The Role of Nucleotides in the Nutrition of Newly Weaned Pigs

by

Samuel Mwangi Waititu

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science

University of Manitoba

Winnipeg

ABSTRACT

A major challenge to the pig industry is to find potential alternatives for in-feed sub-therapeutic antibiotics (used as antimicrobial growth promoters, AGP) in starter diets of weaner pigs to mitigate weaning-associated growth stasis. The effect of a nucleotide-rich yeast extract (NRYE) as an in-feed AGP alternative was investigated using four studies. The first study investigated the effect of supplementing 0.1 and 0.2% NRYE without or with the recommended AGP dosage on growth performance and nutrient utilization. Supplementing AGP without or with 0.1% NRYE improved growth performance compared with control, whereas supplementing 0.1 or 0.2% NRYE without AGP resulted in similar growth performance as AGP. The second study investigated the potential of 0.1% NRYE to fully or partially substitute in-feed AGP in piglet diets supplemented with graded levels (25, 50, 75 and 100%) of the recommended AGP dosage. Supplementing NRYE improved growth performance compared to control, increasing AGP level reduced the beneficial effect of NRYE in piglets receiving more than 25% of recommended AGP. The third study investigated the effect of NRYE on growth performance, immune response, gut structure and gut microbiota using a sanitary challenge model. Supplementing 0.1% NRYE to piglets under unsanitary conditions improved ileal immune response by upregulating inflammatory cytokines, and positively modulating proliferation of beneficial gut bacteria and suppression of harmful ones in both clean and unclean rooms. In the fourth study, an E. coli lipopolysaccharide challenge model was used to study the effect of NRYE without or with carbohydrases (ENZ) on growth performance, blood profile, immune response and gut structure. Lipopolysaccharide-challenged piglets receiving NRYE or ENZ + NRYE showed beneficial responses in gut structure, blood cell profile and immunity commensurate with AGP, though the latter had better overall growth performance. Taken together, the results show that NRYE may improve growth performance of weaner pigs by modulating the

immune system and gut microbiota, and preventing weaning-associated gut atrophy. Furthermore, the results indicate that the effect of NRYE on the immune system was more pronounced, hence, suggesting that future studies should target to elucidate the mechanism by which supplemental nucleotides modulate the immune system during weaning.

DEDICATION

This thesis is dedicated to the grace of God and the love, prayers and support from my wife Leila Nyakio, my parents, Mr. Ngugi Waititu and Mrs. Grace Wambui, my siblings Maina, Wangui, Wanjugu and Tito, and my dear brothers and sisters in Christ Jesus.

ACKNOWLEDGMENTS

I wish to express sincere gratitude to my advisor, Dr. C. M. Nyachoti for the great opportunity to study under his supervision. I warmly appreciate his support, advice, generosity and encouragement throughout my PhD studies. I would like to thank the members of my committee, Drs. J.C. Rodriguez-Lecompte, B.A. Slominski, D. Weihrauch and E. Khafipour for their fruitful discussions and suggestions during our meetings.

Support for this research from Natural Sciences and Research Council of Canada, Manitoba Pork Council and Canadian Bio-systems Inc., (Calgary, AB, Canada) is gratefully acknowledged.

Many thanks to Drs. J.M. Heo, J. Sands, H. Alemu and Y. Fugui for the expert advice you provided to shape the focus of my studies. It is hard to forget the invaluable technical assistance I received from R. Stuski, D. Ramos, and A. Karamanov, and the administrative support from Margaret Ann, Mei Ding, Gwen Band, Catherine Plouffe, Kathy Graham and Valerie Smith during the course of my studies.

The fun, rough and serious moments I shared with Atta, Deepak, Chandini, Bala, Nijitha, Kola, Boonjin, Jong, Saymore, Roseline, Roisbel, Pratima, Roshan, Bishwo, Peris, Deborah, Alex, Harold, Mohammed, and other graduate, undergraduate and visiting students at the Department of Animal Science is a precious memory that is highly appreciated.

I would like to express special thanks to my wife, Leila, my parents and siblings, and to my dear family in Christ Jesus at Winnipeg for their inspiration, support and constant encouragement throughout my studies.

Finally, my gratefulness to God is indescribable. Surely, could we with ink the ocean fill, and were the skies of parchment made, were every stalk on earth a quill and every man a scribe by trade, to write the love of God above would drain the ocean dry nor could the scroll contain the whole, though stretched from sky to sky.

FOREWORD

This thesis was written in a manuscript format and it is composed of four manuscripts prepared from data obtained from four studies that were conducted to achieve the objectives of the thesis research. The manuscripts were written according to the guidelines for the Journal of Animal Science manuscript preparation. The first manuscript has been published in the Canadian Journal of Animal Science and the authors are Samuel. M. Waititu, Jung. M. Heo, Rob Patterson and Charles. M. Nyachoti. The second manuscript has been published in Animal Nutrition Journal and the authors are Samuel. M. Waititu, Jung M. Heo, Rob Patterson and Charles. M. Nyachoti. The third manuscript has been submitted for peer review to the Animal Journal and the authors are Samuel M. Waititu, Fugui Yin, Rob Patterson, Alexander Yitbarek, Juan C. Rodriguez-Lecompte and Charles M. Nyachoti. The fourth manuscript has been submitted for peer review to the Journal of Animal Science and Biotechnology and the authors are Samuel M. Waititu, Fugui Yin, Rob Patterson, Juan C. Rodriguez-Lecompte and Charles M. Nyachoti.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF COPYRIGHTED MATERIALS FOR WHICH PERMISSION WAS OBTAINED

LIST OF ABBREVIATIONS

AGP	Antibiotic growth promoters
ADFI	Average daily feed intake
ADG	Average daily gain
ATTD	Apparent total tract digestibility
CP	Crude protein
DM	Dry matter
G:F	Gain to feed ratio
GE	Gross energy
ENZ	Carbohydrase enzymes
ETEC	Enterotoxigenic *Escherichia coli*
IFN- γ	Interferon gamma
IL-1β	Interleukin-1 beta
IL-10	Interleukin 10
IL-6	Interleukin 6
LPS	Lipopolysaccharide
NRYE	Nucleotide-rich yeast extract
NSP	Non-starch polysaccharides
MLN	Mesenteric lymph nodes
PUN	Plasma urea nitrogen
TNF-α	Tumor necrosis factor alpha

CHAPTER ONE

GENERAL INTRODUCTION

Weaning is meant to be a gradual process of replacement of milk feeding regimen to an *ad libitum*, dry feeding regimen. However, in commercial settings, weanling piglets are abruptly separated from the sow and offered dry feeds for economic reasons. The abrupt withdrawal of sow's milk during weaning deprives the piglet of essential nutrients, immune cells and bioactive compounds necessary for enhancing growth, survival and health (Xu and Cranwell, 2003). Coupled with the psychosocial stress due to separation from the sow, introduction to a new environment and mixing with pigs from other litters, weaning negatively affects growth and health of the piglets (Kim et al., 2012).

Piglets are highly susceptible to enteric disorders during the immediate period after weaning (Pluske et al., 1997; Lallès et al., 2007). During this stage, the piglets have immature digestive and immune systems, and a tendency for proliferation of enteropathogenic bacteria. The immature digestive system suggests that the digestive capacity of the piglet gut is impaired during weaning. This is characterized by post-weaning diarrhea and reductions in villus height and activity of brush border digestive enzymes in the small intestine (Pluske et al., 1997; Heo et al., 2013). Such enteric disorders limit the development of the digestive capacity in young pigs, which leads to poor growth performance. The immature intestinal immune system and tendency for the proliferation of enteropathogenic bacteria further predisposes piglets to enteric infections such as post-weaning collibacilosis, which worsens growth stasis and may be lethal if not checked.

Post-weaning associated challenges are an important concern in swine production because they affect economic gains (Whittemore and Green, 2001). Consequently, different

nutritional and non-nutritional strategies have been employed to mitigate these challenges. The non-nutritional strategies include all-in all-out management, age-segregation, sanitation, biosecurity and vaccination, whereas nutritional strategies can be classified in terms of those aiming to reduce the concentration of anti-nutritional factors in diets or supplementing growth promoting additives (Cromwell, 2002; Kim et al., 2012; Heo et al., 2013).

Since 1949, the non-therapeutic use of antibiotics in livestock production as growth promoters has proved very effective in mitigating post-weaning associated challenges (Jukes, 1972). In-feed antibiotics have been reported to positively modify the composition of gut microbiota (Looft et al., 2012) and enhance intestinal mucosa integrity (Owusu-Asiedu et al., 2003; Nyachoti et al., 2012), immune responses (Nau and Tauber, 2008), nutrient digestibility (Thymann et al., 2007; Choi et al., 2011) and growth performance (Han and Thacker, 2010; Choi et al., 2011; Han et al., 2011; Yan et al., 2011a; Li et al., 2012; Yan et al., 2012; Zhou et al., 2015). However, public pressure to eliminate the use of in-feed antibiotics has intensified because of their potential association with the development of microbial antibiotic resistance in humans and livestock (Heuer et al., 2006), and environmental contamination (Carlson et al., 2004). Consequently, pork products sourced from pigs raised under antibiotic-free regimen are on high demand in several major international markets. Therefore, there is an urgent need for viable alternatives to dietary antibiotics that are acceptable to the consumer, environmentally sound and can mitigate the post-weaning challenges (Choct, 2001; Heo et al., 2013; Gong et al., 2014).

Research on antibiotic alternative feed additives (such as spray dried porcine plasma, organic acids, high levels of zinc and copper salts, probiotics, prebiotics, egg yolk antibodies, herbs, spices, bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides) and dietary interventions (such as dietary protein and carbohydrate modulation) has been ongoing (Choct, 2001; Heo et al., 2013; Gong et al., 2014). To date, the major concern in the

development of new additives and adoption of existing ones is the lack of knowledge of the mechanisms through which additives that are considered potential in-feed antibiotic alternatives improve health and growth of the piglets.

Dietary nucleotides have been proposed as potential in-feed antibiotic alternative. This was inspired by findings of studies with human infants fed nucleotide supplemented formula milk showing that dietary nucleotides have beneficial effects on the small intestine (maturation and recovery) and gut microbiota, lipid and hepatic metabolism and on the immune system (Boza, 1998). Nucleotides are low-molecular-weight intracellular compounds that participate in numerous biochemical processes and are the building block monomers of nucleic acids (DNA and RNA). With adequate supply of amino acids and energy, nucleotides can be synthesized de novo. However, they may become essential when the endogenous nucleotide supply is insufficient for normal function. Conditions under which nucleotides become essential include certain disease states, periods of limited nutrient intake or rapid growth, and the presence of regulatory or developmental factors that interfere with full expression of the endogenous synthetic capacity (Carver and Walker, 1995). All these conditions are present in weaned pigs and therefore, it is expected that they have high tissue requirement for nucleotides during this period. Consequently, nucleotides can be perceived as "conditionally essential" nutrients for piglets.

Studies on supplementation of dietary nucleotides to piglets' diet show their beneficial effects on growth and gut health during (Domeneghini et al., 2004; Martinez-Puig et al., 2007; Weaver and Kim, 2014) and after (Carlson et al., 2005) the weaning period. The mechanism by which nucleotides promote growth and gut health has been suggested to mainly involve the promotion of cellular functions associated with cell proliferation. By influencing the cellular functions, nucleotides enhance structural growth and maturation of

important tissues of the immune system and the intestinal mucosa. In this regard, the effect of dietary nucleotides may be deemed long-lasting (Córdoba et al., 2008).

Therefore, it was hypothesized that supplementing weaned pigs with a nucleotide-rich yeast extract under sanitary and disease challenge will mitigate post-weaning stress and maintain growth performance. Furthermore, yeast-based nucleotides will act synergistically with carbohydrase enzymes in mitigating post-weaning stress and maintaining growth performance. These effects will be mediated through enhanced immune system activation and maintenance of a stable intestinal microbial composition.

CHAPTER TWO

LITERATURE REVIEW

2.1 Defining gut health

Clear-cut criteria for defining gut health are lacking (Rijkers et al., 2011). This is not surprising because even the definition of health by WHO (1946) as being a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity, sparked much controversy. According to Huber et al. (2011), the critics of the WHO definition of health were mostly against the absoluteness of the word “complete” in relation to wellbeing. In animal care, health status is primarily diagnosed through appearance of clinical signs using subjective measures such as appetite, body condition, feces and urine properties, respiratory difficulties, tumors and behavior. In this regard, the lack of clinical signs of disease would be accepted as a healthy status but this may not be a true indicator of a healthy gut. For instance, dysbiosis in commensal gut bacteria is not part of the routine welfare assessment in commercial swine holdings. However, human studies have shown that dysbiotic intestinal bacterial population is linked to pathogenesis of various diseases including those not directly associated with the gastrointestinal tract such as asthma (Azad and Kozyrskyj, 2012), diabetes (Qin et al., 2012), disorders of the immune system (Wen et al., 2008; Brown et al., 2012), arthritis (Gomez et al., 2012) and pregnancy (Santacruz et al., 2010). Furthermore, some gut disorders may develop slowly and therefore lead to delayed clinical signs and symptoms (Ford et al., 1983; Subasinghe et al., 2011).

The complexity of the GIT makes it difficult to properly define a healthy gut without a detailed consideration of the unique functions of the key components that constitute the gut's ecosystem. In this context, gut health can be inferred from two perspectives: the proper functioning of each component and a prevailing protective homeostasis at every interaction

between the intestinal mucosa and the external environment (mainly microbiota and food particles), which is broadly termed as the gut barrier. This approach is consistent with that recommended by Ayres and Schneider (2012) who noted the difficulty of measuring fitness or wellness and suggested that health status be inferred from measurement of physiological parameters that are important to the subject so long as those responses are readily measurable and have a broad dynamic range. Only such an approach may provide a more acceptable and overarching definition of gut health that addresses the complex nature of the GIT compartments, their physiological processes and adaptations, and their interaction with the external environment as highlighted by gut studies in different disciplines.

The first aspect of the suggested approach, the functions and adaptations of the piglet gut, were comprehensively reviewed by Pluske et al. (1997), Lallès et al (2004) and Heo et al. (2013). In the next parts of this review, the second aspect of the suggested approach, measurement of factors that fortify the gut barrier, will be discussed as indicators of gut health.

2.1.1 Gut pH as an indicator of gut health

The stomach wall is lined with parietal cells which secrete gastric juice that contains hydrochloric acid that helps to keep its pH low (Yen, 2000). Exposure to low pH values (i.e. less than 3.5) is bactericidal for many pathogenic bacteria, including *Escherichia coli* (Zhu et al., 2006). Therefore, maintenance of a low gastric pH value is essential and may indicate a healthy gut because this can help to reduce the passage of pathogenic bacteria into the small intestine (Nyachoti et al., 2006).

2.1.2 Gut morphology as an indicator of gut health

Villus height and crypt depth are used as indicators of small intestine integrity (Hampson, 1986a; Spreeuwenberg, 2002). The villi are finger-like projections made up of epithelial cells called enterocytes found on the mucosal layer that help to increase the surface

area for digestion and absorption processes, whereas the crypts are sub mucosal glands that repopulate the epithelial cells (Zhang and Xu, 2003). For optimal function of the small intestine, long villi and deeper crypts are desirable. A deeper crypt is a sign of more active cellular turnover to maintain villi height (Yason et al., 1987; Smith, 1992). Generally, increases in the ratio of villi height to crypt depth indicates better digestive and absorptive capacity of the small intestine (Kelly and King, 2001). Changes in gut morphology influence secretion of brush border enzymes, hence, weaning-associated damage to enterocytes may decrease digestive enzyme activity thereby limiting the digestive and absorptive capacity of the gut, which may lead to malabsorption, dehydration, enteric infection, and diarrhea.

2.1.3 Gut permeability as an indicator of gut health

In both humans and animals, malnutrition alters the function of the intestinal barrier resulting in increases in intestinal transport of macromolecules, intestinal ionic conductance and small solute permeability (Heyman et al., 1984; Carey et al., 1994; Rodriguez et al., 1996; Kim et al., 2012). Gut paracellular permeability to solutes is regulated by the tight junctions, which are made up of a complex of several proteins that seal the paracellular space between epithelial cells (Berkes et al., 2003). Tight junctions show both, size selectivity and charge selectivity for transport of substances, and these properties may be regulated by physiological stimuli such as alterations of tight junction protein formation and distribution, and pathophysiological stimuli such as translocation of enteric pathogens and endotoxins (Berkes et al., 2003). Gut health status can be inferred from parameters such as the concentration or level of gene expression of tight junction proteins (Hamard et al., 2010), and paracellular permeability rates measured using either a selective marker of paracellular permeation of tight junctions (Barreau et al., 2004) or short-circuit current and transepithelial resistance measured in Ussing chambers (Carey et al., 1994; Hamard et al., 2010).

2.1.4 Gut hormones as an indicator of gut health

The presence of dietary nutrients in the intestinal lumen evokes the release of numerous hormones from enteroendocrine cells interspersed among the epithelial cells lining the intestine, which not only affect gut motility, hunger, discomfort and satiation but also the gut barrier function (Suzuki et al., 2010; Kaji et al., 2013). For instance, when ingested food arrives in the stomach, gastrin is released by the pyloric glands and stimulates the parietal cells to secrete gastric juice which contains hydrochloric acid that lowers the stomach pH and consequently killing bacteria that cannot survive the low pH (Schubert and Peura, 2008). In addition, activation of nutrient receptors in the duodenum (Kawamata et al., 2003; Akiba et al., 2009) triggers intracellular signaling mechanisms that stimulate enteroendocrine cells to release hormones such as glucagon-like peptides 1 and 2, which are associated with glycemic control and increased bicarbonate and mucus secretion and mucosal blood flow (Wang et al., 2011), respectively. Kaji et al. (2013) suggest that the dysfunction of the chemosensing system may be implicated in the pathogenesis of foregut mucosal injury due to acid-pepsin secretion. In this context, measuring the concentration of gut hormones in the portal vein may be indicative of gut health status.

2.1.5 Gut immunity as an indicator of gut health

The gut mucosa is the major site of lymphocyte contact with antigens in the entire body. Immunological stress has a detrimental effect on the nutritional status of animals because nutrients are diverted away from growth to support tissues involved in the immune response (Spurlock, 1997). There is clear evidence that over expression of proinflammatory cytokines such as interleukin (IL-) 6, IL-1 and tumor necrosis factor alpha (TNF-α) in the small intestine results in villus atrophy and reduced performance of pigs (Johnson, 1997; Spurlock, 1997; McLamb et al., 2013). Furthermore, the first step in many microbial infections is colonization and invasion of a mucosal surface (Jimenez-Valera and Ruiz-Bravo,

2011). Presence of secretory immunoglobulin A (sIgA) on the intestinal mucosa can prevent such infections by blocking colonization (Wilson et al., 2011).

2.1.6 Gut bacteria as an indicator of gut health

The importance of gut microbiota in the development of diseases was reviewed by Sekirov et al. (2010). The reviews of Calder et al. (2006) and Wu and Wu (2012) on studies associating gut microbial colonization in neonates with the maturation of the gut and gut-associated immune system concluded that the regulation of the mucosal immune response is dependent on the normal gut microbiota. Other studies have reported that an immature or dysbiotic gut microbiota results in decreased intestinal surface area (Romick-Rosendale et al., 2014), reduced capacity for inflammatory responses, and abrogation of oral tolerance (Sudo et al., 1997). Furthermore, the review of Berg (1996) highlights the morphological, physiological and immunological deleterious effects of the immature gut microbiota of germ free rodents (Table 2.1).

2.2 Post-weaning stress

Weaning is a stressful period to the piglet and results in intestinal, immunological, and behavioral changes (Hampson, 1986a; Heo et al., 2013). The stressors include the abrupt separation from the sow, transportation and handling, transition from liquid to solid food, formation of a new social hierarchy as they co-mingle with pigs from other litters, a different physical environment, and increased exposure to antigens from the diet or environment. The physiological, environmental, psychological and social challenges experienced by piglets post-weaning predisposes them to diseases and other production losses. To be productive and efficient, the piglet must adapt to all of these stressors rapidly. In the absence of weaning, the piglets mitigate these challenges by deriving immune system boosting nutrients, cells and bioactive compounds from the sow milk.

Table 2.1. Characteristics of germfree rodents without an indigenous gastrointestinal microbiota compared with conventional rodents with indigenous gastrointestinal microbiota

Morphological characteristics
– Increased cecum size
– Decreased weight of intestinal wall
– Decreased intestinal surface area
– Shorter intestinal villi
– Thinner lamina propria
– Decreased size of liver, heart, adrenals, etc.
– Decreased blood volume
Physiological/biochemical characteristics
– Decreased intestinal motility or peristalsis (i.e. increased transit time)
– Decreased rate of villus epithelial cell renewal
– Altered mucosal enzyme patterns (i.e. increased trypsin, decreased β-glucuronidase)
– Lower pH of intestinal contents
– Increased oxygen levels (i.e. higher oxidation-reduction potential)
– Decreased basal metabolic rate
– Decreased cardiac output
– Decreased regional blood flow (to intestines, liver, etc.)
– Decreased synthesis of vitamin K and vitamin 8 complex
– No bacterial aided bile acid degradation in the gut
– Lack of short chain fatty acids or coprostanol
Immunological characteristics
– Decreased lymph node and spleen size
– Decreased Peyer's patch size
– Decreased serum gammaglobulin levels
– Decreased numbers of IgA producing lymphocytes in lamina propria
– Decreased numbers of intraepithelial T cells
– Decreased inflammatory response
– Decreased blood clearance of microorganisms
– Delayed immune response after antigenic challenge

Adapted from Berg (1996)

2.2.1 Sow's milk nutrition

In addition to having highly digestible nutrients, the sow's colostrum and milk contain a rich supply of leukocytes comprising macrophages, eosinophils, neutrophils and lymphocytes, and bioactive compounds such as hormones, cytokines, immunoglobulins, growth factors, and digestive enzymes, and immune cells that support growth and survival of the neonatal pig (Xu and Cranwell, 2003). Generally, these bioactive compounds and immune cells carryout important functions including antimicrobial protection, immune modulation (to suppress inflammatory responses), digestive enzyme supplementation, stimulation of cell division and differentiation in the small intestine, endocrine modulation, and metabolic regulation (Xu et al., 2000).

The neonatal pig derives its energy from metabolism of body protein, glycogen, and fat reserves (Le Dividich et al., 2005). However, at birth, the body energy reserves of neonatal piglets are very low, averaging between 8-25% liver glycogen, 1-2% fat, and approximately 12% protein (Le Dividich et al., 1991; Pluske et al., 1995; Kuralkar and Kuralkar, 2010). Therefore, colostrum/milk fat and lactose are the major energy source for neonates (Le Dividich et al., 1994). In addition, colostrum/milk casein is the main source of essential amino acids, calcium and phosphorus to the young pig (Holt and Sawyer, 1988).

The bioactive compounds are mostly contained in both casein and whey proteins of colostrum/milk (Silva and Malcata, 2005; Khan and Bhati, 2010). Nguyen et al. (2007) reported the presence of cytokines including IL-6, TNF-α, IFN-γ, IL-12, IL-10, IL-4, and TGF-β1in sow's colostrum and milk. Colostrum/milk cytokines and antibodies (IgA and IgM) enhance the passive immune protection of neonates against pathogens and modulate the development of the neonatal innate and adaptive immune system during the colonization of the GIT by commensal bacteria (Salmon, 2002; Pomorska-Mól et al., 2010). Insulin, insulin-like growth factor I and II and epidermal growth factor are examples of growth-promoting

peptides in sow's colostrum /milk, which not only accelerate proliferation and maturation of the GIT in young pigs but also that of other organs such as liver, pancreas, kidney, spleen, and skeletal muscle (Simmen et al., 1990; Donovan and Odle, 1994; Burrin et al., 1997; Kelly and King, 2001).

On the other hand, prior to the development of high gastric acidity which occurs after the first several days of life, colostrum/milk leukocytes are absorbed through intracellular spaces in the duodenum and jejunum of young pigs and enter blood circulation where they influence immune related functions such as cell mediated immune response, development of antigen tolerance, stimulation of active immune development, and provision of passive immune protection (Williams, 1993; Pomorska-Mól et al., 2010). Therefore, the withdrawal of sow's milk during weaning deprives the piglet of essential nutrients, immune cells and bioactive compounds necessary for enhancing growth, survival and health. Coupled with the psychosocial stress due to separation from the sow, introduction to a new environment and mixing with pigs from other litters, weaning negatively affects growth and health of the piglets.

2.2.2 Effect of weaning on the gut morphology and physiology

Weaning induces villi atrophy and crypt hyperplasia in the intestinal mucosa tissues and these changes may be transient or long-lasting (Pluske et al., 1997; Boudry et al., 2004). The first 2 d post-weaning are marked by transitory anorexia that causes intestinal inflammation, which leads to villi atrophy by either increasing the rate of cell loss among enterocytes lining the villi or reducing the rate of cell renewal by the crypt (Pluske et al., 1997; McCracken et al., 1999). The pre-weaning villi and crypt integrity may be achieved between 3 to 5 d post-weaning upon resumption of feed intake but long-lasting changes may prevail for up to 2 wk post-weaning (Pluske et al., 1997). Impairment of the villi and crypt

integrity reduces the gut absorptive capacity and activities of the brush-border digestive enzymes (Kelly et al., 1991).

2.2.3 Effect of weaning on gut microbioata

The piglet gut is supposedly sterile in the uterus but during and after birth becomes colonized by bacteria from the sow, sow's milk and feces, and other environmentally sourced bacteria (Mackie et al., 1999; Martín et al., 2009). During the suckling period, the gut microbiota constituting the first colonizers is underdeveloped, remains relatively stable in terms of species composition and are dominated by lactobacilli and streptococci, which are well adapted to utilize nutrient substrates in sow's milk (Swords et al., 1993). The stability of first colonizers is easily disrupted by introduction of the solid diet during weaning (Gaskins, 2001). The weaning associated anorexia is followed by a fast consumption of the new diet (Hopwood and Hampson, 2003), which results in an increase of undigested fermentable carbohydrate and protein in the hind gut owing to the reduced digestive capacity of the small intestines, which provides a substrate for microbial fermentation and bacterial overgrowth (Williams et al., 2005; Jeaurond et al., 2008).

The main outcome of the disruption of the underdeveloped initial colonizers immediately after weaning is a decline of beneficial bacteria and an overgrowth of N-utilizing pathogenic bacteria such as *Clostridium perfringens* and *Clostridium difficile,* which results in scours, decreased growth performance, and pre-weaning mortality (Songer and Uzal, 2005; Konstantinov et al., 2006). Increased fermentation of undigested protein in the hind gut is associated with a higher incidence of post weaning diarrhea, proliferation of potential pathogenic proteolytic bacteria such as enterotoxigenic strains of *E. coli* (ETEC), and production of higher amounts of potentially toxic metabolites such as ammonia, amines, phenols and indoles (Pluske et al., 2002; Heo et al., 2009; Rist et al., 2013). Enteric bacterial infections are economically significant and their clinical signs normally include: diarrhea,

weight loss, decreased growth rate and death of neonatal, weaning, and growing-finishing pigs. The most common etiological agents of these infections are *Clostridium difficile, Clostridium perfringens, E. coli, Salmonella enterica, Brachyspira hyodysenteriae, Brachyspira pilosicoli* and *Lawsonia intracellularis* (Moxley and Duhamel, 1999; Menin et al., 2008).

2.2.4 Effect of weaning on gut immunity

The piglet is immunodeficient at birth and depends on immune factors present in sow's colostrum and milk for immune protection (Lallès et al., 2007). At weaning, the piglet gut immune system is relatively immature and evidence suggests that it matures between 4 to 6 wk post-weaning (King et al., 2003; Bailey et al., 2005). The maturation of the piglet GIT immune system is mainly affected by the development of gut microbiota, as evidenced in studies using germfree animals (Gaskins, 2001). Weaning exposes the piglet to new microbial and dietary antigens thus activating immune responses in the gut.

Increases in T-cells populations in lamina propria (King et al., 2003), plasma concentrations of acute-phase proteins and inflammatory cytokines, fibrinogen and glucagon (McCracken et al., 1995), and expression of genes for inflammatory cytokines in the piglet gut (Pié et al., 2004) has been reported soon after weaning (McCracken et al., 1995; King et al., 2003). This sudden activation of the immune system at weaning may be due to the compromised intestinal integrity allowing antigens from the lumen to penetrate or an overreaction of the immature GIT due to its inability to differentiate between harmful and innocuous antigens (Stokes et al., 2004). Evidence suggests that weaning-associated intestinal inflammation is due to overproduction of proinflammatory cytokines such as IL-1, IL-6, and TNF-α (McCracken et al., 1999; Pié et al., 2004) and results in a redistribution of nutrients away from growth processes in aid of the immune system (Wannemacher, 1977; Johnson, 1997), a factor that leads to growth stasis during weaning.

2.2.5 Effect of weaning on growth performance

Weaning growth stasis and mortality is due to the weaning-associated compromise of the GIT mucosa barrier function following alterations on gut morphology, physiology, microbiota and immunity (Pluske et al., 1997). In the small intestines, damage to the villi reduces the absorptive capacity of the gut, whereas damage to the crypts reduces turnover rate of enterocytes and mucus production, the latter exposing the mucosa to corrosion by hydrochloric acid present in gastric secretion and pathogens (Pluske et al., 1997). Furthermore, a loss of enterocytes which secrete brush-border enzymes, such as aminopeptidases, and various carbohydrases reduces digestive capacity of the gut (Miller et al., 1986). These factors coupled with weaning-associated anorexia that limits the piglet from meeting maintenance energy requirements reduce the availability of essential nutrients required for growth. Therefore, an immature and inflamed gut immune system and proliferation of pathogenic bacteria in the hind gut makes the piglet more susceptible to enteric diseases such as post-weaning collibacilosis, which worsens growth stasis and may be lethal if not checked.

Taken together, post-weaning associated challenges are an important concern in swine production because they affect economic gains by increasing housing and feed costs due to an increase in days to market, cost of disease treatment and management, and loses due to mortality (Whittemore and Green, 2001). Consequently, different nutritional and non-nutritional strategies have been employed to mitigate these challenges. The non-nutritional strategies include all-in all-out management, age-segregation, sanitation, biosecurity and vaccination, whereas nutritional strategies can be classified in terms of those aiming to reduce the concentration of anti-nutritional factors in diets or supplementing growth promoting additives (Cromwell, 2002; Kim et al., 2012; Heo et al., 2013).

2.3 Role of in-feed antibiotics in mitigating post-weaning stress

Since 1949, the non-therapeutic use of antibiotics in livestock production as growth promoters proved very effective in curbing the spread of bacterial infections on commercial farms to extents not possible before, and also unnaturally stimulated growth and productivity (Jukes, 1972). Thus, the non-therapeutic use of antibiotics in livestock production became established worldwide. Antibiotics have been used in the swine industry for over 6 decades (Cromwell, 2002) to treat bacterial infections (therapeutic use) and to prevent and control bacterial infection or promote growth (non-therapeutic use) of weaned, grower and finisher pigs (Jukes, 1972).

