

ABSTRACT

QIU, RONGSHENG. DFM/Probiotics Effects on Gastrointestinal Development and Immune Function in Broiler Chicken. (Under the direction of Dr. Matthew Koci and Dr. Jim Croom).

Direct fed microbial (DFM) or probiotics are live microbial supplements, which are intentionally administered to animals and human to beneficially improve intestinal function by affecting the composition of the GI microflora. Supplementation of DFM/probiotics have demonstrated to have very promising effects on promoting resistance to diseases, reducing allergic responses, alleviating gut disorders and boosting immunity. The mechanisms, however, are still unclear. Regulation of the composition of microbial communities in the GI tract is a multi-factorial process in which any or all of these numerous forces may come into play. Therefore, it is important to use well defined strains of probiotic bacteria and appropriate biomarkers to evaluate their effects on a specific animal model. Primalac™ is a defined bacteria consortium, which has been reported to modulate GI tract structure and energy metabolism in this laboratory. This study is to investigate the mechanisms, by which DFM communicate with GI tract so as to affect histology, immune function, and energy metabolism using the rapidly growing broiler chick model.

The present studies demonstrated a faster rate of antigen specific IgG production and an increase in total sIgA secretion, suggesting a stimulation of Th2 type response. These effects may be attributed to more energy that has been repartitioned to immune system following DFM supplementation. DFM supplementation also has demonstrated a protective effect on GI epithelia, especially to esophagus and crop, and a stimulatory effect on the growth of small intestine, ileal villi as well as microvilli. The increased mucous secretion which may be induced by increased number of bacterial colonization observed may contribute to these

beneficial effects. The stimulated Th2 immune function may also help protect epithelium from damage either by pathogens or inflammation. To further understand the molecular mechanisms which may be involved in DFM-mediated changes in the gut, we assayed both ileum and cecum tissues for differences in gene expression using a focus oligo nucleotide array. Analysis of gene array networks indentified the IL-27 pathway as the most likely one that may be associated with DFM-mediated changes in the host gene expression in both ileum and cecum.

In summary, it is likely that supplementation of DFM Primalac™ can stimulate the development of immune system by modulation of energy repartition and induce protective effects on the GI tract development which result in improved gut morphology and function.

DFM/Probiotic Effects on Gastrointestinal Tract Development and Immune Function in
Broiler Chicken

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Nutrition

Raleigh, North Carolina

2009

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DEDICATION

TO MY FAMILY

BIOGRAPHY

Rongsheng Qiu (Rocky) was born on September 17, 1975, in a small village in southern China. He is the older son of Chengzhong Qiu and Saizhu Wang. Because of the environment he grew up, Rocky has a fascination with animals and plants, and was interested in creating a harmony between animals, human and the environment. To learn more about animals, animal nutrition became his major. In 1998, he received his B.S. degree from China Agricultural University. After graduation, he joined some companies that deal with different problems for the animals. To gain more experience, he tried different positions such as marketing, product management, inventory management and even delivery system. Then he was involved in an Alltech project to study the organic mineral effects on antioxidation properties in animals under the direction of Dr. Yuming Guo. He received his M.S. degree in animal nutrition in 2003 from China Agricultural University. After that, he worked as a technical manager in Beijing Zhenya Animal Nutrition Co., Ltd (Beijing, China) for about 2 years before he came to USA. In Apr., 2006, Rocky was lucky enough to meet Dr. Koci and Dr. Croom and got support from them, starting pursuing his PhD degree in nutrition. His academic research has focused on the effects of probiotics on the gastrointestinal development and immune functions of chickens.

ACKNOWLEDGEMENTS

It is impossible for me to this step without the integrated support and ideas of many people that had an influence on my life. First, I would like to thank Dr. Matt Koci, the chairman of my committee for his guidance, attention, assistance and friendship during the journey that resulted in this dissertation. Second, I want to show my especial gratitude to Dr. Jim Croom, the co-chairman of my committee for his supports and help. Discussions with him gave me inspiration for many research ideas. I'd also like to thank him for his treating me like a member of family, helping me adjust to live in USA. I also would like to express my sincere gratitude to other members of my committee, Dr. Jerry Spears and Dr. Jack Odle, for their constructive advices on my research and critical reviews on this document. In addition, I would like to thank Dr. Hosni Hassan in Department of Microbiology and Valerie Knowlton for their help on my sample measurement. I cannot forget to mention the extensive help provided by the members of Dr. Croom's Nutrition Lab and Dr. Koci's Immunology Lab, the technician Rizwana Ali, Jillene Wock, and Linda Daniel, undergraduate student Robert Ryan Meyerhoff, as well as labmate Carson. Without their help, I would not be able to perform all of these experiments. Furthermore, I want to show my appreciation to many friends who greeted me so warmly in North Carolina, Dr. Jiashon Shih, Ping, Tad, Kiano, Kim, Diego, Megan and Ni, without whose help I would never survive. Finally, my sincere gratitude is expressed to all the friends and relatives that have supported me and have not been mentioned here.

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CHAPTER I

LITERATURE REVIEW

PROBIOTIC EFFECTS ON HUMAN AND ANIMALS: THE RATIONAL

1.1 INTRODUCTION

The gastrointestinal (GI) tract, together with its accessory digestive organs, constitutes the digestive system. The major function of the digestive system is to break down large dietary polymers into smaller molecules and then to absorb these for subsequent distribution throughout the body for use as energy sources and substrates that support maintenance and growth (Whittow, 2000). Therefore, the status of gut health would directly affect human and animal health. The lumen of the GI tract can be regarded as an external environment with the epithelium responsible for allowing the absorption/transport of nutrients from the external space into the host while at the same time preventing pathogens, toxins, and carcinogen from entering. As a consequence of these competing tasks, the GI tract has developed into the largest immune organ within the body and contains approximately 65% of the immune tissue and cells. It is also responsible for up to 80% of the total immunoglobulins produced in the body (Bengmark, 1999). In addition, the GI contains numerous commensal bacterial which has been demonstrated to be essential for the maturation of immune system (Helgeland *et al.*, 1996; Shroff *et al.*, 1995). There is growing scientific evidence that the maintenance of a healthy gut microflora may provide protection against GI disorders including infections, inflammatory bowel diseases, and even cancer (Haenel and Bendig, 1975; Mitsuoka, 1982). Furthermore, it has been found that modification of GI microflora by ingestion of beneficial bacteria, like probiotics, may also promote barrier functions or prevent atopic disease (Isolauri *et al.*, 1999; Kalliomaki *et al.*, 2001). Therefore, a better understanding of the interaction between the gut epithelium and commensal microbes is needed to find new methods of protecting humans and animals from diseases.

1.2 GI TRACT AND GI MICROFLORA

1.2.1 Gastrointestinal Tract Anatomy and Structure

The GI tract consists of several anatomically and functionally distinct regions: the oral cavity, esophagus, stomach, small intestine (duodenum, jejunum, and ileum) and the large intestine (cecum, colon, and rectum). It can be considered to be a continuous exogenous tube open to the external environment from the oral cavity and extending to the anus (Schnell and Herman, 2009).

The broiler chicken shares all the basic structures of the digestive tract with other vertebrates, except a few notable adaptations (Whittow, 2000). Special characteristics of the avian digestive tract include a crop, division of the secretory and triturative functions of the stomach into two distinct compartments, and the paired cecal sacs (Figure 1.1).

The environmental conditions (pH, O₂ tension, substrate availability and temperature) within each region of the GI tract are very different, and therefore each has a very distinctive microflora community (Whittow, 2000). Generally, the GI tract (except oral cavity) consists of a tube comprised of four layers of tissue (Figure 1.2). The innermost layer is the mucosa, which consists of an epithelium surrounded by connective tissue and a thin layer of muscle known as the muscularis mucosae. Mucosa-associated lymphoid tissue is also present here. Mucosal epithelial surface of GI tract is the site at which the host encounters a large variety of microorganisms derived from the environment, and through which the pathogens invade the host and colonize to initiate infection. Therefore, they play a very important role in host defense system (DeWitt and Kudsk, 1999). Muscular in this layer contractions cause folding of the mucosa, which increases its surface area, thereby aiding digestion and absorption of

