EFFECTS OF DIETARY SUPPLEMENTATION OF *LACTOBACILLUS*-BASED PROBIOTICS ON GROWTH AND GUT ENVIRONMENT OF NURSERY PIGS

by

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A THESIS

IN

ANIMAL SCIENCE

Submitted to the Graduate Faculty
of Texas Tech University in
Partial Fulfillment of
the Requirements for
the Degree of

MASTER OF ANIMAL SCIENCE

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December, 2007

ACKNOWLEDGEMENTS

I wish to express my gratitude to the following:

First and foremost, I would like to give thanks to my God. Without Your unconditional support none of this could have been possible.

Dr. Sung Woo Kim and Dr. Kris Wilson, your guidance as the co-chairs of my graduate committee was superb. You were very helpful in directing, correcting and giving positive criticism of the manuscript. Your thorough and thoughtful reading of this thesis unearthed many imperfections which when corrected resulted in this thesis.

Dr. Scot Dowd, your unwavering positive attitude is infectious, and I am fortunate to have known you. I would like to thank you for your help with the microarray analysis and advice on chapter four of this thesis.

Dr. Mindy Brashears, your unselfish dedication to this work was unparalleled. I would like to thank you for your help with the microbial population analysis in this thesis.

I would like to express a special thank you to my beautiful wife, Hellen. You willingly agreed to stay behind thousands of miles away to take care of the children when I was gone from home for years for studies. Your support has been incredible. My children, Grace and Emmanuel though young and needed daddy around, you had to contend with my absence. God bless you all.

I would like to express thanks to my mother for emphasis on the value of education and instilling it in me and ensuring that I got it. You've been such an inspiration to me Mama.

My brother, Charles thank you very much for your guidance and support in all spheres of life.

I would also like to thank Stanley Harris and Edward Carrasco for all the assistance and help they provided me at the Texas Tech Research Farm.

I also acknowledge the help of my fellow graduate students, but special thanks to Ronaldo Mateo for being a great help in setting up this study.

If I failed to include your name above, but you helped me in one way or another, my sincere thanks goes out to you.

This work is dedicated to my wife Hellen and children, Grace and Emmanuel.

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ABSTRACT

Two experiments were conducted to evaluate the effects of dietary supplementation of *Lactobacillus*-based probiotics on the growth and gut health of newly weaned pigs.

In Exp. 1, 20 nursery pigs weaned at 21-d of age (6.68 ± 0.27 kg BW) were allotted to 2 treatment groups representing: (1) CON (probiotic-free; corn-soy diet) and (2) PB (test group fed a diet containing 0.2% *lactobacillus* based probiotics). Pigs were housed individually in metabolism crates and fed the diets for 15-d. At d-15, all pigs were euthanized to collect gut tissues and digesta. There were no differences ($P > 0.05$) in body weight, ADG, ADFI and FE between the treatments during 15-d period. Analysis of *E. coli, Bifidobacteria, Lactobacillus spp*., and total anaerobe populations in colon digesta between the treatments did not show any difference ($P > 0.05$). Major VFAs were acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate. Acetate accounted for more than 60% of the total VFAs in both treatments while isobutyrate accounted for less that 0.5% of the total VFAs in both treatments. There was no difference ($P > 0.05$) observed in amount of VFAs in both treatments. There was also no difference ($P > 0.05$) in the apparent ileal digestibility of amino acids in the diets. Villi height was greater ($P < 0.05$) in the treatment group as compared to control group.

A separate study was conducted to investigate the effects dietary supplementation with *Lactobacillus*-based probiotics elicits on the immune status of the nursery pig. Differential blood counts were determined on whole blood samples collected on d 13 of the study period. Total RNA was isolated from mesenteric lymph nodes. Gene expression

was determined using a 12,000+ pig specific custom microarray, results of which were validated with quantitative polymerase chain reaction (**PCR**). There were no differences ($P > 0.05$) in lymphocyte, neutrophil and monocyte, white blood cell (**WBC**) count, red blood cell (**RBC**) count, hematocrit, and hemoglobin among treatment groups. Microarray results identified significant difference in the expression of 80 genes that were altered by *Lactobacillus*-based probiotics supplementation. Of these genes, nine were comparatively induced (> 2.0 fold) and the rest were comparatively repressed (> 2.0 fold). Functional analyses of these genes identified 25 distinctly enriched functional categories. One of the primary functional categories identified showed significant repression of catalytic activity genes, which represented the majority of repressed transcripts (24.4%). Analyses indicated that *Lactobacillus*-based probiotics supplementation altered genes responsible for carbohydrate transport activity, cellular physiology process, defense to pathogens and immune response.

Collectively, dietary supplementation of *Lactobacillus*-based probiotics at 0.2% may have beneficial effects by positively interacting with the intestinal mucosa and the microflora if the length of feeding was increased. Nonetheless, further studies are required to confirm and to elucidate the responsible mechanisms. In addition, the extent to which altered gene expression may affect the animal's response to actual immune challenge conditions should be determined.

Key Words: Growth, Gut flora, Immunity, *Lactobacillus*, Probiotics, nursery pigs

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CHAPTER I

INTRODUCTION

In pig production, a focus has been on finding alternatives to subtherapeutic antibiotic supplementation in order to reduce the emergence of antibiotic resistance and to favor consumer desires while obtaining the same level of performance as those fed with antibiotics. One such alternative is the use of probiotic supplementation of which has been suggested to benefit the host animal by stimulating appetite (Nahashon et al., 1994), improving both intestinal microbial population balance (Fuller, 1989) and digestion (Collins et al., 1999). Furthermore, studies have also suggested a role for probiotics in stimulating the immune system (Collins et al., 1999; Perdigon et al., 1999).

However, results from different studies have been conflicting. For example, Harper et al. (1983) found no differences in growth performance of weanling pigs fed either a control diet or the control diet supplemented with *Lactobacillii*. On the other hand, Jasek et al. (1992) reported that addition of *Lactobacilli* to weanling pig diets improved growth performance and decreased the incidence of *Escherichia coli* in feces. The authors also reported that *Lactobacilli* in grower-finisher diets also improved growth performance. Similarly, Gombo et al. (1995) reported that feeding a combination of *Lactobacilli* and yeast to growing pigs improved ADG and feed efficiency. Differences in scientific literature can be attributed to several factors including the strain of the probiotic, the dosage of the probiotic and the length of feeding.

The gut represents a complex and dynamic microbial ecosystem in which microorganisms in the intestine (microflora) have important and specific metabolic and

protective functions (Massi et al., 2006). *Lactobacilli acidophilus* are found to modify gut microbial populations, suggesting the capacity to control gut bacterial growth (Gaon et al., 2002). Normal gut structure and function is as a result of complex interactions between the host animals and microorganisms colonizing the gut (Massi et al., 2006) which may have profound effects on animal health. Indeed, studies have shown that dietary supplementation with *Lactobacillus acidophilus* exerts a wide range of effects that benefit the health of animals (Cross et al., 2002).

Diet supplementation with lactic acid producing bacteria generally results in a decrease in gut pH resulting in altered gut microfloral populations. This is supported by studies indicating that dietary supplementation with lactic acid bacteria leads to reduced fecal pH. For example, Foo et al. (2003) reported that fecal pH was lower in rats when given lactic acid bacteria via drinking water. Shah (2001) suggested that the decrease in fecal pH was due to the lactic acid produced by lactic acid bacteria. In addition, the authors also suggested that the decrease in fecal pH due to gastric lactic acid produced from probiotics might kill or prevent microorganism populations.

However, it is not known whether or not this reduction in pH is beneficial to the health of the pigs. We hypothesized that similar results might occur in swine reducing potential disease transmission in swine production. Therefore, this study was conducted to investigate the effects of probiotic supplementation on GI tract microflora and intestinal morphology in nursery pigs.

CHAPTER II

LITERATURE REVIEW

Probiotics

Maintaining intestinal microflora balance in animals is important to prevent diseases by controlling the overgrowth of potentially pathogenic bacteria. Therefore, control of infections through a non-antibiotic approach is urgently needed. Natural bacterial flora (e.g. probiotic bacteria) represents a promising alternative therapy. A probiotic is defined as live microbial feed supplement that beneficially affects the host animal by improving its intestinal balance (Fuller, 1989) and has beneficial effects on the health of the host (Lee et al., 1999a).

Lactobacillus and *Bifidobacterium* species have been used most extensively in humans, whereas species of *Bacillus, Enterococcus,* and *Saccharomyces* yeast have been the most common organisms used in livestock (Salminen et al., 1998). Probiotic supplementation of intestinal microflora in poultry, especially with *Lactobacillus* species, showed beneficial effects on resistance to infectious agents such as *Escherichia coli* (Jin et al., 1996), *Salmonella sp*. (Pascual et al., 1999), and more recently, *Eimeria acervulina* (Dalloul et al., 2003). Proposed mechanisms of pathogen inhibition by probiotic microorganisms include competition for nutrients, production of antimicrobial conditions and compounds (volatile fatty acids, low pH, and bacteriocins), competition for binding sites on the intestinal epithelium, and stimulation of the immune system (Rolfe, 2000).

These are not mutually exclusive mechanisms, and some microorganisms may effect the change due to a single mechanism whereas others may use several mechanisms.

Effects of Probiotic Supplementation in Pigs

Dietary supplementation of probiotics such as of *Lactobacillus spp*. has been shown to cause several beneficial effects in livestock (Fuller, 1992a). Some of those benefits include: increased resistance to infectious diseases, particularly of the intestine (Fernandes et al., 1987), decreased duration of diarrhea (Saavedra et al., 1994), reduction in blood pressure (Sawada et al., 1990), reduction in serum cholesterol concentration (Gilliland et al., 1990), reduction in allergy (Isolaurie et al., 1993), stimulation of phagocytosis by peripheral blood leucocytes (Schiffrin et al., 1995), modulation of cytokine gene expression (Delneste et al., 1998), adjuvant effects (Bloksma et al., 1979; Perdigon et al., 1995), regression of tumors (Kato et al., 1981) and reduction in carcinogen or co-carcinogen production (Goldin et al., 1980).

Prebiotics can improve the efficiency of probiotics. Both prebiotics and probiotics have been shown to increase the colonization of commensal bacteria at the lower intestinal tract. Probiotic microorganisms inhibit growth of potentially pathogenic microorganisms by competitive exclusion **(CE)**. Competitive exclusion of commensal microflora against pathogens include lowering pH through production of lactate, lactic acid and volatile fatty acids (**VFA**), competing for gut lining attachment and available nutrients, producing bacteriocins and stimulating the gut associated immune system through cell wall components (Nousiainen et al., 1998). Others include increasing the production of VFA, which have bacteriostatic and bactericidal properties (Fuller, 1977)

and stimulating intraepithelial lymphocytes, and natural killer cells (Ishizuka et al., 2002; Ishizuka et al., 2004). Most commercial probiotic products are composed of pure defined cultures of one or more micro-organisms. Probiotics have some disadvantages in comparison to other modulators of enteric microflora (Fooks et al., 1999; Patterson and Burkholder, 2003; Isolauri et al., 2004). For example, relatively few species of microorganisms can be considered for use in probiotics products due to their limited knowledge of culturability and required conditions for application and storage, such as extreme anaerobiosis. Probiotics have a short shelf-life and most are labile to excessive heat and pressure during feed processing. Some probiotic microorganisms may be reduced or eliminated by low pH in the gut, and thus have little effect in the lower intestinal tract where pathogens pose problems. Coating technology has helped with some of these concerns, but more research is needed in this area.