2.3.1 Mechanism of growth promotion by antibiotics

At least four mechanisms have been proposed to explain how antibiotics promote growth (Francois, 1961; Visek, 1978; Gaskins et al., 2002): (1) inhibition of sub-clinical infections, thereby reducing the metabolic costs of the immune system, (2) reduction of growth-depressing microbial metabolites, (3) reduction of microbial use of nutrients, and (4) enhanced uptake and use of nutrients because the intestinal wall of antibiotic-fed animals are thinner. Together, the four mechanisms share the common hypothesis that both commensal and pathogenic gut bacteria may depress animal growth directly or indirectly through their metabolic activities (Gaskins et al., 2002). This hypothesis is supported by studies showing that oral antibiotics do not enhance the growth of germfree animals (Coates et al., 1963) and may inhibit the bacterial catabolism of urea and amino acids and the fermentation of carbohydrates both *in vitro* and *in vivo* (Dierick et al., 1986; Nagaraja et al., 1987). Recently, Niewold (2007) proposed a fifth mechanism based on the hypothesis that antibiotics promote growth by reducing intestinal inflammation. This suggestion is based on the observation that most antibiotics get accumulated in inflammatory cells where they possibly enhance the intracellular killing of bacteria, and inhibit parts of the innate immune response (van den

Broek, 1989; Labro, 1998). According to Niewold (2007), this anti-inflammatory role of antibiotics conserves energy for production.

2.3.2 Effect of antibiotics on gut microbioata

In-feed antibiotics, also known as antimicrobial growth promoters (AGP), modify the composition of gut microbiota. For instance, Looft et al. (2012) performed metagenomic analysis on feces collected from 32 d old weaned pigs fed diets without or with a feed grade AGP product (ASP 250; containing chlortetracycline, sulfamethazine, and penicillin) for 14 d and reported a shift in microbial community membership. Antibiotic receiving pigs showed a decrease in the abundance of Bacteroidetes, members of *Anaerobacter*, *Barnesiella*, *Papillibacter*, *Sporacetigenium*, and *Sarcina* genera. In addition, they reported increase in the abundance of members of the *Deinococcus-Thermus* and *Proteobacteria* phyla as well as *Succinivibrio* and *Ruminococcus* genera. The most notable shift was a 10% increase in *Proteobacteria* abundance in pigs fed the medicated diet compared to control, of which 62% was constituted by *E. coli* population.

Thymann et al. (2007) reported that Augmentin (amoxicillin plus clavulanic acid) administered in-feed and intramuscularly reduced small intestinal microbial diversity, and specifically reduced *E. coli* population compared with control. Looft et al. (2012) explained that results on AGP effect on gut microbiota are equivocal and demonstrate the varying collateral effects of different AGP. For instance, *E. coli* are both commensal and pathogenic inhabitants of mammalian GIT, thus, an increase in *E. coli* could be beneficial or harmful, either to the host or to the food chain.

Piglets receiving in-feed AGP and challenged with ETEC were shown to have reduced counts of members of Bacteroidetes and Firmicutes phyla (Bhandari et al., 2008), reduced total count of harmful coliform (Namkung et al., 2004; Nyachoti et al., 2012), reduced the count of *E. coli* K88+ attached to ileal mucosa and in colon digesta (Nyachoti et

al., 2012), reduced the count of *E. coli* attached to colon mucosa (Bhandari et al., 2009), reduced ileal digesta count of members of orders *Lactobacillales, Clostridiales* and *Enterobacteriales* (Kiarie et al., 2011), and increased richness (number of distinct species present) and diversity (abundance of distinct species) of microbial species (Bhandari et al., 2008).

2.3.3 Effect of antibiotics on intestinal mucosa integrity

Antibiotic use in swine diets has been associated with beneficial effects on the intestinal mucosa integrity. Studies have reported that piglets receiving in-feed AGP had longer villi, higher villi/crypt depth ratio and greater small intestinal weights than control after an ETEC challenge (Owusu-Asiedu et al., 2003; Nyachoti et al., 2012). Furthermore, Thymann et al. (2007) reported that AGP-receiving piglets had increased mucosal goblet cell area (suggesting improved mucus secretion hence protection of mucosal epithelia against pathogens), reduced colonic tissue weight by 23%, and lower concentration of colonic short chain fatty acids (suggesting reduced hindgut fermentation hence an indicator of stimulation of digestive enzymes and nutrient absorption in the small intestine) compared with control.

2.3.4 Effect of antibiotics on immune responses

There is growing evidence that certain AGP exert their beneficial effects not only by killing or inhibiting the growth of bacterial pathogens but also indirectly by immunomodulation (Nau and Tauber, 2008). For instance, macrolides achieve high intracellular concentrations and have good activity against Gram-positive bacteria, influence the release of cytokines such as IL-8 and TNF as well as mediators of inflammation such as nitric oxide (Nau and Tauber, 2008). Swine studies have shown that in-feed AGP have ability to modulate immune response. For instance, pigs receiving in-feed AGP have shown reduced serum IgG (Namkung et al., 2004), IgA (Wu et al., 2012) and TNF-α (Nyachoti et al., 2012), which suggests suppression of immune response. However, other studies have reported that

in-feed AGP increased serum antibodies IgA, IgG and IgM, increased serum cytokine concentrations of IL-1β and IL-6, increased concentration secretory IgA on jejunum mucosa (Wu et al., 2012), proliferation of lymphocytes (Yan et al., 2011b; Li et al., 2012; Yan et al., 2012; Zhou et al., 2015) and higher lymphocyte transformation ratio (Zhou et al., 2015) in piglets. Improved serum antibody level indicates enhanced humoral immunity, whereas increased lymphocyte population and transformation ratio indicates enhanced cell-mediated immunity.

2.3.5 Effect of antibiotics on nutrient digestibility

Thymann et al. (2007) reported that AGP-fed piglets had improved amylase, trypsin and small intestinal aminopeptidase A and N activities, thus suggesting improved protein digestive function. Moreover, AGP supplementation in piglet diets has been associated with increased digestibility of dry matter (Huang et al., 2010; Li et al., 2012; Yan et al., 2012; Zhou et al., 2015), starch (Bhandari et al., 2009), crude protein (Huang et al., 2010; Choi et al., 2011; Yan et al., 2011a; Li et al., 2012; Zhou et al., 2015), energy (Huang et al., 2010; Wu et al., 2012; Yan et al., 2012; Zhou et al., 2015), fat and Ca (Hahn et al., 2006).

2.3.6 Effect of antibiotics on growth performance

Compared with grower and finisher pigs, in-feed AGP are mostly employed in weaner pigs as growth promoters because young pigs have an immature immune system that makes them highly susceptible to pathogens. A meta analysis of 453 experiments involving 13,632 weaned pigs (7 to 25 kg) conducted at universities or other research stations in the US showed that AGP improved average daily gain (ADG) by an average of 16% and gain to feed ratio (G:F) by 6.9% (Cromwell, 2001), whereas these beneficial effects were doubled in pigs raised under farm conditions due to increased microbial load (Cromwell, 2001). Other studies have reported increased ADG, feed intake and G:F after antibiotics supplementation in weaned pig diets (Han and Thacker, 2010; Choi et al., 2011; Han et al., 2011; Yan et al.,

2011a; Li et al., 2012; Yan et al., 2012; Zhou et al., 2015). Furthermore, Wu et al. (2012) reported that ETEC challenged piglets receiving in-feed AGP had higher ADG and feed efficiency than control.

2.3.7 The ban on the use of in-feed antibiotics

In 2006 and 2011, European Union countries and South Korea, respectively, banned the use of in-feed AGP in livestock as a part of their food safety strategy. While the use of in-feed AGP is still legal in North America, public pressure to eliminate the practice has intensified because of their potential association with the development of microbial antibiotic resistance in humans and livestock (Heuer et al., 2006), and environmental contamination (Carlson et al., 2004).

2.3.8 Challenges of raising pigs without in-feed antibiotics

The use of in-feed AGP became prevalent when industrialized pork production became necessary in meeting the rising demand for meat protein. There is sufficient evidence that AGP use has been more beneficial in commercial farms with large herds and poor management conditions. For instance, Cromwell (1999) estimated a saving of $2.92 per pig in feed and housing costs in terms of increased production efficiency. Therefore, a ban on AGP use in swine production poses challenges on animal welfare, nutrient utilization, manure production, and economic sustainability.

Recent studies suggest that the impact of the cost of the ban will be greater in piglet production compared to grower-finisher pigs production (Thomke and Elwinger, 1998; Kjeldsen et al., 1999) because of the need to mitigate early-weaning *E. coli* associated diarrhoea and swine dysentery contamination. Table 2.2 summarizes the potential economic effects of AGP ban at animal, farm, and market levels (Teillant and Laxminarayan, 2015), which show the multifaceted challenges of raising pigs without AGP.

Table 2.2. Potential economic costs of AGP restrictions at animal, farm, and market levels

Potential Animal-Level Effects
– Decreased growth rate, decreased feed efficiency.
– Short term higher mortality rate (especially of young animals), increased morbidity.
– Fewer animals born per litter
– Increased variability of product.
Potential Farm-Level Effects
– Increased time to market and decreased stocking densities.
– Increased input costs: feed (non AGP), young animals purchased.
– Cost of more biosecurity measures and adjustments in housing to compensate for AGP termination.
– Increased veterinary costs (more treatment of disease).
– Higher labor costs if alternatives to AGP are more labor-intensive
– Increased variability of product.
Potential Market-Level Effects
– Less output for each level of input
– Increase in wholesale and retail price of meat
– Variation in producers revenues (increase or decrease)

Adapted from Teillant and Laxminarayan (2015)

2.3.9 Characteristics of a potential alternative to antibiotics

Recent surveys suggests that U.S consumers are increasingly concerned about food safety, environmental degradation and animal welfare, and are willing to place substantial premiums on pork produced without AGP (Lusk et al., 2006; Nilsson et al., 2006). Therefore, despite the challenges producers may face in raising pigs without AGP, they have no option but to seek alternative additives and management measures that will sustain profitability of their farms. Consequently, research on alternative feed additives (such as organic acids, probiotics, prebiotics, nucleotides, bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides) and dietary interventions (such as dietary protein and carbohydrate modulation) have been proposed (Choct, 2001; Heo et al., 2013; Gong et al., 2014). A major concern in the development of new strategies is the lack of knowledge of the mechanisms through which additives that are AGP alternatives can protect intestinal cells from the damage induced by pathogens and thereby improve health and growth of the pig. Thus, the better knowledge of how in-feed AGP alternatives work is important in order to understand their role in intestinal cell protection and to finally select those able to provide such protection in piglets.

Based on the earlier discussion on gut health indicators and the effectiveness of AGP, a suitable alternative to in-feed AGP must have multifaceted characteristics and beneficial effects to the piglet which include: (1) ability to modulate gut microbiota by suppressing proliferation of pathogenic communities and promoting that of beneficial strains, (2) ability to modulate gut microbiota without causing microbial antibiotic resistance, (3) ability to modulate gut immune response especially by controlling excessive inflammation after pathogen clearance, (4) ability to maintain the intestinal mucosa integrity by promoting pathogen clearance, (5) ability to maintain the intestinal mucosa integrity by promoting growth and maturation of tissues, (6) ability to promote growth performance, (7) ability to

promote nutrient utilization, (8) ability to induce response within a relatively short period of time compared with antibiotics, (9) ability to interact synergistically with other feed additives, (10) should have minimal impact on feed cost, and (11) ability to maintain effectiveness after subjection to conventional feed processing and storage standards. In the next parts of the present review, the use of dietary nucleotides as an alternative to in-feed AGP will be discussed.

2.4 Nucleotides

Nucleotides are low-molecular-weight intracellular compounds that participate in numerous biochemical processes and are the building block monomers of nucleic acids (DNA and RNA). Nucleotides are also used in formation of ATP, GTP and co-enzymes.

2.4.1 Structure and types of nucleotides

Nucleotides are composed of a nitrogenous base linked to a pentose (ribose or deoxyribose) sugar to which one, two or three phosphate groups are attached. A nucleoside consists of a nitrogenous base covalently attached to a pentose sugar but without the phosphate group. Nucleotides can be classified as either pyrimidines or purines based on their nitrogenous base. The pyrimidines comprise cytosine, uridine or thymine, whereas the purines comprise adenine, guanine and hypoxanthine. Purine and pyrimidine bases are hydrophobic and relatively insoluble in water at the near-neutral pH of the cell but their solubility in water increases at acid or alkaline pH because the bases become charged (Nelson et al., 2008). Illustrations of the structure of nucleotides, purines, pyrimidines and some nucleotide-based compounds are shown in Figure 1.

2.4.2 Sources of nucleotides

In mammalian cells, the three potential sources of nucleotides are *de novo* synthesis, salvage pathways, and the diet (Boza, 1998; Cosgrove, 1998). To synthesize nucleotides by the *de novo* pathways, the cells require substantial amounts of ATP energy, aspartate,

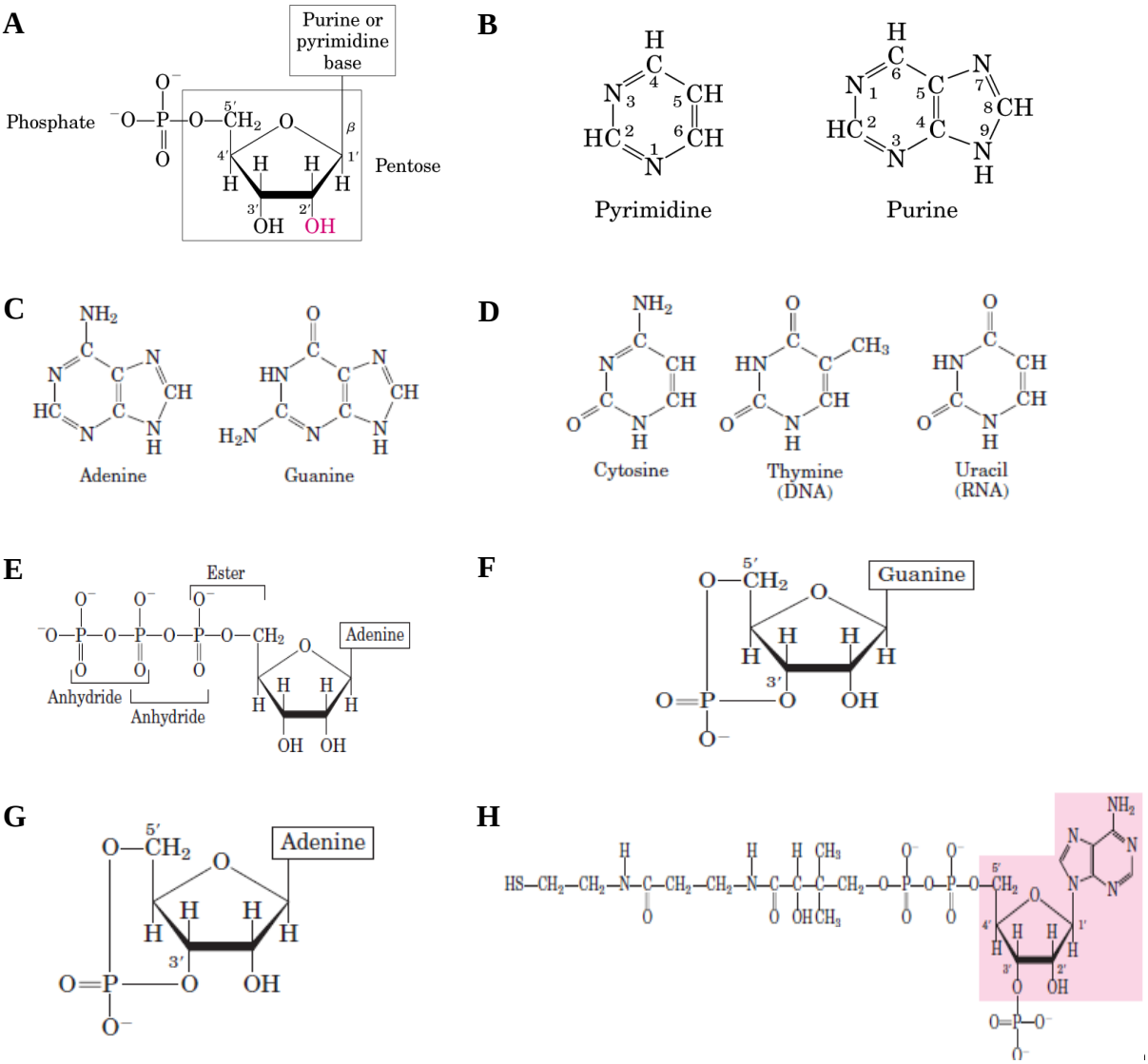

Figure 1. Structure of nucleotides and some nucleotide-based compounds.

(A) General structure of nucleotides, (B) parent compounds of the pyrimidine and purine bases of nucleotides and nucleic acids, (C) major purine bases of nucleic acids, (D) major pyrimidine bases of nucleic acids (E) adenosine 5'-triphosphate (ATP), (F) guanosine 3,5-cyclic monophosphate (cGMP), (G) adenosine 3',5'-cyclic monophosphate (cAMP) and (H) coenzyme A. Adapted from Nelson et al. (2008).

glutamine and glycine thus making the process metabolically costly (Carver and Walker, 1995). The salvage pathways maintain the cellular nucleotide pools by recycling approximately 90% or more of the bases and nucleosides that are formed during degradation of RNA and DNA, hence, the salvage pathway requires less energy than *de novo* synthesis of nucleotides (Carver and Walker, 1995). Tissues such as the intestinal mucosa, the hematopoietic cells of the bone marrow, leucocytes, erythrocytes and lymphocytes preferentially utilize the salvage pathway because of their limited capacity for *de novo* synthesis of nucleotides (Sanderson and He, 1994; Uauy et al., 1994). The *de novo* synthesis pathway and the salvage pathway are inversely related, the former being activated by the absence of nucleotides and the latter stimulated by their presence in the diet (LeLeiko et al., 1983). Hence, dietary nucleotides contribute to the salvage pathway by providing preformed nucleosides and nitrogenous bases.

Dietary nucleotides exist in the form of nucleoproteins. The release of nucleosides and nitrogenous bases from dietary nucleoproteins was reviewed by Sauer et al. (2011). Briefly, after ingestion, proteases in the GIT liberate nucleic acids from dietary nucleotides. Pancreatic nucleases degrade nucleic acids into a mixture of mononucleotides, dinucleotides, trinucleotides and polynucleotides. Phophoesterases supplement the action of nucleases producing mononucleotides. Intestinal alkaline phosphatase converts the nucleotides to nucleosides thus releasing free phosphate. Finally, nucleosidases release the pentose sugar from the nucleosides thus giving free nitrogenous bases. The mixture of nucleosides and nitrogenous bases is offered to the enterocytes for absorption (Uauy et al., 1994).

2.4.3 Absorption and metabolism of dietary nucleotides

Nucleosides are the main form in which nucleotides are absorbed in intestinal cells. The duodenum has the most developed absorptive capacity for nucleosides, which has been suggested to be an adaptive response of duodenal cells since they have a lower capacity for

de novo synthesis of nucleotides compared to the ileum and jejunum (Savaiano and Clifford, 1978). The absorption of nucleosides involves the combination of a highly efficient Na^+-dependent active transport and facilitated diffusion (Bronk and Hastewell, 1987; Jarvis, 1989). Using human intestinal Caco-2 cell line, Sanderson and He (1994) reported differences in the absorption rate of the different nucleosides, with guanosine being taken up most rapidly compared to adenosine, inosine, cytidine, uridine and thymidine.

Sonoda and Tatibana (1978) fed mice with chow containing radiolabeled RNA or DNA at purine or pyrimidine bases and examined the metabolic fate of the their constituents. Their findings suggest that (1) ingested nucleotides are quantitatively and rapidly absorbed (more than 70% after 1 h post-feeding), (2) metabolism of the absorbed nucleotides is carried out principally by enterocytes and liver tissues, (3) immediately after absorption, most of the nucleosides are removed from the portal circulation by both metabolism and utilization before entry into the systemic circulation, hence, most are utilized in the GIT and the liver, whereas only a small amount reach other tissues, and (4) the *de novo* synthesis of nucleotides in other tissues is not significantly affected by the exogenous bases except when a large dose of purines or pyrimidines is ingested at one time. Savaiano and Clifford (1978) also observed that 90% of radiolabeled purine bases were absorbed by mice but 24 h thereafter only 5% were incorporated into tissues, whereas the remainder were excreted in the urine. These observations imply that the high absorption rate of nucleotides is not proportional to its assimilation into tissues, and this strengthens the suggestion that nucleotides are conditionally essential nutrients. Savaiano and Clifford (1978) also observed that adenine was more effectively incorporated into the tissues compared to guanine, hypoxanthine or xanthine. This implies that the effectiveness of nucleotides source products may differ based on the constituent nucleotides profile.

Gil et al. (2007) demonstrated the ability of the piglet intestinal epithelium to hydrolyze RNA and free nucleotides. They cultured jejunal explants from suckling piglets in a medium supplemented with either a mixture of nucleosides, nucleotides or RNA. They reported a rapid decrease in concentration of other nucleosides except cytidine in the nucleosides culture medium, suggesting that they were efficiently taken up by the explants. Furthermore, when explants were incubated in the presence of nucleotides, the total concentration of these compounds decreased while the total concentration of nucleosides increased, suggesting that enterocytes efficiently hydrolyze nucleotides into nucleosides. Likewise, when explants were incubated in the presence of RNA, the total concentration of both nucleotides and nucleosides increased, indicating that intestinal explants are able to hydrolyze RNA to nucleotides and then to nucleosides in the absence of luminal enzymes.

Absorbed nitrogenous bases that are not metabolized or utilized by enterocytes enter the hepatic portal vein and are carried to the hepatocytes for further metabolism. From the liver, they are released into systemic circulation and enter muscle tissue. If they are not re-utilized for nucleotide synthesis or remain unabsorbed, the purine bases are catabolized into uric acid, whereas the pyrimidine bases are catabolized into ß-alanine or ß-aminoisobutyrate (Chu, 1991). In primates, uric acid is excreted via the urine, whereas in other mammals it is further catabolized into allantoin via the enzyme uricase to be excreted into the urine. On the other hand, the catabolic products of pyrimidine bases (ß-alanine or ß-aminoisobutyrate) are further metabolized into ammonia, carbon dioxide and acetyl coenzyme A.

2.4.4 Nucleotides dependent biochemical processes

The comprehensive review of Sauer et al. (2011) on this subject shows that nucleotides mostly influence biochemical processes associated with cellular functions. For instance, being precursors of nucleic acids (RNA and DNA) they influence the cell cycle. In addition, nucleotides also influence phagocygotic activity, lymphokine production,

lymphocyte maturation (Paubert-Braquet et al., 1992; Carver, 1994) and act as carriers of phosphate (ATP and UTP) in enzymatic reactions involved in the transfer of chemical energy (Cosgrove, 1998). Furthermore, nucleotides are constituents of important co-enzymes such as NAD, NADP, FAD and CoA (Lodish et al., 2000), which act as activated intermediates in the synthesis of lipids, carbohydrates and protein.

2.5 Dietary nucleotides as antibiotics alternative in piglet nutrition

The study of nucleotides as AGP alternative in piglet nutrition was inspired by findings of studies with human infants fed nucleotide supplemented formula milk. As reviewed by Boza (1998), results from rodent and human studies show that dietary nucleotides seem to have beneficial effects on the small intestine (maturation and recovery) and gut microbiota, lipid and hepatic metabolism and on the immune system. Porcine milk is the best source of nucleotides for the piglet. The nucleotide/nucleoside profile of porcine milk shows a substantial predominance for pyrimidines as compared to purines (Mateo et al., 2004). Pyrimidine nucleotides are more absorbed and incorporated into tissue RNA compared to purine nucleotides (Pizzini et al., 1990). In most animal studies conducted to validate the numerous benefits of dietary nucleotides, the most significant finding attributed to nucleotide supplementation is its effect in modulating the immune system. It is in this context that dietary nucleotides have been studied to determine their helpfulness in mitigating weaning-associated challenges in piglets raised in AGP-free regimens.

2.5.1 Nucleotides as "conditionally essential" nutrients

Immediately after weaning, feed intake is very low hence the pig is deprived of energy and nutrients necessary for the rapid growth experienced at this stage (Pluske et al., 1995). The gut and immune system are not fully developed by the time of weaning and their tissues have a rapid turnover (Grimble and Westwood, 2001). In the absence of nutrients and immunity enhancing factors previously supplied via sow milk, the deprivation of energy and

nutrients due to weaning-associated anorexia and increase in pathogen load from the environment leads to growth stasis (Heo et al., 2013). In such situations, nucleotides are deemed conditionally essential nutrients since RNA synthesis precedes any changes in protein synthesis (Grimble and Westwood, 2001). In this context, the term 'conditionally essential' denotes a dietary component whose endogenous supply is insufficient to fully support normal function, but for which there is no classical clinical deficiency syndrome (Boza, 1998).

Although there is paucity of studies concerning effect of supplemental nucleotides in pigs, the available studies give evidence of the beneficial effect of supplemental nucleotides in pigs. In the next parts of the present review, results from swine studies showing effect of dietary nucleotides will be discussed.

2.5.2 Effect of dietary nucleotides on growth performance

Most of the studies in which dietary nucleotides were supplemented to weaner diets have reported equivocal results on improvement of growth performance in pigs. Carlson et al. (2005) reported higher ADG and average daily feed intake (ADFI) after supplementing 5.0% NRYE with AGP in phase 1 diets (d 1 to 14) and 2.5% NRYE without AGP in phase 2 diets (d 15 to 28) and overall (d 29 to 130). In addition, Zomborszky-Kovacs et al. (2000) and Weaver and Kim (2014) reported that supplemental pure nucleotides improved growth performance of piglets. On the contrary, some studies have not shown effect of supplemental nucleotides on improvement of growth performance (Di Giancamillo et al., 2003; Domeneghini et al., 2004; Lee et al., 2007; Martinez-Puig et al., 2007; Šperanda et al., 2008; Moore et al., 2011; Sauer et al., 2012a).

Studies in which dietary nucleotides were supplemented to sows' diet during late gestation and lactation also report equivocal results. Plante et al. (2011) reported no increase of nucleotide concentrations in milk and sow or nursing piglet performances. On the contrary,

Vitagliano et al. (2014) reported increased litter size and litter weight at weaning, and total RNA concentration in milk. In addition, Vitagliano et al. (2014) and Hung (2015) reported decreased birth mortality. Furthermore, Hung (2015) reported increased body weight, ADG and ADFI during the total nursery period in pigs from sows fed nucleotide-containing diets. The reasons explaining the inconsistent results of nucleotides supplementation on growth performance of nursery pigs are discussed in section 2.5.6.

2.5.3 Effect of dietary nucleotides on intestinal morphology

Dietary or salvage nucleotides synthesized from the liver are important for enterocytes because of their high cell turnover rate and limited capacity for *de novo* synthesis (LeLeiko et al., 1983; Grimble and Westwood, 2000). Moore et al. (2011) and Sauer et al. (2012a) did not observe differences in villus height, crypt depth and villus/crypt ratio between control and pigs receiving 0.2% or 1.34 g/d pure nucleotides in their diets, respectively, after 20 d post-weaning. Moreover, Sauer et al. (2012b) reported that supplementing weaned piglets with 0.1% NRYE for 36 d neither affected activities of ileal α-amylase, leucine amino peptidase, maltase and lactase nor ileal dry matter, crude protein and crude fibre digestibilities. On the other hand, Di Giancamillo et al. (2003) and Domeneghini et al. (2004) reported beneficial effects of supplementing 0.05% dietary NRYE on intestinal gut morphology as indicated by an increase in villus height, crypt depth and decrease of villus to crypt ratio. Similar results were obtained by Martinez-Puig et al. (2007) after supplementing 0.1 or 0.2% dietary nucleotides to piglet diets. Di Giancamillo et al. (2003) reported a thicker adherent mucous gel in the ileum of nucleotide fed piglets compared with control, which implies enhanced protection of epithelial cells from digestive enzymes and penetration of pathogens. Moreover, Lee et al. (2007) reported that supplemental nucleotides increased the small intestine weight and gastric pepsin activity, whereas Carlson et al. (2005)

reported that 5% NRYE reduced the duodenal wall thickness compared with control, suggesting lowered intestinal hyperplasia.

2.5.4 Effect of dietary nucleotides on modulation of the immune system

Dietary nucleotides have been reported to improve immune responses in weaned piglets. Domeneghini et al. (2004) reported a higher number of intraepithelial lymphocytes and macrophages in the ileal tissues of piglets receiving dietary nucleotides. Likewise, Šperanda et al. (2008) reported an immunostimulatory effect of NRYE in terms of increasing the percentage of lymphocytes, especially CD4 and CD8 subpopulation of T cells. Intraepithelial lymphocytes are associated with modulation of immune responses and elimination of damaged or infected cells (Cerf-Bensussan and Guy-Grand, 1991), whereas macrophages act as the first line of the host defense against viral infections (Ma et al., 2003).

Supplemental nucleotides have also been reported to increase IgA levels in plasma (Lee et al., 2007; Sauer et al., 2012a) and bile (Lee et al., 2007). A high concentration of IgA in the intestinal mucosa may indicate stronger humoral mucosal immunity (Macpherson et al., 2008). Hung (2015) observed that pigs from nucleotide-supplemented sows had higher total IgA level in the serum at weaning and 1 wk post-weaning. Martinez-Puig et al. (2007) reported a reduction in the mortality rate and diarrhea incidence that was associated with a beta-haemolytic strain of *E coli* in piglets supplemented with 0.075 and 0.1% dietary nucleotides compared with the control group.

Furthermore, Carlson et al. (2005) observed that 5% NRYE supplementation reduced the lamina propria area 28 d post-weaning. The lamina propria contains B lymphocytes, mature plasma cells, T cells, macrophages, and mast cells, hence, a thinner lamina propria suggests suppressed immune stimulation. Salobir et al. (2005) induced oxidative stress in pigs by feeding them a diet with a high proportion of dietary polyunsaturated fat (Quiles et al., 1999). Oxidative stress is known to induce genotoxic effects on immune cells (Lang et al.,

2013). They reported that supplementing 0.44% of NRYE reduced blood lymphocyte DNA damage, suggesting that nucleotides have an immunonutritive effect through their involvement in the mechanisms of excision and repair of fragmented DNA molecules.

Moreover, beneficial effects of dietary nucleotides have been reported in studies using disease challenge models. For instance, Hung (2015) observed that dietary nucleotide supplementation reduced the concentration of serum TNF-α and IL-6 after 2 and 4 hours, respectively, following a 50 μg/kg body weight of *E. coli* lipopolysaccharide injection to piglets, , which suggests a stimulation of macrophage and B cell proliferation, respectively.

2.5.5 Effect of dietary nucleotides on gut microbiota

This is a less researched but extremely important area regarding the effect of supplemental nucleotides in piglets. Sauer et al. (2010) studied the *in vitro* effect of supplementing individual nucleotides on growth response of selected bacterial strains dominating the small intestine of piglets. They observed that individual nucleotides exerted differential effects on the growth of tested *E. coli* strains. An important observation they made was that compared with control medium, the *in vitro* growth of pathogenic *E. coli* strain PS 37 was suppressed by nucleotides, whereas that of non-pathogenic *E. coli* DSM 2840 and pathogenic strain PS79 were enhanced. In addition, they observed no effect of nucleotides on the growth of *Lactobacillus reuteri*, *Lactobacillus amylovorus*, *Enterococcus faecium* and *Enterococcus faecalis*. Their results imply that both pathogenic and non-pathogenic microbes are capable of utilizing nucleotides for their growth, hence, effects under *in vivo* conditions will most likely be determined by competitive exclusion among species with similar survival requirements. This implication is supported by the results of the *in vitro* study of Mateo (2005) who added nucleosides to broths containing bacteria isolated from feces of piglets and observed an increase in total coliform counts that was associated with a decrease in *Clostridium perfringens* counts.