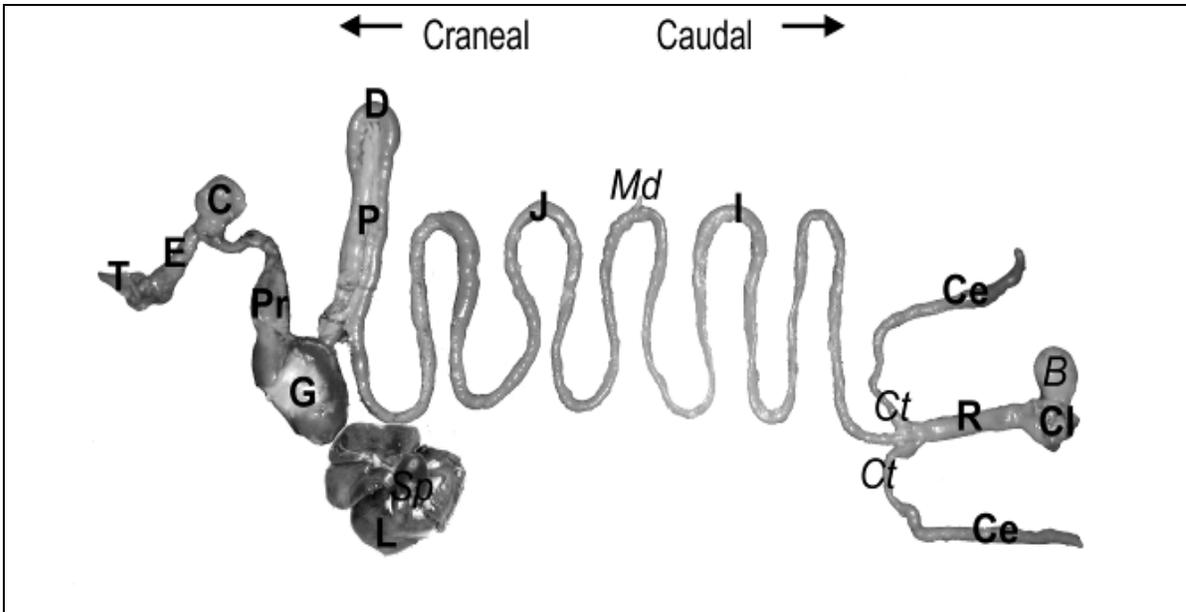


Figure 1.1 Chicken digestive tract. The avian digestive tract primarily consists of esophagus (E) which expands into the crop (C), gastric stomach which is developed into two distinct compartments: proventriculus (Pr) and gizzard (G). This leads to the small intestine [duodenum (D), jejunum (J), and Ileum (I)] and large intestine [ceca (Ce) and rectum (R)]. Note: T, tongue; L, liver; P, pancreas; Md, Meckel's diverticulum; Ct, caecal tonsils; B, bursa of Fabricius; Cl, cloaca. (Photo by D.V. Bohórquez).

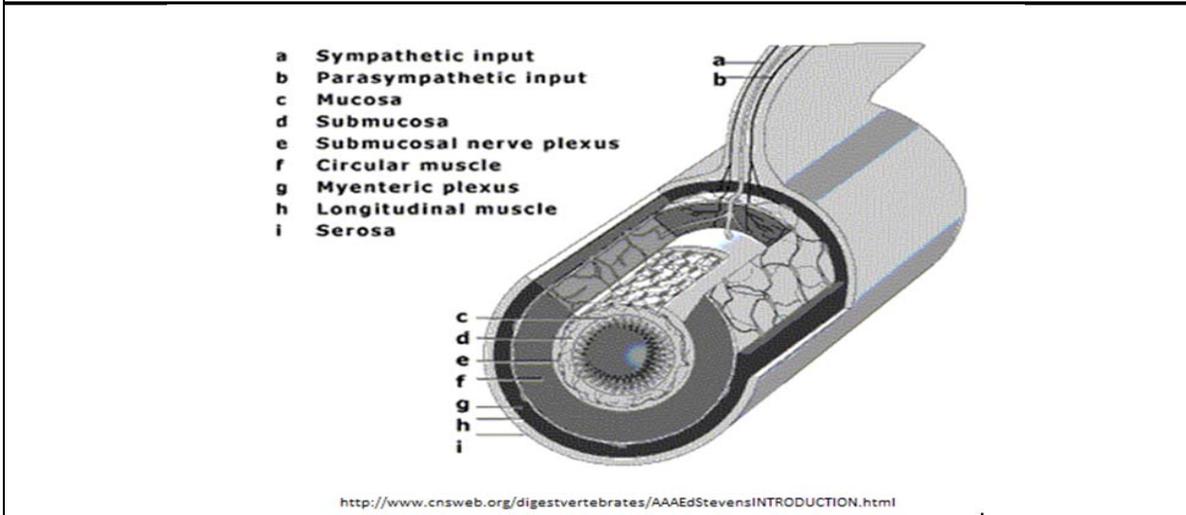


Figure 1.2 Structure of gastrointestinal (GI) tract. GI tract consists of four layers: mucosa, submucosa, muscularis, and serosa. Mucosa is responsible for absorption and secretion and important for barrier defense. Submucosa is a layer of dense irregular connective tissue that supports the mucosa. Muscularis is composed of several thin layers of smooth muscle fibers, keeping the mucosal surface and underlying glands in a constant state of gentle agitation to expel contents of glandular crypts and enhance contact between epithelium and the contents of the lumen. The serosa consists of a thin layer of loose connective tissue covered by mesothelium. It is the place of the GI tract facing the peritoneal cavity.

the resulting products (Whittow, 2000). It is estimated that the total surface area of the mucosa is 200-300 m² (human- about the surface are of a doubles tennis court), which makes it the largest body surface in contact with the external environment (Gordon and Bruckner-Kardoss, 1961).

1.2.1.1 Pregastric

The esophagus, sometimes known as the gullet, is an organ with high peristaltic activity. It consists of a muscular tube through which food passes from the pharynx to the stomach (Whittow, 2000). Esophagus is covered with a stratified squamous epithelium layer (Figure

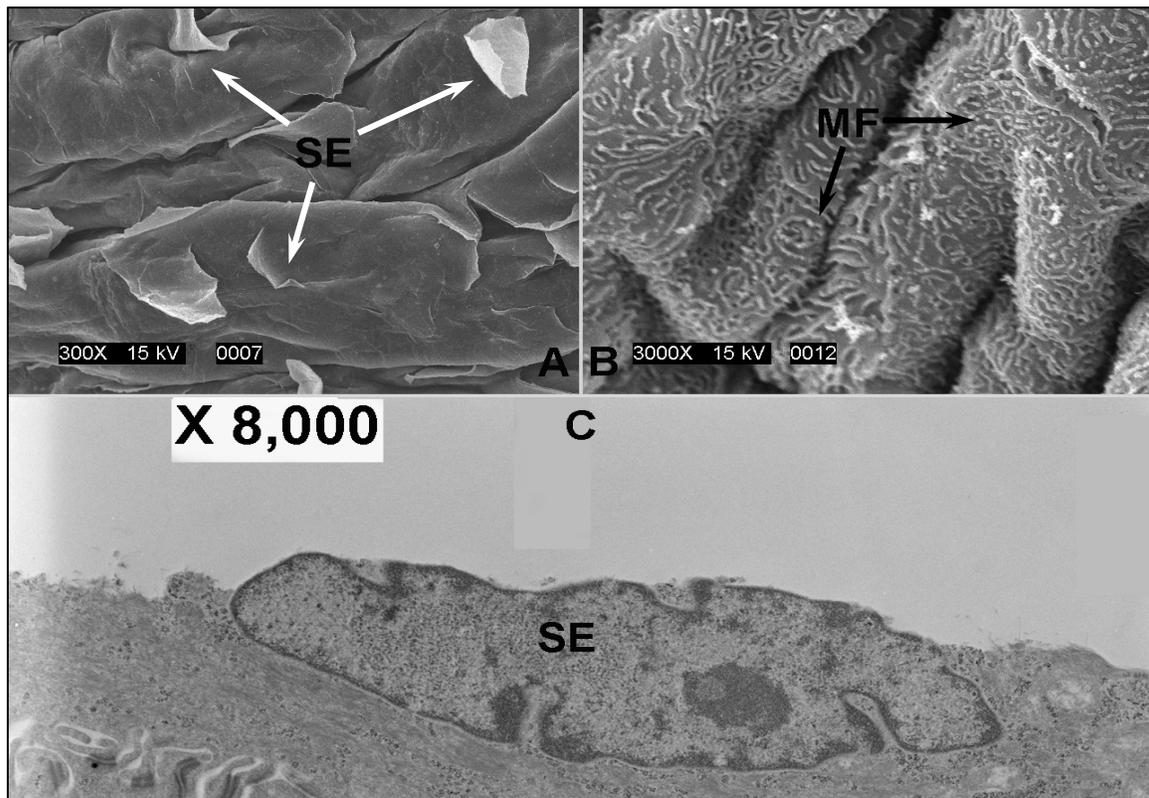


Figure 1.3 Surface of chicken esophagus. A. SEM showing surface of esophagus is covered with stratified squamous epithelial cells (SE). B. SEM showing the stratified epithelia are covered with numerous microfolds (MF), the function of which is still unknown. C. TEM showing the spatial position of stratified epithelial cells. (Photo by R. Qiu)

1.3A and C), and a large number of mucous goblet cells which together with saliva and mucous act as a mechanical barrier to pathogens (Pearson and Brownlee, 2005). The mucous contains a high concentration of immunoglobulin, which contributes to prevention of infection (Tomasi Jr, 1970). With the aid of saliva and mucus, food entering the esophagus is propelled towards the stomach by peristalsis, where digestion of the nutrients begins. The esophagus of chickens, the most proximal tubular portion of the gastrointestinal tract, channels food distally from the beak. The distal portion of the esophagus expands and merges with the crop (Ziswiler and Farner, 1972). Crop is used for the storage of food prior to digestion. It is one of the major sites for bacterial fermentation in chicken. The stratified epithelia on the surface of chicken esophagus and crop (Figure 1.3B) are covered with numerous microfolds, the function of which is still unknown.