In the livestock industry, the use of probiotics aims to improve intestinal health which can then lead to a better general health and productivity (Blok et al., 2002). However, obtained results from studies are inconsistent. This may be the result of varying conditions when probiotics are fed, as well as differences in preparation's validity. Some reports show that feeding lactic acid bacteria improve growth performance in sucking pigs (Abe et al., 1995), weanling pigs (Jasek et al., 1992), grower pigs (Baird, 1977) and finishing pigs (Jonsson et al., 1992, Hong et al., 2002). However, other studies have found no differences in growth performance of pigs (Apgar et al., 1993). Supplementation of *Bacillus* species has also suggested improving growth rate and feed efficiency in piglets (Kyriakis et al., 1999) and grower pigs (Succi et al., 1995). Live

yeast supplementation to the diet of pigs has resulted in improvements in growth rate (Mathew et al., 1998) and reductions of pathogenic bacteria (Anderson et al., 1999). However, there are still some studies that have reported no improvements in the performance of pigs fed diets supplemented with yeast compared to pigs fed a control diet (Jost et al, 2000).

Positive effects were observed by Maxwell et al. (1983) and Hong et al. (2002) as a result of supplementation with lactobacillus based probiotics on nutrient digestibility. However, other reports show no effect on digestibility of Dry Matter (DM), Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), amino acid when pigs were fed probiotics containing *Lactobacillus* or *Bacillus* cultures (Hale et al., 1979; Kornegay et al., 1996). Stimulation of immunity of pigs was observed by several authors (Toruero et al., 1995; Takahashi et al., 1998). However, there still a large number of studies indicating no effects of probiotics supplementation on immune system of pigs (Kluber et al., 1985; Apgar et al., 1993).

The efficacy of probiotics under different conditions may be due to the probiotic preparation itself or some other factors. Low survival rate of strains, stability of the strain, low dosage and frequency of administration, interactions with some medicines, health and nutritional status of the animal and the effect of age, stress, genetics and type differences of animals (Bomba et al., 2002). Research experience points to the fact that probiotics are most effective in animals during microflora development or when microflora stability is impaired (Stavric et al., 1995). It has however been pointed out that

the effects of probiotics appear to be more consistent and positive in piglets rather than in grower-finisher pigs (William, 2000).

Due to the manipulation influence of intestinal microbial population by probiotics, recent concepts suggest that the noxious gas emission from pigs manure can also be reduced. Such beneficial effects were observed by Jeon et al. (1996), Hong et al., (2002) and Ji et al., (2002).

Effects of Probiotic Supplementation on Growth Potential of Piglets

The growth performance of pig is economically paramount. Swine growth rate is one of the key indicators affecting the profitability of pork production. Improvement in growth rate and feed to gain ratio will result in improved profitability due to greater output and reduction in overhead costs (Campbell, 1997). It is well known that the age and weight at weaning are closely related to post weaning growth rates (Quiniou et al., 2002; Mahan et al., 1998). Many studies have demonstrated that weaning weight influences post weaning growth performance and also influences performance during the subsequent grower and finisher phases. An increase in pig weight at weaning of 1kg will result in a pig which reaches slaughter weight at least 10 days faster (Cole et al., 2001). It is also accepted that average daily gain during the first week post-weaning has a major impact on subsequent growth performance (Tokach et al., 1992). The use of growth promoting antibiotics in pigs results in a 4-6.5% increase of their growth and feed efficiency (Muirhead et al., 1997). Rosell, (1987) reported improved feed efficiency in calves fed *Lactobacillus acidophilus*, *S. faecium,* and a yeast culture. Piglets showed

improved weight gain and feed efficiency when fed lactobacillus based probiotics (Abe et al., 1995).

Several studies have shown that dietary supplementation of Lactobacillus sp. causes an increase in the growth performance of nursery pigs (Lessard et al., 1987; Ogle et al., 1987; Jasek et al., 1992). In addition, studies have shown beneficial supplementation of Bacillus sp. (Kyriakis et al., 1999; Collinder et al., 2000). However, these results have not been consistent with some studies showing no response in the growth performance of the nursery pigs in response to supplementation with Lactobacillus sp. (Hale et al., 1979; Pollmann et al., 1980; Harper et al., 1983), or with Bacillus sp. (Jonsson et al., 1992). The mode of action of the probiotics in regard to their effects on the growth performance of the animals is not clear. Probiotics are beneficial to the host animal by increasing competition for adhesion receptors and nutrients with the pathogenic bacteria in the gut besides producing antibacterial substances which helps in controlling the composition of pathogenic gut microflora (Fuller, 1992). However, some other factors can complicate the effects of probiotics, which include the environmental conditions of the research facility, handling of the animals, genetic background of the animals, different stress factors, composition of gut microflora in the animals, and chances for cross-contamination (Jonsson et al., 1992). It can be complicated to specify with certainty how these factors contributed to the growth performance effects of probiotics to animals (Jonsson et al., 1992). Types of microorganisms and carriers in probiotics can also cause modifications in gut microorganism populations and as a result intestinal health modifications (Jin et al., 1998).

Effect of Probiotics Supplementation on Pig Gut Microbiology

Weaning of pigs is associated with the change of diet from sow's milk to a solid weaner diet and other post-weaning stressors. The major intestinal flora of the pig is *Lactobacilli, Bifidobacteria, Streptococci, Bacteriodes, Clostridium perfringes* and *E. coli*. The microflora dynamics change with age. It has been suggested that it may take 4 to 6 weeks to establish a stable flora (Mul et al., 1994). When piglets are weaned, the intestinal microflora of piglets is altered (Jensen, 1998). It has been well recognized that *E. coli* populations, especially haemolytic *E. coli*, are markedly increased in the anterior small intestine after weaning, and enteropathogenic *E. coli* is the major infection agent for post weaning diarrhea (Hopwood et al., 2003). Withdrawal of sow's milk that contains natural immunoglobulin will cease to prevent the proliferation of haemolytic *E. coli*. (Deprez et al., 1986). The dietary and environmental change after weaning may also be associated with changes in intestinal microflora (Jensen, 1998). It is assumed that probiotic supplementation in weaner diets may help to smooth the transition of intestinal microflora after weaning by stimulation of beneficial bacteria such as bifidobacteria and lactobacilli. This may stimulate their growth (Modler et al., 1990).

Small Intestine Morphology of a Pig

The gastrointestinal tract **(GIT)** is the main digestive and absorptive organ in the animal. The small intestine has a huge absorptive surface. The gastrointestinal tract permits the uptake of dietary substances into systemic circulation and it also excludes pathogenic compounds simultaneously (Gaskins, 1997). The presence of villi and microvilli in the small intestine results in a much larger surface area than that of a

cylindrical tube (Caspary, 1992) largely because the shape of healthy villi in pigs is finger-like (Mouwen, 1972). The total absorptive surface area in the small intestine of a ten day old piglet is 114m^2 (Buddle et al., 1992). Moon (1971) found that the villous epithelium in the small intestine in 3 week old pigs is replaced more rapidly (in 2-4 days) than that of one day old piglets (in 7-10 days). There is a reduction in villous height (villous atrophy) and an increase in crypt depth (crypt hyperplasia) at weaning (Pluske et al., 1996). Villous atrophy was associated with either an increased rate of cell loss from the villous apex or a reduced rate of cell renewal (Pluske et al., 1997). These changes are clear at 5 days post weaning and continued in the first to second week after weaning (Kelly et al., 1991). The villous height was reduced to 50- 75% of pre-weaning values (Hampson, 1996; Kelly et al., 1991). Several other reports demonstrated that higher feed intake immediately after weaning reduced the histological changes of small intestinal morphology (Spreeuwenberg, 2002; Pluske et al., 1996; Verdonk et al., 2001). Thus, it is important to increase feed intake immediately after weaning. Low feed intake is usually seen during the first few days after weaning. This is associated with decreased villous height and low brush border enzyme activities. The result is decreased nutrient absorption, post weaning growth check and high incidence of diarrhea in pigs in commercial early weaning facilities. A nutritional approach may be used to help solve the post weaning stress and growth check. Probiotics may be part of the potential alternatives to antimicrobial growth promoters.

Gastrointestinal Microbiota

There is growing attention to the fact that supplementation of lactobacillus based probiotics to pig feed might improve growth performance (Geary et al.,1996), and that supplementation might also influence the bacterial ecology of the GIT. These improvements may be observed when supplemented in particular by members of the family Enterobacteriaceae, including Salmonella spp. (Urlings et al., 1993; Van den Elzen et al., 1993). Lactobacillus based probiotics produce high concentrations of lactic acid resulting in reduced gut pH. Fermentation of feed materials in the gut also results in production of volatile fatty acids. These two parameters can have an effect on bacterial ecology of the GIT. Lactic acid and VFA are also produced by the indigenous microflora in the GIT (Argenzlo et al., 1974). Non-dissociated form of lactic acid and VFA can have a bactericidal or bacteriostatic effect (Russell et al., 1998). The exact concentrations of these non-dissociated acids and the possible effect on bacterial groups in the content of the GIT in pigs are not known. It is reported that lactobacillus based probiotics may reduce pH in the entire GIT (Ravindran et al., 1993) and thus modifying GIT microflora.

The composition and activity of the GIT microflora has a significant impact on the health of the host because it influences nutrition, intestinal physiology, immunity, and consequently resistance to pathogen colonization (Montagne et al., 2003; Denise et al., 2004). Attempts have been made to bolster host defenses by using feed ingredients that favor the growth of bacteria generally regarded as beneficial (Apajalahti et al., 2001). Numerous modulators of the GIT ecology have been proposed (Ferket et al., 2005), and many are available for enhancing the performance and intestinal health of livestock. The

intestinal microflora comprises a diverse collection of cultivable and uncultivable microbial species. The normal microflora of the gastrointestinal tract of the pigs consists of bacteria such as *Lactobacillus sp., Streptococcus sp., E.coli, Eubacterium sp., Clostridium sp., Peptostreptococcus sp.,* and *Bacteroides sp.* (Tannock, 1999). The obligate anaerobic bacteria are generally present in the large intestine (Tannock, 1999). However, large proportions of *Lactobacillus sp*. bacteria are generally present adhered to the stratified squamous epithelial lining in the proximal regions of the digestive tract (Tannock, 1999). These *Lactobacillus sp*. bacteria are very important for maintaining the low pH of the stomach and the proximal portion of the digestive tract by producing lactic acid and other organic acids that favor effective enzyme secretion and digestion of the nutrients (Cranwell et al., 1976; Barrow et al., 1977). These *Lactobacillus sp*. bacteria are continuously replicated to form a thick layer over the gastric epithelium and then they are shed into the digesta continuously as the digesta passes the stomach and small intestine (Tannock, 1999). However, as the digesta reaches the large intestine the numbers of obligatory anaerobic bacteria outnumber *Lactobacillus sp*. bacteria. In this environment, pH is higher thus favoring the growth of obligatory anaerobic bacteria (Tannock, 1999). Obligatory anaerobic bacteria and the *Lactobacillus sp*. are then shed along with the feces (Tannock, 1999). *Bacillus subtilis* and *Bacillus licheniformis* bacteria could also be component of gut microflora (Jonsson et al., 1992b). Their spores are believed to germinate in the proximal portion of the gastrointestinal tract of the pigs (Ozawa et al., 1981). The probiotic actions of these bacteria are considered to be due to the preformed antibiotic substances in their spores (Jonsson et al., 1992b). The major probiotic actions

of these bacteria include competitive exclusion of pathogenic bacteria such as *E.coli* (Ozawa et al., 1981), an increase in the Lactobacillus sp. population in the proximal portions of the digestive tract, suppression of the pathogenic bacteria that produce compounds such as ammonia and amines, and a decrease in the incidence of Bacteriodes sp. in the large intestine (Jonsson and Conway, 1992b).