The *in vivo* study of Sauer et al. (2012a) failed to show effects of supplemental dietary nucleotides on the composition of small and large intestine gut microbiota of piglets. On the contrary, Mateo (2005) fed piglets a control diet without or with 30 or 150% more nucleoside content as that of sow milk for 14 d post-weaning and reported that pigs receiving nucleosides had lower fecal counts of *Clostridium perfringens* and higher fecal counts of *L. acidophilus* and *Bifidobacterium* spp compared with control. The results suggest that dietary nucleotides modulate proliferation of beneficial and pathogenic bacteria in the gut of piglets as also observed in human studies (Gil et al., 1986; Singhal et al., 2008).

2.5.6 Reasons for inconsistent results on supplemental nucleotides effect in piglets

The inconsistent results of the reported effects of dietary nucleotides in pigs may be due to differences in the constitution and concentration of nucleotides used in the trials (Sauer et al., 2011; Hung, 2015). For instance, Savaiano and Clifford (1978) observed that adenine was more effectively incorporated into the tissues compared to guanine, hypoxanthine or xanthine. In addition, Pizzini et al. (1990) observed that pyrimidine nucleotides are more absorbed and incorporated into tissue RNA compared to purine nucleotides. Sauer et al. (2012a) mimicked the individual nucleotides' concentration in sow milk using a purified mixture of nucleotides, whereas all the studies using yeast based nucleotides (Mateo, 2005; Lee et al., 2007; Martinez-Puig et al., 2007; Sauer et al., 2012b) only mimic total concentration but not individual nucleotides' composition because it is impossible to balance for individual nucleotides in yeast extracts.

Moore et al. (2011) and Lee et al. (2007) used the same commercial product but at different inclusion rates, hence, making it difficult to compare their results. Although Weaver and Kim (2014) and Carlson et al. (2005) reported improved growth performance after nucleotides' supplementation, they used different products and nucleotides' concentration. Martinez-Puig et al. (2007) and Sauer et al. (2012b) used similar inclusion rate of the same

commercial product but did not measure identical response criteria, hence, making it difficult to substantiate their results.

Furthermore, Sauer et al. (2011) suggested that the concentration and constitution of nucleotides may be altered by those nucleotides contained in dietary ingredients, hence, affecting results of different trials. Moreover, the experimental environment may also affect the results because nucleotide requirements increase in challenge conditions (Grimble and Westwood, 2001). For instance, Martinez-Puig et al. (2007) reported that a sudden diarrhea outbreak associated with a beta-haemolytic strain of *E coli* during the trial demonstrated a reduction in the mortality rate and diarrhea incidence in piglets receiving NRYE.

It is worth noting that yeast extracts also contain variable amounts of viable cells, cell wall components, and parts of the medium on which the yeast cells were grown, which may influence growth and immune responses. For instance, cell wall polysaccharides may help in the modulation of mucosal immunity and provision of binding sites for pathogens hence mediating their elimination from the gut (Kogan and Kocher, 2007). Therefore, the beneficial effects of NRYE may be multi-faceted and cannot be exclusively attributed to the effect of nucleotides present in these products (Sauer et al., 2012b). Therefore, further study is needed to substantiate the effect of individual, pure mixtures and yeast-based nucleotides supplementation.

2.5.7 Time of response of dietary nucleotides

As discussed earlier, an effective AGP alternative should have a relatively shorter time of response in piglets. This is because some of the negative effects of post-weaning stress appear as early as 3 d after weaning (Heo et al., 2013), especially when the weaning-associated anorexia is severe. Results from the studies of Vitagliano (2014) and Hung (2015) suggest that supplementing nucleotides to sow diet during late gestation may be beneficial to the neonatal pig at birth, through the nursing period, and during and after weaning. Studies on

supplementation of dietary nucleotides to piglet diets also show their beneficial effects during (Domeneghini et al., 2004; Martinez-Puig et al., 2007; Weaver and Kim, 2014) and after (Carlson et al., 2005) the weaning period. The ability of dietary nucleotides to exhibit effects in both cases is based on their role in influencing the cellular functions as discussed earlier. Since dietary nucleotides enhance structural growth and maturation of important tissues of the immune system and the intestinal mucosa during the weaning period, their effects have been described as being long-lasting (Córdoba et al., 2008).

2.5.8 Interaction of dietary nucleotides with other additives

Studies that investigated the interactive effect of dietary nucleotides with other feed additives such as exogenous enzymes, organic acids, probiotics, prebiotics and antibiotics are scarce. Nevertheless, owing to the low concentration of nucleotides in piglet diets (as DNA and RNA in cellular components of the diet), interactive effects with other additives are expected at optimal nucleotides dosage synonymous with that of sow milk. For instance, supplementing NRYE with AGP improved ADG and ADFI of weaned pigs (Carlson et al., 2005). Furthermore, supplementing nucleotides with L-glutamine resulted in an increase in villus height and crypt depth, decrease in villus height/crypt depth ratio, increase of mitosis and decrease in apoptosis in lymphatic follicles, increase in percentage of macrophages and intraepithelial lymphocytes in ileal tissue (Domeneghini et al., 2004). Further research is needed to elucidate the synergistic or antagonistic effect of dietary nucleotides with other commonly used feed additives.

2.5.9 Impact of dietary nucleotides on feed cost and processing

Use of pure nucleotides in animal nutrition may not be feasible because of the high cost involved in purifying nucleotides. However, new developments have made it possible to use yeast extract as the delivery vehicle for nucleotides, hence, making NRYE to be available at an affordable cost. Although no econometric studies have been done on nucleotide

supplementation in pigs, prevailing market prices as of December 2015 show that the cost of supplementing nucleotides is estimated at $10 per tonne of treated feed, which is equal to that of supplementing antibiotics such as lincomycin or aureomycin. Assuming that the performance of pigs fed nucleotides or antibiotics is the same, then nucleotides can replace antibiotics at no extra feed cost. In addition, producers in Canada and USA can get a premium of 5 to 10% for pigs raised without AGP (Grannis and Thilmany, 2001, 2002; Wheatley, 2003), hence, there would be a significant economic advantage for nucleotide supplementation.

Moreover, nucleotides are easily incorporated into feed as they are stable at temperatures (100 to 120˚C; Motono, 1982) higher than the optimum temperature range used for pelletization or extrusion of feed (65 to 95˚C; Furuta et al., 1980; Thomas et al., 1997). Therefore, the delivery of supplemental nucleotides into feed has no negative impact on the commonly used feed processing methods.

2.6 Summary of literature review

Weaning is a stressful period for pigs and leads to a growth stasis that may reduce profitability or increase mortality rate if not checked. These negative results are due to the effect of weaning on gut health outcomes which have been herein reviewed. In-feed AGP have been successful in mitigating the weaning-associated challenges and promoting growth. However, the association of AGP with the development of drug resistance in pathogenic bacteria and contamination of both the environment and pork products with their residues has resulted in the ban of their usage or low prices of products from AGP-fed pigs. Nucleotides have been suggested as potential alternatives to antibiotics in mitigating weaning-associated challenges. Nucleotides are deemed "conditionally essential" nutrients in situations of rapid growth, tissue injury, disease and limited nutritional supply such as during early weaning. The mechanism by which nucleotides promote growth and gut health has been suggested to

mainly involve the promotion of cellular functions associated with cell division. It has been suggested that the metabolic cost of *de novo* and salvage pathways of nucleotide synthesis can be lowered by supplementing dietary nucleotides. Nucleotides sourced from yeast extracts are more affordable than purified sources, hence, are easily incorporated into practical diets. However, the reported effects of dietary nucleotides are equivocal and further studies are needed to substantiate the beneficial effects attributed to nucleotides sourced from both purified products and yeast extracts. In addition, there is need to study the effects of dietary nucleotides under commercial barn and disease settings to substantiate the claim of nucleotides as "conditionally essential" nutrients for piglets.

CHAPTER THREE

HYPOTHESES AND OBJECTIVES

In this thesis, it was hypothesized that:

1) Supplementing weaned pig diets with NRYE under sanitary and disease challenge will mitigate post-weaning stress and improve growth performance.
2) Supplemental NRYE will act synergistically with carbohydrase enzymes in mitigating post-weaning stress and maintaining growth performance.
3) The beneficial effects of supplemental NRYE will be mediated through enhanced immune system activation and maintenance of a stable intestinal microbial composition.

The overall objective of this study was to determine the effect of supplementing a nucleotide-rich yeast extract on growth performance, gut digestive and immune systems, and gut microbiota in weaned pigs. The specific objectives of this study were:

1) To determine the effect of supplemental NRYE on growth performance and apparent total tract digestibility (ATTD) of dry matter (DM), crude protein (CP) and gross energy (GE).
2) To determine the interaction effect of graded levels of antimicrobial growth promoters with supplemental NRYE on growth performance and ATTD of DM, GE and CP, and to establish whether NRYE supplementation can completely or partially replace AGP in diets for weaned pigs.
3) To determine the effect of supplemental NRYE on growth performance, gut structure, immunity and microbiota on piglets raised in sanitary and unsanitary conditions.
4) To determine the interaction effect of supplemental NRYE and feed enzymes versus antimicrobial growth promoters on performance, blood cell profile, immune response, and gut structure after an *E. coli* lipopolysaccharide challenge.

CHAPTER FOUR

MANUSCRIPT I

Dietary yeast-based nucleotides as an alternative to in-feed antibiotics in promoting growth performance and nutrient utilization in weaned pigs[1]

S. M. Waititu[1], J. M. Heo[1,a], R. Patterson[2], and C. M. Nyachoti[1],

[1]Department of Animal Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2; and [2]Canadian Bio-systems Inc., Calgary, AB, Canada T2C 0J7.

Present address:

[a]Department of Animal Science and Biotechnology, Chungnam National University, Daejon 305-764, South Korea.

Author contributions:

SMW wrote the research proposal. All author's participated in the review and approval of the design of the experiment. SMW carried out the animal studies, sample collection and analysis. JMH assisted in monitoring animals and sample collection. SMW carried out statistical analysis and prepared the manuscript. All authors participated in the interpretation of the data, review and approval of the final manuscript.

[1] The material presented in chapter four of this thesis has been accepted for publication in the Canadian Journal of Animal Science.

4.1 ABSTRACT:

Weaning deprives piglets of nucleotides present in sow milk. Some studies have suggested that supplemental nucleotides are potential alternatives to antibiotics (AGP) and are beneficial in mitigating post-weaning associated growth stasis. Therefore, it was hypothesized that supplemental nucleotide-rich yeast extracts (NRYE) would have similar beneficial effect as in-feed AGP on growth performance of piglets, the NRYE effect on growth performance would be more pronounced at a higher NRYE concentration and supplementing both NRYE and AGP would have an additive effect on growth performance. Ninety piglets (6.79 ± 0.5 kg body weight) were fed corn-wheat-dry whey-canola meal-soybean meal based-diets containing 0, 0.1, or 0.2% NRYE without or with antimicrobial growth promoters (AGP) for 21 d to determine NRYE effect on performance and apparent total tract digestibility (ATTD) of DM, GE and CP. Supplementing AGP without or with 0.1% NRYE increased ADG ($P < 0.05$), AGP × NRYE interactions were observed for all ATTD measurements ($P < 0.05$), and increasing NRYE level in AGP diets linearly decreased ($P < 0.05$) ATTD of DM, CP and GE. In conclusion, although pigs receiving AGP had better ADG than control, supplementation of 0.1 or 0.2% NRYE to control diet resulted in similar growth performance as AGP group. The results suggest that NRYE can mitigate weaning-associated growth stasis.

Key words: antimicrobial growth promoters, digestibility, nucleotide-rich yeast extract, growth performance, piglet

4.2 INTRODUCTION

The continuous sub-therapeutic use of AGP has been associated with development of antimicrobial resistance (Heuer et al., 2006) and environmental contamination (Kümmerer, 2003). Hence, effective alternative therapies to in-feed AGP to mitigate piglet post-weaning stress are needed (Heo et al., 2013).

Nucleotides are “conditionally” essential nutrients in periods of stress, rapid growth or limited nutritional supply such as early weaning (Grimble and Westwood, 2001). Starvation due to weaning anorexia depletes energy and glutamine supply that limits *de novo* nucleotides synthesis. This impedes nucleotide-dependent physiological processes like cell division, co-enzyme activation and stimulation of cell and humoral immunity because nucleotides are building blocks of RNA, DNA, ATP, GTP and cAMP (Grimble and Westwood, 2001; Sauer et al., 2011).

Nucleotides concentration in sow’s milk (2715 ppm) is relatively constant during the last phase of lactation and many times greater than that in weaner diets (71 ppm) as reported by Mateo et al. (2004). Weaning deprives piglets of nucleotides, which have been associated with beneficial effects such as minimizing morphological changes in intestinal tissues, growth promotion of beneficial gut microbiota, reducing incidence of post-weaning diarrhea, immune system modulation and improving growth performance (Superchi et al., 2012).

Nucleotides can be supplemented in pure forms or as NRYE, the latter being more affordable than the former. Studies examining effect of supplemental NRYE in pigs are scarce, whereas those that simultaneously examine NRYE and AGP effect are lacking. Therefore, it was hypothesized that supplemental NRYE would have similar beneficial effect as in-feed AGP on growth performance of piglets, the NRYE effect on growth performance would be more pronounced at a higher NRYE concentration and supplementing both NRYE and AGP would have an additive effect on growth performance. The study aimed to compare the effects of two levels of NRYE supplementation in pig starter diets without or with AGP on growth performance and apparent total tract digestibilities (ATTD) of dry matter (DM), crude protein (CP) and gross energy (GE).

4.3 MATERIALS AND METHODS

All experimental procedures were reviewed and approved by the University of Manitoba Animal Care Committee (protocol number F08-033/1/2/3), and pigs were handled in accordance with guidelines described by the Canadian Council on Animal Care (2009).

The NRYE supplement, Maxi-Gen Plus, was supplied by Canadian Bio-Systems Inc. (Calgary, AB, Canada) and contained cell wall polysaccharides (21.6%), CP (32.7%), carbohydrates (14.3%) and a mixture of 5 nucleotides (1.1%; adenosine monophosphate, cytosine monophosphate, inosine monophosphate, uridine monophosphate and guanosine monophosphate), with 1 g of the NRYE additive supplying approximately 1000 ppm of mixed nucleotides. The AGP supplements, Aueromycin and Tiamulin, were supplied by Bio Agri Mix LP (Mitchell, ON, Canada). The AGP were supplemented in the diet according to the recommended dosage of 55 mg of Aueromycin (chlortetracycline) and 31.2 mg of Tiamulin per kg of diet by the Canadian Food Inspection Agency.

Ninety [Duroc × (Yorkshire × Landrace)] male and female piglets, weaned at 21d and with an initial average body weight of 6.79 ± 0.5 kg were used in a 3-wk study. Pigs were housed in an environmentally controlled nursery building with pens equipped with a feeder, a nipple drinker, and plastic-covered expanded metal floors. The room temperature was maintained at 29 ± 1°C in wk 1, and then gradually decreased by 1ºC every wk thereafter. The pigs were acclimatized to the experimental environment for 4 d, during which they were fed the basal starter diet without NRYE or AGP (Table 4.1). Thereafter, they were randomly assigned to 1 of 6 dietary treatments based on initial BW with 3 pigs per pen and 5 replications per treatment. The treatments were arranged as a 2 x 3 factorial of 2 AGP groups (+ or -) and 3 NRYE levels (0, 0.1 or 0.2%). All piglets were fed a corn-wheat-dry whey-canola meal-soybean meal basal starter diet (Table 4.1) without or with AGP. Diets were supplemented with 0, 0.1 or 0.2% NRYE. All diets were formulated to meet or exceed the

Table 4.1. Composition of the basal diets (as-fed basis)

Item	Basal diet
Ingredient, %	
Corn	25.90
Wheat	13.75
Canola meal	10.00
Soybean meal	20.00
Fish meal	5.00
Dried whey	20.00
Vegetable oil	2.50
Limestone	0.60
Monocalcium phosphate	0.60
Iodized salt	0.25
Vitamin-trace mineral premix[1]	1.00
L-Lys·HCl	0.10
Titanium dioxide	0.30
Calculated nutrient content	
DE, kcal/kg	3 472
CP, %	22.7
Ca, %	0.80
Total P, %	0.70
Available P[2], %	0.50
SID[3] AA, %	
Lys, %	1.40
Met, %	0.40
Thr, %	0.90
Analyzed composition	
CP, %	22.1
Ca, %	0.90
Total P, %	0.75

[1]Supplied the following per kilogram of complete diet: 9,000 IU of vitamin A; 1,500 IU of vitamin D_3; 18 mg of vitamin E; 1.5 mg of vitamin K; 250 mg of choline; 30 mg of niacin; 27.5 mg of calcium pantothenate; 9.4 mg of riboflavin; 2 mg of pyridoxine; 25 µg of cyanocobalamin; 80 µg of biotin; 0.5 mg of folic acid; 18 mg of Cu from copper sulfate, 110 mg of Zn from zinc oxide, 0.2 mg of I from calcium iodide, 110 mg of Fe from ferrous sulfate, 50 mg of Mn from manganese dioxide, and 0.3 mg of Se from sodium selenite.

[2]Available P = % of ingredient in diet × % total P in each ingredient × % P bioavailability in each ingredient

[3]Standardized ileal digestible

NRC (2012) recommendation for all nutrients. The NRYE and AGP supplements were top-dressed to the basal diet. The diets were fed as mash and contained 0.3% titanium dioxide as an indigestible marker for estimation of ATTD. Feed disappearance and body weight were recorded on d 0 and 21. Representative freshly voided fecal samples were collected from each pen on d 19, 20 and 21, and stored at -20°C until required for analysis.

Fecal samples were dried in an oven at 60°C for 4 d and pooled for each pen and along with diet samples, they were finely ground to pass through a 1 mm screen using a Cyclotec 1093 Sample Mill (FOSS North America, Eden Prairie, MN, USA), and Thomas-Wiley mill (Thomas Scientific Swedesboro, NJ, USA), respectively, and thoroughly mixed before being analyzed for DM, GE, CP, and Ti.

Dry matter was determined according to AOAC (1990) method 925.09 and GE was determined using an adiabatic oxygen bomb calorimeter (Parr Instrument Co., Moline, IL, USA) which had been calibrated using benzoic acid as a standard. Crude protein (N × 6.25) was determined according to method 990.03 of AOAC (1990) using a combustion analyzer (model CNS-2000;Leco Corp., St. Joseph, MI, USA). Dietary total Ca and P were analyzed following AOAC (1990) procedures (method 990.08) using a Varian inductively coupled plasma mass spectrometer (Varian Inc., Palo Alto, CA). Samples for Ti analysis were ashed and digested as described by Lomer et al. (2000) and were measured by inductively coupled plasma mass spectrometer (Varian Inc., Palo Alto, CA, USA).

The digestibility of nutrients was calculated using the following equation:

% Apparent nutrient digestibility $= \{1 - [(T_d/T_f) \times (N_f/N_d)]\} \times 100$ where T_d and T_f are the titanium dioxide concentration in the diet and feces, respectively, and N_f and N_d are the nutrient concentration in the feces and diet, respectively.

Data were analyzed using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC) for a 2 × 3 factorial arrangement of treatments. Pre-planned contrasts were used to evaluate dietary treatment effects. Linear and quadratic effects for NRYE were determined using orthogonal polynomial contrasts. Significance was defined as $P < 0.05$ and $0.05 < P < 0.10$ was considered a trend.

4.4 RESULTS AND DISCUSSION

Increasing dietary NRYE level had no effects ($P > 0.05$) on the ADFI, ADG and G:F independent of AGP inclusion for 21 d after weaning (Table 4.2). Compared with control, supplementing AGP without or with 0.1% NRYE improved ADG ($P < 0.05$). Independent of NRYE supplementation level, pigs receiving AGP tended to have higher ADG ($P = 0.072$) than those receiving non-AGP diets. There was an interaction between AGP and NRYE on the ATTD of DM ($P = 0.011$), CP ($P = 0.009$) and GE ($P = 0.014$). Firstly, the ATTD values were numerically higher in pigs receiving AGP with 0 or 0.1% NRYE than in those not receiving AGP. Secondly, when 0.2% NRYE was supplemented with AGP the ATTD values were reduced, whereas when the same level was supplemented in the absence of AGP, the ATTD values were increased. Increasing NRYE level in diets with AGP linearly decreased ($P < 0.05$) ATTD of DM ($P = 0.018$), CP ($P = 0.005$) and GE ($P = 0.014$), whereas in non-AGP diets it tended to quadratically increase ATTD of DM ($P = 0.058$) and GE ($P = 0.067$).

In this study, supplementing 0.1% NRYE without AGP had no effect on growth performance. This is consistent with results of Lee et al. (2007) and Martinez-Puig et al. (2007), who reported no effect of supplementing NRYE at 0.075 to 0.1% on growth performance of piglets. Sauer et al. (2012b) fed pigs a mixture of pure nucleotides (5'AMP, 5'CMP, 5'GMP, 5'IMP, and 5'UMP) and reported improvements in ADFI but not on ADG, whereas Zomborszky-Kovacs et al. (2000) using a product having only purified adenine and

Table 4.2. Growth performance and ATTD of DM, CP and GE of pigs fed diets containing 0, 0.1 or 0.2% NRYE without or with AGP

			Growth performance criteria[1]			ATTD, %		
Diet	AGP	NRYE (%)	ADFI, g/d	ADG, g/d	G:F, g/g	DM	CP	GE
1	-	0	515	365	0.71	79.8	75.7	78.8
2	-	0.1	571	439	0.77	77.9	74.2	76.6
3	-	0.2	561	417	0.74	81.0	77.2	79.7
4	+	0	618	460	0.75	81.3	78.9	80.4
5	+	0.1	614	463	0.76	81.1	78.8	80.1
6	+	0.2	571	425	0.74	78.8	74.7	77.5
SEM			38.4	27.2	0.027	0.84	1.11	0.92
AGP × NRYE			NS	NS	NS	0.011	0.009	0.014
Contrasts[2]								
AGP -	Linear		NS	NS	NS	NS	NS	NS
	Quadratic		NS	NS	NS	0.058	NS	0.067
AGP +	Linear		NS	NS	NS	0.018	0.005	0.014
	Quadratic		NS	NS	NS	NS	0.078	NS
Diet 1 vs. 2			NS	NS	NS	NS	NS	NS
Diet 1 vs. 3			NS	NS	NS	NS	NS	NS
Diet 1 vs. 4			0.092	0.033	NS	NS	0.057	NS
Diet 1 vs. 5			NS	0.029	NS	NS	0.062	NS
Diet 1 vs. 6			NS	NS	NS	NS	NS	NS
Diet 2 vs. 3			NS	NS	NS	0.015	0.007	0.024
Diet 2 vs. 4			NS	NS	NS	0.008	0.007	0.007
Diet 3 vs. 4			NS	NS	NS	NS	NS	NS

[1] ADFI = average daily feed intake; ADG = average daily gain; G:F = gain to feed ratio

[2] AGP - = diets without AGP; AGP + = diets with AGP; Linear and quadratic contrasts were determined for AGP - and AGP + treatments with 0, 0.1 or 0.2% NRYE; NS = not significant at $P > 0.10$.

uracil bases and Weaver and Kim (2014) using a semi-purified product rich in inosine 5'monophosphate reported significant improvements in both ADFI and ADG of piglets. The inconsistency of the results may be due to use of products with different nucleotides profiles in these studies. Moreover, the experimental environment may also affect the results because nucleotide requirements increase in challenged conditions (Grimble and Westwood, 2001).

In this study, it was hypothesized that NRYE would have similar effect on growth performance as in-feed AGP and supplementing both NRYE and AGP may have a synergistic effect on growth performance when supplemented together. Unlike AGP supplementation, compared with control, NRYE supplementation had no significant effect on ADG. However, 0.1% and 0.2% NRYE supplementation led to a 20 (439 vs. 365 g/d) and 14% (417 vs. 365 g/d) numerical improvement over control, respectively, making piglets receiving NRYE have statistically similar ADG as those receiving AGP. Therefore, further studies, with more replicates than those used in this study are required to substantiate the effect of NRYE on growth performance of piglets. The mechanism by which nucleotides improve performance responses in piglets is not clear but it is hypothesized that they enhance development of the immune system and the intestinal mucosa because they are building blocks of DNA, RNA and ATP which are required during cell division (Grimble and Westwood, 2001; Sauer et al., 2011). Dietary supplementation with nucleotides is more important for enterocytes and other cells with a high rate of replication and a low level of *de novo* nucleotide synthesis (Cosgrove, 1998). By enhancing cell growth, the intestinal mucosa and immune system mature faster thus reducing the stress that the animals may suffer as a result of various enteric diseases or post-weaning stress.

In this study, the effect of supplementing NRYE and AGP on the ATTD of CP, DM and GE was investigated because transition from milk to solid feed imparts stress to the development and function of the immature gut, which may result in slow maturation of the

gut mucosa and consequently insufficient secretion of brush border digestive enzymes. Moreover, the secretion of digestive enzymes by the pancreas is influenced by the nucleotide pool in pancreatic cells since nucleotides are needed for RNA synthesis which precedes protein synthesis (Schulz and Stolze, 1980). Together, these factors may result in poor digestion and absorption of nutrients thus contributing to the growth depression observed after weaning. Studies have shown that AGP (Jensen et al., 2014) and NRYE (Martinez-Puig et al., 2007) increase villous height and villous/crypt ratio in the small intestine of pigs thereby enhancing nutrient absorption. The results of this study show an interaction between AGP and NRYE on the ATTD of DM, CP and GE suggesting that increasing the NRYE level in the diet in the presence of AGP reduced the ATTD values of DM, CP and GE, whereas increasing the NRYE level in the diet in the absence of AGP increased the ATTD values of DM, CP and GE. Supplementing both AGP and 0.1% NRYE to the diet tended to improve the ATTD of CP but studies where AGP and NRYE were simultaneously studied in pigs are not available to compare with our results. Moreover, digestibility studies involving supplemental NRYE in pigs are not available to compare with our results except that of Sauer et al (2012) which reported no effects of pure nucleotides on apparent ileal digestibilities of CP, DM and crude fiber in piglets.

It is worth noting that a yeast extract was the nucleotides' source in this study. The product also contains yeast cell wall polysaccharides (21.6%) which may help in modulation of mucosal immunity and provision of binding sites for pathogens hence mediating their elimination from the gut (Kogan and Kocher, 2007). Therefore, the beneficial effects of the NRYE may be multi-faceted and cannot be exclusively attributed to the effect of nucleotides present in the product (Sauer et al., 2012b).

In conclusion, compared with control, supplementing AGP without or with 0.1% NRYE improved ADG, supplementing AGP with 0.1% NRYE tended to improve ADFI,

whereas supplementing 0.1% or 0.2% NRYE without AGP resulted in similar growth performance as AGP. Further studies using nutritional, sanitary or disease challenge models, which may increase nucleotides requirements in the piglets are needed to substantiate the importance of supplemental NRYE in improving piglet growth performance post-weaning and their effectiveness towards feeding AGP-free diets. These results show the importance of supplemental NRYE in improving piglet growth performance post-weaning and may be beneficial to producers and nutritionists desiring to adapt AGP-free feeding programs.

CHAPTER FIVE

MANUSCRIPT II

Dose-response effects of in-feed antibiotics on growth performance and nutrient utilization in weaned pigs fed diets supplemented with yeast-based nucleotides[2]

S. M. Waititu[1], J. M. Heo[1,a], R. Patterson[2], and C. M. Nyachoti[1],

[1]Department of Animal Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2; and [2]Canadian Bio-systems Inc., Calgary, AB, Canada T2C 0J7.

Present address:

[a]Department of Animal Science and Biotechnology, Chungnam National University, Daejon 305-764, South Korea.

Author contributions:

SMW wrote the research proposal. All author's participated in the review and approval of the design of the experiment. SMW carried out the animal studies, sample collection and analysis. JMH assisted in monitoring animals and sample collection. SMW carried out statistical analysis and prepared the manuscript. All authors participated in the interpretation of the data, review and approval of the final manuscript.

[2] The material presented in chapter five of this thesis has been published in Animal Nutrition: Waititu, S. M., J. M. Heo, R. Patterson, and C. M. Nyachoti. 2015. Dose-response effects of in-feed antibiotics on growth performance and nutrient utilization in weaned pigs fed diets supplemented with yeast-based nucleotides. Animal Nutrition. 1(3), 166-169.

5.1 ABSTRACT

Dietary nucleotides are bioactive compounds with the potential to mitigate weaning-associated challenges in piglets. An experiment was conducted to determine the interaction effect of AGP and NRYE on growth performance and ATTD of DM, CP and GE, and to establish whether NRYE supplementation may completely or partially replace AGP in diets for weaned pigs. In phase 1 and 2, corn, wheat, canola meal and soybean meal based diets, which were formulated to contain 0.0 or 0.1% NRYE with 0, 25, 50, 75 or 100% of the recommended AGP dosage, were fed to 108 twenty-one day old piglets (initial body weight 7.11 ± 0.9 kg; mean ± SD) from d 1 to 14 and 15 to 28, respectively. Overall, increasing AGP level in NRYE supplemented diets linearly decreased ADG (P = 0.002) and G:F (P = 0.007); and quadratically decreased ATTD of DM (P = 0.001), CP (P = 0.003) and G:F (P = 0.017) during phase 2. Compared with control and pigs fed NRYE with 100% of recommended AGP dosage, pigs fed 0.1% NRYE without AGP had greater (P < 0.05) ADG and G:F in phase 2 and overall. In conclusion, supplementing 0.1% NRYE improved growth performance of pigs but this beneficial effect was reduced by increasing dietary AGP dosage.

Key words: antimicrobial growth promoters; digestibility; growth performance; piglet; nucleotide-rich yeast extract

5.2 INTRODUCTION

Sub-therapeutic levels of AGP have been supplemented in pig starter diets to mitigate proliferation of enteropathogenic bacteria associated with post-weaning diarrhea (Heo et al., 2013). However, public pressure to eliminate the use of in-feed AGP from livestock feed has intensified because of their potential association with the development of microbial antibiotic resistance in humans and livestock (Heuer et al., 2006), and environmental contamination (Carlson et al., 2004). Furthermore, pork products sourced from pigs raised under AGP-free

regimen are on high demand in several major international markets. Therefore, there is an urgent need for viable alternatives to dietary antibiotics (Choct, 2001; Heo et al., 2013; Gong et al., 2014).

Dietary nucleotides have been suggested as a potential alternative therapy to in-feed AGP in mitigating post-weaning stress in piglets. Sauer et al. (2011) reviewed the role of dietary nucleotides in piglets and showed that they may have beneficial effects on intestinal morphology and function, intestinal microbiota, immune function, nutrient metabolism, and hepatic morphology and function. Zomborszky-Kovacs et al. (2000) and Weaver and Kim (2014) reported that dietary nucleotides supplementation improved the growth performance of weaned piglets. However, studies that simultaneously compare the effectiveness of dietary nucleotides and AGP are lacking thereby making it difficult to substantiate the role of nucleotides as alternatives to AGP. Nucleotides can be supplemented in pure forms or as NRYE, the latter being more affordable than the former.