1.2.1.2 Gastric Stomach

The stomach serves as a site to store, mix and digest the food. In most mammals, the stomach is a hollow, muscular organ of the GI tract, between the esophagus and the small intestine (Whittow, 2000). The epithelium of the stomach forms deep gastric pits, where columnar secreting epithelium is located. These secreting epithelium including parietal cells releasing gastric acid and goblet cells producing mucous gel layer which protects gastric epithelium from adverse factors like gastric acid (Johnson and Gerwin, 1997). The secretion of gastric acid keeps the gastric lumen at a very low pH level, which can rise from 1.8 in empty stomach to 6.4 after eating (Tyssandier *et al.*, 2003). Low pH makes gastric stomach to be an environment unfavorable for bacterial colonization.

Chicken gastric stomach consists of the proventriculus and gizzard (Figure 1.1). Proventriculus is generally a glandular part of the stomach, lined by fundic glands (Whittow, 2000). Gizzard is used for grinding up food, constructed of muscular walls lined by pyloric glands. The gastric juice secreted in the proventriculus has a pH of 0.2 to 1.2, while pH measured in the gizzard ranges from 0.7 to 2.8 (Ziswiler and Farner, 1972).

1.2.1.3 Small Intestine

The small intestine is the main site of digestion and absorption. The small intestine comprises the proximal (duodenum), mid (jejunum), and distal (ileum) areas. The epithelial surface of small intestine is covered by a mucus blanket 10-250 μ m thick (Johnson and Gerwin, 1997). The epithelium consists mainly of columnar enterocytes and mucus-secreting cells (goblet cells) (Figure 1.4 C and D, Figure 1.5 A and B). The intestinal mucosa is highly folded to produce permanent ridges, which project into the lumen, and has numerous finger-like projections known as villi (Figure 1.4B, C, and D), of which there are 20 to 40 per mm^2 (Johnson and Gerwin, 1997). The maintenance and renewal of the villi cells depends on a small number of stem cells that reside at the bottom of the crypts of Lieberkühn (Figure 1.4D). In birds, it appears there is a second type of epithelial cell at the apical end of the villi that retain the capacity for cell division and differentiation (Uni *et al.*, 1998). Villi possess highly undulated apical membranes forming small, but numerous protuberances called microvilli. These protuberances for each cell are collectively known as the “brush border” and dramatically increase the surface area for absorption of digestion products in the lumen (Figure 1.5). Villi increase the area by 25 folds while microvilli increase it by 60 folds (Strocchi and Levitt, 1993). This increases the efficiency of nutrient absorption. Each villi

enterocyte contains constituent digestive enzymes that form an integral part of the apical membranes and microvilli (Chaves *et al.*, 1987). Therefore, the assessment of the structure of villi (height and width), microvilli (height and density) as well as the depth of the Crypt of Lieberkühn can be used as indicators of intestinal health, which is essential for proper nutrient absorption.

The chicken small intestines appear to be generally shorter than in mammals like pigs (Ziswiler and Farner, 1972) and the length may increase as much as 40% when switched to high fiber diet (Karasov, 1996). The chicken has a distinct duodenal loop, and the yolk stalk (formally called Meckel's diverticulum) is often used as a landmark to separate the jejunum and ileum (Whittow, 2000).

1.2.1.4 Large Intestine

Generally, the large intestine consists of the cecum and colon in mammals. Its function is to absorb water from the remaining indigestible food matter, and then to pass useless waste material from the body. The wall of the large intestine is lined with simple columnar epithelium with many goblet cells and invaginations (the intestinal glands) but no villi (Johnson and Gerwin, 1997).

Chicken large intestine consists of cecum, rectum (or colon) and cloaca (Figure 1.1). Chicken ceca are more tubular than saccular, and they contain prominent villi (Whittow, 2000; Planas *et al.*, 1987). Given their higher surface to volume ratio, they seem designed for surface-related processes such as hydrolysis and absorption in addition to, lumen related microbial processes such as fermentation (Joefiak *et al.*, 2004). While the chicken rectum has numerous flat villi and relatively few goblet cells (Clauss *et al.*, 1991).

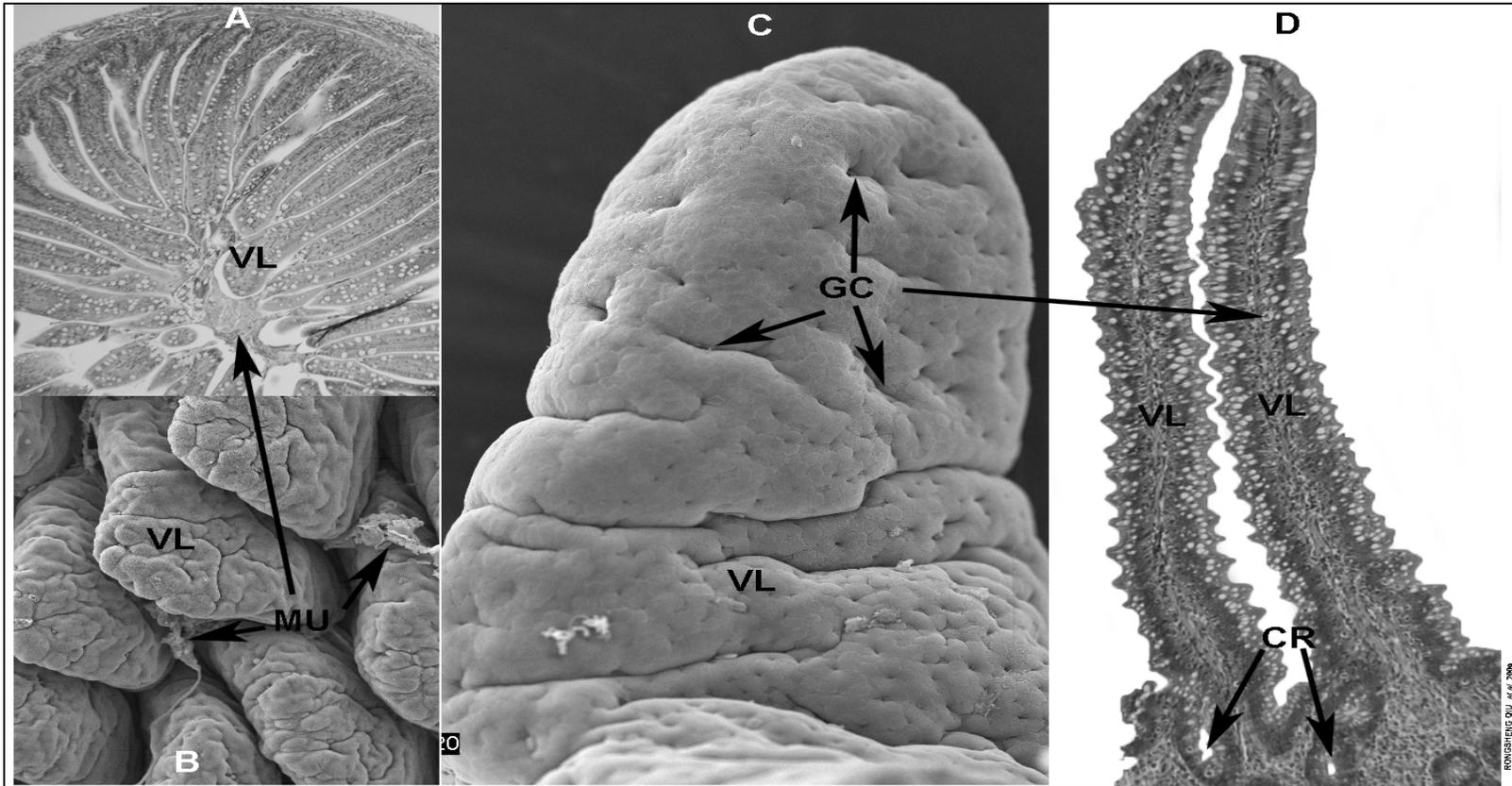


Figure 1.4 The shape and spatial position of ileal villi in chicken (d7). **A.** Light micrograph ($\times 60$) showing the overview of villi (VL) in the ileum projecting into the lumen. **B** ($\times 150$) and **C** ($\times 500$) scanning electron microscopy showing the VL from different angles. Villi are covered with a thick layer of mucus (MU), which is produced and secreted by the goblet cells (GC). **D.** Light micrograph ($\times 200$) showing the cross section of VL. Stem cells in the crypts (CR) produce the four main cell lineages that form the intestinal epithelia. Except for Paneth cells, which migrate towards the bottom of the crypts, the other three lines of cells, enterocytes (EC), GC, and enteroendocrine (not shown) migrate towards the luminal end of the villi. (Photo by R. Qiu)