Most of the knowledge concerning intestinal bacterial species has been determined by cultural methods (i.e. cultivation of samples in selective media, generation of pure cultures and subsequent taxonomic identification of the unknown bacterium). Although cultivation-based techniques have been useful for analysis of specific groups of bacteria, it has several limitations for surveying the intestinal ecosystem (McCracken et al., 2001). In addition to being time and labor intensive, the use of selective media specific for different types of bacteria dictates the types of bacteria that can be enumerated (MacCracken et al., 2001). Furthermore, estimation of cultivability of bacteria in the GIT varies from 20 to 50% (Zoetendal et al., 1998). Thus, up to 80% of intestinal bacterial species may not be detected using cultivation-based techniques (Suau et al., 1999; Vaughan et al., 2000).

Table 2.1. Summary of the influence probiotic strains on the gastrointestinal microflora in pig

Animal	Probiotics	Effects	Reference
Suckling piglet	*B. thermophilum & B. pseudolongum*	Reinforced the normal intestinal flora and alleviated clinical symptoms of scouring	Kimura et al.,1983
Neonate	*L. reuteri*	Lactobacilli (↑), E. coli (↓).	Ratcliffe et al.,1986
Piglet	*Strectococci, Ent. Faecum cernelle*	Faecal E. coli and haemolytic E. coli (↓)	Deprez et al., 1986
Weanling	*Ent. Faecalis*	Faecal E. coli (↓)	Danek, 1986
Piglet	*L. acidophilus*	Lactobacillus and E. coli in stomach (↑), but no influence in other digestive tract	Pollman et al.,1980b
Weanling	*Lactobacillus spp.*	Scouring (↓).	Hale et al., 1979
Weanling	*Bacillus cereus Lactobacillus spp.*	No influence on mortality, clinical symptoms and fecal hemolytic E. coli	Cupere et al.,1992
Suckling Piglet	*Lactobacillus*	Faecal lactobacillus (↑)	Jonsson, 1986
Weanling	*Bifidobacterium Globosum*	No consistent effect on scour scores, fecal or gastrointestinal pH and cell mediated immune response	Apgar et al., 1993
Finisher pig	*Bacillus spp.*	No influence on intestinal microflora	Spriet et al., 1987
Weanling	*Bacillus subtilis*	Streptococci and bifidobacteria (↑), Bacteroides (↓).	Ozawa et,1983
Suckling Piglet	*Lactobacillus*	Coliforms (↓), lactobacillus (No effect)	Newman, 1990
Suckling Weanling	*Bifidus bifidum*	Less incidence of diseases	Ervolder et al., 1985

Adopted from: Verdonk et al., 2002. Nutrient intake level affects histology and permeability of the small intestine in newly weaned piglets
(↓) and (↑) are either significantly increased or decreased.

Probiotics and Immune Response

Lactic acid bacteria supplementation has been speculated to have immunomodulating effects. Stimulation as well as suppression of T helper (Th1) mediated immune responses, have been described for various strains (Baken et al., 2006). Baken et al. (2006) also reported enhancement of innate immune responses of Th1 mediated immune reactivity in an experiment involving use of Lactobacillus casei Shirota (LcS). Isolauri et al. (1995) demonstrated in healthy infants that Lactobacillus GG administration has an immune stimulating effect on oral rotavirus vaccination 8 d post vaccination. However, it is not well established in what way Lactobacillus consumption may play a protective role immediately during the diarrheal period. Lactobacillus consumption may reinforce the integrity of the mucosa and prevent the reinfection of intestinal villi throughout the intestinal tract. The results obtained in animal models are in agreement with studies conducted in infants suffering rotavirus diarrhea and supplemented with Lactobacillus strains. In a separate study involving 10-d-old rats inoculated with a group B rotavirus, Isolauri et al. (1993) showed that the intestinal dysfunction characterized by increase of ionic conductance and macromolecule permeability was counteracted by daily feeding of *Lactobacillus rhamnosus GG* through a tube. One speculation is that alterations in the intestinal microbial population may decrease the production of negative growth factors such as inflammatory cytokines, which have been documented to decrease feed intake and negatively alter metabolic growth processes (Spurlock, 1997). The gastrointestinal tract of animals is the site of complex interactions between the host immune system and various dietary factors, their

breakdown products, as well as microorganisms, parasites, and exogenous toxins (Gaskins, 2001). Studies on colonization of the intestinal tract of gnotobiotic animals with either defined enteric bacteria or incompletely defined normal gut microflora have revealed that the microbial population drives gut immune system development (Cebra, 1999). These microbes have an immense influence on nutritional, physiological, and immunological processes in the host animal (Zoetendal, 2004), and many of the benefits to animal growth performance resulting from diet manipulation are likely due to alterations in the gastrointestinal microflora. The administration of probiotics such as lactobacilli may be more beneficial in altering the microbial flora.

The intestinal mucosa is known to be provided with one of the largest immunological organs of the body which, through complex mechanisms, is able to remove pathogens, eliminate infected cells and can induce a more rapid response against a subsequent exposure to antigens (Roselli et al., 2005). The gut-associated lymphoid tissue **(GALT)** comprises follicles or groups of them defined as Peyer's patches, surrounded by specialized M cells, responsible for the transport in the patches of antigens and bacteria coming from the intestinal lumen (Stokes et al., 2004). The GALT also includes diffuse lymphoid tissue in the lamina propria, intraepithelial lymphocytes, mesenteric lymph nodes and appendix (Roselli et al., 2005). After antigen presentation by antigen presenting cells, lymphocytes leave the Peyer's patches (inductive sites of the intestinal mucosa), and migrate to effector sites, such as the spleen, lungs, respiratory and urogenital tracts (Roselli et al., 2005). The intestinal immune system must protect the mucosa against pathogens and noxious substances, but has to be able to avoid

hypersensitivity reactions against food proteins, normal microflora and innocuous macromolecules present in the intestine.

Probiotics are able to prevent intestinal diseases through, both humoral and cell mediated immune modulation (Erickson et al., 2000). Perdigon et al. (1995) reported that probiotics may lead to an increased IgA production and stimulation of macrophage. Moreover, several studies have reported that probiotics are able to regulate both anti- and pro-infammatory cytokine production (Matsuzaki et al., 2000; Chiang et al., 2000). However, the results are quite conflicting and the mechanisms of action of probiotics on cytokine expression are still not understood. In pigs, weaning is associated with a transient inflammatory response in the gut, as indicated by the up regulation of several inflammatory cytokines, such as IL-1â, tumor necrosis factor (TNF)-á and IL-6 (Pollmann et al., 1980), which might be implicated in the development of post-weaning diarrhea. Some studies reported that treatment of piglets with B. lactis increases blood leukocyte phagocytic and T-lymphocyte proliferative responses (Shu et al., 2001). Similar results have been observed with gnotobiotic piglets, where feeding with L. acidophilus increases the white blood cells (Pollmann et al., 1980), and the antibody level (Lehto et al., 1997).

In conclusion, it is evidence that the complex microbial flora present in the gastrointestinal tract of all warm-blooded animals is effective in providing resistance to disease. It should also be noted that probiotics are not a single entity. Different probiotics contain different micro organisms which may behave differently. Even different strains of the same species may have different metabolic activities which affect the result when

they are used as probiotics. Therefore, it is necessary to evaluate the efficacy of different probiotic preparations as well as the optimum supplementation strategies. More attention suggested to be paid on the application of probiotics due to a large potential for the probiotics in farm animal feed industry. We would therefore suggest further investigation into efficiency of different probiotic preparations used for pigs and determine what the optimal feeding strategy for those cultures is.

CHAPTER III

EFFECTS OF DIETARY SUPPLEMENTATION OF *LACTOBACILLUS*-BASED PROBIOTICS ON GROWTH AND GUT ENVIRONMENT OF NURSERY PIGS

Introduction

Several studies have shown that dietary supplementation of *Lactobacillus spp*. (Lessard and Brisson, 1987; Ogle and Inborr, 1987; Jasek et al., 1992) and *Bacillus* sp. (Kyriakis et al., 1999; Collinder et al., 2000) can enhance growth performance of nursery pigs. There is growing attention to the theory that supplementation of *Lactobacillus*-based probiotics to pig feed influences the bacterial ecology of the gastrointestinal tract **(GIT)**, in particular members of the family *Enterobacteriaceae*, including *Salmonella* spp., *Lactobacilli, Bifidobacteria, Streptococci, Bacteriodes, Clostridium perfringes and E. coli.* (Urlings et al., 1993; Van den Elzen et al., 1993) which may be related to improved gut health and growth performance (Geary et al.,1996). The composition and activity of the GIT microflora have a significant impact on the health of the host because it can influence nutrient digestion (Montagne, et al., 2003), intestinal physiology (Yan, et al., 2004), immunity (Simon et al., 2004), and consequently resistance to pathogen colonization in GIT (Andrew et al., 2004; Denise et al., 2004). However, it is not well understood how dietary *lactobacillus* supplementation affects gut microflora and therefore host immunity, gut health, and finally growth. This study aims to test the hypothesis that supplementation of *lactobacillus*-based probiotics may modify gut microflora affecting gut immune status and growth performance of the nursery pigs.

Materials and Methods

Animals and Diets

A total of 20 newly weaned pigs (Camborough 22 × PIC boar, Pig Improvement Company, Franklin, KY) from 5 litters (4 pigs from each litter) were selected from Texas Tech University Swine Teaching and Research Unit (New Deal, TX) and weaned at 21 d-of-age for this study. Immediately after weaning, pigs were randomly assigned to 2 dietary treatments within their litter origin and body weights at weaning. Pigs were housed individually in metabolism crates (0.46 x 1.07 m) meeting the space requirement by Ag Guide (1999) and thus each treatment had 10 nursery pigs. Dietary treatments were (1) CON; a control group of pigs fed a corn-soybean meal basal diet without probiotics and (2) PB; a test group of pigs fed a corn-soybean meal basal diet supplemented with 0.2 % *lactobacillus* based probiotics in the diet to provide 2.4×10^6 cfu *Lactobacillus piantarumcase* and 2.4×10^6 cfu *Lactobacillus salivaruscase* per kilogram of complete feed for the PB diet. Both diets contained 1.50% total lysine and 3.4% Mcal ME/kg diet as fed basis which meet the suggested nutrient requirements by NRC (1998). Both diets contained 0.25% chromium oxide as an indigestible external marker to calculate ileal amino acid digestibility. The assigned experimental diets were fed for 15 d. Pigs had access to diets and water *ad libitum* during the entire 15-d period. Body weight and feed intake of each pig was recorded every third day at 0800 throughout the study. Fresh feed was provided every third day at 0900 to ensure pigs had fresh diet without contamination. The animal care and use protocol was approved by the Texas Tech University Animal Care and Use Committee.

Test Materials

The probiotic used in this study contained live cultures of *Lactobacillus piantarumcase* (1.2×10^6 cfu/g) and *Lactobacillus salivaruscase* (1.2×10^6 cfu/g) in liquid form. This probiotic has been prepared from fruit fermentation and has been used in swine production and generally recognized as safe (GRAS) to be used in swine feed. This probiotic was supplemented at 0.2% in the diet to provide 2.4×10^6 cfu *Lactobacillus piantarumcase* and 2.4×10^6 cfu *Lactobacillus salivaruscase* per kilogram of complete feed for the PB diet

Sampling of Gastrointestinal Contents

After 15 d of feeding, all the pigs were euthanized by carbon dioxide suffocation in a chamber followed by exsanguinations. Pigs were then dissected collecting gut tissues and digesta samples. The entire GIT was removed carefully including digesta. The GIT was dissected into jejunum, ileum, cecum, colon, and rectum. The stomach was tied off at the esophageal and pyloric sphincters. The small intestine was tied off at the ileocecal junction. Digesta from jejunum, ileum (incision of 20 cm from the ileocecal junction), cecum, colon and rectum were collected into plastic containers for measuring volatile fatty acid content, chromium content and digesta pH. The procedure of digesta collection followed the procedure described by Kim et al, (2003).