In Manuscript I, it was concluded that supplementing 0.1 or 0.2% NRYE without AGP resulted in similar growth performance as AGP. Hence, 0.1% NRYE inclusion level was chosen for the succeeding studies. The aim of this study was to determine the effect of supplementing 0.1% NRYE to diets containing graded levels of AGP on piglet growth performance and ATTD of DM, CP and GE, and to establish whether NRYE supplementation may completely or partially replace AGP in piglet starter diets.

5.3 MATERIALS AND METHODS

All experimental procedures were reviewed and approved by the University of Manitoba Animal Care Committee (protocol number F08-033/1/2/3), and pigs were handled in accordance with the guidelines described by the Canadian Council on Animal Care (2009).

Animals and housing

One-hundred and eight [Duroc × (Yorkshire × Landrace)] male and female piglets, weaned at 21d and with an initial average body weight of 7.11 ± 0.9 kg were used in a 4-wk study. The pigs were housed in an environmentally controlled nursery building with pens equipped with a feeder, a nipple drinker, and plastic-covered expanded metal floors. The room temperature was maintained at 29 ± 1°C in wk 1, and then gradually decreased by 1ºC every week thereafter.

Experimental design

On d 1, pigs were randomly assigned to the dietary treatments based on the initial body weight with 3 pigs per pen and 6 replications per treatment. Piglets were fed a standard phase 1 and 2 diet (Table 5.1) without or with 0.1% NRYE from d 1 to 14, and d 15 to 28, respectively. Diets with 0.1% NRYE were supplemented with 0, 25, 50, 75 and 100% of the recommended AGP dosage in each phase. The NRYE supplement, Maxi-Gen Plus, was supplied by Canadian Bio-Systems Inc. (Calgary, AB, Canada) and had similar composition as that described in Manuscript 1. The AGP supplements, Aueromycin and Tiamulin, were supplied by Bio Agri Mix LP (Mitchell, ON, Canada) and were included following the Canadian Food Inspection Agency recommended dosage of 55 mg of Aueromycin (chlortetracycline) and 31.2 mg of Tiamulin per kg of diet. All diets were fed as mash and contained 0.3% titanium dioxide as an indigestible marker. Feed disappearance and body weight were recorded weekly. Representative freshly voided fecal samples were collected over the last 3 d of wk 2 and 4, and stored at -20°C until required for analysis.

Sample preparation and chemical analyses

Fecal and diet samples were processed and analyzed for DM, GE, CP, Ca (diets only), total P (diets only) and Ti content as described in Manuscript I, except that the feces were pooled for each pen and week of collection.

Table 5.1. Ingredient and chemical composition of the basal diets, as-fed basis

Item	Phase 1[1]	Phase 2[2]
Ingredient		
Corn	25.90	38.35
Wheat	13.75	21.90
Canola meal	10.00	10.00
Soybean meal	20.00	23.85
Fish meal	5.00	0.00
Dried whey	20.00	0.00
Vegetable oil	2.50	2.35
Limestone	0.60	0.80
Monocalcium phosphate	0.60	1.00
Iodized salt	0.25	0.25
Vitamin-trace mineral premix[3]	1.00	1.00
L-Lys·HCl	0.10	0.20
Titanium dioxide	0.30	0.30
Calculated nutrient content		
DE, kcal/kg	3,472	3,450
CP, %	22.7	20.9
Ca, %	0.80	0.70
Total P, %	0.70	0.69
Available P	0.50	0.33
SID[4] AA, %		
Lys, %	1.40	1.24
Met, %	0.40	0.34
Thr, %	0.90	0.78
Analyzed composition		
CP, %	22.1	20.8
Ca, %	0.90	0.79
Total P, %	0.75	0.70

[1] Phase 1 diet was fed to pigs from d 1 to 14.
[2] Phase 2 diet was fed to pigs from d 15 to 28.
[3] Premix provided per kilogram of complete diet: 9,000 IU of vitamin A; 1,500 IU of vitamin D_3; 18 mg of vitamin E; 1.5 mg of vitamin K; 250 mg of choline; 30 mg of niacin; 27.5 mg of calcium pantothenate; 9.4 mg of riboflavin; 2 mg of pyridoxine; 25 µg of cyanocobalamin; 80 µg of biotin; 0.5 mg of folic acid; 18 mg of Cu from copper sulfate, 110 mg of Zn from zinc oxide, 0.2 mg of I from calcium iodide, 110 mg of Fe from ferrous sulfate, 50 mg of Mn from manganese dioxide, and 0.3 mg of Se from sodium selenite.
[4] SID = standardized ileal digestible.

Calculations and statistical analysis

The digestibility of DM, CP and GE were calculated using the equation described in Manuscript I. Data were analyzed using the mixed procedure of SAS (SAS Inst., Inc., Cary, NC). Initial body weight was used as a covariate for analyses of ADG data. Pre-planned contrasts were used to compare pigs receiving 0.1% NRYE with control and those offered 100% recommended AGP dosage. Linear and quadratic effects were determined using orthogonal polynomial contrasts and differences were considered significant at $P < 0.05$.

5.4 RESULTS AND DISCUSSION

The 0.1% inclusion level of NRYE was selected based on the study of Martinez-Puig et al. (2007) and preliminary studies in our laboratory (Waititu et al., 2013) showing that supplementing NRYE at levels above 0.1% does not have any added benefits to the performance of piglets. In this study, graded levels of the AGP recommended dosage were used to study the potential of NRYE to partially or completely replace AGP in piglet diets.

In phase 2 and overall, pigs fed 0.1% NRYE without AGP had higher ($P < 0.05$) ADG and G:F than control pigs (Table 5.2). This observation contradicts the studies of Lee et al (2007) who supplemented 0.1% NRYE and Martinez-Puig et al. (2007) who supplemented 0.075 and 0.1% NRYE and reported no effect of supplementing NRYE on growth performance. However, the results support the studies of Zomborszky-Kovacs et al. (2000) who supplemented pure uracil and adenine at 0.5% each and Weaver and Kim (2014) who supplemented a mixture of nucleotides high in inosine at 0.02, 0.05, or 0.1% inclusion level and reported significant linear improvement in ADG and ADFI as dietary nucleotides increased. Nucleotides are thought to improve performance responses in piglets by enhancing the development of the immune cells and the intestinal mucosa because they are building blocks of DNA, RNA and ATP, which are required during cell division (Grimble and Westwood 2001; Sauer et al. 2011). Dietary supplementation of nucleotides is more

Table 5.2. Growth performance of pigs fed diets without or with 0.1% NRYE with graded levels of AGP, phase 1 is from d 1 to 14, phase 2 is from d 15 to 28[1]

			ADFI, g/d			ADG, g/d			G:F, g/g		
Diet	NRYE, %	AGP, %[2]	Phase 1	Phase 2	Overall	Phase 1	Phase 2	Overall	Phase 1	Phase 2	Overall
1	0	0	369	701	535	282	442	362	0.77	0.64	0.67
2	0.1	0	416	685	551	314	560	437	0.76	0.83	0.80
3	0.1	25	415	662	538	328	491	410	0.79	0.77	0.77
4	0.1	50	416	716	566	345	408	376	0.83	0.58	0.67
5	0.1	75	416	691	554	310	469	389	0.75	0.69	0.71
6	0.1	100	395	670	533	278	412	345	0.70	0.62	0.65
Pooled SEM			22.8	38.2	21.4	19.7	32.6	21.2	0.028	0.06	0.04
Contrasts [3]											
Linear			NS	NS	NS	NS	0.002	0.002	NS	0.007	0.006
Quadratic			NS	NS	NS	0.069	NS	NS	0.012	NS	NS
Diet 2 vs. 1			NS	NS	NS	NS	0.017	0.019	NS	0.035	0.040
Diet 2 vs. 6			NS	NS	NS	NS	0.004	0.005	NS	0.021	0.016

[1] ADFI = average daily feed intake; ADG = average daily gain; G:F = gain to feed ratio; SEM = standard error of mean; NS = not significant.
[2] 100% of recommended AGP dosage = 55 mg of Aueromycin (chlortetracycline) and 31.2 mg of Tiamulin per kg of diet.
[3] Linear and quadratic contrasts were determined for treatments supplemented with 0.1% NRYE; preplanned contrasts were determined between Diet 2 vs. 1 and Diet 2 vs. 6.

important for enterocytes and other cells with a high rate of replication and a low level of *de novo* nucleotide synthesis (Cosgrove, 1998). By enhancing cell growth, the intestinal mucosa and immune system mature faster thus reducing the stress that the animals may suffer as a result of various enteric diseases or post-weaning stress. Additionally, because nucleotides aid in tissue development, their effect is considered long-lasting (Singhal et al., 2010).

During phase 2 and overall, pigs fed 0.1% NRYE without AGP had greater ($P < 0.05$) ADG and G:F than pigs fed 0.1% NRYE with 100% of recommended AGP dosage (Table 5.2). This implies that increasing AGP level in NRYE supplemented diets suppressed the beneficial effects of NRYE. This is further supported by the results showing that increasing AGP level in diets with 0.1% NRYE linearly decreased ADG and G:F during phase 2 (P = 0.002 and 0.007, respectively) and overall (P = 0.002 and 0.006, respectively). To the best of our knowledge, we could not find studies to compare with these observations because studies that simultaneously examine the effect of AGP and NRYE are lacking.

The beneficial effect of AGP was observed in phase 1 showing that increasing AGP level in diets with 0.1% NRYE quadratically increased ADG (P = 0.069) and G:F (P = 0.012; Table 5.2), and in phase 2 showing that increasing AGP level in diets with 0.1% NRYE, quadratically increased ATTD of DM (P = 0.001), CP (P = 0.001) and GE (P = 0.017; Table 5.3). In both cases, the highest values of the measured parameters were associated with 25 and 50% supplementation of the recommended AGP dosage implying that supplementing 0.1% NRYE can replace in-feed AGP dosage by 75% without suppressing performance and nutrient utilization. The effect of supplementing NRYE and AGP on the ATTD of CP, DM and GE was investigated because transition from milk to solid feed imparts stress to the development and function of the immature gut (van Beers-Schreurs et al., 1998), which may result in slow maturation of the gut mucosa and consequently insufficient secretion of

Table 5.3. Apparent total tract digestibility of DM, CP and GE of pigs fed diets without or with 0.1% of a NRYE with graded levels of AGP, phase 1 is from d 1 to 14, phase 2 is from d 15 to 28[1]

Diet	NRYE (%)	AGP[2] (%)	ATTD of DM, %		ATTD of CP, %		ATTD of GE (%)	
			Phase 1	Phase 2	Phase 1	Phase 2	Phase 1	Phase 2
1	0	0	77.2	77.7	70.0	72.6	74.8	76.7
2	0.1	0	73.5	76.3	66.7	70.9	71.7	74.9
3	0.1	25	74.0	78.9	62.8	76.5	71.6	77.4
4	0.1	50	77.0	78.1	65.7	74.0	75.3	76.9
5	0.1	75	71.7	77.3	61.7	73.3	69.3	75.8
6	0.1	100	73.8	75.8	65.4	70.5	71.6	74.5
Pooled SEM[3]			1.52	0.83	2.15	1.17	1.73	0.92
Contrast[3]								
Linear			NS	NS	NS	NS	NS	NS
Quadratic			NS	0.001	NS	0.003	NS	0.017
Diet 2 vs. 1			NS	NS	NS	NS	NS	NS
Diet 2 vs. 6			NS	NS	NS	NS	NS	NS

[1] SEM = standard error of mean; NS = not significant.
[2] 100% of recommended AGP dosage = 55 mg of Aueromycin (chlortetracycline) and 31.2 mg of Tiamulin per kg of diet.
[3] Linear and quadratic contrasts were determined for treatments supplemented with 0.1% NRYE; preplanned contrasts were determined between Diet 2 vs. 1 and Diet 2 vs. 6.

digestive enzymes. Together, these factors may result in poor digestion and absorption of nutrients thus contributing to the growth depression observed after weaning.

It is worth noting that the NRYE product used in this study also contained yeast cell wall polysaccharides (21.6%) which may help in the modulation of mucosal immunity (Kogan and Kocher, 2007). Therefore, the beneficial effects of the NRYE may be multi-faceted and cannot be exclusively attributed to the effect of nucleotides present in the product (Sauer et al., 2012b). Furthermore, although the findings of this study shows that AGP dosage can be reduced when NRYE is supplemented to diets, there are concerns that lower antibiotic dosages may enhance development of drug resistance (Olofsson and Cars, 2007). However, the interactive effect of NRYE and reduced AGP dosage on gut microbioata and drug resistance has not been investigated. Moreover, pharmacological studies have not fully established optimal antibiotic dosing strategies that can be used to simultaneously treat bacterial infections and prevent emergence of resistance (Olofsson and Cars, 2007). Consequently, the concerns of reducing the AGP dosage when NRYE are supplemented requires substantiation by further studies.

In conclusion, dietary NRYE supplementation improved growth performance but increasing AGP level reduced the beneficial effect of NRYE. The NRYE can be supplemented alone or with 25% of the recommended AGP dosage without affecting growth performance and nutrient utilization in weaned pigs. However, further studies with more replicates are needed to substantiate these results and the importance of supplemental NRYE in improving piglet growth performance post-weaning and their effectiveness towards feeding AGP-free diets. Overall, these results show the importance of supplemental NRYE in improving piglet growth performance post-weaning and may be beneficial to producers and nutritionists desiring to adapt AGP-free feeding programs.

CHAPTER SIX

MANUSCRIPT III

Dietary supplementation with a nucleotide-rich yeast extract modulates gut immune response and microbiota in weaned pigs in response to a sanitary challenge[3]

Samuel M. Waititu[1], Fugui Yin[1], Rob Patterson[2], Alexander Yitbarek[3], Juan C. Rodriguez-Lecompte[4] and Charles M. Nyachoti[1*]

[1]Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada R3T 2N2, [2]Canadian Bio-systems Inc., Calgary, AB, Canada T2C 0J7, [3] Department of Pathobiology, University of Guelph, Guelph, ON, Canada, N1G 2W1, [4]Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Prince Edward Island, Canada C1A 4P3.

Authors' contributions:

SMW wrote the research proposal. All author's participated in the review and approval of the design of the experiment. SMW carried out the animal studies, sample collection and analysis. FY and AY assisted in carrying out the qPCR analysis. SMW carried out statistical analysis and prepared the manuscript. All authors participated in the interpretation of the data, review and approval of the final manuscript.

[3] The material presented in chapter six of this thesis has been submitted to the Animal Journal.

6.1 ABSTRACT

An experiment was carried out to evaluate the effect of supplementing a NRYE on growth performance, gut structure, immunity and microbiota of piglets raised in sanitary and unsanitary conditions. Unsanitary conditions were created in this study to mimic an environment of increased microbial load such as that often present in commercial farms, which could induce stress to the immature piglet immune system and consequently enhance the effect of supplemental nucleotides. Eighty-four, 21-d old piglets housed 3 per pen were fed a corn-soybean meal based diet without or with 0.1% NRYE. Feed disappearance and body weight were recorded on d 1, 14 and 28. On d 14, one pig per pen was euthanized to collect ileum, mesenteric lymph nodes (MLN) and spleen tissues, and cecum and colon digesta. Overall, NRYE supplementation improved ADFI (P = 0.034) in the clean room, kidney weight in both clean (P = 0.0002) and unclean room (P < 0.0001) and tended to improve the villus height/crypt depth ratio in the clean room (P = 0.073). Ileal programmed cell death gene-1 (P = 0.0003), IL-1β (P < .0001), IL-6 (P = 0.0003), IL-10 (P < 0.0001) and TNF-α (P < 0.0001) were upregulated in pigs receiving NRYE in the unclean room. Supplementing the NRYE in the clean room suppressed growth of cecal Enterobacteriacea (P < 0.001) members and colonic *Enterococcus* spp. (P < 0.019), improved proliferation of cecal *Lactobacillus* (P < 0.002) and colonic *Clostridium* cluster IV (P < 0.011) and XVIa members (P < 0.0002). Supplementing the NRYE in the unclean room improved proliferation of cecal *Clostridium* cluster IV (P < 0.026) and suppressed proliferation of colonic *Enterococcus* spp. (P < 0.037). In conclusion, supplementing the NRYE to piglets under unsanitary conditions improved ileal immune response by upregulating inflammatory cytokines, and positively modulated proliferation of gut bacteria considered beneficial to gut health and suppression of bacteria considered harmful to gut health.

6.2 INTRODUCTION

Dietary nucleotides are bioactive compounds with the potential to mitigate weaning-associated challenges in piglets (Sauer et al., 2011). The presence of nucleotides in sow milk has stimulated research on their importance in regulating growth performance, and development of gut immunity and microbiota in piglets. Various studies have suggested that under stressful conditions such as diseases or malnutrition, the beneficial effects of dietary nucleotides on growth performance and development of gut immunity and a healthy microbiota will be more pronounced (Grimble and Westwood, 2001; Yu et al., 2002; Sauer et al., 2011). Hence, nucleotides are viewed as conditionally essential nutrients in tissues requiring rapid cell replication (Van Buren and Rudolph, 1997). Dietary nucleotides may be more essential for intestinal epithelium and lymphoid cells, which lack significant capacity for *de novo* synthesis of nucleotides (Uauy et al., 1994). In addition, it has been reported that dietary nucleotides may positively transform the gut microbial profile of piglets. For instance, Mateo (2005) reported higher fecal counts of beneficial *Lactobacillus* and *Bifidobacteria* and lower counts of *Clostridum perfringens* in piglets fed diets supplemented with nucleotides.

Weaning deprives piglets of essential nutrients, immunoglobulins and nucleotides found in sow milk. Compounded feeds have lower concentrations of nucleotides compared with sow milk (Mateo, 2005). Consequently, the physiological systems of the piglet dependent on nucleotides such as cell division, ATP energy and co-enzymes activation are negatively impacted (Sauer et al., 2011). In this regard, nucleotides are deemed conditionally helpful in mitigating the challenges piglets are predisposed to immediately after weaning (Grimble and Westwood, 2001; Mateo, 2005; Sauer et al., 2011). However, there is a paucity of studies showing the effects of dietary nucleotides in weaned pigs raised in stressful, unsanitary or disease conditions, which represent conditions pigs face in conventional farms. Unsanitary conditions were created in this study to mimic an environment of increased

microbial load such as that often present in commercial farms, which could induce stress to the immature piglet immune system and consequently enhance the effect of supplemental nucleotides as suggested by Grimble and Westwood (2001), and Sauer *et al.* (2011). Therefore, it was hypothesized that in pigs raised under unsanitary conditions, supplemental NRYE will enhance their growth performance, gut structure, immunity and microbiota composition. The aim of the present study was to evaluate the effect of dietary NRYE supplementation on growth performance, gut structure, immunity and microbiota in response to a sanitary challenge.

6.3 MATERIALS AND METHODS

All experimental procedures were reviewed and approved by the University of Manitoba Animal Care Committee (protocol number F13-002), and pigs were handled in accordance with the guidelines described by the Canadian Council on Animal Care (2009).

Animals and housing

A total of eighty-four [Duroc × (Yorkshire × Landrace)] piglets (male and female), weaned at 21d of age with an initial average body weight of 7.11 ± 0.90 kg, were randomly allocated on the basis of body weight to four treatments in a 2 x 2 factorial arrangement of diet (0 or 0.1% NRYE) and sanitation (clean or unclean room). There were 7 replicate pens per treatment each with 3 piglets. One group was raised in a room designated as the clean room that was washed once per week, whereas the second group was raised in a room designated as the unclean room in which 7 kg of manure from a sow herd was spread on each pen floor on d 1 and d 7 and the room was not washed throughout the experiment. Each room had 14 pens equipped with a feeder, a suspended water line fitted with a low-pressure nipple, and plastic-covered expanded metal floors. Temperature in both rooms was maintained at 29 ± 1°C in wk 1, and then gradually decreased by 1ºC every wk thereafter.

Dietary treatments

The dietary treatments were a corn-soybean meal-based-diet fed without or with 0.1% NRYE. The NRYE supplement, Maxi-Gen Plus, was supplied by Canadian Bio-systems Inc (Table 6.1). (Calgary, AB, Canada) and contained cell wall polysaccharides (21.6%), CP (32.7%), carbohydrates (14.3%) and a mixture of 5 nucleotides (1.1%; adenosine monophosphate, cytosine monophosphate, inosine monophosphate, uridine monophosphate and guanosine monophosphate), with 1 g of the NRYE additive supplying approximately 1000 ppm of mixed nucleotides. The diets were formulated to meet or exceed the NRC (2012) requirement specifications for weanling pigs, and were fed in a mash form.

Sampling and measurements

Feed consumption and body weight were recorded biweekly. On d 14, one pig per pen was euthanized. The spleen, liver and kidneys were removed, blotted dry and weighed. A 2 cm section of distal ileum was collected and fixed with 10% buffer neutral formalin for morphological evaluation. Approximately 2 g of spleen and mesenteric lymph node (MLN) and another 2 cm of distal ileum were collected and immediately frozen in liquid nitrogen, and stored at -80°C for RNA extraction and gene expression analysis. About 5 g of the cecum and colon digesta were also collected and frozen in liquid nitrogen, and transferred to -80°C until required for bacterial genomic DNA extraction.

Sample preparation and chemical analyses

Dietary samples were processed and analyzed for CP, Ca and total P as described in manuscript I (see section 4.3).

Histomorphology measurements

The formalin-fixed ileum tissues were processed at the Veterinary Diagnostic Services Laboratory (Winnipeg, Manitoba, Canada). Villus height and crypt depth were measured at 10× magnification using Axiostar Plus microscope (Carl Zeiss, Oberkochen,

Table 6.1. Ingredient and chemical composition of the basal diets, as-fed basis

Item	Phase 1[1]	Phase 2[2]
Ingredient		
Corn	25.25	35.00
Wheat	19.00	22.00
Soybean meal	20.00	26.00
Canola meal	5.00	10.00
Fish meal-H	5.00	0.00
Dried Whey	20.00	0.00
Vegetable Oil	3.00	3.50
Limestone	0.83	1.10
Monocalcium phosphate	0.14	0.62
Iodized Salt	0.26	0.26
Vitamin-trace mineral premix[3]	1.00	1.00
L-Lys·HCl	0.35	0.35
DL-Methionine	0.10	0.10
Threonine	0.07	0.07
Calculated nutrient content		
DE (kcal/kg)	3573	3537
CP, %	22.3	22.4
Ca, %	0.80	0.70
Total P, %	0.65	0.60
Available P, %	0.43	0.33
SID Lys, %	1.36	1.25
SID Met, %	0.43	0.41
SID Thr, %	0.79	0.74
Analyzed composition		
CP, %	23.2	23.4
Ca, %	0.84	0.65
Total P, %	0.78	0.62

[1]Phase 1 diet: was fed to pigs from d 1 to 14.
[2]Phase 2 diet: was fed to pigs from d 14 to 28.
[3]Provided the following per kilogram of complete diet: 9,000 IU of vitamin A; 1,500 IU of vitamin D_3; 18 mg of vitamin E; 1.5 mg of vitamin K; 250 mg of choline; 30 mg of niacin; 27.5 mg of calcium pantothenate; 9.4 mg of riboflavin; 2 mg of pyridoxine; 25 µg of cyanocobalamin; 80 µg of biotin; 0.5 mg of folic acid; 18 mg of Cu from copper sulfate, 110 mg of Zn from zinc oxide, 0.2 mg of I from calcium iodide, 110 mg of Fe from ferrous sulfate, 50 mg of Mn from manganese dioxide, and 0.3 mg of Se from sodium selenite.

Germany) equipped with a camera (Canon Canada Inc., Mississauga, Ontario, Canada) and ImageJ software (National Institutes of Health, Bethesda, MD) in at least 15 well oriented villus and crypt columns of each tissue sample. Villi height was measured from the top of the villus to the start of the crypt, whereas crypt depth was measured from the bottom end of the villi to the start of the mucosa. The villus height/crypt depth ratio was calculated.

Bacterial DNA extraction

Bacterial DNA was extracted from cecal and colonic digesta for the determination of the relative abundance of target bacteria at the genus level. Cecum and colon digesta samples were thawed before analyses and DNA was extracted (QIAmp DNA Stool mini Kit; QIAGEN, Hilden, Germany) according to the manufacturer's protocol for stool pathogen detection as described by Li et al. (2003).

Total RNA extraction and cDNA synthesis

Total RNA of ileum, MLN and spleen samples was extracted. Briefly, approximately 100 mg of tissue sample was placed in a 2 ml tube containing 0.5 ml of 0.1 mm silica beads and 1 ml TRIZOL reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada) and subjected to bead beating with PowerLyzer (MO BIO Laboratories, Inc., Canada) for 2 min at homogenization level 2,500, and then chilled on ice for 5 min. The above procedure was repeated once. Total RNA was then extracted, washed, and eluted following the manufacturer's instructions for the TRIZOL reagent. RNA concentration and purity was measured using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). To remove genomic DNA contamination, total RNA was treated with DNase I according to the manufacturer's protocol. Two micrograms of total RNA with an optical density 260/280 ratio between 1.8 and 2.0 was used for cDNA synthesis using a High Capacity cDNA Reverse transcription Kit (Applied Biosystems, Foster City, CA, USA) with RNase inhibitor according to the manufacturer's instructions.

Quantitative PCR

Quantitative PCR for DNA and cDNA templates was carried out using a Bio-Rad CFX Real-time system (BioRad, Hercules, CA, USA). Each reaction mixture was run in duplicate using optical 96-well reaction plates (BioRad, Hercules, CA, USA). Each amplification reaction was carried out with 12.5 µl of iTaq SYBR Green Supermix (BioRad, Hercules, CA, USA) mixed with 1 µl of each primer set (Table 6.2 and 6.3), 1 µl of genomic DNA or cDNA and 9.5 µl of nuclease-free water. Negative controls were created by replacing DNA or cDNA with nuclease-free water. Amplification consisted of initial denaturation for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, primer annealing at their individual optimal temperatures (Table 6.2 and 6.3) for 30 s, and an extension step at 72°C for 30 s. After the amplification, a melting curve analysis with a temperature gradient of 0.1°C per second from 70 to 95°C was performed to confirm that only specific products were amplified. The DNA templates were pooled and serially diluted eightfold to evaluate the efficiency (E) of the amplification of each primer set (Khafipour et al., 2009; Ivarsson et al., 2014). Amplification efficiency was calculated from the slope of the standard curve generated by plotting the threshold cycle versus logarithmic values of different DNA concentrations using the equation: $E = 10^{(-1/slope)}$ (Rasmussen, 2001).

Calculations and statistical analysis

The *Sus scrofa* beta-actin gene and 16S rRNA gene of *Eubacteria* were used to normalize target genes transcript levels for immune cytokines and gut microbial groups, respectively. Nygard et al. (2007) reported that the expression of beta-actin was stable in 17 different pig tissues. The relative abundance of each target gene was analyzed using the $2^{-\Delta\Delta Ct}$ method (Arocho et al., 2006). All data were analyzed using the Mixed procedure of SAS (SAS Inst., Inc., Cary, NC). For all data analyses, significance was defined as $P < 0.05$ and $0.05 < P < 0.10$ was considered a trend.

Table 6.2. Primers used for qPCR analysis of immune cytokines

Gene	GenBank accession Reference no.	Amplicon size (bp)	T_m [1] (°C)	Primer sequence (5'-3')
IFN-γ	NM_213948.1	231	60	F: GGCCATTCAAAGGAGCATGG
				R: GCTCTCTGGCCTTGGAACAT
IL-1β	NM_214055.1	131	60	F: GCCCATCATCCTTGAAACGTG
				R: GGAGAGCCTTCAGCATGTGT
IL-6	NM_214399.1	149	60	F: TAAGGGAAATGTCGAGGCCG
				R: TCCACTCGTTCTGTGACTGC
IL-10	NM_214041.1	179	60	F: TCCGACTCAACGAAGAAGGC
				R: AACTCTTCACTGGGCCGAAG
TNF-α	NM_214022.1	120	60	F: ATTCAGGGATGTGTGGCCTG
				R: CCAGATGTCCCAGGTTGCAT
PD-1	NM_213838.1	123	60	F: ATGGTCCTGTTACCTGTGCC
				R: ACAGGTGCCGATCTGTTTCA
Beta-actin	XM_005670976.1	179	60	F: CGAGGCTCAGAGCAAGAGAG
				R: GGTTGGCCTTAGGGTTCAGG

[1] Annealing temperature.

Table 6.3. Primers used for qPCR analysis of gut bacteria groups

Target bacterial group	Amplicon size (bp)	T_m[1]	Primer sequence	Reference
Total Eubacteria	180–220	58	F: CGGYCCAGACTCCTACGGG R: TTACCGCGGCTGCTGGCAC	Lee et al. (1996)
Lactobacillus spp.	341	62	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	Walter et al. (2001)
Enterococcus spp.	144	60	F: CCCTTATTGTTAGTTGCCATCATT R: ACTCGTTGTACTTCCCATTGT	Rinttilä et al. (2004)
Enterobacteriacea	189	63	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	Bartosch et al. (2004)
Bifidobacterium spp.	243	63	F: TCGCGTCYGGTGTGAAAG R: CCACATCCAGCRTCCAC	Rinttilä et al. (2004)
Clostridium cluster XIVa	438–441	50	F: AAATGACGGTACCTGACTAA R: CTTTGAGTTTCATTCTTGCGAA	Matsuki et al. (2002)
Clostridium cluster IV	240–244	50	F: GCACAAGCAGTGGAGT R: CTTCCTCCGTTTTGTCAA	Matsuki et al. (2004)
Clostridium Cluster I	231	63	F: TACCHRAGGAGGAAGCCAC R: GTTCTTCCTAATCTCTACGCAT	Matsuki et al. (2002)

[1]Annealing temperature, °C.

6.4 RESULTS

Growth performance

All piglets in both clean and unclean conditions appeared healthy throughout the experiment. Overall, no diet × sanitation interactions were observed except for ADFI (P = 0.018; Table 6.4) showing that NRYE improved ADFI in the clean room (P = 0.034).

Carcass characteristics and ileal histomorphology

Diet × sanitation interactions were observed for villus height/crypt depth ratio (P = 0.028) showing that NRYE tended to improve the villus height/crypt depth ratio (P = 0.073) in the clean room (Table 6.5). Compared to control, supplementing NRYE increased kidney weight of piglets in both clean (P = 0.0002) and unclean (P < 0.0001) rooms.

Immune response in the ileum, mesenteric lymph nodes and spleen

Diet × sanitation interactions were only observed for the ileal expression of programmed cell death gene-1 (PD-1; P = 0.0003), IL-1β (P < 0.0001), IL-6 (P = 0.0003), IL-10 (P < 0.0001) and TNF-α (P < 0.0001) mRNA (Table 6.6). Taken together, the interactions show that NRYE supplementation upregulated the expression of these genes only in the unclean room.