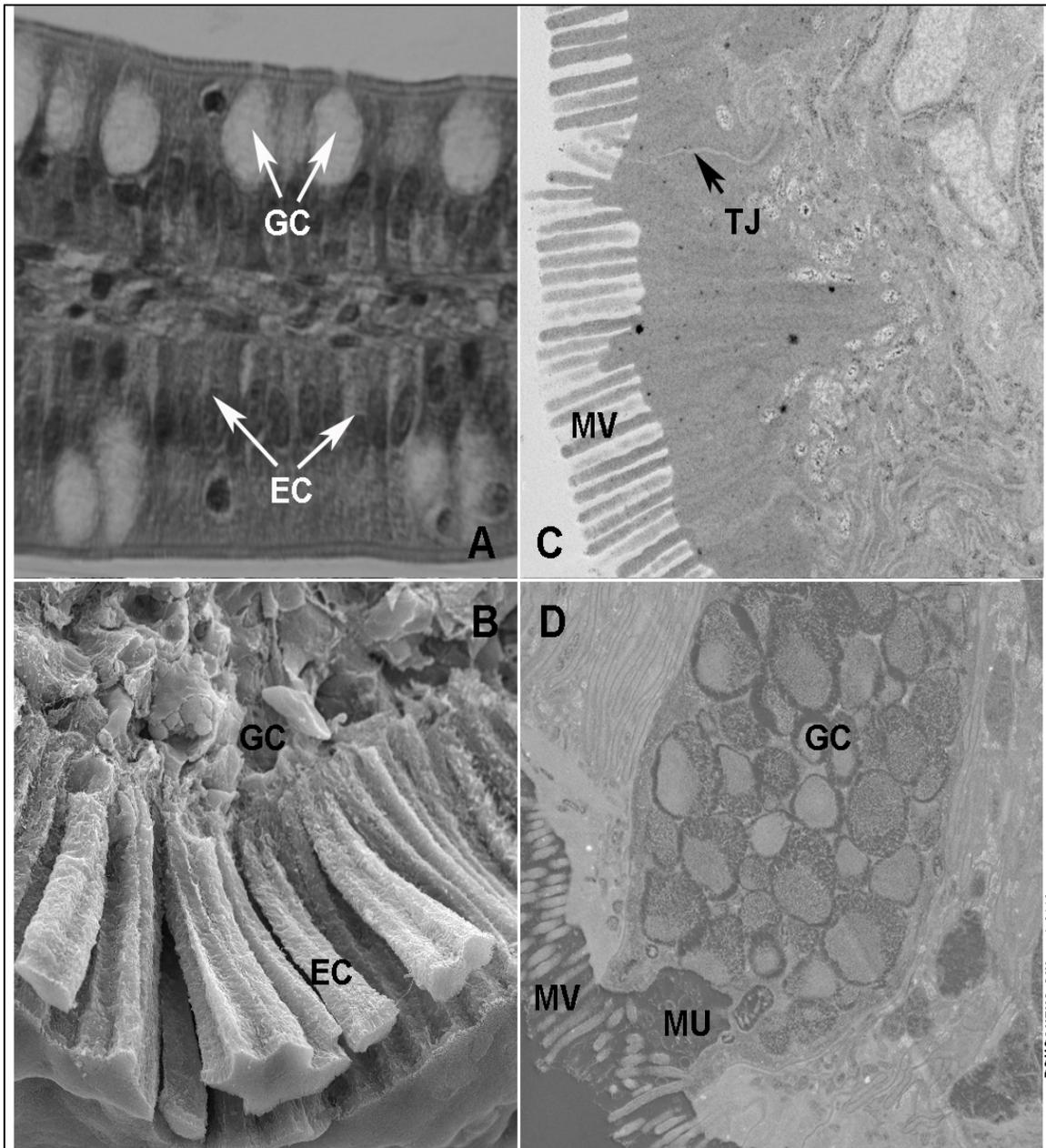


Figure 1.5 Shape and spatial position of enterocyte and goblet cells in ileum. **A.** Light micrograph ($\times 400$) showing a part of the villi (VL). The epithelium primarily consists of enterocyte (EC) and goblet cells (GC). **B.** Scanning electron micrograph showing the shape of enterocyte and goblet cells. **C.** Transmission electron micrograph showing the membranes of two enterocytes join together forming tight junction (TJ), which is impermeable barrier to fluid and epithelial cells covered with microvilli (MV). **D.** Transmission electron micrograph showing a goblet cells releasing mucous (MU) into the lumen. (Photo by R. Oiu)

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1.2.2 Microbial Flora Colonization in Gastrointestinal Tract

Immediately after birth, the GI tract of human and animals begin to be colonized by exogenous microorganisms that inhabit the reproductive and GI tract of the mother, as well as external surfaces and aerosols in the environment (Ley *et al.*, 2006). The final indigenous gut microflora is a very complex and diverse collection of microbes. In humans, for instance, there are about 100 trillions different micro-organisms representing an estimated 400 different types of bacteria (Moore and Holdeman, 1974). Among these, only 30 to 40 anaerobic bacterial species constitute ~ 99% of the total microbial biomass, with the rest consisting of other facultative organisms (Finegold *et al.*, 1983). It is interesting to note that though minor members in terms of numbers, *Lactobacilli*, are relatively numerous in the proximal region of the gut of mice, rats, pigs, and chickens. This is especially true for the surfaces of orally proximate portions of the GI tract, such as the rodent stomach, the avian crop (Figure 1.3) A and C, and the porcine pars oesophagea, which are covered with a non-secretory stratified squamous epithelium (Barrow *et al.*, 1980; Fuller and Brooker, 1974; Savage *et al.*, 1968).

The total cell population in an adult human has been estimated at about 10^{13} cells while the total microbial population at over 10^{14} (Savage, 1977). Therefore, both humans and the animals can be regarded as a biological organism composed of both eukaryotic animal cells and eukaryotic and prokaryotic microbial cells (Savage, 1986). This concept is supported by data describing how microbial and animal components of the body interact in both physiological and biochemical ways (Bry *et al.*, 1996). It is supported as well by the comparative nutrition research

findings revealing that adult mammals, including humans, are dependent on microbial processes in the alimentary canal for some nutrients (Savage, 1986).

In adult animals, the microbial communities occupy many environmental niches in habitats throughout the gut. These microenvironments include attachment to epithelial surfaces, colonization of the crypts in the mucosa as well as the epithelial mucous blanket, attachment to food particles, or free colonies within the aqueous milieu of the intestinal lumen (Berg, 1996). Major sites of colonization within the GI tract can only occur in regions of relative stasis, where digesta transit time is long enough to provide substrate for significant microbial growth. Furthermore, the only microbes that can successfully colonize regions of fast digesta transit are those that attach to epithelial surfaces (Figure 1.6D).

1.2.2.1 Pregastric

The mouth and the oropharynx predominantly harbor Gram-positive organisms (Williams, 2001) such as *Streptococci*, *Neisseria*, *Veillonella*, *Fusobacteria*, *Bacterioides*, *Lactobacilli*, *Staphylococci*, *Yeasts*, and *Enterobacteria* (Galatola *et al.*, 1991). Bacteria can be introduced into the esophagus by the swallowing of food, by resident oral microbiota or by reflux from the stomach (Pei *et al.*, 2004).

The crop in chicken serves several functions, including as a site for microbial fermentation. Scanning electron micrographs (SEM) reveal a large population of bacteria on the wall of the chicken crop as well as bacteria attached to food particles stored within that organ. These bacteria may contribute to the digestion of nutrients, such as starch (Champ *et al.*, 1981) and fiber (Shetty *et al.*, 1990). *Lactobacilli* are the predominant microbial genus in the chicken crop (Fuller and Brooker, 1974). Since a mucus layer is absent from the surface

of the crop, *Lactobacilli* can be found adhering directly to epithelial cells, forming a layer of bacterial cells across the crop epithelium (Figure 1.6A). The *Lactobacillus* colonizing the crop luminal surface become established within a few days after hatching and the specific adherence of avian associated *Lactobacilli* on the crop epithelium play a role in the colonization and the regulation of the distal intestinal microflora (Fuller, 1973). It has been demonstrated that freshly isolated *Lactobacilli* from the intestinal tract of chickens are able to adhere to crop epithelium surface when introduced orally (Edelman *et al.*, 2002). After colonization, these bacteria can replicate (Figure 1.6B) and then migrate with the digesta to colonize the remainder of the gut (Fuller and Brooker, 1974). The mechanism by which the *Lactobacillus* strains adhere to the epithelia has not yet been determined, however, preliminary *in vitro* investigation have demonstrated that both carbohydrate and protein molecules (like surface layer protein) are involved (Fuller, 1975; Suegara *et al.*, 1975; Greene and Klaenhammer, 1994; Hynonen *et al.*, 2002; Schneitz *et al.*, 1993). Bacteria in crop can form a filamentous web of unknown composition directly connecting to the crop epithelial cells and with other as well bacterial cells (Figure 1.6 C and D; Fuller and Brooker, 1974). The filamentous web may serve as an anchor for the *Lactobacilli* and could be important for the colonization of these bacteria in the crop.

1.2.2.2 Gastric Stomach

The mammalian stomach is lined with columnar secreting epithelium located within gastric pits. Normally, most of the bacterial in the stomach are killed because of the low pH levels, and the typical numbers cultured are less than 10^3 CFU/ml (Franklin and Skoryna, 1966). The normal resident microbiota of the stomach consists mainly of Gram-positive

aerobic bacteria, such as *Streptococci*, *Staphylococci*, and *Lactobacilli* (Simon and Gorbach, 1986; Franklin and Skoryna, 1966; Simon and Gorbach, 1984). Lactic acid producing bacteria are commonly isolated from the human gastric digesta, especially when strict anaerobic culture techniques are employed (Mackie *et al.*, 1997).