Ten mL of colon digesta was stored in 15 mL sterile centrifuge tubes and then delivered to the Food Microbiology Laboratory at Texas Tech University within 4 h for microbial counting. Tissue samples from colon and GIT lymph were obtained and placed in an RNA extraction tube (Qiagen Inc., Valencia, CA) in order determine the amount of

genes expressed using a microarray chip at the USDA-ARS Livestock Issues Research Unit.

Microbiological Analysis

Digesta samples were serially diluted in peptone dilution water (0.01%) and plated onto according to standard procedures outlined by FDA (1996). The microflora enumerations were expressed as $\log_{10}$ colony forming units (cfu) per gram. *Lactobacillus* species were propagated in LBS agar (Sigma Chemical Co., St. Louis, MO) under anaerobic conditions at 30^{o}C for 5 d; *Bifidobacteria* species in Columbia agar (Sigma Chemical Co., St. Louis, MO) with 5 mL/L propionic acid and 2 mg/L of dicloxacillin under aerobic conditions at 37^{o}C for 3 d; *E. coli* (biotypen*1*) in MAC agar (Sigma Chemical Co., St. Louis, M) under aerobic conditions at 37^{o}C for 24 h and total anaerobic bacteria in aerobic plate counts (APC) agar (Sigma Chemical Co., St. Louis, MO) under anaerobic conditions at 37^{o}C for 2 to 3 d. Digesta samples were weighed before and after freeze drying to calculate content of water disappeared. Dried digesta samples were finely ground using a commercial blender (Sunbeam Products Inc., Florida) and stored at 4^{o}C. Volatile fatty acid in dried digesta sample from colon was quantified by GLC (model CP-3380; Varian, Walnut Creek, CA) at North Carolina State University (Eun et al., 2004). Dried digesta sample from ileum was used to measure amino acid content using high performance liquid chromatography (HPLC) as described by Van Winsen et al. (2001). Chromium content in the diets and ileal digesta were measured at the University of Missouri Ag Lab using an atomic absorption spectophotometer (Model 5000, Perkin-Elmer, Shelton, CT).

Measurement of Depth of Crypts and Height of Villi

Tissue samples from the small intestines were obtained from about the middle of the small intestine. Samples were fixed in 10% formaldehyde (Protocol Fisher Scientific) to determine villi height and crypt depth. The samples were processed and histological evaluation done as described by Kim et al. (2006).

Calculations and Statistical Analysis

The apparent ileal digestibilities of amino acids in the experimental diets were calculated according to this equation:

$D_D = 100\% - [(I_D \times A_F) / (A_D \times I_F)] \times 100\%$

where D_D = apparent ileal amino acid digestibility in the assay diet (percentage), I_D = marker concentration in the assay diet (percentage), A_F = amino acid concentration in ileal digesta (percentage), A_D =amino acid concentration in the assay diet (percentage), and I_F = marker concentration in ileal digesta (percentage).

All data were analyzed as a completely randomized design using the General Linear Model (PROC GLM) procedure in SAS/STAT software (SAS Inst. Inc., Cary, NC). The animal was the experimental unit. Least square means, probability of difference, and standard errors were used to evaluate the differences among the treatment groups. Mean differences were considered significant at $P < 0.05$.

Results

Table 3.2 shows growth performance results. There was no difference ($P > 0.05$) in body weight, ADG, ADFI and gain: feed among the treatments.

The microbial populations in the GIT contents are shown in Table 3.3. In general, there was no difference ($P > 0.05$) in the *E. coli, Bifidobacteria, Lactobacilli* and total anaerobic counts among treatments.

Table 3.4 shows VFA analysis results. The major VFAs analyzed were acetate, propionate, isobutyrate, butyrate and valerate. Acetate accounted for more than 60% of the total VFAs in both treatments while isobutyrate accounted for less that 0.5% of the total VFAs in both treatments. There was no difference ($P > 0.05$) observed in amount of VFAs in both treatments.

Histology results of the ileum are presented in Table 3.5. The supplementation of lactobacillus based probiotics had significant effects on villi height. Villi height was greater ($P < 0.05$) in the treatment group as compared to control group.

The apparent ileal digestibility of amino acids in the diets is presented in Table 3.6. There was no difference ($P > 0.05$) in the apparent ileal digestibility of amino acids in the diets.

Table 3.1. Composition of diets fed to the pigs in this study

Ingredient, (%)	CON[a]	PB[b]
Corn grain	55.6	55.5
Soybean meal, dehulled	25.0	25.0
Fish meal, Menhaden	5.0	5.0
Dried whey	10.0	10.0
Salt	0.3	0.3
Vitamin-mineral premix[c]	1.5	1.5
Restaurant grease	1.0	1.0
Dicalcium phosphate	1.3	1.3
Limestone	0.3	0.3
Supplement	0.0	0.1
Total	100.0	100.0
Chemical composition:		
DM, (%)	90.28	90.20
ME Mcal/kg	3.31	3.31
CP, (%)	20.89	20.89
Lys, %	1.23	1.23
Cys + met (%)	0.71	0.71
Trp (%)	0.25	0.25
Thr (%)	0.83	0.83
Calcium (%)	0.91	0.91
Available phosphorus (%)	0.52	0.52

[a] Without any probiotic (n=10).

[b] Probiotic treatment containing *Lactobacillus piantarumcase* (1.2×10^6 cfu/g) and *Lactobacillus salivaruscase* (1.2×10^6 cfu/g) in liquid form (Genebiotech Inc., 0.2%) (n=10).

[c] Vitamin-mineral premix contained the amounts per kilogram of premix: Vit. A, 503,742 IU as vitamin A acetate; Vit. D3, 54,996 IU; Vit. E, 4,125 IU; Vit. B12, 3.6 mg; Choline as choline chloride, 110,470 mg; Riboflavin, 915.4 mg; Niacin, 3,657.4 mg; D-Pantothenic acid as calcium pentathonate, 2,926.8 mg; Menadione, 182.5 mg; Folic acid, 110.1 mg; Thiamine, 365.2 mg; Pyridoxine, 365.6 mg; D-Biotin, 18.3 mg; Ca., 3.17 mg; P., 0.15 mg; Vit. K., 0.18 mg as menadione sodium bisulfite, ; Mg., 3.11 mg; Na., 0.02 mg; Cl., 1.68 mg; Fe., 5,000 mg; Mn., 3,340 mg; Se., 15 mg; S., 4,160 mg; Zn., 6,920 mg; Cu., 632 mg/kg; I., 47.8 mg.

Table 3.2. Growth performance of pigs fed diets with or without *Lactobacillus*-based probiotics during 15 d.

	Treatments			
Item	CON[a]	PB[b]	SEM[c]	*P*-Value
Initial BW, kg	6.76	6.59	0.27	0.677
d 1-3	6.89	6.89	0.29	0.629
d 4-6	7.11	7.08	0.30	0.945
d 7- 9	7.43	7.41	0.32	0.976
d 10-12	8.49	8.44	0.43	0.945
d 13-15	9.09	9.32	0.51	0.765
ADG, g/d				
d 1-3	43.846	31.297	26.221	0.742
d 4-6	74.238	131.088	32.756	0.248
d 7- 9	107.352	112.642	35.423	0.918
d 10-12	351.532	340.949	58.548	0.901
d 13-15	201.847	291.053	46.313	0.203
Overall	155.763	181.405	27.424	0.524
ADFI, g/d				
d 1-3	155.000	128.500	15.576	0.257
d 4-6	247.000	245.000	19.793	0.944
d 7- 9	267.000	260.000	30.512	0.874
d 10-12	387.000	345.000	44.286	0.518
d 13-15	416.500	456.500	25.894	0.300
Overall	294.500	287.000	17.573	0.769
Gain:feed				
d 1-3	0.05	-1.16	0.84	0.334
d 4-6	0.26	0.50	0.13	0.217
d 7- 9	0.40	0.38	0.11	0.902
d 10-12	0.87	0.98	0.18	0.658
d 13-15	0.48	0.65	0.12	0.348
Overall	0.51	0.60	0.08	0.450

[a] Without any probiotic (n=10).
[b] Probiotic treatment containing *Lactobacillus piantarumcase* (1.2×10^6 cfu/g) and *Lactobacillus salivaruscase* (1.2×10^6 cfu/g) in liquid form (Genebiotech Inc., 0.2%) (n=10)
[c] Values are means with pooled SEM, n = 10

Table 3.3. Microbial counting in digesta from colon of pigs fed diets with or without *Lactobacillus*-based probiotics during 15 d.

	Treatments			
Item	CON[a]	PB[b]	SEM[c]	*P*-Value
Microbial Count (Log_{10} CFU/g)				
E. Coli	6.916	6.178	0.588	0.389
Bifidobacteria	7.462	7.116	0.455	0.587
Lactobacillus spp.	9.021	9.633	0.315	0.175
Total anaerobic	7.942	7.928	0.578	0.986
Percentage of total anaerobic)				
E. Coli	87.5	84.4	8.4	0.776
Bifidobacteria	94.9	98.3	4.1	0.528
Lactobacillus spp.	112.3	114.6	9.6	0.860

[a] Without any probiotic (n=10).
[b] Probiotic treatment containing *Lactobacillus piantarumcase* (1.2×10^6 cfu/g) and *Lactobacillus salivaruscase* (1.2×10^6 cfu/g) in liquid form (Genebiotech Inc., 0.2%) (n=10)
[c] Values are means with pooled SEM, n = 10

Table 3.4. Percentage volatile fatty acids in digesta from colon of pigs fed diets with or without *Lactobacillus*-based probiotics during 15 d.

	Treatments			
Item	CON[a]	PB[b]	SEM[c]	*P*-value
Colon				
Acetate	64.6	61.7	2.0	0.493
Propionate	21.5	21.8	2.0	0.941
Isobutyrate	0.4	0.3	0.0	0.469
Butyrate	10.7	12.9	0.3	0.122
Valerate	2.2	2.6	0.2	0.417
Total VFA	100.0	100.0		

[a] Without any probiotic (n=10).
[b] Probiotic treatment containing *Lactobacillus piantarumcase* (1.2×10^6 cfu/g) and *Lactobacillus salivaruscase* (1.2×10^6 cfu/g) in liquid form (Genebiotech Inc., 0.2%) (n=10)
[c] Values are means with pooled SEM, n = 10

Table 3.5. Villi height and crypt depth of pigs fed diets with or without *Lactobacillus*-based probiotics during 15 d.

	Treatments			
Item	CON[a]	PB[b]	SEM[c]	*P*-Value
Hitology				
Villi height	392.41	470.52	0.00	0.029

[a] Without any probiotic (n=10).
[b] Probiotic treatment containing *Lactobacillus piantarumcase* (1.2×10^6 cfu/g) and *Lactobacillus salivaruscase* (1.2×10^6 cfu/g) in liquid form (Genebiotech Inc., 0.2%) (n=10)
[c] Values are means with pooled SEM, n = 10

Table 3.6. Apparent ileal amino acid digestibility of pigs fed diets with or without *Lactobacillus*-based probiotics during 15 d.