Relative abundance of bacterial groups in the cecum and colon digesta

Diet × sanitation interactions were observed for changes in abundance of cecal Enterobacteriacea members (P = 0.001), *Lactobacillus* spp. (P = 0.002), and colonic *Clostridium* cluster XIVa (P = 0.002), whereas there was a tendency towards interaction for *Clostridium* cluster IV members (P = 0.002; Table 6.7). Taken together, these interactions show that NRYE supplementation suppressed growth of cecal Enterobacteriacea members (P < 0.001) and colonic *Enterococcus* spp. (P < 0.019), and improved proliferation of cecal *Lactobacillus* (P < 0.002) and colonic *Clostridium* cluster IV (P < 0.011) and XVIa (P <0.0002) members in pigs raised in the clean room. Compared with control, supplementing

Table 6.4. Growth performance of piglets fed diets without or with NRYE and raised in clean or unclean conditions

	Treatments[2]							
	Clean room		Unclean room			*P*-value[3]		
Item[1]	CON	NRYE	CON	NRYE	SEM	D × S	1	2
Phase 1								
ADFI (g/d)	262	285	280	255	16.3	NS	NS	NS
ADG (g/d)	184	202	211	195	16.9	NS	NS	NS
G:F (g/g)	0.69	0.71	0.75	0.77	0.031	NS	NS	NS
Phase 2								
ADFI (g/d)	773	844	833	795	28.7	0.075	0.086	NS
ADG (g/d)	439	459	541	537	29.7	NS	NS	NS
G:F (g/g)	0.56	0.54	0.65	0.68	0.025	NS	NS	NS
Overall								
ADFI (g/d)	518	565	557	525	15.1	0.018	0.034	NS
ADG (g/d)	311	331	376	366	11.9	NS	NS	NS
G:F (g/g)	0.63	0.62	0.70	0.72	0.014	NS	NS	NS

[1]ADG, average daily gain; ADFI, average daily feed intake; G:F, gain to feed ratio.

[2]CON = control diet; NRYE = diet containing 0.1% NRYE

[3]D × S = Diet × sanitation interaction effect; 1 = effect of NRYE in the clean room; 2 = effect of NRYE in the unclean room; NS = not significant.

Table 6.5. Live body weight (d 14), empty carcass weight, organ weights, and ileal morphology of weaner pigs fed diets without or with NRYE and raised in clean or unclean conditions

	Treatments[2]							
	Clean room		Unclean room			P-value[3]		
Item[1]	CON	NRYE	CON	NRYE	SEM	D × S	1	2
Live body weight (LBW, kg)	10.3	9.8	10.3	9.4	0.39	NS	NS	NS
Empty carcass weight (kg)	7.2	6.8	7.4	6.7	0.34	NS	NS	NS
Organ weights, g/kg of LBW								
Empty gut	92.5	91.7	88.7	94.0	1.94	NS	NS	0.064
Liver	35.2	38.3	34.5	37.5	1.86	NS	NS	NS
Kidneys	6.3	7.7	5.9	7.5	0.22	NS	0.0002	<.0001
Spleen	3.6	3.4	3.5	2.8	0.40	NS	NS	NS
Ileal morphology								
VH, µm	349	405	411	399	26.4	NS	NS	NS
CD, µm	369	371	360	393	20.3	NS	NS	NS
VH:CD	0.97	1.14	1.14	1.01	0.065	0.028	0.073	NS

[1] VH = villus height, CD = crypt depth, VH:CD = villus height/crypt depth ratio.

[2]CON = control diet; NRYE = diet containing 0.1% NRYE

[3]D × S = Diet × sanitation interaction effect; 1 = effect of NRYE in the clean room; 2 = effect of NRYE in the unclean room; NS = not significant.

Table 6.6. Mesenteric lymph node (MLN), ileal and splenal cytokine gene expression (log_2) of weaner pigs fed diets without or with NRYE and raised in clean or unclean conditions

	Treatments[1]							
	Clean room		Unclean room			P-value[2]		
Item	CON	NRYE	CON	NRYE	SEM	D × S	1	2
MLN								
IFN-γ	2.65	3.42	2.78	1.23	1.307	NS	NS	NS
PD-1	0.53	0.43	2.49	2.50	0.390	NS	NS	NS
IL-1β	0.67	0.49	0.19	0.13	0.213	NS	NS	NS
IL-6	1.79	1.56	1.04	0.83	0.232	NS	NS	NS
IL-10	1.42	0.73	1.19	0.44	0.557	NS	NS	NS
TNF-α	1.08	0.71	1.23	0.97	0.502	NS	NS	NS
Ileum								
IFN-γ	1.35	0.29	2.47	3.66	0.547	0.066	NS	NS
PD-1	1.04	1.45	1.29	3.23	0.270	0.015	NS	0.0003
IL-1B	0.73	0.48	1.77	19.00	0.586	<.0001	NS	<.0001
IL-6	1.09	1.32	1.46	6.87	0.670	0.002	NS	0.0003
IL-10	1.09	0.39	1.83	11.20	0.933	0.000	NS	<.0001
TNF-α	0.94	0.48	3.08	17.30	0.917	<.0001	NS	<.0001
Spleen								
IFN-γ	0.56	0.66	0.92	1.07	0.214	NS	NS	NS
PD-1	1.80	1.61	0.86	0.66	0.592	NS	NS	NS
IL-1B	1.70	2.42	1.84	4.73	1.121	NS	NS	NS
IL-6	1.32	1.24	1.09	1.16	0.339	NS	NS	NS
IL-10	2.24	2.45	1.58	3.92	0.958	NS	NS	NS
TNF-α	1.44	1.36	1.26	1.92	0.401	NS	NS	NS

[1]CON = control diet; NRYE = diet containing 0.1% NRYE

[2]D × S = Diet × sanitation interaction effect; 1 = effect of NRYE in the clean room; 2 = effect of NRYE in the unclean room; NS = not significant.

Table 6.7. Bacteria abundance (log_2) in the cecal and colonic contents of weaner pigs fed diets without or with NRYE and raised in clean or unclean conditions

	Treatments[1]							
	Clean room		Unclean room			P-value[2]		
Item	CON	NRYE	CON	NRYE	SEM	D × S	1	2
Cecum								
Enterococcus	1.56	0.27	0.88	0.61	0.398	NS	0.041	NS
Bifidobacteria	2.84	2.82	0.71	2.19	0.636	NS	NS	NS
Enterobacteriacea	5.72	0.53	2.50	2.29	0.862	0.017	0.001	NS
Lactobacillus	0.04	1.25	0.41	0.36	0.195	0.009	0.002	NS
Clostridium I	1.04	0.72	1.37	1.26	0.204	NS	NS	NS
Clostridium IV	0.38	0.46	1.18	2.01	0.244	NS	NS	0.026
Clostridium XIVa	0.59	0.44	0.57	0.90	0.393	NS	NS	NS
Colon								
Enterococcus	2.29	0.27	2.16	0.73	0.473	NS	0.019	0.037
Bifidobacteria	1.70	2.36	0.53	0.92	0.666	NS	NS	NS
Enterobacteriacea	4.96	1.04	5.15	3.51	2.079	NS	NS	NS
Lactobacillus	3.26	3.85	3.85	5.51	1.144	NS	NS	NS
Clostridium I	1.30	1.93	1.28	1.01	0.267	NS	NS	NS
Clostridium IV	6.89	11.84	1.75	2.40	1.056	0.070	0.011	NS
Clostridium XIVa	0.24	4.25	0.61	0.89	0.427	0.002	0.0002	NS

[1]CON = control diet; NRYE = diet containing 0.1% NRYE

[2]D × S = Diet × sanitation interaction effect; 1 = effect of NRYE in the clean room; 2 = effect of NRYE in the unclean room; NS = not significant.

the NRYE to pigs in the clean room also suppressed proliferation of cecal *Enterococcus* spp. ($P = 0.041$), whereas supplementing the NRYE to pigs in the unclean room improved proliferation of cecal *Clostridium* cluster IV ($P < 0.026$) and suppressed proliferation of colonic *Enterococcus* spp. ($P < 0.037$).

6.5 DISCUSSION

The aim of the present study was to evaluate the effect of supplementing NRYE on growth performance, gut structure, immunity and microbiota in response to a sanitary challenge. An unsanitary condition was created in this study to mimic an environment of increased microbial load such as that present in commercial farms, which will induce stress to the immature piglet immune system and consequently enhance the effect of supplemental nucleotides as suggested by Grimble and Westwood (2001), Yu et al. (2002) and Sauer et al. (2011).

In this study, supplementing NRYE did not influence weight gain and feed efficiency in both sanitary conditions. To the best of our knowledge, we could not find studies to compare with observations made in the unsanitary condition because studies testing effect of nucleotides in piglets under a sanitary challenge are lacking. However, results from the clean conditions are consistent with those of Sauer et al. (2012b) who supplemented 0.1% of a mixture of pure nucleotides, and Andrés-Elias et al. (2007) who supplemented 0.15% of yeast-based nucleotides and reported no effect of the supplements on piglet performance.

Regardless of sanitation condition, NRYE supplemented pigs had higher kidney weights than control which may indicate improved cell division due to presence of nucleotides which are DNA and RNA monomers. Kartha and Toback (1985) and Toback *et al.* (1990) reported that DNA synthesis in cultured renal epithelial cells could be stimulated by exogenous purine nucleosides and nucleotides but similar effect was not observed in fibroblasts (cells of connective tissues). The villus height/crypt depth ratio of the NRYE

receiving pigs in the clean room was increased by 18% compared to the control in the clean room. This is supported by the observation that ADFI of the NRYE receiving pigs in the clean room was 8.4% higher than that of the control in the clean room. This implies that supplemental NRYE has beneficial effects on development of gut structure partly associated with the observed increase in ADFI (Kelly et al., 1991; Pluske et al., 1997; McCracken et al., 1999). However, this beneficial effect was not observed in the pigs receiving NRYE under a sanitary challenge possibly due to the reduced ADFI (Kelly et al., 1991; Pluske et al., 1997; McCracken et al., 1999). Generally, weaning stress lowers voluntary feed intake which is partially responsible for triggering a reduction in villus height and in the villus height/crypt depth ratio (Pluske et al., 1996).

Dietary nucleotides have been suggested as immune system stimulants (Kulkarni et al., 1994; Grimble and Westwood, 2001). The mechanism by which nucleotides stimulate gut immune system is unclear, but it is mostly hypothesized that being building blocks of ATP, DNA and RNA, nucleotides promote cell division and development of the immune system of enterocytes which generally lack the capacity for *de novo* synthesis of nucleotides (Cosgrove, 1998). Kulkarni *et al.* (1994) postulated that a nucleotide-free diet causes functional impairment of T-lymphocytes in mice, possibly due to the defect in the differentiation and maturation of T lymphocytes associated with immunodeficiency.

In the present study, the effect of NRYE supplementation on the expression of IL-1β, IL-6, IL-10, TNF-α and PD-1 was localized in the ileum of pigs raised in the unsanitary conditions thus suggesting an immunostimulatory effect of NRYE. In addition, the observed upregulation of ileal IL-1β and IL-6 in NRYE receiving pigs in the unclean room suggests that under the sanitary challenge supplementing NRYE is beneficial to piglets in enhancing the expression of pro-inflammatory cytokines. This immunoresponse leads to activation of lymphocytes thereby improving host defense mechanisms that accelerate the process of

pathogen clearance (Sherman and Kalman, 2004; Medzhitov, 2007; D'Elia et al., 2013). In addition to pro-inflammation, upregulation of IL-1β is also associated with differentiation of epithelial cells at weaning (Pié et al., 2004). Interleukin-6 enhances differentiation of macrophages and type 2 T helper cells, which engulf pathogens and enhance antibody secretion, respectively (Pescovitz et al., 1998). The upregulation of ileal IL-10 in unclean room NRYE-receiving pigs suggests that the NRYE supplement has immunoregulatory effects because IL-10 inhibits the production of proinflammatory mediators and also augments the production of anti-inflammatory factors (Pescovitz et al., 1998; Khan, 2008). In addition, the upregulation of PD-1 expression as observed in unclean room NRYE-receiving pigs is an indicator of improved immunoregulation after lymphocyte activation. Generaly, PD-1 is expressed on the surface of activated T cells, B cells, and macrophages to regulate immune responses by increasing T-cell death (Sharpe et al., 2007; Peng et al., 2010). An inability to express PD-1 is associated with multiple lymphoproliferative, uncontrolled, immune responses, leading to several types of severe autoimmune disorders (Latchman et al., 2001; Liang et al., 2003).

Dietary nucleotides have been suggested to promote proliferation of beneficial gut microbiota such as *Bifidobacteria* spp. and *Lactobacillus* spp. as demonstrated by *in vitro* (Tanaka and Mutai, 1980) and *in vivo* studies with human subjects (Gil et al., 1986) and rodents (Uauy et al., 1990). Mateo (2005) reported an increase in *Bifidobacteria* spp. and *Lactobacilli* spp. and a reduction in *Clostridium* spp. in feces of piglets fed a nucleoside-containing diet. In the current study, supplementing NRYE reduced the abundance of Enterobacteriacea members in the clean room and *Enterococcus* spp. in both the clean and unclean rooms. Enterobacteriacea family members (such as *Salmonella* sp., *E. coli*, *Mycobacterium* sp., *Streptococcus* sp., *Staphylococcus* sp.) and *Enterococcus* spp. are mostly opportunistic pathogens that have adverse effects on gut health and are increasingly resistant

to antimicrobial agents (Fisher and Phillips, 2009; Baer et al., 2013). On the other hand, NRYE supplementation improved proliferation of *Lactobacillus* and *Clostridium* cluster XVIa members in pigs raised in the clean room and *Clostridium* cluster IV members in both clean and unclean room pigs. *Clostridium* cluster IV and XIVa members are butyrate producing bacteria (Levine et al., 2013). Butyrate is the main fuel for the colonocytes, has been reported to regulate gene expression leading to anti-inflammatory effects (Hamer et al., 2008) and also to posses antibacterial properties (Van Deun et al., 2008). Thus, the results imply that supplementation of 0.1% NRYE was associated with proliferation of beneficial gut bacteria and suppression of pathogenic ones, a factor attributable to both the nucleotides and yeast cell wall polysaccharides present in the NRYE supplement. This observation supports recent findings of Li et al. (2015) showing that in piglets orally challenged with *E. coli* K88, the NRYE-receiving groups had higher *Lactobacillus* fecal count and lower *E. coli* fecal count than control.

In summary, supplementing NRYE to pigs in the sanitary conditions improved feed intake and consequently the villus/crypt depth ratio. Supplementation of NRYE had no effect on weight gain and feed efficiency in both sanitary conditions. Regardless of the sanitary condition, pigs receiving NRYE had heavier kidneys, which is attributable to enhancement of cellular growth by nucleotides. Supplementing NRYE to pigs in unsanitary conditions was associated with stimulation of both pro- and anti-inflammatory cytokines and PD-1 in the ileum, which indicates enhanced and homeostatic immune response. In addition, NRYE supplementation was associated with proliferation of beneficial bacteria in pigs raised in the clean room and suppression in the growth of harmful gut bacteria in pigs raised in the unclean room, which indicates improved gut immunity and health.

In conclusion, these results show that supplementing NRYE as a source of dietary nucleotides to weaned pigs raised in unsanitary condition positively modulated their immune response and composition of gut microbiota.

CHAPTER SEVEN

MANUSCRIPT IV

Short-term effect of dietary yeast-based nucleotides on performance, immune response, blood parameters and gut structure of weaned pigs challenged with *Escherichia coli* lipopolysaccharide[4]

Samuel M. Waititu[1], Fugui Yin[1], Rob Patterson[2], Juan C. Rodriguez-Lecompte[3] and Charles M. Nyachoti[1*]

[1]*Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada R3T 2N2,* [2]*Canadian Bio-systems Inc., Calgary, AB, Canada T2C 0J7,* [3]*Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Prince Edward Island, Canada C1A 4P3.*

Authors' contributions:

SMW wrote the research proposal. All author's participated in the review and approval of the design of the experiment. SMW carried out the animal studies, sample collection and analysis. FY assisted carrying out the qPCR analysis. SMW carried out statistical analysis and drafted the manuscript. All authors participated in the interpretation of the data, review and approval of the final manuscript.

[4] The material presented in chapter seven of this thesis has been submitted to the Journal of Animal Science and Biotechnology.

7.1 ABSTRACT

This study investigated the response of piglets receiving a nucleotide-rich yeast extract (NRYE) without or with feed enzymes (ENZ) and antimicrobial growth promoters (AGP) on performance, blood cell profile, serum and ileum cytokines, and gut structure after an *E. coli* lipopolysaccharide (LPS) challenge. The LPS challenge was used to induce an immunological stress that is equivalent to a moderate clinical disease so as to evaluate the effect of dietary NRYE supplementation on piglet health and performance during the immediate post-weaning period. Thirty-six pigs were allotted to 6 diets including: a non-challenged control (NCC); LPS-challenged control (CC); CC + AGP; CC + NRYE; CC + ENZ; and CC + ENZ + NRYE. On day 7, pigs were bled and thereafter injected with LPS or sterile saline. Blood samples were collected at 6, 48, and 96 h post-challenge (hpc). After 96 hpc, pigs were euthanized to obtain duodenum, jejunum and ileum tissues. Overall (d 1 to 11), NRYE supplementation tended to attenuate LPS-induced reduction in G:F ($P = 0.096$), but CC + AGP pigs had higher body weight ($P = 0.04$) and ADG ($P = 0.03$) than CC + NYRE pigs. At 6 hpc, CC + NRYE and CC + ENZ + NRYE pigs had lower ($P < 0.05$) plasma urea N (PUN) and higher ($P < 0.05$) platelet count than CC pigs. At 96 hpc, LPS-induced villus atrophy in the jejunum and duodenum was reduced ($P < 0.05$) in CC + ENZ + NRYE pigs. At 6 hpc, serum TNF-α and IL-10 concentration was reduced ($P < 0.05$) and increased ($P < 0.05$), respectively, in CC + AGP, CC + NRYE, and CC + ENZ + NRYE pigs compared with CC pigs. At 96 hpc, compared with CC, ileal TNF-α expression was lowered in CC + NRYE ($P = 0.01$) and CC + ENZ + NRYE ($P = 0.01$) pigs, whereas ileal IL-1β and IL-10 expression was lower in all other treatments ($P < 0.05$). In conclusion, LPS challenged piglets receiving NRYE or ENZ + NRYE showed beneficial responses in gut structure, blood cell profile and immunity commensurate with antibiotics, though the latter had better overall growth performance.

7.2 INTRODUCTION

Following the ban on sub-therapeutic use of antibiotics in animal feed in the European Union (Casewell et al., 2003) and the rising pressure for the same action in USA and Canada (Kondro, 2002), the need for potential antibiotic alternatives that can promote piglet growth and health has soared up. The abundance of nucleotides in sows milk has necessitated research on their role in piglet growth and development (Sauer et al., 2011). Supplementation of nucleotides in piglet starter diet is deemed prudent in replenishing the nucleotide pool sourced from sows milk before weaning (Mateo et al., 2004).

During early weaning, the piglet requires sufficient supply of nutrients which were previously supplied through sow milk to fully develop its digestive and immune system. Nucleotides have been suggested to be conditionally essential nutrients during early weaning (Domeneghini et al., 2006) because weaned pigs undergo rapid growth of tissue and organ systems, a process that is heavily dependent on availability of DNA, RNA, and ATP energy, whose synthesis depends on availability of nucleotides. The piglet enterocytes and immune cells have a high rate of proliferation but this is hampered by their limited capacity for *de novo* synthesis of nucleotides (Cosgrove, 1998), the low concentration of nucleotides in compounded feed (Mateo, 2005), and low voluntary feed intake by the piglet due to the transition from liquid to solid feed (Pluske et al., 1997). This may result in gut atrophy and slow maturation of both the digestive and immune system, which compromises the ability of the piglet to utilize nutrients for growth and to combat pathogens (Pluske et al., 1997).

There is a paucity of studies on the effect of nucleotides on piglet growth and development, and especially studies that simultaneously compare the effects of dietary nucleotides with AGP supplementation. In addition, it has been suggested that carbohydrases enhance gut health by hydrolyzing complex non-starch polysaccharides (NSP), thereby not only increasing nutrient availability but also inducing a prebiotic effect by releasing

fermentable oligosaccharides that are utilized by host gut microbiota as an energy source (Kiarie et al., 2007; Kiarie et al., 2008; Masey-O'Neill et al., 2014). To the best of our knowledge, there are no published studies investigating the effect of simultaneous supplementation of multi-enzyme mixtures (ENZ) and nucleotides on growth performance and gut health of weaned piglets. Since supplementation of nucleotides to piglet diets has been suggested to enhance the maturation of their digestive and immune systems (Sauer et al., 2011), it was hypothesized that given an immune challenge, piglets receiving supplemental nucleotides will have better immune responses that suppress potentially harmful inflammation compared to challenged control pigs. Consequently, the suppression of harmful inflammation will prevent gut atrophy, encourage voluntary feed intake, and mitigate growth stasis. Moreover, it was hypothesized that a simultaneous supplementation of nucleotides and ENZ will have a synergistic effect of enhancing growth performance of the piglet. Therefore, in the current study, the aim was to determine the response of piglets receiving 0.1% of a nucleotide-rich yeast extract (NRYE) without or with ENZ compared with AGP to an immune system challenge with *E. coli* lipopolysaccharide (LPS) on growth performance, immune status, and gut structure during the immediate post-weaning period.

7.3 MATERIALS AND METHODS

All experimental procedures were reviewed and approved by the University of Manitoba Animal Care Committee (protocol number F13-002/1), and pigs were handled in accordance with the guidelines described by the Canadian Council on Animal Care (2009).

Animals and housing

Thirty-six [Duroc × (Yorkshire × Landrace)] male and female piglets, weaned at 21d and with an initial average body weight of 6.89 ± 0.5 kg were used in an 11-day study. Pigs were individually housed in pens in an environmentally controlled nursery building. Each pen

was equipped with a feeder, a nipple drinker, and plastic-covered expanded metal floors. The room temperature was maintained at 29 ± 1°C throughout the study.

Experimental design

On d 1, pigs were randomly assigned to 6 dietary treatments based on the initial body weight resulting in 6 replications per treatment. Treatments included: (1) non-challenged control (NCC; pigs fed the basal diet and injected with sterile saline on day 7); (2) LPS-challenged control (CC; pigs fed the basal diet and challenged by injection with *E. coli* LPS on day 7); (3) CC + AGP (pigs fed the basal diet supplemented with antibiotic growth promoters (AGP) and challenged with *E. coli* LPS on day 7); (4) CC + NRYE (pigs fed the basal diet supplemented with NRYE and challenged with *E. coli* LPS on day 7); (5) CC + ENZ (pigs fed the basal diet supplemented with feed enzymes (ENZ) and challenged with *E. coli* LPS on day 7); and (6) CC + ENZ + NRYE (pigs fed the basal diet supplemented with ENZ and NRYE, and challenged with *E. coli* LPS on day 7). The LPS (*E. coli* serotype 055: B5; Sigma Chemical Inc., St Louis, MO, USA) was dissolved in sterile saline and administered intramuscularly at 60µg/kg body weight, whereas an equivalent amount of sterile saline was administered to CON pigs. The LPS dosage of 60µg/kg body weight was based on findings of Rakhshandeh and de Lange (2012), which reported that an intramuscular injection of LPS at 60µg/kg body weight resulted in an immune system stimulation in pigs.

The basal diet (Table 7.1) was formulated to meet or exceed the National Research Council (2012) requirements for all nutrients. The NRYE supplement, Maxi-Gen Plus, was supplied by Canadian Bio-systems Inc. (Calgary, AB, Canada) and had similar composition as described in Manuscript 1. The AGP supplement, Aureomycin (Pfizer Inc., New York City, NY, USA), supplied 0.10 g chlortetracycline, 0.10 g sulfamethazine and 0.05 g penicillin per kg of feed. The multi-carbohydrase enzymes supplement, Superenzyme-OM

Table 7.1. Composition of the basal diet, as-fed basis

Item	%
Ingredient	
Corn	25.25
Wheat	19.00
Soybean meal	20.00
Canola meal	5.00
Fish meal-H	5.00
Dried Whey	20.00
Vegetable Oil	3.00
Limestone	0.83
Monocalcium phosphate	0.14
Iodized Salt	0.26
Vitamin-trace mineral premix[1]	1.00
L-Lys·HCl	0.35
DL-Methionine	0.10
Threonine	0.07
Calculated nutrient content	
DE (kcal/kg)	3573
CP, %	22.3
Ca, %	0.80
Total P, %	0.65
Available P, %	0.43
SID Lys, %	1.36
SID Met, %	0.43
SID Thr, %	0.79
Analyzed composition	
CP, %	23.5
Ca, %	0.86
Total P, %	0.77

[1]Provided the following per kilogram of complete diet: 9,000 IU of vitamin A; 1,500 IU of vitamin D_3; 18 mg of vitamin E; 1.5 mg of vitamin K; 250 mg of choline; 30 mg of niacin; 27.5 mg of calcium pantothenate; 9.4 mg of riboflavin; 2 mg of pyridoxine; 25 µg of cyanocobalamin; 80 µg of biotin; 0.5 mg of folic acid; 18 mg of Cu from copper sulfate, 110 mg of Zn from zinc oxide, 0.2 mg of I from calcium iodide, 110 mg of Fe from ferrous sulfate, 50 mg of Mn from manganese dioxide, and 0.3 mg of Se from sodium selenite.

(Canadian Bio-systems Inc., Calgary, AB, Canada), supplied 2800 U of cellulase, 400 U of mannanase, 50 U of galactanase, 1000 U of xylanase, 600 U of glucanase, 2500 U of amylase and 200 U of protease per kg of diet.

Growth performance

From day 1 to 7 and 9 to 11, pigs had free access to feed and water but were pair-fed for 48 h (day 7 to 9) after LPS or sterile saline injection to exclude the possible effects of LPS-induced food intake reduction on gastrointestinal characteristics of the pigs. Feed disappearance and body weight were recorded on day 7 and day 11.

Blood sampling and rectal temperature measurement

Whole blood samples (10 mL per pig) were collected via jugular vein puncture into ethylenediaminetetraacetic acid tubes on day 7 (before LPS challenge), and after 6, 48 and 96 hours post LPS or saline injection and were placed on ice until they were processed further. In addition, on day 7 (before LPS challenge) and after 6 hours post LPS or saline injection, rectal temperature of each pig was measured and 10-mL blood sample from each pig was collected into a non-heparinized Vacutainer tube (Becton Dickinson, Rutherford, NJ) and incubated at room temperature for 1 hour before being centrifuged at 3,000 × g for 10 min at 4°C to obtain serum. Serum samples were stored at −80°C until required for analysis.

Ileal, duodenal and jejunal tissue sampling

On d 11 (96 hpc), all pigs were euthanized. A 5 cm section of duodenum, ileum and jejunum was fixed by immersion in 10% buffer neutral formalin, whereas 5g samples of ileum were also collected, stored in RNA later solution (Qiagen , Hilden, Germany), and thereafter stored at -80°C until required for analysis.

Sample preparation and chemical analyses

Dietary samples were processed and analyzed for CP, Ca and total P as described in manuscript I.

Histomorphology measurements

The formalin-fixed duodenum, ileum and jejunum tissues were analyzed as described in Manuscript III.

Red blood cell, white blood cell and platelet counts, and PUN measurement

Whole blood and plasma samples were sent to the Manitoba Veterinary Services Laboratory (Winnipeg, MB, Canada) for complete blood count and PUN analysis, respectively. Complete blood count was measured using an automated analyzer (Orthoclinical Johnson & Johnson VITROS 250 Chemistry System; Diamond Diagnostics, Holliston, MA), whereas PUN was measured using a Technicon Autoanalyzer System (Technicon Autoanalyzer Systems, Tarrytown, NY). Both analyses were measured in duplicate.

Serum TNF-α and IL-10

Serum samples were used to measure the concentration of IL-10 and TNF-α using the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique using porcine IL-10 and TNF-α immunoassay kits (R & D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. The optical densities were read on a spectrophotometer (Soft Max Pro 3.1.1; Molecular Devices, Abingdon, Oxfordshire, UK). For each cytokine, the absorbance was measured at 450 nm with the correction wavelength set at 540 nm. Cytokine concentrations were calculated from standards using a 4-parameter logistic curve fit.

Total RNA extraction and reverse transcription

Ileal tissues total RNA extraction and cDNA reverse transcription materials and methods are similar as those described in Manuscript III.

Real-time PCR

The materials and methods for qPCR for cDNA templates are identical to those described in Manuscript III (see section 6.3). The primer sets used for amplification reactions are shown in Table 7.2.

Statistical analysis

All data were analyzed using the Mixed procedure of SAS (SAS Inst., Inc., Cary, NC). Preplanned contrasts were used to compare CC with NCC to determine LPS effect, and to compare CC with other treatments, AGP with NRYE and AGP with ENZ to determine the effect of the supplements before and after LPS challenge. For all data analyses, significance was defined as $P < 0.05$ and $0.05 < P < 0.10$ was considered a trend.

7.4 RESULTS

Growth performance

During day 1 to 7 (pre-challenge), there was no difference in ADG and G:F among treatments except that CC + AGP pigs tended to have higher ADFI than CC + NRYE (P = 0.06; Table 7.3) pigs.

During day 7 to 11 (post-challenge), NCC pigs had higher ADG (P = 0.001) and ADFI (P = 0.003) than CC pigs and tended to have higher G:F (P = 0.07) than CC pigs. In addition, CC + AGP pigs tended to have higher ADG (P = 0.07) than CC pigs.

Overall (day 1 to 11), LPS challenge resulted in a 13% loss in body weight (P = 0.02), 56% reduction in ADG (P = 0.004), a 37% reduction in ADFI (P = 0.02) and a 26% reduction in G:F (P = 0.02) compared to NCC pigs. Compared to CC pigs, supplementation of AGP improved body weight (P = 0.02), ADG (P = 0.004), ADFI (P = 0.03) and G:F (P = 0.01), whereas ENZ and NRYE supplementation tended to improve ADG (P = 0.08) and G:F (P = 0.096), respectively. In addition, CC + AGP pigs had higher body weight (P = 0.04) and

Table 7.2. Primers used for qPCR analysis of immune cytokines

Gene	GenBank accession Reference no.	Amplicon size (bp)	T_m [1] (°C)	Primer sequence (5'-3')
IFN-γ	NM_213948.1	231	60	F: GGCCATTCAAAGGAGCATGG
				R: GCTCTCTGGCCTTGGAACAT
IL-1β	NM_214055.1	131	60	F: GCCCATCATCCTTGAAACGTG
				R: GGAGAGCCTTCAGCATGTGT
IL-6	NM_214399.1	149	60	F: TAAGGGAAATGTCGAGGCCG
				R: TCCACTCGTTCTGTGACTGC
IL-10	NM_214041.1	179	60	F: TCCGACTCAACGAAGAAGGC
				R: AACTCTTCACTGGGCCGAAG
TNF-α	NM_214022.1	120	60	F: ATTCAGGGATGTGTGGCCTG
				R: CCAGATGTCCCAGGTTGCAT
Beta-actin	XM_005670976.1	179	60	F: CGAGGCTCAGAGCAAGAGAG
				R: GGTTGGCCTTAGGGTTCAGG

[1] Annealing temperature

Table 7.3. Effect of a nucleotide-rich yeast extract without or with feed enzymes on growth performance of weaned piglets challenged with *E. coli* lipopolysaccharide

		LPS treatments[3]						Contrast						
Item[1]	NCC[2]	CC	AGP	NRYE	ENZ	ENZ + NRYE	SEM	NCC *vs* CC	CC *vs* AGP	CC *vs* NRYE	CC *vs* ENZ	CC *vs* ENZ + NRYE	AGP *vs* NRYE	AGP *vs* ENZ
Day 1 BW, kg	6.89	6.9	6.88	6.89	6.91	6.86	0.237	NS	NS	NS	NS	NS	NS	NS
Day 1 to 7														
ADG, g/day	159	150	212	140	196	164	31.7	NS	NS	NS	NS	NS	NS	NS
ADFI, g/day	191	168	225	160	214	172	24	NS	NS	NS	NS	NS	0.06	NS
G:F, g/g	0.82	0.77	0.93	0.83	0.91	0.87	0.062	NS	NS	NS	NS	NS	NS	NS
Day 7 to 11														
ADG, g/day	323	130	226	174	170	160	33.2	0.001	0.07	NS	NS	NS	NS	NS
ADFI, g/day	366	214	288	229	264	249	29.8	0.003	NS	NS	NS	NS	NS	NS
G:F, g/g	0.88	0.59	0.8	0.75	0.64	0.66	0.1	0.07	NS	NS	NS	NS	NS	NS
Day 1 to 11														
BW, kg	9.3	8.07	9.27	8.39	8.9	8.78	0.319	0.02	0.02	NS	NS	NS	0.04	NS
ADG, g/day	241	112	239	162	192	190	27.3	0.004	0.004	NS	0.08	NS	0.03	NS
ADFI, g/day	280	177	273	204	250	210	26.3	0.02	0.03	NS	NS	NS	0.06	0.08
G:F, g/g	0.85	0.63	0.88	0.79	0.77	0.76	0.061	0.02	0.01	0.096	NS	NS	NS	NS

[1]BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; G:F, gain to feed ratio

[2]NCC, non-challenged control pigs that were fed the basal diet and injected with sterile saline on day 7

[3] Pigs fed the basal diet without any additive (CC, challenged control) or with AGP, NRYE, ENZ or ENZ + NRYE and injected with *E. coli* LPS on day 7

ADG (P = 0.03) than CC + NYRE pigs and tended to have higher ADFI than CC + NYRE (P = 0.06) and CC + ENZ + NRYE (P = 0.08) pigs.