Candida and other yeast species have also been detected. Bacteria isolated from gastric digesta are considered transient with a short residence time. These bacteria have been passed down from habitats above the stomach or have been present in ingested materials (Savage, 1977).

Chicken gastric stomach consists of the proventriculus and gizzard (Figure 1.1). These parts of the GI tract may retain their contents long enough for microbes to grow, however, growth would presumably be limited by a low pH (Ziswiler and Farner, 1972). Even in this acidic environment, relatively large number of bacteria adhering to the gut wall are possible because the attached mucous layer can shelter bacteria from the ambient pH and because microbial urease can produce NH_3 that neutralizes acidity in the immediate microenvironment (Ouweland and Vaughan, 2006). Also, uneven mixing of stomach contents allows areas of relatively high pH to persist. Although relatively high SCVFA concentrations occur in the gizzard of some avian species (Swart *et al.*, 1993), it is not a major site of microbial SCVFA production (the total anaerobes is around 3×10^6 CFU/g digesta).

1.2.2.3 Small Intestine:

An example of microbial species isolated from the small intestine is listed in Table 1.1.

These microbes include those descending from habitats above the small intestine such as the

mouth, and ingested food. The velocity of the intra-luminal content of the small intestine decreases from the duodenum to the ileum with an increase in the density of microbiota towards the distal small intestine (Johnson and Gerwin, 1997). The upper two thirds of the small intestine (duodenum and jejunum) contain only small numbers of the same microorganisms, which range from 10^3 to 10^5 CFU/ml (Simon and Gorbach, 1986). Culturing studies indicated that acid- and facultative Gram-positive species such as *Lactobacilli* and *Streptococci* dominate in the proximal portion, while distally, anaerobic, and more Gram-negative bacteria are dominant (Johnson and Gerwin, 1997). The rich microbiota milieu within the contents of the cecal digesta can enter the ileum through the ileocecal valve, via reverse peristalsis. With decreased intra-luminal transit, decreased acidity, and lower oxidation-reduction potentials, the ileum maintains more diverse and numerous microbial communities (Savage, 1977).

In chicken small intestine since it appears to be generally shorter than in mammals, the transit time of digesta is usually shorter than in mammals (Ziswiler and Farner, 1972). Therefore, in most instances, the lumen is unsuitable for colonization by microbes. Facultative anaerobic microbiota (*Streptococci*, *Lactobacilli* and *E. coli*) comprise the predominant microbiota of the small intestine and these bacteria may represent 60-90% of the bacteria colonizing the intestinal tract (Salanitro *et al.*, 1978). The epithelium of the small intestine of domesticated chickens is colonized by an unidentified segmented, filamentous bacterium that attach to the apical membranes of enterocytes, beginning in the upper third of the small intestine (Berg, 1996). In previous studies from this laboratory, these organisms appeared preferentially associated with goblet cells of the ileum and cecum, however, the

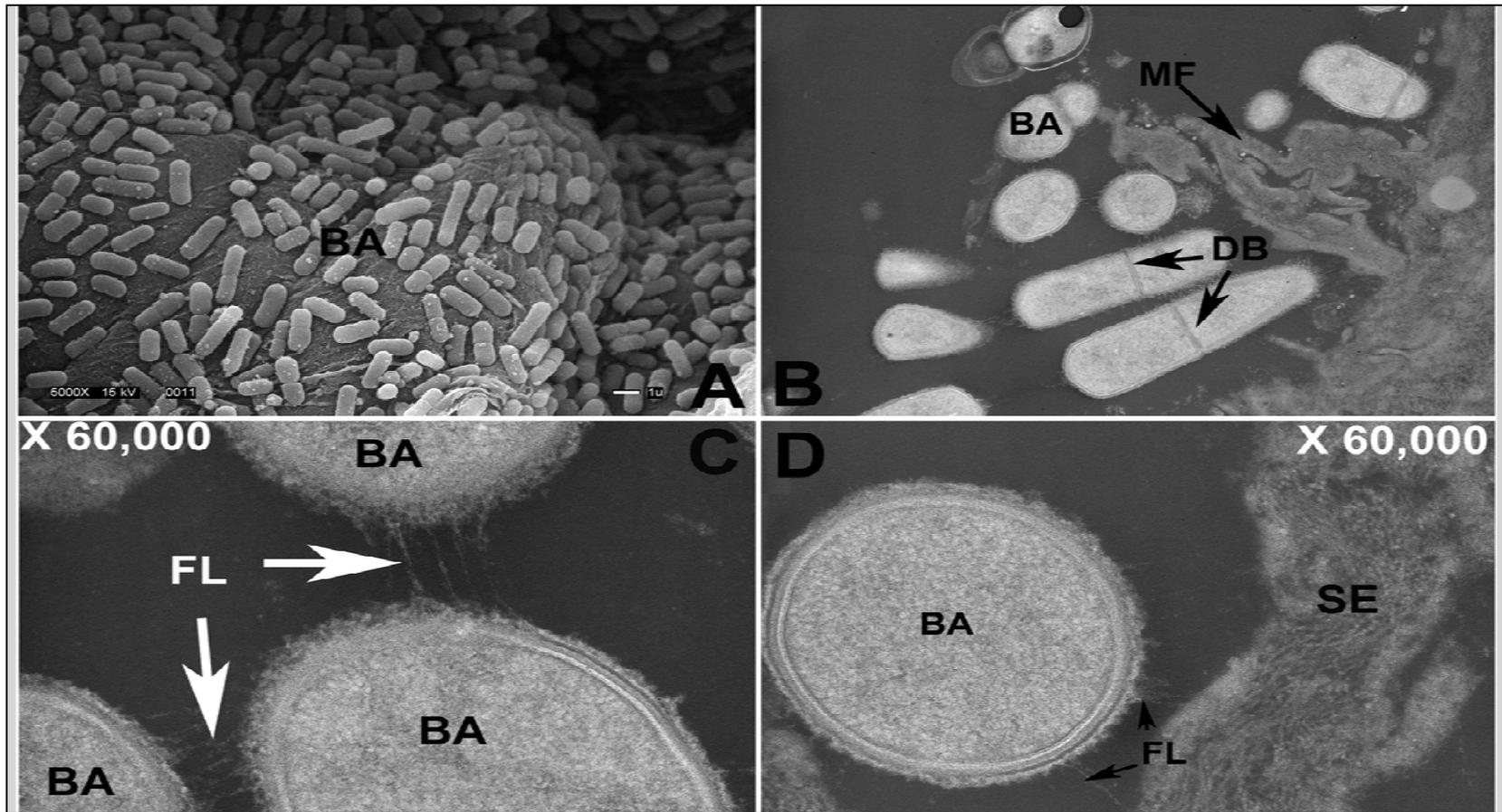


Figure 1.6 Bacteria in chicken crop **A.** SEM showing rod-shaped bacteria (BA) forming a bacterial blanket on the surface of crop. **B.** TEM showing bacteria around microfolds in chicken crop. Some dividing bacteria (DB) were noted as well. **C.** TEM showing bacteria in chicken crop can form filamentous (FL, arrow) web between each other. **D.** TEM showing bacteria in chicken crop attaching to stratified epithelial cells (SE) through filamentous structures (arrow). (Photo by: R. Qiu)

numbers of these bacteria was found to be decreased by the introduction of DFM (Chichlowski *et al.*, 2007b).

Table 1.1 Microorganisms cultured from the small intestinal tract¹

Microbe	duodenum and proximal jejunum	distal jejunum and ileum
<i>Lactobacilli</i>	+	
<i>Streptococci</i>	+	
<i>Bifidobacteria</i>		+
<i>Clostridia</i>		+
<i>Coliforms</i>		
<i>Bacteroides</i>		+
<i>Veillonellae</i>	+	
Gram positive		
Nonsporing		
<i>Anaerobes</i>		
<i>Staphylococci</i>	+	
<i>Actinobacilli</i>	+	
<i>Fusobacterium</i>		+

¹ Source: (Simon and Gorbach, 1986; Ouwehand and Vaughan, 2006). ‘+’ denotes the primary bacterial found in the gut.

1.2.2.4 Large Intestine

Generally, the large intestine harbor over 500 species of bacteria, mainly obligate anaerobes with 10^{11} to 10^{12} CFU/g (Simon and Gorbach, 1986). *Bacteroides*, *Bifidobacteria*, *Eubacteria*, *Clostridia*, and *Enterobacteriaceae* can predominantly be found in the colon of humans (Table 1.2). Enormous microbial populations can develop in the lumen of the large bowel, and especially in that of the cecum, because these areas have a relative slow digesta transit times. Bacteria in food, when found in large concentrations, are known to pass into human feces. Bacteria from habitats above the large bowel pass down into the distal GI lumen too.