	Treatments			
Item	CON[a]	PB[b]	SEM[c]	*P*-Value
Aspartic Acid	70.02	71.00	2.34	0.777
Threonine	63.71	65.81	3.15	0.654
Serine	66.21	67.04	2.82	0.842
Glutamic Acid	70.69	69.51	3.35	0.811
Proline	55.90	61.62	5.43	0.484
Glycine	49.03	47.64	4.93	0.849
Alanine	63.90	62.31	4.62	0.806
Cysteine	59.44	56.54	4.12	0.636
Valine	70.85	71.05	3.10	0.965
Methionine	76.13	73.47	3.88	0.646
Isoleucine	73.74	73.90	2.68	0.967
Leucine	72.71	71.15	4.28	0.805
Tyrosine	70.91	70.77	3.44	0.978
Phenylalanine	73.48	72.20	3.39	0.799
Lysine	74.29	74.85	1.99	0.847
Histidine	75.45	72.98	3.21	0.605
Arginine	82.02	83.64	1.89	0.546
Tryptophan	77.51	78.81	2.28	0.702
Total Amino acid	69.30	69.49	2.77	0.963
Essential amino acids	74.11	76.17	2.28	0.546

[a] Without any probiotic (n=10).
[b] Probiotic treatment containing *Lactobacillus piantarumcase* (1.2×10^6 cfu/g) and *Lactobacillus salivaruscase* (1.2×10^6 cfu/g) in liquid form (Genebiotech Inc., 0.2%) (n=10)
[c] Values are means with pooled SEM, n = 10

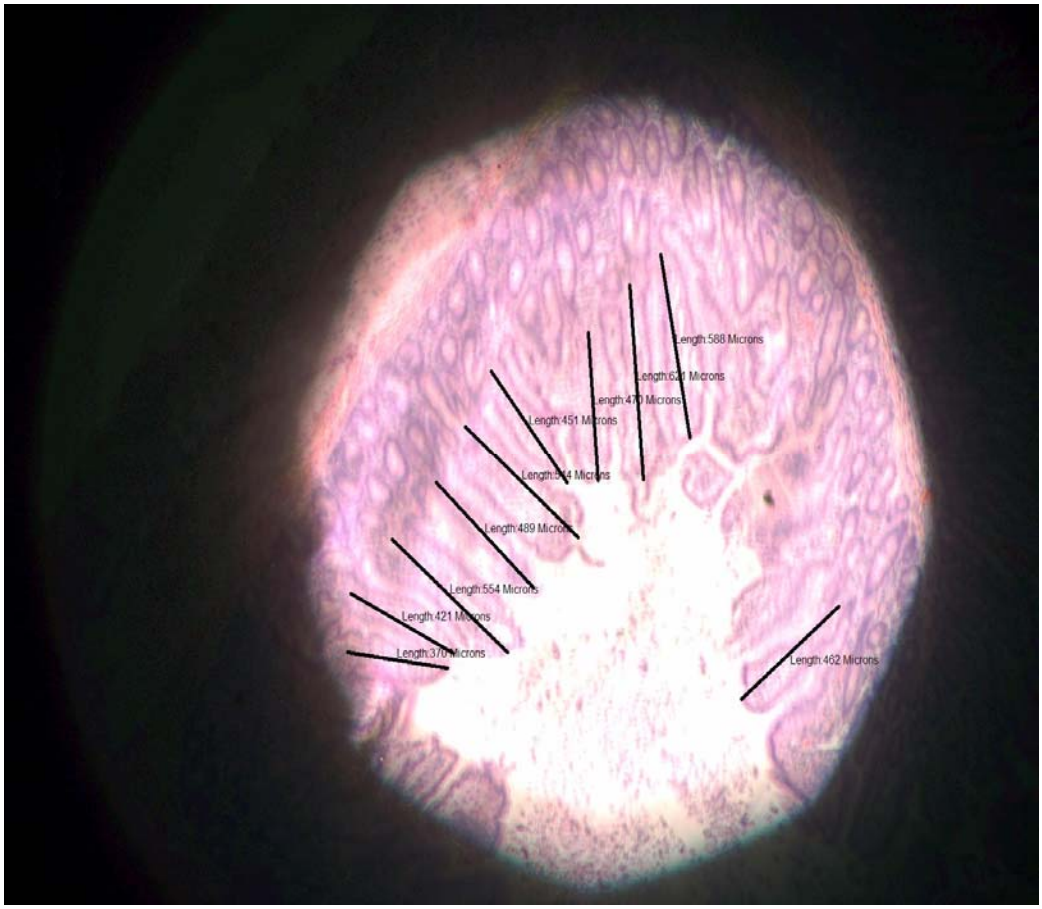

Fig 1: Photomicrograph of histological section of ileum of pig fed control diet without *Lactobacillus*-based probiotics during 15 d. Villi heights shown.

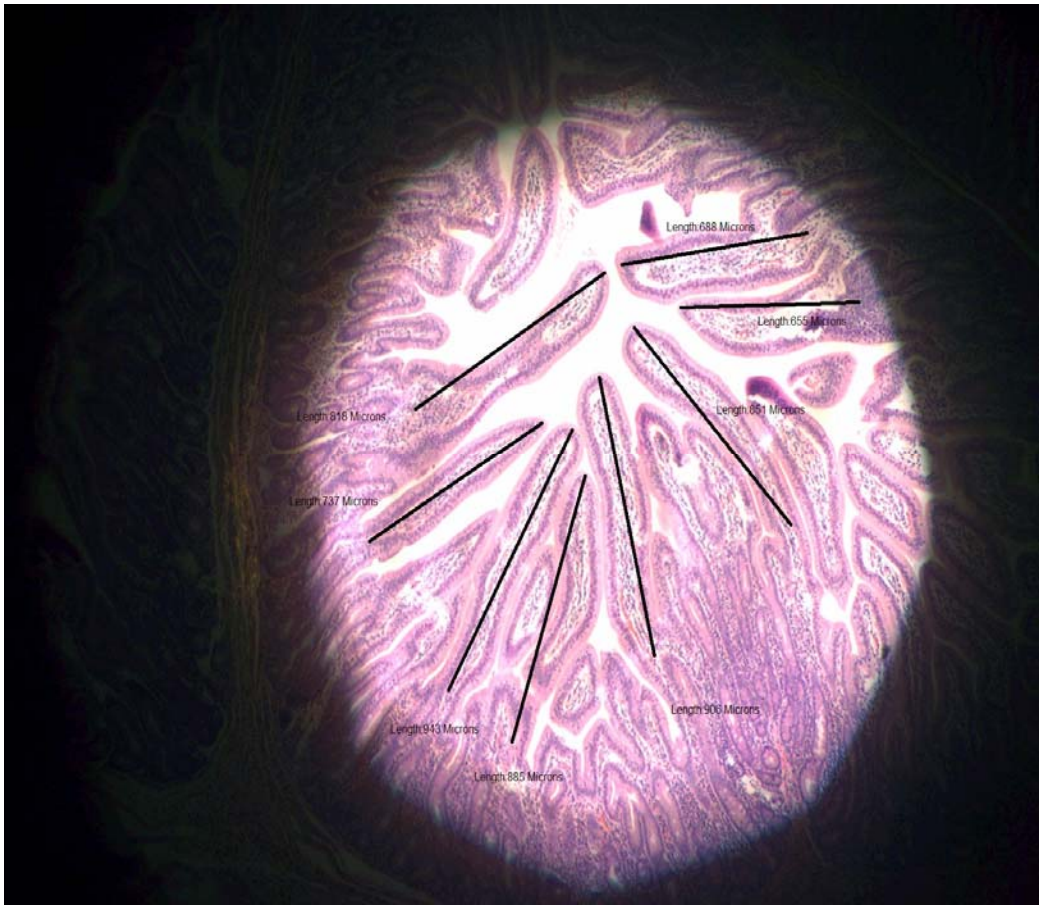

Fig 2: Photomicrograph of histological section of ileum of pig fed diets with 0.2% *Lactobacillus*-based probiotics during 15 d. Villi heights shown.

Discussion

There was a trend towards improvements in overall body weight, ADG, ADFI and FE between the treatment group and the control group. These results are consistent with those found by (Watkins et al., 1983 and Watkins et al., 1984) who reported no differences in weight gain of chicken fed diets with *Lactobacillus* cultures. Some studies have reported that probiotics have no benefit in growth performance (Heugten, et al., 2003). In contrast, previous researches have demonstrated positive effects of probiotics on piglet growth. For example, treatment with cultures of *Streptococcus faecium* suppressed *Escherichia coli* induced diarrhea and improved growth in gnotobiotic pigs (Underdahl, 1983). Growth rate of newborn piglets was also increased by a combination of bifidobacteria and lactic acid bacteria (Abe et al., 1995). The dichotomous results of the current experiment and previous researches could be due to different probiotics utilized and the specific environment of the study. Turner et al. (2001) noted that the specific production environment, including cleanliness of the facility, history of disease on the premises, and health status of treated pigs, greatly influences improvements in growth performance observed in response to performance enhancing agents.

We hypothesized that dietary supplementation of lactobacillus based probiotics would help the beneficial microflora by stimulating the good microflora or by adding beneficial microbes in the gut. This might improve gut health and in that aspect indirectly cause an increase feed intake. As a result animals may have increased growth because they eat more. Heugten et al., (2003) reported that dietary supplement with probiotics could potentially alter gut microflora by selectively stimulating growth of beneficial

bacteria while suppressing the growth of pathogenic bacteria. Microbial analysis in this study revealed that *Lactobacillus spp.* was numerically higher in the treatment group and *E. coli* tended to be reduced ($P \geq 0.05$). This could be as a result of the reason given by Shu et al. (2002) who reported that fed *L. rhammnosus* could increase mice intestinal anti-*E. coli* IgA responses and blood leucocyte phagocytic activity, and reduce the severity of *E. coli* infection

Volatile fatty acids are end products of the fermentation of soluble carbohydrates and other nutrients by anaerobes in particular. Anaerobic microorganisms are present in high numbers in the intestines especially in the cecum. Volatile fatty acid concentration is affected by composition and metabolic activity of the anaerobic flora (Yang et al., 1970). The quantity of VFA produced in the large intestine depends on the amount and composition of the substrate and on the microflora present (MacFarlane and MacFarlane, 2003).

We hypothesized that supplementation of lactobacillus based probiotics can prevent to a certain extent the decrease in height of villi in the small intestine of nursery pigs. The positive effect of lactobacillus based probiotics supplementation on villi height can improve the nutrient uptake from intestine, which could result in improved growth performance. The greater villi height in pigs may reflect the higher level of energy absorbed in the small intestines, as a result of the volatile fatty acids formed by fermentation. This is in agreement with results obtained by others authors, who reported a positive relationship between dietary probiotic supplementation and higher intestinal villi in piglets (Spencer et al., 1997) and rabbits (Mourão et al., 2006). It is therefore possible

that probiotics could stimulate the development of the intestinal villi. Therefore our results confirm the hypothesis that lactobacillus based probiotics supplementation have a positive effect on villus height.