Plasma urea N content and blood cells count

No effect of dietary treatment was observed for PUN and counts of white blood cells, red blood cells and platelets pre-challenge (Table 7.4). After 6 hpc, CC pigs had higher PUN than NCC (P = 0.01), CC + AGP (P = 0.02), CC + NRYE (P = 0.02) and CC + ENZ + NRYE (P = 0.03) pigs. In addition, CC pigs had lower white blood cell count than NCC (P = 0.0004) and CC + ENZ + NRYE (P = 0.01) pigs, and tended to have lower white blood cell count than CC + NRYE pigs (P = 0.07), whereas CC + AGP pigs tended to have lower white blood cell count than CC + NRYE pigs (P = 0.07). The red blood cell count of CC + AGP pigs was lower (P = 0.01) than of CC + NRYE pigs. The platelet count of CC pigs was 53% lower than of NCC pigs (P = 0.0002). Compared with CC pigs, the platelet count was 58 and 79% higher in CC + AGP (P = 0.04) and CC + NRYE (P = 0.01) pigs, respectively.

After 48 hpc, CC pigs tended to have higher PUN than NCC (P = 0.08), CC + AGP (P = 0.02), CC + NRYE (P = 0.001), CC + ENZ (P = 0.01) and CC + ENZ + NRYE (P = 0.02) pigs. In addition, the CC + NRYE pigs tended to have lower PUN than CC + AGP pigs (P = 0.07), whereas treatments had no effect on white blood cell count. The red blood cell count of CC + AGP pigs tended to be lower (P = 0.07) than of CC + NRYE pigs, whereas their platelet count was 21% lower (P = 0.05) than of NCC pigs.

After 96 hpc, CC pigs had higher PUN than NCC (P = 0.01) and CC + AGP (P = 0.02) pigs, and tended to have higher PUN than CC + NRYE (P = 0.08) pigs, whereas CC + ENZ + NRYE pigs tended to have higher PUN than CC + AGP pigs (P = 0.09). Compared to NCC pigs, red blood cell count was lower in CC pigs (P = 0.02), whereas treatments had no effect on white blood cell and platelet counts.

Table 7.4. Effect of supplementing AGP, feed enzymes and NRYE without or with feed enzymes on PUN (mmol/L) and count of white blood cells (WBC, 10^9/L), red blood cells (RBC, 10^{12}/L) and platelets (10^9/L) of weaned piglets challenged with *E. coli* lipopolysaccharide

		LPS treatments[2]						Contrast						
Item	NCC[1]	CC	AGP	NRYE	ENZ	ENZ + NRYE	SEM	NCC *vs* CC	CC *vs* AGP	CC *vs* NRYE	CC *vs* ENZ	CC *vs* ENZ + NRYE	AGP *vs* NRYE	AGP *vs* ENZ
Pre-challenge														
PUN	2.34	2.95	2.28	2.08	2.52	2.57	0.412	NS	NS	NS	NS	NS	NS	NS
WBC	16.9	17.8	18	15	14.1	19.3	2.21	NS	NS	NS	NS	NS	NS	NS
RBC	5.96	6.04	5.54	6.09	6.04	6.06	0.228	NS	NS	NS	NS	NS	NS	NS
Platelet	429	450	534	483	447	415	44.8	NS	NS	NS	NS	NS	NS	NS
6 h post-challenge														
PUN	2.12	4.18	2.58	2.5	3.42	2.6	0.478	0.01	0.02	0.02	NS	0.03	NS	NS
WBC	19.7	6.5	10.2	12.2	7.6	15.5	1.98	0.0004	NS	0.07	NS	0.01	NS	0.07
RBC	5.91	6.01	5.68	6.62	6.33	5.97	0.255	NS	NS	NS	NS	NS	0.01	NS
Platelet	413	196	309	350	205	278	37.3	0.0002	0.04	0.01	NS	NS	NS	NS
48 h post-challenge														
PUN	2.16	2.97	1.93	1.24	1.6	1.83	0.285	0.08	0.02	0.001	0.01	0.02	0.07	NS
WBC	18.2	17.3	16.8	17.5	16.2	19.7	1.68	NS	NS	NS	NS	NS	NS	NS
RBC	5.59	5.39	4.96	5.41	5.48	5.25	0.184	NS	NS	NS	NS	NS	0.07	NS
Platelet	445	350	345	338	322	345	29.9	0.05	NS	NS	NS	NS	NS	NS
96 h post-challenge														
PUN	1.66	3.2	1.78	2.1	2.55	2.77	0.384	0.01	0.02	0.08	NS	NS	NS	0.09
WBC	19.6	16.9	16.7	16.3	14.5	18.2	1.3	NS	NS	NS	NS	NS	NS	NS
RBC	5.95	5.32	5.18	5.6	5.46	5.22	0.19	0.02	NS	NS	NS	NS	NS	NS
Platelet	449	386	446	520	427	507	61.3	NS	NS	NS	NS	NS	NS	NS

[1]NCC, non-challenged control pigs that were fed the basal diet and injected with sterile saline on day 7

[2] Pigs fed the basal diet without any additive (CC, challenged control) or with AGP, NRYE, ENZ or ENZ + NRYE and injected with *E. coli* LPS on day 7.

Duodenum, jejunum and ileum histomorphology

Representative images of the histomorphology of the cross-sections of the duodenal, jejunal, and ileal tissue samples are shown in Figure 2. After 96 hpc, the duodenal villus height for CC pigs was lower than of NCC (P = 0.002), CC + AGP (P = 0.03), CC + ENZ (P = 0.005) and CC + ENZ + NRYE (P = 0.002) pigs, and tended to be lower than of CC + NRYE (P = 0.06) pigs (Table 7.5). The duodenal crypt depth for CC pigs was lower than that of NCC pigs (P = 0.02) and tended to be lower than that of CC + NRYE (P = 0.06) and CC + ENZ + NRYE (P = 0.06) pigs. In addition, CC pigs had shorter jejunal villus height than NCC (P < 0.0001), CC + AGP (P = 0.01), CC + NRYE (P = 0.02), CC + ENZ (P = 0.02) and CC + ENZ + NRYE (P = 0.01) pigs. Moreover, compared with CC pigs, the jejunal villus height/crypt depth (VH:CD) ratio was higher in NCC (P = 0.0003), CC + AGP (P = 0.01) and CC + ENZ + NRYE (P = 0.01) pigs, and tended to be higher in CC + NRYE (P = 0.07) pigs. The ileal villus height of CC pigs was shorter than of NCC pigs (P = 0.04) and tended to be shorter than that of CC + AGP pigs (P = 0.07).

Rectal temperature, serum and ileum cytokines

No effect of treatments was observed for rectal temperature, concentration of serum TNF-α and IL-10 before LPS challenge (Table 7.6). After 6 hpc, rectal temperature was increased in CC pigs (P = 0.003) and in all other treatments compared to NCC pigs. In addition, compared with CC pigs, the concentration of serum TNF-α was lower in NCC (P < 0.0001), CC + AGP (P = 0.07), CC + NRYE (P = 0.008) and CC + ENZ + NRYE (P = 0.016), whereas the serum IL-10 concentration was lower in NCC pigs (P = 0.001) but higher in CC + AGP (P = 0.02), CC + NRYE (P = 0.02) and CC + ENZ + NRYE (P = 0.02) pigs.

After 96 hpc, no effect of treatments was observed for the mRNA expression of ileal IL-6. The CC pigs had higher expression of ileal IFN-γ (P = 0.04), TNF-α (P = 0.0003) and IL-1β (P = 0.01), and tended to have higher expression of ileal IL-10 (P = 0.09) than NCC

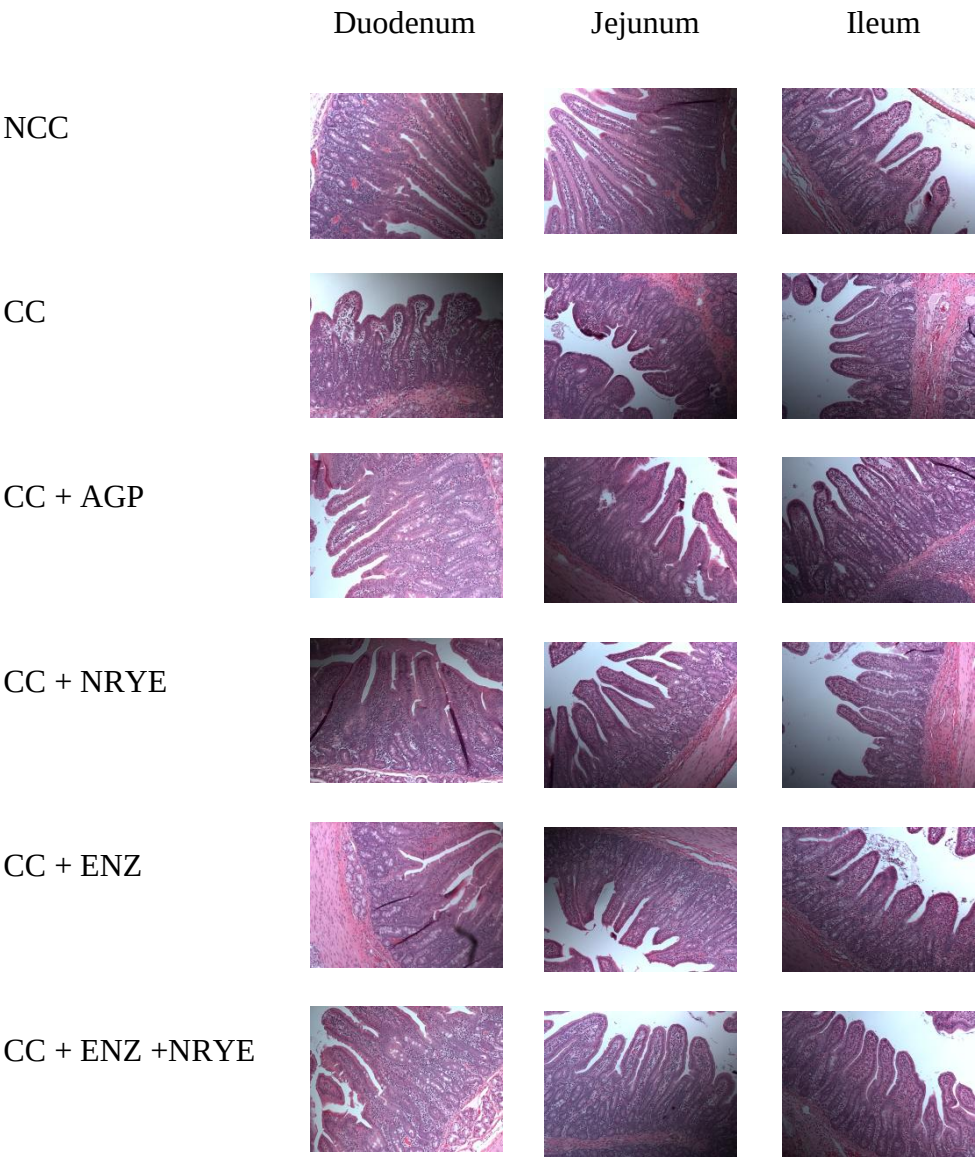

Figure 2. Representative images of the histomorphology of some portions of the cross-sections of the duodenum, jejunum, and ileum tissue samples obtained from piglets.

NCC represents pigs fed the control diet and injected with sterile saline. CC represents pigs fed the control diet and received *E. coli* LPS challenge.

Table 7.5. Effect of supplementing AGP, feed enzymes and NRYE without or with feed enzymes on ileum, jejunum and duodenum histomorphology of weaned piglets after 96 h of challenge with *E. coli* lipopolysaccharide

		LPS treatments[2]						Contrast						
Item[1]	NCC[2]	CC	AGP	NRYE	ENZ	ENZ + NRYE	SEM	NCC *vs* CC	CC *vs* AGP	CC *vs* NRYE	CC *vs* ENZ	CC *vs* ENZ + NRYE	AGP *vs* NRYE	AGP *vs* ENZ
Duodenum, µm														
Villus height	532	410	493	480	520	538	24.7	0.002	0.03	0.06	0.005	0.002	NS	NS
Crypt depth	322	277	308	305	314	316	13.1	0.02	NS	NS	0.06	0.06	NS	NS
VH:CD	1.65	1.50	1.61	1.58	1.68	1.70	0.088	NS	NS	NS	NS	NS	NS	NS
Jejunum, µm														
Villus height	465	293	400	385	385	412	26.3	<.0001	0.01	0.02	0.02	0.01	NS	NS
Crypt depth	277	255	269	275	282	273	13.2	NS	NS	NS	NS	NS	NS	NS
VH:CD	1.70	1.15	1.50	1.40	1.37	1.51	0.092	0.0003	0.01	0.07	NS	0.01	NS	NS
Ileum, µm														
Villus height	329	272	323	294	308	306	18.8	0.04	0.07	NS	NS	NS	NS	NS
Crypt depth	265	244	259	233	268	249	13.6	NS	NS	NS	NS	NS	NS	NS
VH:CD	1.24	1.11	1.26	1.26	1.17	1.23	0.067	NS	NS	NS	NS	NS	NS	NS

[1]VH:CD, villus height/crypt depth

[2]NCC, non-challenged control pigs that were fed the basal diet and injected with sterile saline on day 7

[3] Pigs fed the basal diet without any additive (CC, challenged control) or with AGP, NRYE, ENZ or ENZ + NRYE and injected with *E. coli* LPS on day 7

Table 7.6. Effect of supplementing AGP, feed enzymes and NRYE without or with feed enzymes on the rectal temperature (ºC), concentration of serum (pg/ml) cytokines and fold changes (log_2) of ileal immune cytokines of weaned piglets challenged with *E. coli* lipopolysaccharide

		LPS treatments[2]						Contrast						
Item	NCC[1]	CC	AGP	NRYE	ENZ	ENZ + NRYE	SEM	NCC *vs* CC	CC *vs* AGP	CC *vs* NRYE	CC *vs* ENZ	CC *vs* ENZ + NRYE	AGP *vs* NRYE	AGP *vs* ENZ
Pre-challenge														
Rectal temperature	38.9	39	39.2	39	39.2	39.2	0.13	NS	NS	NS	NS	NS	NS	NS
Serum TNF-α	98	96	88	77	102	93	9.3	NS	NS	NS	NS	NS	NS	NS
Serum IL-10	18	29	16	22	36	27	15.1	NS	NS	NS	NS	NS	NS	NS
6 h post-challenge														
Rectal temperature	39.2	39.9	39.9	39.8	40.1	40.1	0.15	0.003	NS	NS	NS	NS	NS	NS
Serum TNF-α	111	1369	1032	854	1621	933	120.8	<.0001	0.07	0.008	NS	0.016	NS	0.003
Serum IL-10	16	99	157	153	86	156	15.3	0.001	0.02	0.02	NS	0.02	NS	NS
96 h post-challenge														
Ileum														
IFN-γ	1.0	1.46	1.54	1.51	2.15	0.54	0.152	0.04	NS	NS	0.003	0.0002	NS	<.0001
TNF-α	1.0	2.4	2.52	1.37	1.6	1.41	0.244	0.0003	NS	0.01	0.03	0.01	0.002	0.003
IL-1β	1.0	1.63	0.95	1.02	0.77	0.58	0.154	0.01	0.004	0.01	0.0004	<.0001	NS	NS
IL-6	1.0	0.85	0.86	1.16	0.69	1.22	0.203	NS	NS	NS	NS	NS	NS	NS
IL-10	1.0	1.31	0.6	0.74	0.71	0.76	0.123	0.09	0.0003	0.003	0.002	0.004	NS	NS

[1]NCC, non-challenged control pigs that were fed the basal diet and injected with sterile saline on day 7
[2]Pigs fed the basal diet without any additive (CC, challenged control) or with AGP, NRYE, ENZ or ENZ + NRYE and injected with *E. coli* LPS on day 7

pigs. The CC + AGP pigs had lower ileal IL-1β (P = 0.004) and IL-10 (P = 0.0003) expression compared with CC pigs. Compared with CC pigs, CC + NRYE pigs had higher expression of ileal TNF-α (P = 0.01) and IL-1β (P = 0.01) but lower expression of IL-10 (P = 0.003), whereas CC + ENZ pigs had higher expression of ileal IFN-γ (P = 0.003) but lower expression of TNF-α (P = 0.03), IL-1β (P = 0.0004) and IL-10 (P = 0.002) compared with CC pigs. The CC + ENZ + NRYE pigs had a lower expression of ileal IFN-γ (P = 0.0002), TNF-α (P = 0.01), IL-1β (P < 0.0001) and IL-10 (P = 0.004) compared with CC pigs. Compared to CC + AGP pigs, there was a lower expression of ileal TNF-α (P = 0.002) in CC + NRYE pigs and IFN-γ (P <0.0001) and TNF-α (P = 0.003) in CC + ENZ + NRYE pigs.

7.5 DISCUSSION

The aim of this study was to determine the effect of NRYE without or with carbohydrase enzymes in comparison to AGP on the growth performance, blood characteristics, immune response and gut structure in pigs challenged with *E. coli* LPS during the immediate post-weaning period. Lipopolysaccharide is a non-pathogenic immunogen capable of inducing symptoms of acute bacterial infection including anorexia, gut injury, hypersomnia and fever in pigs (Liu et al., 2008; Rakhshandeh and de Lange, 2012). In the current study, the LPS challenge was used to induce an immunological stress that is equivalent to a moderate clinical disease (Rakhshandeh and de Lange, 2012) so as to evaluate the effect of dietary NRYE supplementation on piglet health and performance during early weaning. Studies have reported the beneficial effects of multi-carbohydrases in improving growth performance and nutrient digestibility, and inducing a prebiotic effect in piglets, and currently supplementation of multi-carbohydrases is widespread in the feed industry (Bedford and Schulze, 1998; Kiarie et al., 2008; Woyengo et al., 2008; O'Neill et al., 2014). The ENZ + NRYE treatment was introduced in this study to determine whether simultaneous

supplementation of NRYE and multi-carbohydrases may have synergistic effects on growth performance and gut health.

The AGP, NRYE and ENZ supplements did not affect performance during the first 7 days of weaning. Sauer et al. (2012b) and Andrés-Elias et al. (2007) supplemented 0.1 and 0.15% NRYE in piglet starter diets, respectively, and reported no effect of NRYE on growth performance. Between day 7 and 11 (post-challenge), the LPS challenge reduced ADG, ADFI and G:F of challenged pigs fed the control diet. This observation is consistent with the findings of Liu et al. (2003) and Liu et al. (2008) showing that LPS challenge negatively impacts growth performance in pigs.

Although none of the supplements was able to fully protect the pigs from weight loss or to increase their feed intake between d 7 an 11, the LPS impact on ADG tended to be attenuated in CC + AGP pigs. This beneficial effect of AGP on growth performance was further seen in the overall experimental period (d 1 to 11) with AGP pigs having better ADG, ADFI and GF than LPS-challenged control, and tended to have better G:F than NRYE supplemented pigs. These observations suggest that AGP supplemented pigs had better recovery of weight and feed intake after LPS challenge than NRYE supplemented pigs. Weber et al. (2001) and Skinner et al. (2014) reported that AGP improves ADG during the nursery phase, whereas Wu et al. (2012) reported that piglets receiving in-feed AGP and challenged with enterotoxigenic *E. coli* had higher ADG and G:F than control. Niewold (2007) suggested that AGP improve gut health and growth performance of livestock by reducing the gut pathogen load and competition for nutrients between the host and gut microbes. This effect may also be associated to the immunoregulatory role of AGP (Bode et al., 2014). Although ENZ supplementation tended to improve ADG (d 1 to 11), there was no synergistic effect between NRYE and ENZ on ADG.

The reduced white blood cell and platelet counts in challenged control pigs is due to LPS induced autoimmunity and suggests an increased incidence or severity of infection (Louis and Lambert, 1979; Yeaman, 2014). Platelets play a crucial role in blood clotting and as the first responders to sites of vascular injury or pathogen invasion, platelets play multiple roles in the modulation of both innate and adaptive immunity thereby boosting host defense against infection (Yeaman, 2014). The observation that CC + NRYE pigs maintained higher white blood cell and platelet count than CC pigs and higher red blood cell count than CC + AGP pigs at 6 hpc suggests that NRYE supplementation either had a role in preventing blood cells death or sustained moderate blood cell formation during the challenge period. This can be attributed to the enhanced supply of dietary nucleotides which are a pre-requisite for DNA and RNA replication during mitosis (Grimble and Westwood, 2001) of the stem cells that differentiate into blood cells.

A 100μg/kg dosage of *E. coli* LPS has been shown to induce gut injury in pigs (Liu et al., 2008; Hou et al., 2010). In the present study, gut injury was observed in piglets that received 60μg/kg of *E. coli* LPS. Supplementing NRYE to pigs without or with enzymes reduced villus atrophy in the jejunum and duodenum, and the villus height/crypt depth ratio in the jejunum when compared with the non-challenged control pigs, and the effect was similar to that of AGP supplementation. The increase in villus height and villus/crypt ratio indicates an improvement in the digestion and absorption of nutrients (Hampson, 1986b; Hou et al., 2010). Enterocytes have limited capacity for *de novo* nucleotides synthesis (Uauy et al., 1994; Grimble, 1996) and thus the increased pool of dietary nucleotides by NRYE supplementation promoted intestinal tissue growth and development.

Injection of LPS induces fever in pigs by stimulating the production of cytokines such as IL-1, TNF-α and IFN-γ, which are regarded as endogenous mediators of fever (Kluger, 1991). In this study, 6 hpc CC pigs showed a significant rise in body temperature compared

to NCC pigs as also observed by Pi et al. (2014) and none of the supplements protected the pigs from fever development.

The concentration of serum TNF-α and IL-10 were measured as indicators of systemic pro- and anti-inflammation responses, respectively. Pigs receiving AGP or NRYE without or with ENZ had less concentration of serum TNF-α and high concentration of IL-10 than the challenged control, hence, implying that the supplements lowered pro-inflammation of the immune system. The ileum immune response was also influenced by both LPS and the supplements. The observed upregulation of ileal TNF-α and IL-1β 96 hpc in the CC pigs compared to NCC pigs suggests development of inflammation in CC pigs, which if prolonged leads to tissue damage such as the observed villus atrophy in these pigs (Lomax, 1981). In contrast, pigs in the NRYE supplemented treatments had downregulated levels of expression of these pro-inflammatory cytokines thus suggesting that NRYE induced beneficial immunoregulatory responses post-challenge that was evidenced by lower expression of TNF-α and IL-1β, and consequently that of IL-10 compared to the challenged control group. The overproduction of proinflammatory cytokines is associated with anorexia development, and may explain the observed reduction in ADFI in LPS challenged pigs in this current study (Wannemacher, 1977; Johnson, 1997).

In the present study, challenged control had higher PUN than unchallenged control. This is consistent with the study of Webel et al. (1997) reporting that LPS challenge increases the PUN level in piglets due to muscle proteolysis as a result of increased inflammation. Owusu-Asiedu et al. (2003) suggested that the released amino acids due to inflammation may be channeled to the liver to synthesize acute phase proteins and/or to serve as an energy source. Supplementing NRYE without or with ENZ had similar effects as AGP in reducing PUN level compared with the challenged control. The reduction in PUN level is an indicator of reduced muscle protein breakdown (Webel et al., 1997) and is consistent with the observed

reduced production of proinflammatory cytokines in NRYE and AGP receiving pigs. These observations suggest that supplementing NRYE or AGP was beneficial to piglets and reduced the severity of the LPS challenge. Such a response may promote efficiency in utilization of dietary amino acids for proteinaceous tissue growth (Wannemacher, 1977).

Taken together, after the immune system stimulation using *E. coli* LPS, the growth performance, platelet count, villus height of the jejunum and duodenum, and immune status of challenged control pigs was negatively affected compared with the unchallenged control. Compared to challenged control, supplementing NRYE together with ENZ had synergistic effects on blood cell counts, histomorphological measurements and expression of immune system cytokines but not on growth performance. The most pronounced beneficial effects of NRYE were on lowering PUN, villus atrophy, and levels of pro-inflammatory cytokines, and maintaining blood cell counts close to that of unchallenged control pigs. This suggests that during the post-weaning stress period the nucleotides sourced from NRYE are mostly channeled to influence the development and maturation of important tissues of the immune system and the intestinal mucosa. This supports the suggestion by Gaskins et al. (2002) that the weaned pig first invests substantially in defensive efforts to sequester gut microbes away from the epithelial surface, and second to quickly mount immune responses against microbes that breach epithelial defenses. This re-direction of nutrients from growth towards fortification of the immune system and maturation of gut structures may explain why beneficial responses on growth performance may not be observed within a short term of supplementing nucleotides. However, a faster maturation of the gut and immune system tissues, may in the long-run support faster growth of the pig as also suggested by Córdoba et al. (2010) who described the effect of nucleotides to be long-lasting.

In conclusion, LPS challenged piglets fed diets supplemented with NRYE without or with enzymes expressed similar beneficial responses as those fed diets with AGP in terms of

lowering PUN concentration, reducing duodenal and ileal villi atrophy, and downregulating serum and ileal proinflammatory cytokines. This suggests that supplementation of NRYE can promote the health of piglets during the immediate post-weaning period in antibiotic-free feeding regimens.

CHAPTER EIGHT

GENERAL DISCUSSION

The overall objective of this study was to determine the effects of supplementing a nucleotide-rich yeast extract (NRYE) on growth performance, gut digestive and immune systems, and gut microbiota of weaned pigs. It was hypothesized that the beneficial effects of supplemental nucleotides will be mediated through enhanced immune system activation and maintenance of a stable intestinal microbial composition. A yeast based nucleotide product was used in this study owing to the affordability of such products compared to pure nucleotide sources, an advantage that may promote their use in practical swine diets. However, the use of pure nucleotides for mechanistic explanations of the nucleotides effect is more desirable as it eliminates confounding effects of yeast cell components (such as cell wall polysacchrides and protein) and allow measurement of responses following supplementation of single or mixed nucleotides at various doses. In Manuscript I, the effect of a NRYE on growth performance and nutrient utilization were determined for two inclusion levels, 0.1 and 0.2%, which represent one- and two-times the sow's milk nucleotide concentration at d 21 of lactation (Mateo et al., 2004; Martinez-Puig et al., 2007; Superchi et al., 2012), respectively. In Manuscript II, the potential of NRYE to fully or partially substitute for in-feed AGP in piglet diets was studied using diets supplemented with 0.1% NRYE and graded levels (25, 50, 75 and 100%) of the recommended antibiotic dosage. Manuscripts I and II provide new information in this study area since no published study exists, which investigated the effect of dietary nucleotides compared to in-feed antibiotics on growth performance. In Manuscript III, a sanitary challenge model was used to evaluate the efficacy of NRYE using performance, immune response, gut structure and gut microbiota as response criteria in weaned pigs. In Manuscript IV, an *E. coli* LPS challenge model was used to study the effect of NRYE in a disease state using performance, blood profile, immune

response and gut structure as response criteria. Taken together, the following aspects of NRYE effects as reported in the four manuscripts can be discussed.

8.1. Effect of NRYE on performance and nutrient utilization

In Manuscript I, supplementation of AGP supported better ADFI and ADG than control. This supports studies showing that weaning results in stresses that lead to growth stasis in piglets but AGP supplementation can mitigate and enhance growth performance of piglets during weaning (Han and Thacker, 2010; Choi et al., 2011; Han et al., 2011; Yan et al., 2011a; Li et al., 2012; Yan et al., 2012; Zhou et al., 2015). However, in Manuscript 1, supplementation of NRYE at 0.1 or 0.2% resulted in similar growth performance as did AGP supplementation, whereas in Manuscript II supplementing NRYE at 0.1% resulted in better ADG and G:F than control. This supports studies suggesting that NRYE supplementation can mitigate the weaning-associated growth stasis in piglets (Zomborszky-Kovacs et al., 2000; Carlson et al., 2005; Weaver and Kim, 2014). Nucleotides are thought to improve performance responses in piglets by enhancing the development of the immune system cells and the intestinal mucosa because they are building blocks of DNA, RNA and ATP, which are required during cell division (Grimble and Westwood, 2001; Sauer et al., 2011). By enhancing cell growth, the intestinal mucosa and immune system mature faster thus reducing the susceptibility of piglets to various enteric diseases and other physiological post-weaning stresses.

In Manuscript III, supplementation of NRYE at 0.1% in clean and unclean conditions had no effect on growth performance compared to control. This agrees with results in Manuscript I and II, showing that NRYE may mitigate the growth stasis during weaning but this benefit may or may not be statistically visible. In addition, the results agree with other studies showing no effect of supplemental nucleotides on growth performance of piglets (Di Giancamillo et al., 2003; Domeneghini et al., 2004; Lee et al., 2007; Martinez-Puig et al.,

2007; Šperanda et al., 2008; Moore et al., 2011; Sauer et al., 2012a). In the context of this study, such inconsistencies may have been due to undetectable and uncontrollable variations in animals and experimental conditions.

In Manuscript IV, pigs LPS-challenged pigs receiving 0.1% NRYE tended to have better overall G:F than the LPS-challenged control pigs. Comparing this observation to those of Manuscripts I, II and III, it is evident that the effect of nucleotides on growth performance is conditional and seems to be majorly influenced by stresses exacted upon the immune system. This observation supports the findings of Li et al. (2015) showing that in piglets orally challenged with *E. coli* K88, the NRYE-receiving groups had better growth performance than control. This suggests that future studies on nucleotides effect should be based on disease challenge models.

