment of asthma by its formulation in solid lipid nanoparticles. International Journal of Nanomedicine 3667.

Wang, Y., T. Shan, Z. Xu, J. Liu, and J. Feng. 2006. Effect of lactoferrin on the growth performance, intestinal morphology, and expression of PR-39 and protegrin-1 genes in weaned piglets. Journal of Animal Science 84:2636–2641.

Weaver, A. C., and S. W. Kim. 2014. Supplemental nucleotides high in inosine 5′-monophosphate to improve the growth and health of nursery pigs. Journal of Animal Science 92:645–651.

Wellock, I. J., P. D. Fortomaris, J. G. M. Houdijk, and I. Kyriazakis. 2008. Effects of dietary protein supply, weaning age and experimental enterotoxigenic *Escherichia coli* infection on newly weaned pigs: health. Animal 2:834–842.

Wijtten, P. J. A., J. van der Meulen, and M. W. A. Verstegen. 2011. Intestinal barrier function and absorption in pigs after weaning: a review. British Journal of Nutrition 105:967–981.

Windisch, W., K. Schedle, C. Plitzner, and A. Kroismayr. 2008. Use of phytogenic products as feed additives for swine and poultry. Journal of Animal Science 86:E140–E148.

Wise, R. 2007. An overview of the specialist advisory committee on antimicrobial resistance (SACAR). Journal of Antimicrobial Chemotherapy 60:i5–i7.

Wu, Y., Z. Jiang, C. Zheng, L. Wang, C. Zhu, X. Yang, X. Wen, and X. Ma. 2015. Effects of protein sources and levels in antibiotic-free diets on diarrhea, intestinal morphology, and expression of tight junctions in weaned piglets. Animal Nutrition 1:170–176.

Wu, Y., L. Pan, Q. H. Shang, X. K. Ma, S. F. Long, Y. T. Xu, and X. S. Piao. 2017. Effects of isomalto-oligosaccharides as potential prebiotics on performance, immune function and gut microbiota in weaned pigs. Animal Feed Science and Technology 230:126–135.

Wu, Z., B. Tan, Y. Liu, J. L. Dunn, and P. Ji. 2018. *In Vitro* analysis of antioxidant properties of mint oils. Journal of Animal Science 96:144–145.

Xiong, X., B. Tan, M. Song, P. Ji, K. Kim, Y. Yin, and Y. Liu. 2019. Nutritional intervention for the intestinal development and health of weaned pigs. Frontiers in Veterinary Science 0:46.

Xu, B., W. Qin, Y. Xu, W. Yang, Y. Chen, J. Huang, J. Zhao, and L. Ma. 2021. Dietary quercetin supplementation attenuates diarrhea and intestinal damage by regulating gut microbiota in weanling piglets. Oxidative Medicine and Cellular Longevity.

Xu, R. J., and P. D. Cranwell. 1990. Development of gastric acid secretion in pigs from birth to thirty six days of age: the response to pentagastrin. Journal of Developmental Physiology 13:315-326.

Xu, X., X. Chen, Z. Huang, D. Chen, B. Yu, H. Chen, J. He, Y. Luo, P. Zheng, J. Yu, and J. Luo. 2019. Dietary apple polyphenols supplementation enhances antioxidant capacity and improves lipid metabolism in weaned piglets. Journal of Animal Physiology and Animal Nutrition 103:1512–1520.

Xu, Y. T., Li. Liu, S. F. Long, L. Pan, and X. S. Piao. 2018. Effect of organic acids and essential oils on performance, intestinal health and digestive enzyme activities of weaned pigs. Animal Feed Science and Technology 235:110–119.

Yang, Q., X. Huang, S. Zhao, W. Sun, Z. Yan, P. Wang, S. Li, W. Huang, S. Zhang, and L. Liu. 2017. Structure and function of the fecal microbiota in diarrheic neonatal piglets. Frontiers in Microbiology 8:502.

Yang, R., Q. Hui, Q. Jiang, S. Liu, H. Zhang, J. Wu, F. Lin, and C. Yang. 2019. Effect of Manitoba-grown red-osier dogwood extracts on recovering Caco-2 cells from H_2O_2-induced oxidative damage. Antioxidants.

Yin. 2013. Oxidative stress-mediated signaling pathways: A review. Journal of Food, Agriculture & Environment 11:132–139.

Yin, J., M. M. Wu, H. Xiao, W. K. Ren, J. L. Duan, G. Yang, T. J. Li, and Y. L. Yin. 2014. Development of an antioxidant system after early weaning in piglets. Journal of Animal Science 92:612–619.

Zabielski, R., M. M. Godlewski, and P. Guilloteau. 2008. Control of development of gastrointestinal system in neonates. Journal of Physiology and Pharmacology 59:35–54.

Zasloff, M. 2002. Antimicrobial peptides of multicellular organisms. Nature 415:389–395.

Zentek, J., F. Ferrara, R. Pieper, L. Tedin, W. Meyer, and W. Vahjen. 2013. Effects of dietary combinations of organic acids and medium chain fatty acids on the gastrointestinal microbial ecology and bacterial metabolites in the digestive tract of weaning piglets. Journal of Animal Science 91:3200–3210.

Zhang, L., Y. Q. Xu, H. Y. Liu, T. Lai, J. L. Ma, J. F. Wang, and Y. H. Zhu. 2010. Evaluation of *Lactobacillus rhamnosus* GG using an *Escherichia coli* K88 model of piglet diarrhoea: Effects on diarrhoea incidence, faecal microflora and immune responses. Veterinary Microbiology 141:142–148.

Zhang, W., E. M. Berberov, J. Freeling, D. He, R. A. Moxley, and D. H. Francis. 2006. Significance of heat-stable and heat-labile enterotoxins in porcine colibacillosis in an additive model for pathogenicity studies. Infection and immunity 74:3107–3114.

Zhu, L. H., K. L. Zhao, X. L. Chen, and J. X. Xu. 2012. Impact of weaning and an antioxidant blend on intestinal barrier function and antioxidant status in pigs. Journal of Animal Science 90:2581–2589.