Ileal digestibility is commonly regarded as a reference for reflecting the recovery rate of protein (amino acids) for absorption through the villi of the small intestine. There was no difference ($P > 0.05$) in the apparent ileal digestibility of amino acids in the diets in this study. These results are consistent with that obtained by Kim et al. (1993) who observed no effects on digestibility when finishing pigs were fed diets with *Lactobacillus*-based probiotics supplementation. Similarly, Kornegay et al., (1996), who used two *bacillus* products (Biomate2B®, *Bacillus subtilis, Bacillus licheniformis* and Pelletmate Livestock®, *Bacillus subtilis, Bacillus licheniformis, Bacillus pumilus*) in finishing pigs diets did not find any influences on nutrients amino acid digestibility. In the study conducted by Shon et al. (2005), no improvement in digestibility were observed when both growing and finishing pigs were fed diets supplemented with *Lactobacillus reuteri*-based probiotic (*Lactibacillus reuteri, Lactobacillus salivarius, Lactobacillus plantarum and Saccharomyces cerevisiae*). In nursery pigs, Xuan et al. (2001) found nutrients digestibility was not affected by the addition of a probiotics complex (*Bacillus* spp. *and Saccharomyces cerevisiae*). Other results in agreement with ours include that reported by Spriet et al. (1987) using *Bacillus* products in pig diets. Similar results were reported by Kornegay et al. (1996) using *bacillus* products for finishing pigs and Hale et al. (1979) who used a *Lactobacillus* fermentation product in a corn-based diet. On the

contrary, positive effects on nutrients digestibility were reported by some other authors (Maxwell et al., 1983; Kil et al., 2004; Lim et al., 2004).

Implications

In conclusion, dietary supplementation of lactobacillus based probiotics may have beneficial effects by positively interacting with the intestinal mucosa and the microflora if the duration of feeding and dosage is increased. A more stable and healthy intestinal ecosystem may discourage the colonization of unfavorable microbial communities, leading to improved growth performance.

CHAPTER IV

EFFECTS OF DIETARY SUPPLEMENTATION OF *LACTOBACILLUS*-BASED PROBIOTICS ON THE IMMUNE STATUS OF THE NURSERY PIGS

Introduction

In recent years, scientific knowledge in the field of microbiology has expanded and it has been suggested that the gastrointestinal microflora has a role in maintaining animal health (Filho-Limo et al, 2000). Most studies indicated that the potential therapeutic effects of *Lactobacillus*, including their immunostimulatory effect, are due primarily to induced changes in the gastrointestinal tract (**GIT**) ecology (Prajapati et al, 1986, Link-Amster et al, 1994). The GIT ecology in normal subjects may be altered by *Lactobacillus* supplementation, which has been shown to inhibit the growth and adherence of potentially pathogenic bacteria (Prajapati et al, 1986). The inhibition of growth and adherence in turn contributes to reduced infectivity of pathogens (Fernandes et al, 1987, Fernandes et al, 1990).

There is also increasing evidence that *Lactobacillus* can interact with the gut-associated lymphoid tissue and influence the local mucosal and systemic immune function (Link-Amster et al, 1994). Animal studies have shown that the lymphoid tissues are stimulated by the lactobacillus based probiotics resulting into enhanced production of cytokine and increased mitogenic activity of Peyer's patch (PP) cells and splenocytes (Insoft et al, 1996). However, the mechanisms underlying the benefits derived from biotherapy with *Lactobacillus* have not been sufficiently elucidated. This study was

conducted to investigate the effect of dietary *Lactobacillus* supplementation on expression of immune-related genes in mesenteric lymph tissues of newly weaned pigs.

Materials and Methods

Animals, Design and Diets

The allotment and standard management procedures have been described in Chapter III

Hematological Analyses

Blood samples were collected after feeding via jugular venepuncture using heparinized tubes (Becton-Dickinson Vacutainer Systems, Rutherford, NJ) on d 13 of the study. Differential blood counts were determined on whole blood using an automated hematological analyzer (CELL-Dyn 3200, Abbott Lab, Abbott Park, IL).

Tissue Preparation

Mesenteric lymph nodes tissues was cut into slices less than 0.5 cm thick. The tissues were then completely submerged into the RNA*later* RNA Stabilization Reagent (Qiagen Inc. Valencia, CA) which rapidly permeates tissue and single cells to stabilize and protect cellular RNA in situ. Samples were then delivered within two hours to USDA-ARS Livestock Issues Research Unit, Lubbock, Texas for RNA extraction.

RNA Extraction

Total RNA was isolated from the mesenteric lymph nodes from pigs fed the experimental diets for 15 d after weaning using commercially available kit (RNeasy, Qiagen Inc., Valencia, CA) in combination with DNase treatment (RNase- Free DNase Set, Qiagen Inc.) according to the manufacturer's instructions. To start the procedure the

frozen tissue was chopped into very tiny pieces using sharp scalpels. The pieces were then placed in the lysis buffer and immediately disrupted and homogenized using a rotor–stator homogenizer (Diax 900 10G, Heidolph, Schwabach, Germany). Total RNA was extracted using RNeasy Mini Kit according to manufacturer's instruction (Qiagen Inc. Valencia, CA). DNA was removed using RNase-Free DNase Set (Qiagen Inc., Valencia, CA). RNA was quantified using a Nanodrop ND-1000 device (NanoDrop Technologies, Wilmington, DE) and quality confirmed by electrophoresis. All RNA samples were stored at -80 °C until microarray analysis.

Microarray Design and Protocol

Using the swine unigene set (NCBI 06/07) an 11,938 gene oligonucleotide microarray, in an 8 x 15K high density format, made up of 60 mers, was designed using E-array 4.5 (Agilent Technologies, Santa Clara , CA). At total of 536 control genes (including positive and negative control probes) were also included in the design of the array.

All procedures were performed according to respective manufacturer protocols. For each sample, 10 g of total RNA and 50-fold dilution of Agilent two color spike-in control RNA kit (Agilent Technologies) were labeled with either CyDye3-dCTP or CyDye5-dCTP (Amersham Biosciences, Piscataway, NJ) using the LabelStar kit (Qiagen Inc.) and oligo-dt and Random nonamers (Sigma-Aldrich Inc., St. Louis, MO). Labeled cDNA were hybridized to the Agilent microarrays using Gene Expression Hybridization kit (Agilent Technologies, Santa Clara, CA) following manufacture's protocols (Agilent Technologies, Santa Clara, CA). Arrays were washed with Gene Expression Wash

Buffer kit (Agilent Technologies, Santa Clara, CA). A total of 8 arrays, each array, including a dye swap for each replicate (4 biological replicates), were analyzed to obtain genes that were consistently regulated while limiting false discover rate (FDR) below 5% (Benjamini and Hochberg, 1995).

Microarray Analysis

Microarray images were captured using a Genepix 4000B (Molecular Devices Corporation, Union City, CA) laser scanner and images processed using Genepix 6.0 software (Molecular Devices Corporation, Sunnyvale, CA). Microarray data analyses were performed using Acuity 4.0 software. Slides were normalized using standard methods. Data were analyzed based upon Log ratio (635/532) values. Genes were included in the final dataset that exhibit at least 2.0 fold comparative difference in expression compared to control animals.

Quantitative PCR

Quantitative PCR results for 3 induced and 3 repressed genes were used as a validation of the microarray results. Measurements of relative transcript amounts were performed by quantitative reverse transcription-PCR (qRT-PCR) with the QuantiTect SYBR Green RT-PCR kit (Qiagen Inc. Valencia, CA) according to the manufacturer's instructions. Cycling conditions were based upon the standard settings recommended for the 7500 Sequence Detection System (PE Applied Biosystems, Foster City, CA). In all cases the same RNA samples used for cDNA synthesis and microarrays were subsequent used for quantitative PCR validation. Specific primer pairs were designed using Applied Biosystems Primer Select Software and standard settings (PE Applied Biosystems, Foster

City, CA). The GAPDH gene was analyzed for both control and treatment samples, in order to normalize the qRT-PCR results of selected genes. The reactions were performed on an ABI Prism 7500 Sequence Detection system (PE Applied Biosystems, Foster City, CA). The difference (fold) in the initial concentration of each transcript with respect to the control samples were calculated according to the comparative Ct method using the built in functions of the 7500 system Sequence Detection Software version 1.3 (Applied Biosystems, Foster City, CA). The results of the qRT-PCR correlated with microarray expression (correlation coefficient 0.89, $p < 0.001$, data not shown).

Bioinformatics

The differentially regulated genes from the microarray study were mapped to functional classifications schemes such as Protein Information Resource (PIR) keywords, Gene Ontology terms (Ashburner et al., 2000), KEGG pathways (Ogata et al., 1999), and COG (Tatusov et al., 2000), though the use of High Throughput Gene Ontology and Functional Annotation Toolkit (HTGOFAT; http://liru.ars.usda.gov), which is a functional annotation engine associated with WND BLAST(Dowd, 2005) and the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Dennis et al., 2003). To accomplish this, regulated genes ($FDR < 0.05$) were mapped to UniProt accession numbers and Gene Index numbers using the built in functions of HTGOFAT. These UniProt accessions were then entered into DAVID website (david.abcc.ncifcrf.gov) to evaluate functional clustering and functional category enrichments with *Sus Scrofa* as the background genome.

Statistical Analyses

Statistics algorithms built into Acuity 4.0 were utilized for analyses related to microarrays. Built in algorithms of the ABI Prism 7500 Sequence Detection system software (PE Applied Biosystems) were utilized for all calculations related to qRT-PCR. Built in algorithms of the DAVID's functional annotation tool were utilized to evaluate clustering and categorization statistics. Correlation analyses were performed using multivariate analyses functions of JMP 5.1 (SAS Inst. Inc., Cary, NC).

Hematological data were analyzed using MIXED procedures of SAS (SAS Inst., Inc., Cary, NC) following a randomized complete block design. Separation of means was done using the PDIFF option of SAS. Probability values less than 0.05 were considered statistically significant.

Results

Hematological Analyses

Hematological analyses results are shown in Table 4.1. There were no differences ($P > 0.05$) in lymphocyte, neutrophil and monocyte, white blood cells count, red blood cell count, hematocrit, and hemoglobin among treatment groups due to *Lactobacillus*-based probiotics supplementation.

Microarray Analysis

Analysis of gene expression identified the expression of 80 genes altered (*FDR* < 5%) by dietary supplementation of probiotics consisted of *Lactobacillus piantarumcase* (1.2×10^6 cfu/g) and *Lactobacillus salivaruscase* (1.2×10^6 cfu/g). Of these genes, 9 were comparatively induced (> 2.0 fold; Table 3) and the rest were comparatively

repressed (> 2.0 fold; Table 4). These genes were in 25 distinct functional groups with the catalytic activity genes representing the majority (24.4%)

Altered genes included those with carbohydrate transport activity, cellular physiology process, defense to pathogens and immune response. In addition, genes related to proteolysis including PRSS2 and CTRB and genes involved in lipid metabolism including LIPC and PLA2G1B were also altered by lactobacillus based probiotic supplementation.

Table 4.1. Hematological analyses of nursery pigs fed diets supplemented with or without a *Lactobacillus*-based probiotics during 15 d.