8.2. Effect of NRYE on nutrient utilization

The effect of supplementing NRYE on the ATTD of CP, DM and GE was investigated because transition from milk to solid feed imparts stress to the development and function of the gut of the piglet (van Beers-Schreurs et al., 1998), which may result in slow gut mucosa maturation and consequently insufficient secretion of digestive enzymes. Together, these factors may result in poor digestion and absorption of nutrients thus contributing to the growth depression observed after weaning. Results of both Manuscripts I and II show that supplemental NRYE had no effect on ATTD values of GE, DM and CP compared to control. Digestibility studies involving supplemental nucleotides in pigs are not available to compare with our results except that of Sauer et al. (2012b) which reported no effects of pure nucleotides on apparent ileal digestibilities of CP, DM and crude fiber in piglets. It is worth noting that no challenge model studies have been reported wherein nutrient digestibility was measured after nucleotide supplementation. Therefore, it can be speculated that the beneficial effect of dietary nucleotides on gut physiology may be visible

only in conditions where the gut immune system is inflamed to a level that induces gut atrophy. In this case, the ability of dietary nucleotides to enhance recovery after gut injury (Uauy et al., 1994; Sukumar et al., 1997) may show beneficial effects on nutrient digestibility.

8.3. Effect of NRYE on gut structure and visceral organs

In Manuscript 3, supplemental NRYE tended to increase VH:CD in clean room pigs but had no effect in unclean room pigs. In both sanitary conditions, NRYE-fed pigs had heavier kidneys, whereas in unclean room they had a heavier gut compared to control. Effects of NRYE on gut structure were also observed in Manuscript IV, showing that LPS challenged NRYE pigs had higher jejunal villus height and tended to have higher duodenal villus height and jejunal VH:CD than LPS challenged control. Taken together, the results support the suggestion that the possible mechanism through which supplemental nucleotides impart beneficial effects to the animal is by increasing the nucleotide/nucleoside pool hence affecting all the biochemical processes associated with the cell cycle, which requires more nucleotides for DNA, RNA and ATP synthesis (Paubert-Braquet et al., 1992; Carver, 1994; Cosgrove, 1998; Sauer et al., 2011). The NRYE effect on gut morphology were more conspicuous under LPS challenge, hence, this further supports the suggestion that NRYE are more beneficial when the animal is under immunological stress.

8.4. Effect of NRYE on gut microbiota

There is a paucity of studies on the effect of NRYE on piglet gut microbiota. In Manuscript III, it was observed that supplementing NRYE in the clean room suppressed growth of cecal Enterobacteriacea members and colonic Enterococcus spp., whereas it improved proliferation of cecal *Lactobacillus* and colonic *Clostridium* cluster IV and XVIa members. Supplementing NRYE in the unclean room improved proliferation of cecal *Clostridium* cluster IV and suppressed proliferation of colonic *Enterococcus* spp. Although

this study investigated only a few beneficial and pathogenic bacteria groups, it gives a strong evidence that supplemental nucleotides have potential to beneficially transform gut microbiota by suppressing and enhancing proliferation of pathogenic and beneficial microbes *in vivo*, respectively. The results of this study support the recent findings of Li et al. (2015) showing that in piglets orally challenged with *E. coli* K88, the NRYE-receiving groups had higher *Lactobacillus* fecal count and lower *E. coli* fecal count than control. This effect of nucleotides was observed in human subjects (Gil et al., 1986; Singhal et al., 2008) and in *in vitro* studies (Mateo, 2005; Sauer et al., 2010). However, more research is needed to substantiate these results because the study of Sauer et al. (2012a) did not show effect of supplemental nucleotides on the composition of small and large intestine gut microbiota of piglets.

8.5. Effect of NRYE on immune responses

Manuscript III and IV give strong evidence that NRYE supplementation had beneficial immunoregulatory effects in the cells and tissues associated with the gut and circulatory systems. Furthermore, this response to NRYE supplementation is more conspicuous in both studies when compared to other parameters that were measured and agrees with reports of other studies showing profound beneficial effects of dietary nucleotides on the immune system (Domeneghini et al., 2004; Lee et al., 2007; Martinez-Puig et al., 2007; Šperanda et al., 2008; Li et al., 2015). Under the sanitary challenge, NRYE supplementation was associated with upregulation of both pro- and anti-inflammatory cytokines. This supports the suggestion by Gaskins et al. (2002) that the weaned pig first invests substantially in defensive efforts to sequester gut microbes away from the epithelial surface, and second to quickly mount immune responses against microbes that breach epithelial defenses. Under LPS challenge, NRYE supplementation lowered the concentration and expression of pro-inflammatory cytokines in the serum and ileum, respectively. In

addition, this was associated with lowering of PUN and villus atrophy, and maintenance of blood cell counts close to that of unchallenged control pigs. These responses show a protective effect of NRYE against immune system overstimulation by LPS as also reported by Hung (2015).

8.6. The potential of NRYE to replace AGP in piglet diets

Manuscript I results show that NRYE mitigated the weaning-associated growth stasis and helped the pigs perform similar to those receiving AGP. Manuscript II results show that NRYE supplementation can reduce in-feed AGP dosage by up to 75% without any loses in growth performance and nutrient utilization, however, further studies are needed to address the concerns that reducing AGP dosage with nucleotide supplementation may still enhance drug resistance of enteric bacteria. Manuscript III results show that NRYE can beneficially transform gut microbiota, hence alleviating the potential risks associated with in-feed AGP in the development of microbial antibiotic resistance in humans and livestock (Heuer et al., 2006), and environmental contamination (Carlson et al., 2004). Furthermore, in Manuscript IV, between 6 to 48 h post-LPS challenge, NRYE-receiving pigs had same growth performance, higher RBC count and lower PUN level compared to AGP-receiving pigs. Moreover, after 96 h post-LPS challenge, both NRYE- and AGP-receiving pigs had similar villus height, crypt depth and villus height/crypt depth ratio in the duodenum, jejunum and ileum. Although the superiority of AGP on promoting growth performance was evident in both Manuscript I and overall results of Manuscript IV, in situations where an AGP ban exists or non-AGP raised pork is more profitable, NRYE supplementation with enhanced sanitation seems viable in mitigating weaning associated challenges.

8.7. Synergistic effects of NRYE and carbohydrases

In Manuscript IV, compared to challenged control, supplementing NRYE together with carbohydrases had no synergistic effects on performance. However, 6 h post challenge,

NRYE and carbohydrases acted together to improve white blood cell count, whereas 96 h post-challenge they acted together to increase duodenal villus height and crypt depth, jejunal villus height:crypt depth ratio and downregulated the expression of IFN-α and IL-1β. This is more evidence of the role of both NRYE and carbohydrases in improving gut structure and immunological response system of the piglet. Studies where nucleotides and carbohydrases have been simultaneously supplemented to piglet diets are lacking. However, some studies have indicated synergistic effects of nucleotides with AGP (Carlson et al., 2005) in improving ADG and ADFI of weaned pigs, and with L-glutamine (Domeneghini et al., 2004) in increasing villus height and crypt depth, decreasing villus height/crypt depth ratio, increasing mitosis and decreasing apoptosis in lymphatic follicles, and increasing percentage of macrophages and intraepithelial lymphocytes in ileal tissue. Taken together, these studies show that supplemental nucleotides have potential to improve the beneficial effects of the commonly used feed additives.

CHAPTER NINE

CONCLUSIONS AND FUTURE STUDIES

CONCLUSIONS

The following conclusions can be drawn from the present research:

1. Supplementation of 0.1 or 0.2% NRYE as a source of dietary nucleotides resulted in similar growth performance as in-feed antibiotics.
2. 0.1% NRYE can be supplemented alone or with 25% of the recommended AGP dosage without affecting growth performance and nutrient utilization in weaned pigs.
3. Supplementing 0.1% NRYE to weaned pigs raised in unsanitary condition positively modulated their immune response.
4. Supplementing NRYE to weaned pigs raised in unsanitary condition positively modulated the composition of gut microbiota.
5. Supplementing 0.1% NRYE to LPS challenged piglets expressed similar beneficial responses as in-feed AGP in terms of lowering PUN concentration, reducing duodenal and ileal villi atrophy, and downregulating serum and ileal proinflammatory cytokines.
6. Supplementing 0.1% NRYE and carbohydrases had synergistic effects on improving white blood cell count, duodenal villus height, jejunal villus height:crypt depth ratio and downregulating the expression of IFN-α and IL-1β.

FUTURE STUDIES

Further research is suggested to:

1. Determine the mechanisms by which supplemental nucleotides influence the immune responses and composition of gut microbiota. For instance, investigate the process of immune system activation after nucleotide supplementation and demonstrate changes in cell cycles of different immune cells. In addition, nucleotides effect on Toll-like receptors should be investigated to determine whether pathogen inhibition is a direct result of immune system activation or nucleotide uptake by commensal gut microbes.
2. Establish the succession of gut microbiota communities after supplementation of dietary nucleotides using metagenomic tools.
3. Determine effects of supplemental nucleotides together with enzymes using nutrient/energy deficient diets to investigate effect on nutrient/energy digestibility.
4. Determine effects of supplemental nucleotides together with probiotics and prebiotics in enhancing the gut health and function.

CHAPTER TEN

REFERENCES

Akiba, Y., C. Watanabe, M. Mizumori, and J. D. Kaunitz. 2009. Luminal L-glutamate enhances duodenal mucosal defense mechanisms via multiple glutamate receptors in rats. Am. J. Physiol-Gastr. L. 297: G781-G791.

Amit-Romach, E., Z. Uni, and R. Reifen. 2010. Multistep mechanism of probiotic bacterium, the effect on innate immune system. Mol. Nutr. Food Res. 54: 277-284.

Andres-Elias, N., J. Pujols, I. Badiola, and D. Torrallardona. 2007. Effect of nucleotides and carob pulp on gut health and performance of weanling piglets. Livest. Sci. 108: 280-283.

AOAC. 1990. Official methods of analysis of the AOAC. 15th ed. Association of Official Analytical Chemists, Washington, DC.

Arocho, A., B. Chen, M. Ladanyi, and Q. Pan. 2006. Validation of the 2-ΔΔCt calculation as an alternate method of data analysis for quantitative PCR of BCR-ABL P210 transcripts. Diagn. Mol. Pathol. 15: 56-61.

Ayres, J. S., and D. S. Schneider. 2012. Tolerance of infections. Annu. Rev. Immunol. 30: 271-294.

Azad, M. B., and A. L. Kozyrskyj. 2012. Perinatal Programming of Asthma: The Role of Gut Microbiota. Clin. Dev. Immunol. 2012: 9.

Baer, A. A., M. J. Miller, and A. C. Dilger. 2013. Pathogens of interest to the pork industry: a review of research on interventions to assure food safety. Compr. Re.v Food Sci. Food Saf. 12: 183-217.

Bailey, M., K. Haverson, C. Inman, C. Harris, P. Jones, G. Corfield, B. Miller, and C. Stokes. 2005. The development of the mucosal immune system pre-and post-weaning: balancing regulatory and effector function. Proc. Nutr. Soc. 64: 451-457.

Barreau, F., C. Cartier, L. Ferrier, J. Fioramonti, and L. Bueno. 2004. Nerve growth factor mediates alterations of colonic sensitivity and mucosal barrier induced by neonatal stress in rats. Gastroenterol. 127: 524-534.

Bartosch, S., A. Fite, G. T. Macfarlane, and M. E. McMurdo. 2004. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl. Environ. Microbiol. 70: 3575-3581.

Bedford, M. R., and H. Schulze. 1998. Exogenous enzymes for pigs and poultry. Nutr. Res. Rev. 11: 91-114.

Berg, R. D. 1996. The indigenous gastrointestinal microflora. Trends Microbiol. 4: 430-435.

Berkes, J., V. Viswanathan, S. Savkovic, and G. Hecht. 2003. Intestinal epithelial responses to enteric pathogens: effects on the tight junction barrier, ion transport, and inflammation. Gut 52: 439-451.

Bhandari, S., C. Nyachoti, and D. Krause. 2009. Raw potato starch in weaned pig diets and its influence on postweaning scours and the molecular microbial ecology of the digestive tract. J. Anim. Sci. 87: 984-993.

Bhandari, S., B. Xu, C. Nyachoti, D. Giesting, and D. Krause. 2008. Evaluation of alternatives to antibiotics using an K88 model of piglet diarrhea: Effects on gut microbial ecology. J. Anim. Sci. 86: 836-847.

Bode, C., B. Diedrich, S. Muenster, V. Hentschel, C. Weisheit, K. Rommelsheim, A. Hoeft, R. Meyer, O. Boehm, and P. Knuefermann. 2014. Antibiotics regulate the immune response in both presence and absence of lipopolysaccharide through modulation of Toll-like receptors, cytokine production and phagocytosis in vitro. Int. Immunopharmacol. 18: 27-34.

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. J. Nutr. 134: 2256-2262.

Boza, J. 1998. Nucleotides in infant nutrition. Monatsschrift Kinderheilkunde 146: S39-S48.

Bronk, J., and J. Hastewell. 1987. The transport of pyrimidines into tissue rings cut from rat small intestine. J. Physiol. 382: 475-488.

Brown, K., D. DeCoffe, E. Molcan, and D. L. Gibson. 2012. Diet-induced dysbiosis of the intestinal microbiota and the effects on immunity and disease. Nutrients 4: 1095-1119.

Burrin, D. G., T. A. Davis, S. Ebner, P. A. Schoknecht, M. L. Fiorotto, and P. J. Reeds. 1997. Colostrum enhances the nutritional stimulation of vital organ protein synthesis in neonatal pigs. J. Nutr. 127: 1284-1289.

Calder, P. C., S. Krauss-Etschmann, E. C. de Jong, C. Dupont, J.-S. Frick, H. Frokiaer, J. Heinrich, H. Garn, S. Koletzko, and G. Lack. 2006. Early nutrition and immunity–progress and perspectives. Br. J. Nutr. 96: 774-790.

Canadian Council on Animal Care. 2009. CCAC guidelines on: the care and use of farm animals in research, teaching and testing. Canadian Council on Animal Care, Ottawa, ON, Canada.

Carey, H. V., U. L. Hayden, and K. Tucker. 1994. Fasting alters basal and stimulated ion transport in piglet jejunum. Am. J. Physiol. Regul. Integr. Comp. Physiol. 267: R156-R163.

Carlson, M., T. Veum, and J. Turk. 2005. Effects of yeast extract versus animal plasma in weanling pig diets on growth performance and intestinal morphology. J. Swine Health Prod. 13: 204.

Carlson, M. S., C. A. Boren, C. Wu, C. E. Huntington, D. W. Bollinger, and T. L. Veum. 2004. Evaluation of various inclusion rates of organic zinc either as polysaccharide or proteinate complex on the growth performance, plasma, and excretion of nursery pigs. J. Anim. Sci. 82: 1359-1366.

Carver, J. D. 1994. Dietary nucleotides: cellular immune, intestinal and hepatic system effects. J. Nutr. 124: 144S-148S.

Carver, J. D., and W. A. Walker. 1995. The role of nucleotides in human nutrition. J. Nutr. Biochem. 6: 58-72.

Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phillips. 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. J. Antimicrob. Chemother. 52: 159-161.

Cerf-Bensussan, N., and D. Guy-Grand. 1991. Intestinal intraepithelial lymphocytes. Gastroenterol. Clin. North Am. 20: 549-576.

Choct, M. 2001. Alternatives to in-feed antibiotics in monogastric animal industry. ASA Technical Bulletin. AN30: 1-6.

Choi, J., P. Shinde, S. Ingale, J. Kim, Y. Kim, K. Kim, I. Kwon, and B. Chae. 2011. Evaluation of multi-microbe probiotics prepared by submerged liquid or solid substrate fermentation and antibiotics in weaning pigs. Livest. Sci. 138: 144-151.

Chu, S. 1991. Nucleotides: biochemistry and metabolism. Sem Pediat Gastr Nutr 2: 6-11.

Coates, M. E., R. Fuller, G. Harrison, M. Lev, and S. Suffolk. 1963. A comparision of the growth of chicks in the Gustafsson germ-free apparatus and in a conventional environment, with and without dietary supplements of penicillin. Br. J. Nutr. 17: 141-150.

Córdoba, X., E. Borda, and D. Martínez-puig. 2008. The role of nucleotides in piglet nutrition. Pig Progress 24 http://www.pigprogress.net/PageFiles/26477/001_boerderij-download-PP5809D01.pdf Accessed Date Accessed.| doi:DOI|

Cosgrove, M. 1998. Nucleotides. Nutrition 14: 748-751.

Cromwell, G. 1999. Subtherapeutic use of antibiotics for swine: performance, reproductive efficiency and safety issues. In: Proceeding of 40th George A. Young Swine Health and Management Conference, Lincoln. p 69-87.

Cromwell, G. 2001. Antimicrobial and promicrobial agents. Swine nutrition 2: 401-426.

Cromwell, G. L. 2002. Why and how antibiotics are used in swine production. Anim. Biotechnol. 13: 7-27.

D'Elia, R. V., K. Harrison, P. C. Oyston, R. A. Lukaszewski, and G. C. Clark. 2013. Targeting the “cytokine storm” for therapeutic benefit. Clin. Vaccine Immunol. 20: 319-327.

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. Ital. J. Anim. Sci. 2: 364-366.

Dierick, N., I. Vervaeke, J. Decuypere, and H. Henderickx. 1986. Influence of the gut flora and of some growth-promoting feed additives on nitrogen metabolism in pigs. II. Studies in vivo. Livest. Prod. Sci. 14: 177-193.

Domeneghini, C., A. Di Giancamillo, S. Arrighi, and G. Bosi. 2006. Gut-trophic feed additives and their effects upon the gut structure and intestinal metabolism. State of the art in the pig, and perspectives towards humans. Histol. Histopathol. 21: 273-283.

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. An histochemical and histometrical study. Histol. Histopathol. 19: 49-58.

Donovan, S., and J. Odle. 1994. Growth factors in milk as mediators of infant development. Annu. Rev. Nutr. 14: 147-167.

Fisher, K., and C. Phillips. 2009. The ecology, epidemiology and virulence of Enterococcus. Microbiology 155: 1749-1757.

Ford, R. P., D. J. Hill, and C. S. Hosking. 1983. Cows' milk hypersensitivity: immediate and delayed onset clinical patterns. Arch. Dis. Child. 58: 856-862.

Francois, A. 1961. Mode of action of antibiotics on growth. World. Rev. Nutr. Diet. 3: 21.

Furuta, K., I. Oku, and S. Morimoto. 1980. Effect of steam temperature in the pelleting process of chicken food on the viability of contaminating bacteria. Lab. Anim. 14: 293-296.

Gaskins, H. 2001. Intestinal bacteria and their influence on swine growth. In: J. L. Austin and L. L. Southern (eds.) Swine nutrition. p 609-630. CRC Press LLC, Florida.

Gaskins, H., C. Collier, and D. Anderson. 2002. Antibiotics as growth promotants: mode of action. Anim. Biotechnol. 13: 29-42.

Gil, A., E. Corral, A. Martinez, and J. Molina. 1986. Effects of the addition of nucleotides to an adapted milk formula on the microbial pattern of faeces in at term newborn infants. J Clin Nutr Gastroenterol 1: 127-132.

Gil, A., C. Gómez-León, and R. Rueda. 2007. Exogenous nucleic acids and nucleotides are efficiently hydrolysed and taken up as nucleosides by intestinal explants from suckling piglets. Br. J. Nutr. 98: 285-291.

Gomez, A., D. Luckey, C. J. Yeoman, E. V. Marietta, M. E. Berg Miller, J. A. Murray, B. A. White, and V. Taneja. 2012. Loss of sex and age driven differences in the gut

microbiome characterize arthritis-susceptible 0401 mice but not arthritis-resistant 0402 mice. PloS One 7: e36095.

Gong, J., F. Yin, Y. Hou, and Y. Yin. 2014. Review: Chinese herbs as alternatives to antibiotics in feed for swine and poultry production: Potential and challenges in application. Can. J. Anim. Sci. 94: 223-241.

Grannis, J., and D. Thilmany. 2001. Marketing opportunities for natural pork products: Defining the consumers who would purchase natural pork. J. Am. Soc. Farm Manage. Rural Appraisers 23: 93S98.

Grannis, J., and D. Thilmany. 2002. Marketing natural pork: An empirical analysis of consumers in the mountain region. Agribusiness 18: 475-489.

Grimble, G. K. 1996. Why are dietary nucleotides essential nutrients? Br. J. Nutr. 76: 475-478.

Grimble, G. K., and O. M. Westwood. 2000. Nucleotides. In: M. E. Gershwin, J. B. German and L. K. Carl (eds.) Nutrition and immunology: principles and practice. p 135-144. Humana Press, New York.

Grimble, G. K., and O. M. Westwood. 2001. Nucleotides as immunomodulators in clinical nutrition. Curr. Opin. Clin. Nutr. Metab. Care 4: 57-64.

Hahn, T.-W., J. Lohakare, S. Lee, W. Moon, and B. Chae. 2006. Effects of supplementation of β-glucans on growth performance, nutrient digestibility, and immunity in weanling pigs. J. Anim. Sci. 84: 1422-1428.

Hamard, A., D. Mazurais, G. Boudry, I. Le Huërou-Luron, B. Sève, and N. Le Floc'h. 2010. A moderate threonine deficiency affects gene expression profile, paracellular permeability and glucose absorption capacity in the ileum of piglets. J. Nutr. Biochem. 21: 914-921.

Hamer, H. M., D. Jonkers, K. Venema, S. Vanhoutvin, F. Troost, and R. Brummer. 2008. Review article: the role of butyrate on colonic function. Aliment. Pharmacol. Ther. 27: 104-119.

Hampson, D. 1986a. Alterations in piglet small intestinal structure at weaning. Res. Vet. Sci. 40: 32-40.

Hampson, D. J. 1986b. Alterations in piglet small intestinal structure at weaning. Res. Vet. Sci. 40: 32-40.

Han, Y. K., I. H. Hwang, and P. A. Thacker. 2011. Use of a micro-encapsulated eucalyptus-medium chain fatty acid product as an alternative to zinc oxide and antibiotics for weaned pigs. J. Swine Health Prod. 19: 34-43.

Han, Y. K., and P. A. Thacker. 2010. Effects of antibiotics, zinc oxide or a rare earth mineral-yeast product on performance, nutrient digestibility and serum parameters in weanling pigs. Asian Australas. J. Anim. Sci. 23: 1057-1065.

Heo, J., J. Kim, C. F. Hansen, B. Mullan, D. Hampson, and J. Pluske. 2009. Feeding a diet with decreased protein content reduces indices of protein fermentation and the incidence of postweaning diarrhea in weaned pigs challenged with an enterotoxigenic strain of. J. Anim. Sci. 87: 2833-2843.

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. J. Anim. Physiol. Anim. Nutr. 97: 207-237.

Heuer, O. E., A. M. Hammerum, P. Collignon, and H. C. Wegener. 2006. Human health hazard from antimicrobial-resistant enterococci in animals and food. Clin. Infect. Dis. 43: 911-916.

Heyman, M., G. Boudraa, S. Sarrut, M. Giraud, L. Evans, M. Touhami, and J.-F. Desjeux. 1984. Macromolecular transport in jejunal mucosa of children with severe malnutrition: a quantitative study. J. Pediatr. Gastroenterol. Nutr. 3: 357-363.

Holt, C., and L. Sawyer. 1988. Primary and predicted secondary structures of the caseins in relation to their biological functions. Protein Eng. Des. Sel. 2: 251-259.

Hopwood, D., and D. Hampson. 2003. Interactions between the intestinal microflora, diet and diarrhoea, and their influences on piglet health in the immediate post-weaning period. In: J. R. Pluske, J. Le Dividich and M. W. A. Verstegen (eds.) Weaning the pig: concepts and consequences. p 199-217. Wageningen Academic Publishers, Gelderland, The Netherlands.

Hou, Y., L. Wang, B. Ding, Y. Liu, H. Zhu, J. Liu, Y. Li, X. Wu, Y. Yin, and G. Wu. 2010. Dietary α-ketoglutarate supplementation ameliorates intestinal injury in lipopolysaccharide-challenged piglets. Amino Acids 39: 555-564.

Huang, Y., J. Yoo, H. Kim, Y. Wang, Y. Chen, J. Cho, and I. Kim. 2010. Effects of dietary supplementation with blended essential oils on growth performance, nutrient digestibility, blood profiles and fecal characteristics in weanling pigs. Asian Australas. J. Anim. Sci. 23: 607.

Huber, M., J. A. Knottnerus, L. Green, H. van der Horst, A. R. Jadad, D. Kromhout, B. Leonard, K. Lorig, M. I. Loureiro, and J. W. van der Meer. 2011. How should we define health? Br. Med. J. 343: d4163.

Hung, I. F. 2015. The effect of dietary nucleotides in sow and nursery piglet diets on reproduction, growth, and immune response. PhD Thesis, University of Kentucky, Lexington.

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

Jarvis, S. M. 1989. Characterization of sodium-dependent nucleoside transport in rabbit intestinal brush-border membrane vesicles. Biochim. Biophys. Acta 979: 132-138.

Jeaurond, E., M. Rademacher, J. Pluske, C. Zhu, and C. De Lange. 2008. Impact of feeding fermentable proteins and carbohydrates on growth performance, gut health and gastrointestinal function of newly weaned pigs. Can. J. Anim. Sci. 88: 271-281.

Jensen, M. L., T. Thymann, M. S. Cilieborg, M. Lykke, L. Mølbak, B. B. Jensen, M. Schmidt, D. Kelly, I. Mulder, and D. G. Burrin. 2014. Antibiotics modulate intestinal immunity and prevent necrotizing enterocolitis in preterm neonatal piglets. Am. J. Physiol-Gastr. L. 306: G59-G71.

Jimenez-Valera, M., and A. Ruiz-Bravo. 2011. Immunity to Bacterial Infections. Curr. Immunol. Rev. 7: 3-12.

Johnson, R. W. 1997. Inhibition of growth by pro-inflammatory cytokines: an integrated view. J. Anim. Sci. 75: 1244-1255.

Jukes, T. H. 1972. Antibiotics in animal feeds and animal production. Bioscience 22: 526-534.

Kaji, I., Y. Akiba, and J. Kaunitz. 2013. Digestive physiology of the pig symposium: Involvement of gut chemosensing in the regulation of mucosal barrier function and defense mechanisms. J. Anim. Sci. 91: 1957-1962.

Kartha, S., and F. G. Toback. 1985. Purine nucleotides stimulate DNA synthesis in kidney epithelial cells in culture. Am. J. Physiol. Renal Physiol. 249: F967-F972.

Kawamata, Y., R. Fujii, M. Hosoya, M. Harada, H. Yoshida, M. Miwa, S. Fukusumi, Y. Habata, T. Itoh, and Y. Shintani. 2003. AG protein-coupled receptor responsive to bile acids. J. Biol. Chem. 278: 9435-9440.

Kelly, D., and T. King. 2001. Digestive physiology and development in pigs. In: M. A. Varley and J. Wiseman (eds.) The weaner pig: nutrition and management. p 179-206. CABI Publishing, Oxford, UK.

Kelly, D., J. Smyth, and K. McCracken. 1991. Digestive development of the early-weaned pig. Br. J. Nutr. 65: 169-180.

Khafipour, E., S. Li, J. C. Plaizier, and D. O. Krause. 2009. Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis. Appl. Environ. Microbiol. 75: 7115-7124.

Khan, K., and A. Bhati. 2010. Whey protein and its application to human. J. Exp. Sci. 1: 16-20.

Khan, M. M. 2008. Role of Cytokines Immunopharmacology. p 33-59. Springer.

Kiarie, E., S. Bhandari, M. Scott, D. Krause, and C. Nyachoti. 2011. Growth performance and gastrointestinal microbial ecology responses of piglets receiving Saccharomyces cerevisiae fermentation products after an oral challenge with Escherichia coli (K88) 1. J. Anim. Sci. 89: 1062.

Kiarie, E. G., C. M. Nyachoti, B. A. Slominski, and G. Blank. 2007. Growth performance, gastrointestinal microbial activity, and nutrient digestibility in early-weaned pigs fed diets containing flaxseed and carbohydrase enzyme. J. Anim. Sci. 85: 2982-2993.

Kiarie, E. G., B. A. Slominski, D. O. Krause, and C. M. Nyachoti. 2008. Nonstarch polysaccharide hydrolysis products of soybean and canola meal protect against enterotoxigenic *Escherichia coli* in piglets. J. Nutr. 138: 502-508.

Kim, J., C. F. Hansen, B. Mullan, and J. Pluske. 2012. Nutrition and pathology of weaner pigs: nutritional strategies to support barrier function in the gastrointestinal tract. Anim. Feed Sci. Technol. 173: 3-16.

King, M., D. Kelly, P. Morel, and J. Pluske. 2003. Aspects of intestinal immunity in the pig around weaning. In: J. R. Pluske, J. Le Dividich and M. W. A. Verstegen (eds.) Weaning the pig: concepts and consequences. p 219-257. Wageningen Academic Publishers, Gelderland, The Netherlands.

Kluger, M. J. 1991. Fever: role of pyrogens and cryogens. Physiol Rev 71: 93-127.

Kogan, G., and A. Kocher. 2007. Role of yeast cell wall polysaccharides in pig nutrition and health protection. Livest. Sci. 109: 161-165.

Kondro, W. 2002. Canadian scientists urge government to develop antibiotic plan. Lancet 360: 1230.

Konstantinov, S. R., E. Poznanski, S. Fuentes, A. D. Akkermans, H. Smidt, and W. M. De Vos. 2006. Lactobacillus sobrius sp. nov., abundant in the intestine of weaning piglets. Int. J. Syst. Evol. Microbiol. 56: 29-32.

Kulkarni, A. D., F. B. Rudolph, and C. T. Van Buren. 1994. The role of dietary sources of nucleotides in immune function: a review. J. Nutr. 124: 1442S-1446S.

Kümmerer, K. 2003. Significance of antibiotics in the environment. J. Antimicrob. Chemother. 52: 5-7.

Kuralkar, P., and S. Kuralkar. 2010. Nutritional and Immunological Importance of Colostrum for the new born. Vet. World. 3: 46-47.

Labro, M.-T. 1998. Antibacterial agents—phagocytes: new concepts for old in immunomodulation. Int. J. Antimicrob. Agents 10: 11-21.

Lallès, J.-P., P. Bosi, H. Smidt, and C. R. Stokes. 2007. Nutritional management of gut health in pigs around weaning. Proc. Nutr. Soc. 66: 260-268.

Lallès, J.-P., G. Boudry, C. Favier, N. Le Floc'h, I. Luron, L. Montagne, I. P. Oswald, S. Pié, C. Piel, and B. Sève. 2004. Gut function and dysfunction in young pigs: physiology. Anim. Res. 53: 301-316.

Lang, P., H. Xu, M. Grusdat, D. McIlwain, A. Pandyra, I. Harris, N. Shaabani, N. Honke, S. K. Maney, and E. Lang. 2013. Reactive oxygen species delay control of lymphocytic choriomeningitis virus. Cell Death Differ. 20: 649-658.

Latchman, Y., C. R. Wood, T. Chernova, D. Chaudhary, M. Borde, I. Chernova, Y. Iwai, A. J. Long, J. A. Brown, and R. Nunes. 2001. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat. Immunol. 2: 261-268.

Le Dividich, J., T. Esnault, B. Lynch, R. Hoo-Paris, C. Castex, and J. Peiniau. 1991. Effect of colostral fat level on fat deposition and plasma metabolites in the newborn pig. J. Anim. Sci. 69: 2480-2488.