Table 1.2 Microbiota Isolated from the human Large Intestine and Feces by Culturing¹

Microbial type in large intestine		Microbial types in feces	
<i>Lactobacilli</i>	<i>Staphylococcus</i>	<i>Lactobacilli</i>	<i>Coliforms</i>
<i>Streptococci</i>	<i>Coliforms</i>	<i>Streptococci</i>	<i>Bacillus sp</i>
<i>Bifidobacteria</i>	<i>Bacillus sp</i>	<i>Bifidobacteria</i>	<i>Yeasts</i>
<i>Clostridia</i>	<i>Yeasts</i>	<i>Clostridia</i>	<i>Spiral shaped microbes</i>
<i>Propionibacterium</i>	<i>Spiral shaped microbes</i>	<i>Propionibacterium</i>	<i>Peptococcus</i>
<i>Eubacterium</i>	<i>Actinobacillus</i>	<i>Eubacterium</i>	<i>Ruminococcus</i>
<i>Bacteroides</i>	<i>Enterobacteriaceae</i>	<i>Bacteroides</i>	<i>Coprococcus</i>
<i>Fusobacterium</i>	<i>enterococci</i>	<i>Fusobacterium</i>	<i>Acidaminococcus, Succinivibrio, Butyrivibrio, Megasphaera, Gemminger</i>
<i>Veillonella</i>		<i>Veillonella</i>	<i>Catenabacterium</i>
		<i>Staphylococcus</i>	<i>peptostreptococcus</i>

¹Source: (Simon and Gorbach, 1986). Predominant bacteria were marked with bold.

Microbial activity is primarily fermentative and in the chicken, the cecal bacteria are mainly saccharolytic (Savage, 1986b). Large populations of uric acid-degrading bacteria are commonly observed (Mead, 1989). Adherent bacteria cover the walls of the chicken cecum (Fuller and Turvey, 1971; Figure 1.7). The function of the cecum appears to involve mechanism(s) that selectively retain fluid and small particles (including bacteria). In some birds, fluid (urine) is refluxed by antiperistaltic contractions from the cloaca along the usually short colon and into the ceca (Whittow, 2000). This rinses small particles out of the colonic contents and carries them into the cecal (Bjornhag, 1989). Larger particles are left behind to be excreted. What's more, this reflux of urinary nitrogen in birds has been suggested to be involved in osmoregulation (Braun, 1999). Additionally, non-protein N, such as uric acid,

entering the ceca is metabolized by the bacteria into ammonia which can also be used to support microbial protein synthesis (Lavery and Skadhauge, 1999). But the benefit of this activity has not yet been clarified and may be indirect (Mackie *et al.*, 1997).

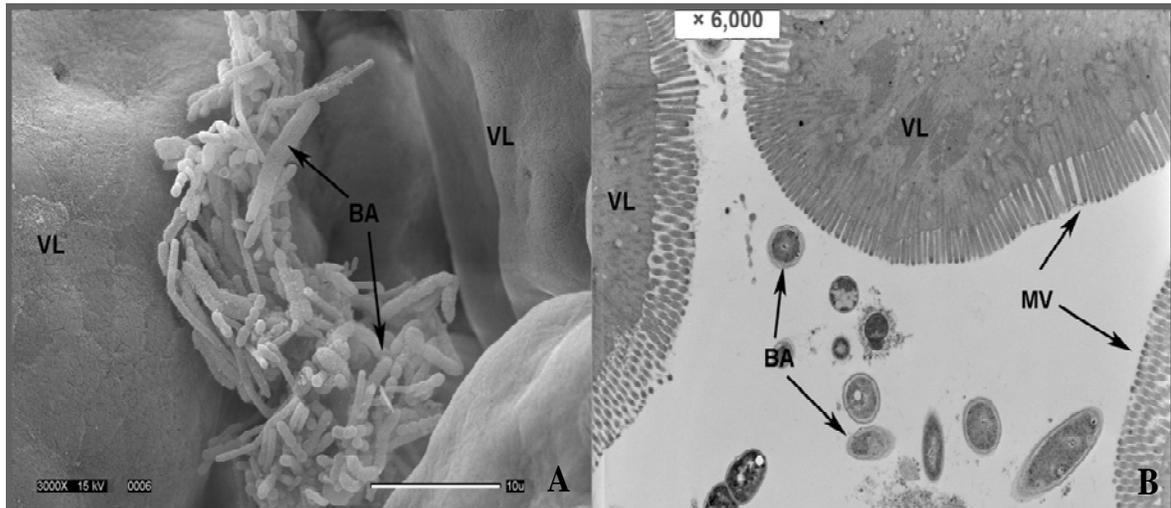


FIGURE 1.7 Bacteria found in chicken cecum. SEM (A) and TEM (B) micrography showing the bacteria (BA) in the cecum. Bacteria normally were found at the bottom site between the gap of villi (VL), where the velocity of the intra-luminal content may be low. They may stay in mucus or attach to the microvilli (MV). (Photo by R. Qiu)

1.2.3 Effect of Microflora on Gut Function

Naturally occurring, symbiotic bacteria of the animal gut have long been appreciated for the benefits they provide to the host. They supply essential nutrients, metabolize indigestible compounds, defend against colonization by opportunistic pathogens and even contribute to the development of the intestinal architecture (Hooper and Gordon, 2001). What's more, commensal microflora byproducts, including SCVFA, polyamines, vitamins, antioxidants,

and amino acids, can also contribute to the health of the intestinal tract and nutrition of the host animal (Macpherson and Harris, 2004).

1.2.3.1 Microbial Contribution to Nutrition

The commensal enteric flora makes significant metabolic contributions to the health of the host. For instance, the production of vitamin K by the commensal flora is essential to normal blood coagulation, and vitamins of the B group play multiple roles in normal hoemostasis (Hill, 1997). In addition, SCVFA production by commensal bacteria supports healthy epithelial cell growth and provides additional energy sources to the host animal (Heerdt et al., 1997, Lupton and Kurtz, 1993). For example, the butyric acid derived from carbohydrate fermentation provides a major energy source for colonocytes in the large intestine (Fleming *et al.*, 1991; Scheppach *et al.*, 1992).

1.2.3.2 Role in Protection from Infection

The Nurmi principle proposes that one can improve the survival of chicks by inoculating them early with an “adult” gut microbiota (Nurmi and Rantala, 1973). It is now well established that one of the essential effects of GI microflora is its ability to prevent colonization by enteric pathogens. Using gnotobiotic animals, it has been demonstrated that introduction of exogenous GI microflora can effectively suppress the colonization of *E. coli* and *Pseudomonas* (Srivastava, 1978) as well as *Salmonella* (Baba *et al.*, 1991; Corrier *et al.*, 1991). Furthermore, inoculated microflora may prevent viral infections. It has been shown that the existence of intestinal microflora may affect the timing, magnitude, and duration of the permeability caused by rotavirus (Heyman *et al.*, 1987).

The precise requirements for successful colonization of microflora for competitive exclusion in the GI tract have not been fully elucidated. Several hypothesis have been proposed including: 1) successful competition for the same substrate, or mucin adhesion receptor sites; 2) production of a physiologically restrictive environment, for instance, with respect to pH, redox potential, hydrogen sulfide production, or production of metabolites toxic to other bacteria; 3) in vivo production of antibiotic substances such as bacteriocins, provided that they are not destroyed by intestinal secretions; and/or 4) production of a signal molecule that acts on the genes encoding for survival (Berg, 1996).

1.2.3.3 Affecting Maturation of the Immune System

Growing evidence suggests that the existence of commensal enteric flora can be the stimulus of infant or neonatal immune system maturity (Umesaki and Setoyama, 2000). Using germfree animals as a model, it has been found that compared to control animals, germfree animals show a large number of immune deficits. Gut associated lymphoid tissue (GALT), in these animals, is sparse and disorganized, failing to demonstrate Peyer's patches (PP) and other lymphoid follicles (Helgeland *et al.*, 1996; Shroff *et al.*, 1995). It has also been found that the germfree animals have a comparatively poor IgA production and the systemic antibody responses are weak (Umesaki and Setoyama, 2000). These inducible structures seem to form normally following the introduction of gut bacteria, suggesting a dynamic relationship between the immune system and the microbiota. As expected, germ-free animals show higher susceptibility to infection, and decreased bacterial clearance has been reported following *Listeria monocytogenes* infection (Zachar and Savage, 1979). In addition, some symbiotic bacterial species have been shown to affect the intestinal

inflammatory status. For example, the growth and capacity to induce inflammation by *S. Typhimurium* can be decreased by the presence of normal commensal intestinal microbiota (Stecher *et al.*, 2007). This suggests the existence of mechanisms that allow intestinal microflora to communicate with the host immune system. Therefore, the intestinal microbiota, and the composition of the bacterial communities in the gut may be intimately linked to the proper functioning of the immune system. This provides a good explanation for the postulated immunomodulation properties of probiotic products.

1.2.3.4 Role in Absorptive and Other Functions

The microflora has been shown to affect the development of intestinal morphology. Intestinal epithelial cells were found to alter their patterns of microvilli formation and decrease rates of cell turnover in germ-free animals compared with wild-type animals (Abrams *et al.*, 1963). It has been found that GI microflora can affect intestinal permeability (Heyman *et al.*, 1987; Beaver and Wostmann, 1962), altering the mechanisms of absorption of nutrients (Mehrazar *et al.*, 1993). Studies have shown a dramatic effect of commensal bacterial on the development of intestinal mucosa (Bry *et al.*, 1996). It was interesting to note that introduction of *Bacteroides thetaiotaomicron*, a component of GI flora, restored the fucosylation pathway that is disestablished in germfree animals (Bry *et al.*, 1996). It indicates that the commensal bacteria can affect the carbohydrate metabolism in the host animals (Bry *et al.*, 1996).