	Treatments			
Item	CON[a]	PB[b]	SEM[c]	*P*-Value
White blood cells, 10^3 cell/ μL	14.591	15.845	1.776	0.624
Neutrophils, 10^3 cell/μL	6.951	5.383	1.128	0.339
Lymphocytes, 10^3 cell/μL	6.625	9.482	1.375	0.159
Monocytes, 10^3 cell/μL	0.849	0.261	0.503	0.419
Eisonophils, 10^3 cell/μL	0.164	0.185	0.048	0.761
Red blood cells,10^3 cell/ μL	7.245	7.071	0.216	0.575
Basophils, 10^3 cell/μL	0.003	0.914	0.661	0.378
Hemoglobin, g/dL	10.928	11.839	0.434	0.155
Hematocrit (Hct), g/dL	32.550	34.810	1.238	0.213

[a] Without any probiotic (n=10).
[b] Probiotic treatment containing *Lactobacillus piantarumcase* (1.2×10^6 cfu/g) and *Lactobacillus salivaruscase* (1.2×10^6 cfu/g) in liquid form (Genebiotech Inc., 0.2%) (n=10)
[c] Values are means with pooled SEM, n = 10

Table 4.2. Functional groups of genes expressed by dietary supplementation of probiotic, *Lactobacillus spp* exposure in nursery pigs

Category	Term	No. of genes	%	P-Value
Sp_pir_keywords	Glycoprotein	16	20.5	0.00
Sp_pir_keywords	Signal	13	16.7	0.00
Up_seq_feature	Active site: Charge relay system	4	5.1	0.00
Sp_pir_keywords	Hydrolase	8	10.3	0.00
Sp_pir_keywords	Serine proteinase	3	3.8	0.01
GO Biological process	Amino acid derivative metabolism	3	3.8	0.01
GO Biological process	Digestion	3	3.8	0.01
GO Biological process	Amine metabolism	5	6.4	0.01
Sp_pir_keywords	Alternative splicing	13	16.7	0.01
Up_seq_feature	Glycosylation site: N-linked	15	19.2	0.01
GO Molecular function	Catalytic activity	19	24.4	0.01
GO Biological process	Nitrogen compound metabolism	5	6.4	0.01
GO Biological process	Lipid metabolism	6	7.7	0.01
Up_seq_feature	Signal peptide	13	16.7	0.01
KEGG_pathway	Arginine and proline metabolism	3	3.8	0.02

Gene expression in nursery pigs mesenteric lymph nodes (MLN)
Gene expression regulation was examined by microarray analysis in the MLN of 21-d old nursery after dietary supplementation of probiotic of *Lactobacillus spp* (n = 10) and a control group (n = 10) for 15 days.

Table 4.2. Continued.

Category	Term	No. of genes	%	P-Value
GO Cellular component	Extracellular space	5	6.4	0.02
Sp_pir_keywords	Serine protease	3	3.8	0.02
GO Molecular function	Hydrolase activity	10	12.8	0.02
GO Biological process	Cellular lipid metabolism	5	6.4	0.03
GO Biological process	Carboxylic acid metabolism	5	6.4	0.03
GO Biological process	Organic acid metabolism	5	6.4	0.03
GO Biological process	Amino acid and derivative metabolism	4	5.1	0.03
GO Biological process	Glycoprotein biosynthesis	3	3.8	0.04
GO Biological process	Glycoprotein metabolism	3	3.8	0.05
GO Molecular function	Serine-type endopeptidase activity	3	3.8	0.05

Gene expression in nursery pigs mesenteric lymph nodes (MLN)
Gene expression regulation was examined by microarray analysis in the MLN of 21-d old nursery after dietary supplementation of probiotic of *Lactobacillus spp* (n = 10) and a control group (n = 10) for 15 days.

Table 4.3. Expression of genes upregulated by dietary supplementation of probiotic, *Lactobacillus spp* exposure in nursery pigs

Gene Symbol	Gene	Fold
SLC2A11,	Solute carrier family 2, member 11	3.8
KIAA1344,	Kiaa1344	2.8
CLIC6,	Chloride intracellular channel 6	2.3
CP,	Ceruloplasmin (ferroxidase)	2.2
NRAMP1,	Natural resistance-associated macrophage protein	2.1
CP,	Ceruloplasmin	2.0
GPX3,	Glutathione peroxidase 3 (plasma)	2.0
CHIA,	Chitinase, acidic	2.0
CIC,	Capicua homolog (drosophila)	2.0

Gene expression in nursery pigs mesenteric lymph nodes (MLN)
Gene expression regulation was examined by microarray analysis in the MLN of 21-d old nursery after dietary supplementation of probiotic of *Lactobacillus spp* (n = 10) and a control group (n = 10) for 15 days.

Table 4.4. Expression of genes down regulated by dietary supplementation of probiotic, *Lactobacillus spp* exposure in nursery pigs

Gene Symbol	Gene	Fold
C9orf4,	Chromosome 9 open reading frame 4	-2.0
GDPD2,	Glycerophosphodiester phosphodiesterase domain containing 2	-2.0
C5,	Complement component 5	-2.0
FXYD2,	Fxyd domain containing ion transport regulator 2	-2.0
GATA4,	Gata binding protein 4	-2.1
PTPA	Ptpa	-2.2
Klhl10,	Kelch-like 10	-2.2
STM3117,	Putative lactoylglutathione lyase	-2.2
LRP2,	Low density lipoprotein-related protein 2	-2.2
Lrrc18,	Leucine rich repeat containing 18	-2.3
NEUROD1	Neurogenic differentiation 1	-2.4
RNase	Rnase 5	-2.5
MT-II	Mt-ii	-2.5
CRB3,	Crumbs homolog 3 (drosophila)	-2.5
GCG,	Glucagon	-2.6
SUB1,	Sub1 homolog (s. Cerevisiae)	-2.6
KRT18,	Keratin 18	-2.6
C20orf31,	Chromosome 20 open reading frame 31	-2.8
GAS2,	Growth arrest-specific 2	-2.8
CHGA,	Pancreastatin	-2.8
Col12a1,	Procollagen, type xii, alpha 1	-2.8
Col12a1,	Procollagen, type xii, alpha 1	-2.8

Gene expression in nursery pigs mesenteric lymph nodes (MLN)
Gene expression regulation was examined by microarray analysis in the MLN of 21-d old nursery after dietary supplementation of probiotic of *Lactobacillus spp* (n = 10) and a control group (n = 10) for 15 days.

Table 4.4. Continued

Gene Symbol	Gene	Fold
MUC16,	Hypothetical protein flj14303	-2.8
OSBPL8,	Oxysterol binding protein-like 8	-2.9
GYLTL1B,	Glycosyltransferase-like 1b	-3.3
LTC4S,	Leukotriene c4 synthase	-3.3
MUC5AC,	Mucin 5, subtypes a and c, tracheobronchial/gastric	-3.3
EXTL1,	Exostoses (multiple)-like 1	-3.4
PPP1R13B,	Protein phosphatase 1, regulatory (inhibitor) subunit 13b	-3.5
HSD17B13	Hydroxysteroid (17-beta) dehydrogenase 13	-3.5
HPN,	Hepsin (transmembrane protease, serine 1)	-3.7
Sycn,	Syncollin	-3.8
GDF-7	Gdf-7	-3.8
PCSK5,	Proprotein convertase subtilisin/kexin type 5	-3.9
ITSN1,	Intersectin 1 (sh3 domain protein)	-4.1
ANTXR1,	Hypothetical protein flj10601	-4.3
HSD17B2,	Hydroxysteroid (17-beta) dehydrogenase 2	-4.4
LIPC,	Lipase, hepatic	-4.5
CART,	Cocaine and amphetamine responsive transcript	-4.5
GATM,	Glycine amidinotransferase (l-arginine:glycine amidinotransferase)	-4.8
AGMAT,	Agmatine ureohydrolase (agmatinase)	-5.0
ABP1,	Amiloride binding protein 1 (amine oxidase (copper-containing))	-5.5
Ctrb,	Chymotrypsinogen b	-5.5

Gene expression in nursery pigs mesenteric lymph nodes (MLN)
Gene expression regulation was examined by microarray analysis in the MLN of 21-d old nursery after dietary supplementation of probiotic of *Lactobacillus spp* (n = 10) and a control group (n = 10) for 15 days.

Table 4.4. Continued

Gene Symbol	Gene	Fold
GJB1,	Gap junction protein, beta 1, 32 kd [connexin 32]	-6.5
ELA3B,	Elastase 3b, pancreatic	-6.8
DPEP1,	Dipeptidase	-7.5
CLDN10,	Claudin 10 isoform b	-9.8
TM0849,	Chaperone protein dnaj	-9.8
GLS2,	Glutaminase 2 (liver, mitochondrial)	-12.3
RBPSUHL,	Recombining binding protein suppressor of hairless-like	-13.7
PP	Pp	-14.0
PLA2G1B,	Phospholipase a2, group ib (pancreas)	-15.8
ATP4B,	Gastric h+/k+-atpase beta subunit	-16.6
CTRL,	Chymotrypsin-like	-33.1
Ctrb,	Chymotrypsinogen b	-50.8
ELS1,	Pancreatic elastase i	-54.3
PTP,	Pancreatic thread protein	-69.7
Rnase	Ribonuclease pancreatic	-88.5
PNLIPRP2,	Pancreatic lipase-related protein 2	-97.4
ELA2,	Pancreatic elastase ii	-117.4
Ela2,	Elastase 2	-121.4
Pnliprp1,	Pancreatic lipase related protein 1	-137.2
Prss2,	Protease, serine, 2	-156.3
	Anionic trypsin precursor	-480.4
PCPA1,	Carboxypeptidase a1 precursor	-577.8

Gene expression in nursery pigs mesenteric lymph nodes (MLN)
Gene expression regulation was examined by microarray analysis in the MLN of 21-d old nursery after dietary supplementation of probiotic of *Lactobacillus spp* (n = 10) and a control group (n = 10) for 15 days.

Discussion

Effects of *Lactobacillus*-based probiotic supplementation on blood characteristics in finishing pigs are present in Table 4.1. Results from the study did not reveal any differences ($P > 0.05$) in lymphocyte, neutrophil and monocyte, white blood cells count, red blood cell count, hematocrit, and hemoglobin among treatment groups due to lactobacillus based probiotics supplementation. The capacity of probiotics to influence the immune system is one of the more recent developments in this field (Bloksma et al., 1979). Some beneficial effects of probiotics on animal's immune system have been proposed by many authors. Perdigon et al. (1991) suggested *Lactobacillus casei* have immunoadjuvant activity. Takahashi et al. (1998) and Vitini et al. (2000) reported that *Bifidobacterium longum* and other lactic acid bacteria can increase the total amount of intestinal IgA. However, Kluber et al. (1985) reported no effect of *Streptococcus faecium* on the cell-mediated immune response in weaning pigs. Similar results also observed by Apgar et al. (1993). These inconsistent responses by probiotic supplementation in pig diets may be due to varying age and health status of the experimental animals used. The effects of probiotics proposed to be more effective during young age and under stress conditions such as weaning or dietary changes for piglets (Jonsson et al., 1992). Also, our experimental period only lasted 15 d, which might be too short to induce any changes in finishing pigs.

Analysis of gene expression in this study identified changes in the expression of 80 genes ($FDR < 5\%$) in the treatment group. Most of the genes identified in this study are responsible for catalytic activities and therefore play role in digestion and absorption

of nutrients. Digestion and absorption of nutrients needed to meet the metabolic requirements and demands for normal host growth and development is the primary role of the GIT. In addition, the intestinal mucosa provides a protective host defense against the constant presence of antigens from food and pathogens in the gut lumen. The nutritional manipulation of the immune system is becoming well characterized and it brings the promise of using diet and nutrition as innovative powerful tools to reduce illness and death caused by infection.

Probiotics have been shown to have some immune effects. For example *Lactobacillus acidophillus* supplementation has been shown to have immunostimulatory effect, which is dependent on the degree of contact with lymphoid tissues while these bacteria are transiently colonizing the intestinal lumen (Hohmann et al, 1979). Animal studies have shown that gut-associated lymphoid tissue is stimulated by these surviving LABs, resulting in enhanced production of cytokine and antibody [secretory immunoglobulin (sIg) A] and increased mitogenic activity of Peyer's patch (PP) cells and splenocytes. Insoft et al, 1996 reported that gnotobiotic animals in the absence of intestinal colonization exhibit an underdevelopment of the effector components of the mucosal immune system, which makes the host more susceptible to pathologic bacterial infections.