Le Dividich, J., P. Herpin, and R. M. Rosario-Ludovino. 1994. Utilization of colostral energy by the newborn pig. J. Anim. Sci. 72: 2082-2089.

Le Dividich, J., J. Rooke, and P. Herpin. 2005. Nutritional and immunological importance of colostrum for the new-born pig. J. Agric. Sci. 143: 469-485.

Lee, D.-H., Y.-G. Zo, and S.-J. Kim. 1996. Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. Appl. Environ. Microbiol. 62: 3112-3120.

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. J. Anim. Physiol. Anim. Nutr. 91: 508-518.

LeLeiko, N. S., A. D. Bronstein, B. S. Baliga, and H. N. Munro. 1983. De novo purine nucleotide synthesis in the rat small and large intestine: effect of dietary protein and purines. J. Pediatr. Gastroenterol. Nutr. 2: 313-319.

Levine, U. Y., T. Looft, H. K. Allen, and T. B. Stanton. 2013. Butyrate-producing bacteria, including mucin degraders, from the swine intestinal tract. Appl. Environ. Microbiol. 79: 3879-3881.

Li, H., P. Zhao, Y. Lei, T. Li, and I. Kim. 2015. Response to an Escherichia coli K88 oral challenge and productivity of weanling pigs receiving a dietary nucleotides supplement. J. Anim. Sci. Biotechnol. 6: 1-9.

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. J. Microbiol. 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 Australas. J. Anim. Sci. 25: 1617.

Liang, S. C., Y. E. Latchman, J. E. Buhlmann, M. F. Tomczak, B. H. Horwitz, G. J. Freeman, and A. H. Sharpe. 2003. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. Eur. J. Immunol. 33: 2706-2716.

Liu, Y., J. Huang, Y. Hou, H. Zhu, S. Zhao, B. Ding, Y. Yin, G. Yi, J. Shi, and W. Fan. 2008. Dietary arginine supplementation alleviates intestinal mucosal disruption induced by *Escherichia coli* lipopolysaccharide in weaned pigs. Br. J. Nutr. 100: 552-560.

Liu, Y. L., D. F. Li, L. M. Gong, G. F. Yi, A. M. Gaines, and J. A. Carroll. 2003. Effects of fish oil supplementation on the performance and the immunological, adrenal, and somatotropic responses of weaned pigs after an lipopolysaccharide challenge. J. Anim. Sci. 81: 2758-2765.

Lodish, H. F., A. Berk, S. L. Zipursky, P. Matsudaira, D. Baltimore, and J. Darnell. 2000. Molecular cell biology. 5 ed. WH Freeman and Company, New York.

Lomax, L. G. 1981. Porcine proliferative enteritis-characterization of the naturally occurring and experimental disease. PhD Thesis, Iowa State University, Ames.

Lomer, M. C., R. P. Thompson, J. Commisso, C. L. Keen, and J. J. Powell. 2000. Determination of titanium dioxide in foods using inductively coupled plasma optical emission spectrometry. Analyst 125: 2339-2343.

Looft, T., T. A. Johnson, H. K. Allen, D. O. Bayles, D. P. Alt, R. D. Stedtfeld, W. J. Sul, T. M. Stedtfeld, B. Chai, and J. R. Cole. 2012. In-feed antibiotic effects on the swine intestinal microbiome. Proc. Natl. Acad. Sci. 109: 1691-1696.

Louis, J. A., and P. H. Lambert. 1979. Lipopolysaccharides: from immunostimulation to autoimmunity. Springer Semin. Immunopathol. 2: 215-228.

Lusk, J. L., F. B. Norwood, and J. R. Pruitt. 2006. Consumer demand for a ban on antibiotic drug use in pork production. Am. J. Agric. Econ. 88: 1015-1033.

Ma, J., T. Chen, J. Mandelin, A. Ceponis, N. Miller, M. Hukkanen, G. Ma, and Y. Konttinen. 2003. Regulation of macrophage activation. Cell. Mol. Life Sci. 60: 2334-2346.

Mackie, R. I., A. Sghir, and H. R. Gaskins. 1999. Developmental microbial ecology of the neonatal gastrointestinal tract. Am. J. Clin. Nutr. 69: 1035s-1045s.

Macpherson, A., K. McCoy, F. Johansen, and P. Brandtzaeg. 2008. The immune geography of IgA induction and function. Mucosal Immunol. 1: 11-22.

Martín, R., S. Delgado, A. Maldonado, E. Jiménez, M. Olivares, L. Fernández, O. J. Sobrino, and J. M. Rodríguez. 2009. Isolation of lactobacilli from sow milk and evaluation of their probiotic potential. J. Dairy Res. 76: 418-425.

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. Livest. Sci. 108: 276-279.

Masey-O'Neill, H., M. Singh, and A. Cowieson. 2014. Effects of exogenous xylanase on performance, nutrient digestibility, volatile fatty acid production and digestive tract thermal profiles of broilers fed on wheat-or maize-based diet. Br. Poult. Sci. 55: 351-359.

Mateo, C. D. 2005. Aspects of Nucleotide Nutrition in Pigs. PhD Diss., Doctoral dissertation, South Dakota State University, Brookings, USA, Brookings.

Mateo, C. D., D. N. Peters, and H. H. Stein. 2004. Nucleotides in sow colostrum and milk at different stages of lactation. J. Anim. Sci. 82: 1339-1342.

Matsuki, T., K. Watanabe, J. Fujimoto, Y. Miyamoto, T. Takada, K. Matsumoto, H. Oyaizu, and R. Tanaka. 2002. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl. Environ. Microbiol. 68: 5445-5451.

Matsuki, T., K. Watanabe, J. Fujimoto, T. Takada, and R. Tanaka. 2004. Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. Appl. Environ. Microbiol. 70: 7220-7228.

McCracken, B. A., H. R. Gaskins, P. J. Ruwe-Kaiser, K. C. Klasing, and D. E. Jewell. 1995. Diet-dependent and diet-independent metabolic responses underlie growth stasis of pigs at weaning. J. Nutr. 125: 2838-2845.

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. J. Nutr. 129: 613-619.

McLamb, B. L., A. J. Gibson, E. L. Overman, C. Stahl, and A. J. Moeser. 2013. Early weaning stress in pigs impairs innate mucosal immune responses to enterotoxigenic E. coli challenge and exacerbates intestinal injury and clinical disease. PloS One 8: e59838.

Medzhitov, R. 2007. Recognition of microorganisms and activation of the immune response. Nature 449: 819-826.

Menin, Á., C. Reck, D. d. Souza, C. Klein, and E. Vaz. 2008. Enteropathogenic bacterial agents in pigs of different age groups and profile of resistance in strains of Escherichia coli and Salmonella spp. to antimicrobial agents. Cienc. Rural 38: 1687-1693.

Miller, B., P. James, M. Smith, and F. Bourne. 1986. Effect of weaning on the capacity of pig intestinal villi to digest and absorb nutrients. J. Agric. Sci. 107: 579-590.

Moore, K., B. Mullan, J. Pluske, J. Kim, and D. 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. Anim. Feed Sci. Technol. 165: 184-190.

Motono, M. 1982. Flavor nucleotides' usages in foods. In: G. Charalambous and G. Inglett (eds.) Chemistry of Foods and Beverages Recent Developments. p 181-194. Academic Press, New York.

Moxley, R. A., and G. E. Duhamel. 1999. Comparative pathology of bacterial enteric diseases of swine Mechanisms in the Pathogenesis of Enteric Diseases 2. p 83-101. Springer.

Nagaraja, T., M. Taylor, D. Harmon, and J. Boyer. 1987. In vitro lactic acid inhibition and alterations in volatile fatty acid production by antimicrobial feed additives. J. Anim. Sci. 65: 1064-1076.

Namkung, H., M. Li J. Gong, H. Yu, M. Cottrill, and C. de Lange. 2004. Impact of feeding blends of organic acids and herbal extracts on growth performance, gut microbiota and digestive function in newly weaned pigs. Can. J. Anim. Sci. 84: 697-704.

Nau, R., and S. Tauber. 2008. Immunomodulatory properties of antibiotics. Curr. Mol. Pharmacol. 1: 68-79.

Nelson, D. L., A. L. Lehninger, and M. M. Cox. 2008. Chapter 8: Nucleotides and Nucleic Acid. In: D. L. Nelson and M. M. Cox (eds.) Lehninger principles of biochemistry. WH Freeman and Company, New York.

Nguyen, T. V., L. Yuan, M. S. Azevedo, K.-I. Jeong, A.-M. González, and L. J. Saif. 2007. Transfer of maternal cytokines to suckling piglets: in vivo and in vitro models with implications for immunomodulation of neonatal immunity. Vet. Immunol. Immunopathol. 117: 236-248.

Niewold, T. A. 2007. The nonantibiotic anti-Inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. Poult. Sci. 86: 605-609.

Nilsson, T., K. Foster, and J. L. Lusk. 2006. Marketing opportunities for certified pork chops. Can. J. Agric. Econ. 54: 567-583.

NRC. 2012. Nutrient Requirements of Swine. 11th ed. National Academic Press, Washington, DC.

Nyachoti, C., F. Omogbenigun, M. Rademacher, and G. Blank. 2006. Performance responses and indicators of gastrointestinal health in early-weaned pigs fed low-protein amino acid-supplemented diets. J. Anim. Sci. 84: 125-134.

Nyachoti, C. M., E. Kiarie, S. K. Bhandari, G. Zhang, and D. O. Krause. 2012. Weaned pig responses to K88 oral challenge when receiving a lysozyme supplement. J. Anim. Sci. 90: 252-260.

Nygard, A.-B., C. B. Jørgensen, S. Cirera, and M. Fredholm. 2007. Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. BMC Mol. Biol. 8: 1.

O'Neill, H. V. M., J. A. Smith, and M. R. Bedford. 2014. Multicarbohydrase enzymes for non-ruminants. Asian Australas. J. Anim. Sci. 27: 290.

Olofsson, S. K., and O. Cars. 2007. Optimizing drug exposure to minimize selection of antibiotic resistance. Clin. Infect. Dis. 45: S129-S136.

Owusu-Asiedu, A., C. M. Nyachoti, and R. R. Marquardt. 2003. Response of early-weaned pigs to an enterotoxigenic (K88) challenge when fed diets containing spray-dried porcine plasma or pea protein isolate plus egg yolk antibody, zinc oxide, fumaric acid, or antibiotic. J. Anim. Sci. 81: 1790-1798.

Paubert-Braquet, M., N. Hedef, S. Picquot, C. Dupont, and R. Paoletti. 1992. Quantification of nucleotides in human milk and their effects on cytokine production by murine fibroblasts, J774A1 macrophages and human monocytes. In: M. Paubert-Braquet, C. Dupont and R. Paoletti (eds.) Foods, nutrition and immunity: effects of dairy and fermented milk products. p 22-34. S. Karger AG, Basel.

Peng, J. M., Z. J. Tian, H. G. Liu, T. Q. An, Y. J. Zhou, Y. Wang, D.-Y. Li, J. Z. Chen, Y. Q. Yang, and G. Z. Tong. 2010. Cloning and identification of porcine programmed death 1. Vet. Immunol. Immunopathol. 136: 157-162.

Pescovitz, M., R. Pabst, H. Rothkötter, M. Murtaugh, D. Foss, J. Butler, J. Lunney, P. Vogeli, D. Llanes, and I. Trebichavsky. 1998. Immunology of the pig. In: P. P. Pastoret, P. Griebel, H. Bazin and A. Govaerts (eds.) Handbook of vertebrate immunology. p 373-419. Academic Press, London.

Pi, D., Y. Liu, H. Shi, S. Li, J. Odle, X. Lin, H. Zhu, F. Chen, Y. Hou, and W. Leng. 2014. Dietary supplementation of aspartate enhances intestinal integrity and energy status in weanling piglets after lipopolysaccharide challenge. J. Nutr. Biochem. 25: 456-462.

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. J. Nutr. 134: 641-647.

Pizzini, R. P., S. Kumar, A. D. Kulkarni, F. B. Rudolph, and C. T. Van Buren. 1990. Dietary nucleotides reverse malnutrition and starvation-induced immunosuppression. Arch. Surg. 125: 86-90.

Plante, P., J.-P. Laforest, and C. Farmer. 2011. Effect of supplementing the diet of lactating sows with NuPro® on sow lactation performance and piglet growth. Can. J. Anim. Sci. 91: 295-300.

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. Livest. Prod. Sci. 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. Nutr. Res. Rev. 15: 333-371.

Pluske, J. R., M. J. Thompson, C. S. Atwood, P. H. Bird, I. H. Williams, and P. E. Hartmann. 1996. Maintenance of villus height and crypt depth, and enhancement of disaccharide digestion and monosaccharide absorption, in piglets fed on cows' whole milk after weaning. Br. J. Nutr. 76: 409-422.

Pluske, J. R., I. H. Williams, and F. X. Aherne. 1995. Nutrition of the neonatal pig. In: M. A. Varley (ed.) The neonatal pig: development and survival. CAB International, Wallingford, UK.

Pomorska-Mól, M., I. Markowska-Daniel, and D. Bednarek. 2010. Flow cytometric analysis of leukocytes in porcine mammary secretions. Bull. Vet. Inst. Pulawy 54: 188-192.

Qin, J., Y. Li, Z. Cai, S. Li, J. Zhu, F. Zhang, S. Liang, W. Zhang, Y. Guan, and D. Shen. 2012. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature 490: 55-60.

Quiles, Huertas, Mañas, Ochoa, Battino, and Mataix. 1999. Oxidative stress induced by exercise and dietary fat modulates the coenzyme Q and vitamin A balance between plasma and mitochondria. Int. J. Vitam. Nutr. Res. 69: 243-249.

Rakhshandeh, A., and C. F. M. de Lange. 2012. Evaluation of chronic immune system stimulation models in growing pigs. Animal 6: 305-310.

Rasmussen, R. 2001. Quantification on the LightCycler. In: S. Meuer, C. Wittwer and K. I. Nakagawara (eds.) Rapid Cycle Real-Time PCR. p 21-34. Springer, Berlin.

Rijkers, G. T., W. M. De Vos, R.-J. Brummer, L. Morelli, G. Corthier, and P. Marteau. 2011. Health benefits and health claims of probiotics: bridging science and marketing. Br. J. Nutr. 106: 1291-1296.

Rinttilä, T., A. Kassinen, E. Malinen, L. Krogius, and A. Palva. 2004. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J. Appl. Microbiol. 97: 1166-1177.

Rist, V., 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.

Rodriguez, P., N. Darmon, P. Chappuis, C. Candalh, M. Blaton, C. Bouchaud, and M. Heyman. 1996. Intestinal paracellular permeability during malnutrition in guinea pigs: effect of high dietary zinc. Gut 39: 416-422.

Romick-Rosendale, L. E., A. Legomarcino, N. B. Patel, A. L. Morrow, and M. A. Kennedy. 2014. Prolonged antibiotic use induces intestinal injury in mice that is repaired after removing antibiotic pressure: implications for empiric antibiotic therapy. Metabolomics 10: 8-20.

Salmon, H. 2002. Mammary gland immunology and neonate protection in pigs Biology of the Mammary Gland. p 279-286. Springer.

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. Anim. Sci. 81: 135-140.

Sanderson, I. R., and Y. He. 1994. Nucleotide uptake and metabolism by intestinal epithelial cells. J. Nutr. 124: 131S-137S.

Santacruz, A., M. d. C. Collado, L. Garcia-Valdes, M. Segura, J. Martin-Lagos, T. Anjos, M. Marti-Romero, R. Lopez, J. Florido, and C. Campoy. 2010. Gut microbiota composition is associated with body weight, weight gain and biochemical parameters in pregnant women. Br. J. Nutr. 104: 83-92.

Sauer, N., E. Bauer, W. Vahjen, J. Zentek, and R. Mosenthin. 2010. Nucleotides modify growth of selected intestinal bacteria in vitro. Livest. Sci. 133: 161-163.

Sauer, N., M. Eklund, E. Bauer, M. G. Gaenzle, C. J. Field, R. T. Zijlstra, and R. Mosenthin. 2012a. The effects of pure nucleotides on performance, humoral immunity, gut structure and numbers of intestinal bacteria of newly weaned pigs. J. Anim. Sci. 90: 3126-3134.

Sauer, N., M. Eklund, S. Roth, F. Rink, D. Jezierny, E. Bauer, and R. Mosenthin. 2012b. 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. J. Anim. Physiol. Anim. Nutr. 96: 700-708.

Sauer, N., R. Mosenthin, and E. Bauer. 2011. The role of dietary nucleotides in single-stomached animals. Nutr. Res. Rev. 24: 46-59.

Savaiano, D., and A. Clifford. 1978. Absorption, tissue incorporation and excretion of free purine bases in the rat. Nutr. Rep. Int.: 551-556.

Schubert, M. L., and D. A. Peura. 2008. Control of gastric acid secretion in health and disease. Gastroenterol. 134: 1842-1860.

Schulz, I., and H. H. Stolze. 1980. The exocrine pancreas: the role of secretagogues, cyclic nucleotides, and calcium in enzyme secretion. Annu. Rev. Physiol. 42: 127-156.

Sekirov, I., S. L. Russell, L. C. M. Antunes, and B. B. Finlay. 2010. Gut microbiota in health and disease. Physiol. Rev. 90: 859-904.

Sharpe, A. H., E. J. Wherry, R. Ahmed, and G. J. Freeman. 2007. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. Nat. Immunol. 8: 239-245.

Sherman, M. A., and D. Kalman. 2004. Initiation and resolution of mucosal inflammation. Immunol. Res. 29: 241-252.

Silva, S. V., and F. X. Malcata. 2005. Caseins as source of bioactive peptides. Int. Dairy J. 15: 1-15.

Simmen, F., K. Cera, and D. Mahan. 1990. Stimulation by colostrum or mature milk of gastrointestinal tissue development in newborn pigs. J. Anim. Sci. 68: 3596-3603.

Singhal, A., K. Kennedy, J. Lanigan, H. Clough, W. Jenkins, A. Elias-Jones, T. Stephenson, P. Dudek, and A. Lucas. 2010. Dietary nucleotides and early growth in formula-fed Infants: A randomized controlled trial. Pediatrics 126: e946-e953.

Singhal, A., G. Macfarlane, S. Macfarlane, J. Lanigan, K. Kennedy, A. Elias-Jones, T. Stephenson, P. Dudek, and A. Lucas. 2008. Dietary nucleotides and fecal microbiota

in formula-fed infants: a randomized controlled trial. Am. J. Clin. Nutr. 87: 1785-1792.

Skinner, L. D., C. L. Levesque, D. Wey, M. Rudar, J. Zhu, S. Hooda, and C. F. M. de Lange. 2014. Impact of nursery feeding program on subsequent growth performance, carcass quality, meat quality, and physical and chemical body composition of growing-finishing pigs. J. Anim. Sci. 92: 1044-1054.

Smith, M. W. 1992. Diet effects on enterocyte development. Proc. Nutr. Soc. 51: 173-178.

Songer, J. G., and F. A. Uzal. 2005. Clostridial enteric infections in pigs. J. Vet. Diagn. Invest. 17: 528-536.

Sonoda, T., and M. Tatibana. 1978. Metabolic fate of pyrimidines and purines in dietary nucleic acids ingested by mice. Biochim. Biophys. Acta 521: 55-66.

Šperanda, M., M. Đidara, T. Šperanda, M. Domaćinović, H. Valpotić, J. Kovačević, Z. Antunović, and J. Novoselec. 2008. Hydrolyzed brewery yeast product like immun omodulator in weaned piglets. Arch. Zootech. 11: 52-60.

Spreeuwenberg, M. A. M. 2002. Diet composition and gut integrity in weaned piglets. Ph.D. thesis, Wageningen University, Wageningen, The Netherlands.

Spurlock, M. 1997. Regulation of metabolism and growth during immune challenge: an overview of cytokine function. J. Anim. Sci. 75: 1773-1783.

Stokes, C. R., M. Bailey, K. Haverson, C. Harris, P. Jones, C. Inman, S. Pié, I. P. Oswald, B. A. Williams, and A. D. Akkermans. 2004. Postnatal development of intestinal immune system in piglets: implications for the process of weaning. Anim. Res. 53: 325-334.

Subasinghe, D., N. Nawarathna, and D. N. Samarasekera. 2011. Disease characteristics of inflammatory bowel disease (IBD). J. Gastrointest. Surg. 15: 1562-1567.

Sudo, N., S.-a. Sawamura, K. Tanaka, Y. Aiba, C. Kubo, and Y. Koga. 1997. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. J. Immunol. 159: 1739-1745.

Sukumar, P., A. Loo, E. Magur, J. Nandi, A. Oler, and R. A. Levine. 1997. Dietary supplementation of nucleotides and arginine promotes healing of small bowel ulcers in experimental ulcerative ileitis. Dig. Dis. Sci. 42: 1530-1536.

Superchi, P., R. Saleri, P. Borghetti, E. De Angelis, L. Ferrari, V. Cavalli, P. Amicucci, M. C. Ossiprandi, and A. Sabbioni. 2012. Effects of dietary nucleotide supplementation on growth performance and hormonal and immune responses of piglets. Animal 6: 902-908.

Suzuki, K., K. A. Simpson, J. S. Minnion, J. C. Shillito, and S. R. Bloom. 2010. The role of gut hormones and the hypothalamus in appetite regulation. Endocr. J. 57: 359-372.

Swords, W., C.-C. Wu, F. Champlin, and R. Buddington. 1993. Postnatal changes in selected bacterial groups of the pig colonic microflora. Neonatology 63: 191-200.

Tanaka, R., and M. Mutai. 1980. Improved medium for selective isolation and enumeration of Bifidobacterium. Appl. Environ. Microbiol. 40: 866-869.

Teillant, A., and R. Laxminarayan. 2015. Economics of antibiotic use in US swine and poultry production Choices No. 30. p 1-11. Agricultural & Applied Economics Association, Milwaukee, USA.

Thomas, M., D. Van Zuilichem, and A. Van der Poel. 1997. Physical quality of pelleted animal feed. 2. Contribution of processes and its conditions. Anim. Feed Sci. Technol. 64: 173-192.

Thymann, T., K. U. Sørensen, M. S. Hedemann, J. Elnif, B. B. Jensen, H. Banga-Mboko, T. D. Leser, and P. T. Sangild. 2007. Antimicrobial treatment reduces intestinal

microflora and improves protein digestive capacity without changes in villous structure in weanling pigs. Br. J. Nutr. 97: 1128-1137.

Toback, F. G., M. M. Walsh-Reitz, S. R. Mendley, and S. Kartha. 1990. Kidney epithelial cells release growth factors in response to extracellular signals. Pediatr. Nephrol. 4: 363-371.

Uauy, R., R. Quan, and A. Gil. 1994. Role of nucleotides in intestinal development and repair: implications for infant nutrition. J. Nutr. 124: 1436S-1441S.

Uauy, R., G. Stringel, R. Thomas, and R. Quan. 1990. Effect of dietary nucleosides on growth and maturation of the developing gut in the rat. J. Pediatr. Gastroenterol. Nutr. 10: 497-503.

van Beers-Schreurs, H. M., M. J. Nabuurs, L. Vellenga, H. J. Kalsbeek-van der Valk, T. Wensing, and H. J. Breukink. 1998. Weaning and the weanling diet influence the villous height and crypt depth in the small intestine of pigs and alter the concentrations of short-chain fatty acids in the large intestine and blood. J. Nutr. 128: 947-953.

Van Buren, C. T., and F. Rudolph. 1997. Dietary nucleotides: a conditional requirement. Nutrition 13: 470-472.

van den Broek, P. J. 1989. Antimicrobial drugs, microorganisms, and phagocytes. Rev. Infect. Dis. 11: 213-245.

Van Deun, K., F. Haesebrouck, F. Van Immerseel, R. Ducatelle, and F. Pasmans. 2008. Short-chain fatty acids and L-lactate as feed additives to control Campylobacter jejuni infections in broilers. Avian Pathol. 37: 379-383.

Visek, W. 1978. The mode of growth promotion by antibiotics. J. Anim. Sci. 46: 1447-1469.

Vitagliano, L. A. 2014. Nucleotide supplementation in the diet of farrowing sows and its effect on milk quality, litter weight gain, and mortality. In: 2014 ADSA-ASAS-CSAS Joint Annual Meeting

Waititu, S. M., J. M. Heo, R. Patterson, and C. M. Nyachoti. 2013. Effect of supplementing a nucleotide-rich yeast extract without or with in-feed antibiotics on performance and total-tract nutrient digestibilities in weaned piglets. In: ADSA-ASAS Joint Annual Meeting, Indianapolis, IN, USA

Walter, J., C. Hertel, G. W. Tannock, C. M. Lis, K. Munro, and W. P. Hammes. 2001. Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 67: 2578-2585.

Wang, J.-H., T. Inoue, M. Higashiyama, P. H. Guth, E. Engel, J. D. Kaunitz, and Y. Akiba. 2011. Umami receptor activation increases duodenal bicarbonate secretion via glucagon-like peptide-2 release in rats. J. Pharmacol. Exp. Ther. 339: 464-473.

Wannemacher, R. W. 1977. Key role of various individual amino acids in host response to infection. Am. J. Clin. Nutr. 30: 1269-1280.

Weaver, A., and S. Kim. 2014. Supplemental nucleotides high in inosine 5′-monophosphate to improve the growth and health of nursery pigs. J. Anim. Sci. 92: 645-651.

Webel, D. M., B. N. Finck, D. H. Baker, and R. W. Johnson. 1997. Time course of increased plasma cytokines, cortisol, and urea nitrogen in pigs following intraperitoneal injection of lipopolysaccharide. J. Anim. Sci. 75: 1514-1520.

Weber, T. E., A. P. Schinckel, K. L. Houseknecht, and B. T. Richert. 2001. Evaluation of conjugated linoleic acid and dietary antibiotics as growth promotants in weanling pigs. J. Anim. Sci. 79: 2542-2549.

Wen, L., R. E. Ley, P. Y. Volchkov, P. B. Stranges, L. Avanesyan, A. C. Stonebraker, C. Hu, F. S. Wong, G. L. Szot, and J. A. Bluestone. 2008. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. Nature 455: 1109-1113.

Wheatley, W. P. 2003. The natural and organic pork market: A sustainable niche for small-scale producers? A review and analysis of the evidence. Am. J. Alternative Agric. 18: 18-26.

Whittemore, C., and D. Green. 2001. Growth of the young weaned pig. In: M. A. Varley and J. Wiseman (eds.) The weaner pig: nutrition and management. p 1-15. CAB International, Oxford, UK.

Williams, B. A., M. W. Bosch, A. Awati, S. R. Konstantinov, H. Smidt, A. D. Akkermans, M. W. Verstegen, and S. Tamminga. 2005. In vitro assessment of gastrointestinal tract (GIT) fermentation in pigs: Fermentable substrates and microbial activity. Anim. Res. 54: 191-201.

Williams, P. P. 1993. Immunomodulating effects of intestinal absorbed maternal colostral leukocytes by neonatal pigs. Can. J. Vet. Res. 57: 1.

Wilson, B. A., A. A. Salyers, D. D. Whitt, and M. E. Winkler. 2011. The second line of defense: antibodies and cytotoxic T cells. In: B. A. Wilson, A. A. Salyers, D. D. Whitt and M. E. Winkler (eds.) Bacterial pathogenesis: a molecular approach. p 51-72. American Society for Microbiology (ASM), Washington, USA.

World Health Organization. 1946. Preamble to the Constitution of the World Health Organization as adopted by the International Health Conference.[Official Records of the World Health Organization, no. 2, p. 100] 1948 [cited 2012 May 4]. New York, June, Geneva, Switzerland.

Woyengo, T. A., J. S. Sands, W. Guenter, and C. M. Nyachoti. 2008. Nutrient digestibility and performance responses of growing pigs fed phytase-and xylanase-supplemented wheat-based diets. J. Anim. Sci. 86: 848-857.

Wu, H.-J., and E. Wu. 2012. The role of gut microbiota in immune homeostasis and autoimmunity. Gut microbes 3: 4-14.

Wu, S., F. Zhang, Z. Huang, H. Liu, C. Xie, J. Zhang, P. A. Thacker, and S. Qiao. 2012. Effects of the antimicrobial peptide cecropin AD on performance and intestinal health in weaned piglets challenged with *Escherichia coli*. Peptides 35: 225-230.

Xu, R. J., and P. D. Cranwell. 2003. Composition of porcine milk. In: R. Xu and P. Cranwell (eds.) The neonatal pig: gastrointestinal physiology and nutrition. p 213-246. Nottingham University Press, Nottingham.

Xu, R. J., F. Wang, and S. H. Zhang. 2000. Postnatal adaptation of the gastrointestinal tract in neonatal pigs: a possible role of milk-borne growth factors. Livest. Prod. Sci. 66: 95-107.

Yan, L., J. Lee, Q. Meng, and I. Kim. 2011a. Evaluation of the Anion® supplementation on growth performance, nutrient digestibility, blood characteristics and faecal noxious gas content in weaning pigs. J. Appl. Anim. Res. 39: 36-40.

Yan, L., Q. Meng, and I. Kim. 2011b. The effect of an herb extract mixture on growth performance, nutrient digestibility, blood characteristics and fecal noxious gas content in growing pigs. Livest. Sci. 141: 143-147.

Yan, L., Q. Meng, and I. Kim. 2012. Effect of an herb extract mixture on growth performance, nutrient digestibility, blood characteristics, and fecal microbial shedding in weanling pigs. Livest. Sci. 145: 189-195.

Yason, C. V., B. Summers, and K. Schat. 1987. Pathogenesis of rotavirus infection in various age groups of chickens and turkeys: pathology. Am. J. Vet. Res. 48: 927-938.

Yeaman, M. R. 2014. Platelets: at the nexus of antimicrobial defence. Nat. Rev. Microbiol. 12: 426-437.

Yen, J. T. 2000. Anatomy of the digestive system and nutritional physiology. In: A. J. Lewis and L. L. Southern (eds.) Swine nutrition. p 31-64. CRC Press, Boca Raton, Florida, USA.

Yu, I. T., J. F. Wu, P. C. Yang, C. Y. Liu, D. N. Lee, and H. T. Yen. 2002. Roles of glutamine and nucleotides in combination in growth, immune responses and FMD antibody titres of weaned pigs. Anim. Sci. 75: 379-385.

Zhang, Y., and R. Xu. 2003. Anatomy and histology of the gastrointestinal tract. In: R. J. Xu, Cranwell, P. D. (ed.) The neonatal pig: Gastrointestinal physiology and nutrition. p 1. Nottingham University Press, Thrumpton, Nottingham, UK.

Zhou, H., C. Wang, J. Ye, H. Chen, and R. Tao. 2015. Effects of dietary supplementation of fermented Ginkgo biloba L. residues on growth performance, nutrient digestibility, serum biochemical parameters and immune function in weaned piglets. Anim. Sci. 86: 790–799.

Zhu, H., C. Hart, D. Sales, and N. Roberts. 2006. Bacterial killing in gastric juice–effect of pH and pepsin on Escherichia coli and Helicobacter pylori. J. Med. Microbiol. 55: 1265-1270.

Zomborszky-Kovacs, M., L. Bardos, H. Biro, S. Tuboly, E. Wolf-Taskai, A. Toth, and P. Soos. 2000. Effect of beta-carotene and nucleotide base supplementation on blood composition and immune response in weaned pigs. Acta Vet. Hung. 48: 301-311.