Although it is unclear how intestinal bacteria are capable of producing such varied responses in the host, it is suggested the intestinal microflora may cause all these changes via influence gene expression. It has been found that colonization by only one commensal

organism, *Bacteroides thetaiotaomicron*, can induce the activation of a large number of genes in intestinal epithelial cells (Hooper *et al.*, 2001). Some of the genes that play important roles in digestive functions, such as Na⁺/glucose transporter, colipase, and lipase related protein 2 expression were increased significantly. It was also found that with some genes related to detoxification processes, like glutathione S-transferase, were induced as well. In another study, commensal flora was found to contribute to intestinal capillary morphogenesis (Stappenbeck *et al.*, 2002), suggesting that gut bacteria are required for full intestinal blood vessel development.

1.3 MODIFICATION OF INTESTINAL MICROFLORA WITH PROBIOTICS

Colonization of the gut with “beneficial” microflora has been well recognized to contribute to its ability to function normally. Overgrowth of one bacterial species or imbalances in the microflora resulting from a disturbed mucosal layer can alter digestive function, intestinal products, and/or immunological function (Walker, 2000). In addition, a defective epithelial layer can allow bacteria or pathogens to gain entry into the host. This breach can initiate an inflammatory response in the host that has the potential to further alter normal function.

The most exciting implication of an expanded role for the commensal bacterial flora is the possibility that animal health can be positively influenced by manipulation of the luminal bacterial populations. As discussed above, especially in farm animals, the microbiota of the digestive tract plays an important role both in the process of optimal development and growth of the host as well as promoting resistance to diseases (Tancrede, 1992). Adverse factors like abrupt change of diet, weaning, stress, administration of antibiotics and pathogenic

microorganisms can all disturb the stability and composition of the commensal flora, thus disturbing host physiological processes (Sorum and Sunde, 2001). Therefore, it is obvious that in order to minimize the negative effects caused by disease, it is necessary to support to the beneficial microbiota of the digestive tract. This may be achieved by supplementation with probiotic microorganisms.

This supplementation increases numbers of “beneficial microbes” in the GI tract. In a double blind study, the consumption of *L. casei Shirota*-fermented milk demonstrated an increase of the *Lactobacillus* count in the faeces. This was associated with a significant increase in *Bifidobacterium* colonization (Spanhaak *et al.*, 1998). On the other hand, use of *L. casei* was found to decrease the counts of enterotoxigenic *E. coli* O101:K99 adhering to the small intestinal mucosal of gnotobiotic lambs by 99.1% and 76% on day 2 and 4 after inoculation respectively (Bomba *et al.*, 1997). In addition, many studies have suggested that development of the GI microbiota of the young is especially critical (Cebra, 1999; Benyacoub *et al.*, 2003). It may be because it is the period when the danger of diarrhea-accompanied diseases of the digestive tract reaches its maximum.

1.4 PROBIOTIC HISTORY AND DEFINITION

The consumption of fermented milk products dates from pre-biblical times but the probiotic concept was proposed only a century ago with the work of Dr. Metchnikoff at Pasteur Institute in Paris, who first reported the healthful contribution to humans by “friendly” microbes in the intestinal tract (Metchnikoff and Mitchell, 1910). In his best selling book, *The Prolongation of Life*, he suggested the longevity of people in Caucasus mountains was related to the high consumption of fermented milk products. He considered

substitution of gut microbes by yogurt bacteria to be beneficial and that lactic acid production, resulting from sugar fermentation by “lactic acid bacteria” (LAB), to be particularly beneficial.

The term probiotic, meaning "for life," is derived from the Greek language. The expression of “probiotic” was probably first defined by Kollath, when he suggested the term include all organisms as well as organic and inorganic food supplements that promote GI health, in contrast to pharmaceutical substances, such as antibiotics (Kollath, 1953). Since then, numerous definitions have been proposed but none have been completely satisfactory. In 1980 Fuller proposed probiotics be defined as live microbial feed supplements, which beneficially affect the host animal by improving its intestinal microbial balance (Fuller, 1989). This definition was later broadened to include ‘mono- or mixed-cultures of live microorganisms which benefits man or animals by improving the properties of the indigenous microflora’ (Havenaar *et al.*, 1992). Currently, the definition of probiotics accepted by the international scientific community is that proposed by the European Union Expert Group On Functional Foods as “viable preparations in foods or dietary supplements to improve the health of humans and animals” (Diplock *et al.*, 1999). As these definitions have developed, probiotic organisms and commercial probiotic products have come under increasing scrutiny of various regulatory agencies. Some of these groups have begun to develop different rules for commercial products referred to as probiotics versus those referred to as direct fed microbials (DFMs). This primarily a regulatory distinction as there is no biological difference between these terms, for the purposes of this review, these terms refer to the same thing.

Probiotics are currently used to provide protection against GI disorders like gastrointestinal infections and inflammatory bowel diseases (Mitsuoka, 1982). Probiotics are also regarded a potential alternatives to the subtherapeutic use of antibiotics to improve animal productivity (Reid and Burton, 2002; Alvarez-Olmos and Oberhelman, 2001). In fact, in 1994, the World Health Organization deemed probiotics to be the next-most important immune system supplements when commonly prescribed antibiotics are rendered useless by antibiotic resistance (Bengmark, 2000). Better understanding of probiotic bacteria and their communication with host GI tract will help to improve the quality of probiotic products and apply it in a more proper and efficient way.

1.5 PROBIOTIC MICROORGANISMS

Most probiotic bacteria are derived from indigenous non-pathogenic bacteria, including the most common species, *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* (Charteris *et al.*, 1997). The probiotic products may contain only one, or several different (consortium) species of bacteria. The attraction of probiotics containing multiple species is that they have the potential to be active under a wider range of conditions and in a wider range of animal species (Timmerman *et al.*, 2004).

1.5.1 *Lactobacilli*

The genus *Lactobacillus* belongs to the LAB, a definition which groups Gram-positive, catalase-negative bacterial species able to produce lactic acid as the main end-product of the fermentation of carbohydrates (Kandler, 1983). With regard to cellular shape, *Lactobacilli* can occur as rods or coccobacilli. They are strictly fermentative, mostly facultative and chemo-organotrophic, requiring rich media to grow. The *Lactobacilli* appear to be somewhat

more ubiquitous in nature and are found in association with humans, animals, mashes, ensilages, various dairy products, fermented beverages, and living plants (London, 1976). Hence, this species of bacteria has been the most commonly used bacterial species in probiotic products. The justification for the use of *Lactobacilli* comes from studies showing that when the gut flora develops after birth, other components of the flora decrease with the increased number of *Lactobacilli* (Smith, 1965). Experiments with gnotobiotic chicks have demonstrated that *Lactobacilli* inhibit *E.coli* colonization in the GI tract (Fuller, 1978). Similar effects were confirmed in weaned pigs. When *Lactobacilli* were supplemented in sow's milk substitute, there was a significant decrease in the *E. coli* count in the stomach and duodenum of piglet (Barrow *et al.*, 1980). *Lactobacilli* were also found to inhibit the colonization of *Salmonella* (Pascual *et al.*, 1999), and some viruses as well (Hori *et al.*, 2002; Yasui *et al.*, 2004). It has been found that *Lactobacilli* can adhere to epithelial surfaces by interactions occurring between specific molecules on the bacterial cells and on the GI surface of the host (Gusils *et al.*, 1999) in such a manner as to spatially exclude the colonization of pathogens. Furthermore, the organic acids produced by *Lactobacilli* have been demonstrated to have antimicrobial activity against molds, yeasts, and other bacteria (Percival, 1997) which is considered to contribute to their competitive exclusion function.

1.5.2 Bifidobacteria

The genus *Bifidobacterium*, another lactic acid producing bacterium, was discovered in 1889. It was first isolated in 1905 from the gut of breastfed infants (Tissier, 1984), and this finding became the foundation for the use of *Bifidobacteria* as a probiotic species.

Traditionally, *Bifidobacterium* was listed among LAB, however, it has a distinct metabolic

hexose pathway from other LAB (Palframan *et al.*, 2003). *Bifidobacteria* are Gram-positive, polymorphic branched rods, non-spore forming, non-motile and non-filamentous. They are anaerobic and chemo-organotrophs, having a fermentative type of metabolism (Poupard *et al.*, 1973). *Bifidobacteria* produce acids from a variety of carbohydrates but not gas, with the principal end products of fermentation acetate and lactate (Picard *et al.*, 2005). Most of *Bifidobacteria* are catalase negative.