Principally probiotics are purported to enhance mucosal immune defenses (Isolauri 1993). The general mechanisms for enhancing the host immune defenses has been attributed to the probiotic bacteria's ability to prevent or block colonization by pathogenic microorganisms. Such mechanisms involve competition for receptor sites on

the intestinal surface, production of antibiotic substances, enhancement of host immune defenses (adjuvant effect, increased polymeric immunoglobulin A production, and cytokine stimulus), and competition with pathogens for intra-luminal nutrients (Lu et al, 2001, Walker et al, 2000). Probiotics have also been suggested to affect other non-immune intestinal host defenses, including strengthening intestinal tight junctions, increasing mucous secretion, enhancing gut motility, and producing metabolic products (amino acids such as arginine and glutamine and short-chain fatty acids) that secondarily function as protective nutrients (Vanderhoof, 2001; Bengmark 1996). In addition, lactobacilli can secrete antimicrobial compounds called bacteriocins which inhibit a broad spectrum of enteric organisms.

Glucose and Glucagon

In this study, the most upregulated gene as a result of *Lactobacillus piantarumcase* and *Lactobacillus salivaruscase* supplementation was solute carrier family 2 (facilitated glucose transporter), member 11. GLUT11 is a member of class II of the GLUT family, thought to have fructose transport activity (Joost et al, 2001). The glucose transporter (GLUT) family catalyses the entry of hexose sugars into mammalian cells, and thus far, over a dozen GLUT family members have been identified on the basis of sequence similarity (Joost et al, 2001; Joost et al., 2002). These proteins are expressed in a tissue- and cell-specific manner and exhibit distinct kinetic and regulatory properties that reflect their functional and tissue specific roles. There is functional evidence from several studies for multiple members of the GLUT family in articular chondrocytes (Ohara et al., 2001; Mobasheri et al., 2002; Richardson et al., 2003). These proteins are

likely to be involved in the facilitated transport of glucose, fructose, and glucose-derived compounds (e.g., the oxidized form of vitamin C) across the chondrocyte membrane. Some GLUTs may also be involved in intracellular (organellar) glucose transport. It is therefore possible that these genes resulted into the down regulation of glucagon as they play similar role to that of glucagon. There is also some evidence to suggest that GLUTs participate in the transport of glucosamine and N-acetylglucosamine (Rauchman et al., 1992; Cloherty et al., 1995). However, it remains to be seen whether these proteins are also involved in transporting glucosamine and other glucose-derived biochemicals in chondrocytes.

Our study revealed suppression of glucagon by lactobacillus based probiotic supplementation. Toshihiko, (2006) reported that supplementation with *Lactobacillus casei* resulted in a suppression of plasma glucose and glucagon concentrations. Matsuzaki et al., (1997) reported that oral administration of *Lactobacillus casei* induces anti-diabetic effects in a non-insulin-dependent diabetic KK-Ay mouse model. Other findings have suggested that lactobacillus supplementation might affect the 2DG hyperglycemia by acting on autonomic nerves (Ganong1997). It is thought that the probiotics inhibit the adrenal sympathetic nerve and activate the gastric vagal nerve resulting in a decrease in adrenaline secretion, which lowers levels of blood glucagon and glucose (Ganong, 1997; Woods et al, 1974). Therefore, the inhibition of the adrenal sympathetic nerve might be involved in the anti-diabetic effect. These observations may be beneficial to pig industry as lowering plasma glucose may result to lean carcass, which is currently emphasized in

the pig industries due to consumer preference. Commonly used probiotics have also been shown to have beneficial effects on cholesterol metabolism (Richardson et al 2003)

Plasma glutathione peroxidase (GPx-3) was also upregulated. Plasma glutathione peroxidase (GPx-3) is a major antioxidant enzyme in plasma and, as a member of the selenocysteine-containing GPx family, scavenges hydrogen peroxide and organic (lipid) hydroperoxides produced during normal metabolism or after oxidative insult (Spallholz et al 1990).Up regulation of GPx may result into increased concentration selenium. Numerous studies have suggested that deficiency of selenium is accompanied by loss of immunocompetence (Spallholz et al 1990). In addition, selenium supplementation has marked immunostimulant effects, including enhancing proliferation of activated T cells (Kiremidjian-Schumacher et al, 1994). Moreover, activated T cells have been shown to induce upregulation of selenophosphate synthetase activity, (Guimaraes et al, 1996) directed at the synthesis of selenocysteine, the essential building block of selenoproteins. Immune dysfunction has been found to be secondary to a selenium deficiency in animals (Taylor et al, 1995). Selenium deficiency results in an impaired T cell response, decreased lymphocytes (including T cells), impaired phagocytic function and decreased immune cytotoxicity (Taylor et al, 1995). Kupka et al, 2004 reported increased risks of fetal death among animals with low plasma selenium. Beck et al, 1995 also showed that in selenium deficient hosts, harmless viruses can become virulent. If these findings were applicable to other RNA viruses there would be considerable health improvement in pig industry as supplementation of *Lactobacillus*-based probiotics would induce might enhance immunity as a result of up regulation of plasma glutathione peroxidase.

Proteases

This study also revealed that dietary supplementation of probiotics *Lactobacillus piantarumcase* and *Lactobacillus salivaruscase* results into down-regulation of six exocrine pancreas zymogens such as, trypsin precursor, chymotrypsinogen B, chymotrypsinogen 1-like enzyme, and elastase 2. It is well known that trypsinogen is synthesized by acinar cells of the pancrease and then secreted into the intestinal lumen after the ingestion of food, where it is processed to active trypsin by enterokinase. Although the function of trypsin is traditionally related to the digestion of consumed proteins, recent discoveries suggest that it also serves as an activator of the proteinase-activated receptor 2 (PAR-2), which, in turn, induces various biological effects, such as cell growth (Ossovskaya et al, 2004). Digestive organs, such as the pancreas and intestine, highly express PAR-2 (Bohm *et al*., 1996; Ossovskaya et al, 2004). It has been reported that PAR-2 plays multiple physiological roles in various tissues such as ion transport, intestinal mobility, several exocrine secretion, and in the response of tissues to injury, including inflammation, pain, and healing (Ossovskaya et al, 2004). In the gastrointestinal tract, PAR-2 is localized to epithelial, endothelial, muscle, neuronal, and immune cells (Nystedt et al 1995) where it modulates several gastrointestinal functions, including motility and secretion (Kawao et al, 2002). In addition, PAR-2 agonists have been reported to either have a pro-inflammatory or anti-inflammatory role in intestinal inflammation (Cenac et al, 2002). This is important in modulation of intestinal health of the pigs and result into improved performance.

During lactic acid fermentation, proteolysis is carried out mainly by proteolytic enzymes of lactic acid bacteria. Cell-wall-associated proteinases and several types of peptidases have been identified and the possible process of extracellular protein degradation has been proposed (Smid et al, 1991). Cell-wall proteinases from *Lactococcus lactis* sub sp. lactis and *Lactococcus lactis* sub sp. cremoris have been well characterized (Hugenholtz et al, 1984). But little is known about the properties of the proteinase of lactobacilli. Supplementation of lactobacillus based probiotics is thought to induce protection by inactivation of digestive proteases in the gut lumen. This is likely beneficial to the pig as a number of studies have shown that pancreatic proteases play a critical role in mucosal damage induced by hemorrhagic and endotoxic shock (Acosta et al, 2006), stress (Bournous, 1969), acute radiation (Delaney, 1992) and agents such as indomethacin (Kimura et al, 1998). This being the case therefore, gut damage can be alleviated by inhibition of these digestive proteases (Acosta et al, 2006). Inactivation of the digestive proteases may play important protective role against gut damage. This would as a result improve the intestinal health of the pigs resulting into better production performance.

Lipase

Our study revealed an improvement in lipase production. Commonly used probiotics have been shown to have beneficial effects on cholesterol metabolism in vitro (Gilliland et al, 1985). The effect of probiotics on triglyceride and glucose metabolism has been lacking. Lactic acid producing bacteria produce lactic acid and it has been reported that an acid and protease resistant lipase of bacterial origin might improve fat

absorption (Toshihiko et al, 2006). Toshihiko et al, 2006 reported that oral administration of *Lactobacillus johnsonii* to rats through drinking water for 2 weeks inhibited the hyperglycemia induced by intracranial injection of 2-deoxy-D-glucose (2DG). Lactic acid bacteria could be used as new vector to promote expression of lipase in the GI tract. This is potentially a major advancement in pig industry as the lipid metabolism and assimilation would result into improvement of growth performance and a reduction in serum cholesterol levels due to cholesterol assimilation by the *Lactobacillus* cells (Drouault et al, 2002) or coprecipitation of cholesterol with deconjugated bile salts (Klaver and Van der Meer, 1993).

Implications

This study indicates that dietary supplementation of *Lactobacillus*-based probiotic preparation at the level of 0.2% may have marked immunostimulant effects, including an enhancement of the proliferation of activated T cells as a result of up regulation of plasma glutathione peroxidase. GPx may result into increased concentration of selenium. Besides Supplementation of lactobacillus based probiotics may induce gut protection by inactivation of digestive proteases in the gut lumen since pancreatic proteases play a critical role in mucosal damage. This would as a result improve the intestinal health of the pigs leading to better production performance.

CHAPTER V

OVERALL SUMMARY

Two studies were conducted to: (1) to determine the effects of dietary supplementation of *Lactobacillus*-based probiotics on growth and gut environment of nursery pigs; (2) to determine the effects of dietary supplementation of *Lactobacillus*-based probiotics on the immune status of the nursery pigs. It was hypothesized in the project that *Lactobacillus*-based probiotics may beneficially alter gut ecosystem and morphology, improve animal performance and enhance the immune status of the animal. This may be beneficial for growth of young pigs by improving digestion, absorption and assimilation of nutrients.

There was no difference in body weight, ADG, ADFI and FE among the treatments. We expected an improvement in growth performance based on literature reviewed. This was however not the case probably because of feeding duration. We also expected an alteration of microbial population in the colon. The level of fermentation also did not change much as confirmed by almost equal concentration of VFAs in the digesta from the treatment groups. Villi height was greater ($P > 0.05$) in the treatment group as compared to control group. This outcome was expected and confirms our hypothesis that supplementation with *Lactobacillus*-based probiotics could improve intestinal health and improve digestion and assimilation of nutrients.

The objective of the study reported in Chapter IV was to determine the effect of *Lactobacillus*-based probiotics on expression of immune-related genes. Allotment and standard management procedures were similar to study in chapter III. Differential blood

counts were determined on whole blood samples collected on d 13 of the study period. Total RNA was isolated from mesenteric lymph nodes. Gene expression was determined using a 12,000+ pig specific custom microarray, results of which were validated with quantitative PCR. There were no significant differences ($P > 0.10$) in lymphocyte, neutrophil and monocyte, WBC count, RBC count, hematocrit, and hemoglobin among treatment groups. Microarray results identified significant difference in the expression of 80 genes that were altered by lactobacillus-based probiotics supplementation. Of these genes, nine were comparatively induced (> 2.0 fold) and the rest were comparatively repressed (> 2.0 fold).

Overall, we concluded that dietary supplementation of *Lactobacillu*s-based probiotics may be considered a nutritional strategy for modulating intestinal microbiota and gut morphology with possibility of enhancing performance of nursery pigs. We also concluded that it may have some positive effects on stimulating beneficial microflora and improving gut ecosystem of nursery pigs.

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