In terms of health, the most significant bacteria are believed to be the *Bifidobacteria*. They are believed to be a major component of the microbial barrier to pathogenic bacterial infection (Gibson and Roberfroid, 1995). *Bifidobacteria* had been noted to have antimicrobial activity against pathogens through the organic acids they produce (Gibson and Roberfroid, 1995). *Bifidobacteria* can make 1 mol lactic acid, 1.5 mol acetic acid and small amounts of formic acid from 1 mol of glucose (Arunachalam, 1999). In addition, the production of antimicrobial substances like Bifidin (Anand *et al.*, 1984), contribute to their antimicrobial activity. *Bifidobacteria* produce a range of antimicrobial agents that are active against Gram-positive and Gram-negative organisms (Gibson and Wang, 1994). It was also reported that *Bifidobacterial* cells can breakdown the conjugated bile acids to free bile acids which intern inhibit the growth of pathogens (Ferrari *et al.*, 1980).

1.5.3 Enterococci

The genera *Enterococcus* includes Gram-positive, spherical or ovoid cells which are typically arranged in pairs or chains (Schleifer and Kilpper-Balz, 1987). They are non-sporing, facultatively anaerobic, catalase negative, and homo-fermentative (lactic acid) bacteria. For many years, they were considered to belong to the same genus as *Streptococcus*.

Enterococci are included in the broad category of LAB. They are generally considered to be hardy since they can survive a wide range of temperature, pH levels, saline solutions, and environments (Rince *et al.*, 2000). An important distinguishing characteristic of various *Enterococci* is their ability to resist many antibiotics (Chenoweth and Schaberg, 1990). It has been shown that *Enterococcus faecium* NCIMB reduces the pathogenic bacterial load of healthy piglets (Lodemann *et al.*, 2006; Pollmann *et al.*, 2005). *In vitro* investigations have reported that *Enterococcus* strains exhibit inhibitory effects on the growth of *Salmonella* Enteritidis (Theppangna *et al.*, 2006). In the avian, swine and canine gastrointestinal tract, *Enterococcus faecium* has also been shown to influence the composition of the bacterial community (Netherwood *et al.*, 1999; Rinkinen *et al.*, 2003; Taras *et al.*, 2006; Pollmann *et al.*, 2005).

1.6 DESIRABLE CHARACTERISTICS OF PROBIOTIC MICROORGANISMS

Many desirable characteristics have been proposed for probiotic species that are to be used as dietary supplements for gastrointestinal health. The first character of probiotic bacteria would be the ability to survive and divide under different ecological environments (Lee, 2009). There are many factors that may affect the survival of probiotic bacteria, especially anaerobes like *Lactobacilli* and *Bifidobacteria*. For instance, in the feed, oxygen toxicity is an important and critical problem to anaerobic probiotic bacteria. Other factors may include environmental pH (Pochart *et al.*, 1992), bile salts (Tannock *et al.*, 1997), the epithelial/mucous layer, the mechanics of peristalsis and desquamation, and actions of secretory IgA (Gionchetti *et al.*, 2000). It has been reported that as low as 30% of *B bifidum* and 10% of *L acidophilus* orally administered as live organisms could be recovered in the

cecum (Marteau *et al.*, 1997). It may be lower if probiotic products used for animal feeds are subjected to the higher temperatures required for pelleting.

In practice, the desired properties of probiotics are dependent on the host for which administration is intended, the targeted anatomical site within the host (most often the GI tract) and the desired effects at that site (Kohwi *et al.*, 1982; Saavedra *et al.*, 1995). *In vitro* tests based on these selection criteria, although not a definite means of strain selection, may provide useful initial information. In addition, well-characterized and validated model systems may also be of value in strain selection. Ultimately, to be characterized as a probiotic, microorganisms must be demonstrated to exert a beneficial effect on the consuming host; be free of significant adverse side effects; and contain an adequate number of viable cells to confer the health benefits (Sanders *et al.*, 2007).

1.7 PROPOSED MECHANISM OF PROBIOTICS

1.7.1 Competitive Exclusion

The beneficial effects of probiotics likely result from several complex, interacting mechanisms that may differ for different strains of bacteria and sites of action (Mai, 2004). One of the most commonly cited potential mechanisms of probiotic prevention of disease is through preventing colonization of the gut by pathogenic microorganisms (Marteau *et al.*, 2001).

First, probiotics have been described to stimulate mucous production and upregulate intestinal mucin expression (Mack *et al.*, 1999), which is an important defense strategy used by epithelial cells to bind pathogens (Neutra and Forstner, 1987). Second, probiotics can restrict the growth of other organisms through a function of their own biology. Since most of

the probiotic bacteria are Lactic-acid producing bacteria, consumption of probiotic bacteria can produce various organic acids and/or ethanol as a byproduct of fermentation of available sugars (Annuk *et al.*, 2003). The production of these compounds themselves and/or the resulting change in pH of the microenvironment produces conditions unfavorable to pathogenic and opportunistic bacteria (Gionchetti *et al.*, 2000; Percival, 1997). The production of these fermentation byproducts has also been associated with ethanol or organic acid-mediated denaturation of viral envelope proteins. Probiotic organisms also produce bacteriocins, such as Lactacin F (Altermann *et al.*, 2005), as well as other poorly characterized compounds that inhibit growth of other organisms (Bongaerts and Severijnen, 2001). Probiotic bacteria can also interfere with the colonization of pathogenic bacteria through competition for nutrients, oxygen or production of antitoxins (Marteau *et al.*, 2001). Finally, supplementation of probiotics can physically exclude pathogenic microbes that could otherwise colonize the gut (Resta-Lenert and Barrett, 2003) by competing for epithelial attachment sites (Koehler *et al.*, 2002).

1.7.2 Immune modulation

Since the 1930s, Dr. Shirota proposed that many enteric diseases could be prevented if an optimal gut microflora was maintained, antimicrobial properties of probiotics have been suggested as potential protective factors in the digestive gut against different pathogens (Alvarez-Olmos and Oberhelman, 2001). At that time, it seemed that all of these protective effects could be attributed to the competitive exclusion effects of probiotics. When, however, probiotics were found to inhibit tumor growth (Matsuzaki, 1998), enhance systemic antibody production (Macpherson and Uhr, 2004) as well as activate innate immunity (Matsuzaki and

Chin, 2000), systemic immuno-modulatory effects were proposed to be an important mechanism for probiotic function. Probiotic supplementation has been reported to recruit immune cells (Perdigon *et al.*, 2002) and activate immune and/or inflammatory responses by altering chemokine and cytokine synthesis (Cross *et al.*, 2004; Suas *et al.*, 1996). Consequently, probiotic bacteria have been shown to enhance humoral immune responses and thereby promote the intestine's immunologic barrier (Isolauri *et al.*, 1993; Kaila *et al.*, 1992). Moreover, probiotic bacteria have been reported to stimulate nonspecific host resistance to microbial pathogens (Perdigon *et al.*, 1988). Probiotics have also been reported to down-regulate hypersensitivity reactions to antigens which may reduce hyper-inflammatory responses (Sutas *et al.*, 1996).

1.7.3. Improve Mucosa Integrity

Improvement in intestinal structural integrity has been reported to be another way by which probiotics can benefit the host (Chichlowski *et al.*, 2007b; Madsen *et al.*, 2001). Administration of *Lactobacillus* to interleukin-10 knockout mice decreased translocation of bacteria to extra-intestinal sites and reduced myeloperoxidase concentrations (Madsen *et al.*, 1999). In another study, probiotics were found to inhibit translocation of bacteria across the GI into the bloodstream and enhanced intestinal barrier function in the proximal small bowel, distal small bowel, and colon (Mao *et al.*, 1996). The observation that probiotics can specifically affect intestinal barrier function was demonstrated through reduced intestinal permeability to mannitol following supplementation with probiotic bacteria (Vanderhoof and Young, 1998). These effects may also relate to the anti-inflammatory effects of probiotics by lowering the production of pro-inflammatory cytokines (Madsen *et al.*, 1999).

1.8 PROBIOTIC MODEL-PRIMALAC™

Primalac™ is a commercial probiotic consortium containing four species of bacteria: *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bifidobacterium thermophilum* and *Enterococcus faecium* and is the probiotic model used in our laboratory. *L. acidophilus* has an ideal pH range from 5.5 to 7.0, and *L. Casei* and *Enterococcus Faecium* exhibits activity in pH ranges from 4.0-9.0. All species have the ability to produce lactic acid and *L. acidophilus* species are able to implant in the intestinal epithelium.

Primalac™ has been reported to improve growth performance and feed efficiency of broiler chickens and turkey (Nayebpor *et al.*, 2007; Talebi *et al.*, 2008; Grimes *et al.*, 2008), and increase egg production (Davis and Anderson, 2002). These improvements may be related to the increase in villus height and perimeter (Chichlowski *et al.*, 2007b) which may enhance intestinal area surface for absorption of nutrients and improved feed efficiency. This increased intestinal area surface may also enhance absorption of SCVFAs. It has been found that the concentration of SCVFA was lower in cecum from Primalac™ fed chicks (Croom *et al.*, 2009). The reduced energy requirement for maintenance in digestive tract may also contribute to this effect (Chichlowski *et al.*, 2007a). In another study, Primalac™ was found to modulate processes of mucin biosynthesis and/or degradation mediated via changes in the intestinal bacterial populations (Smirnov *et al.*, 2005). These modifications in mucin dynamics influence gut function and health and may change nutrient uptake. Primalac™ has also been reported to increase the goblet cell number and mucous secretion in small intestine of turkey, which may protect GI epithelia from different adverse factors including pathogens (Rahimi *et al.*, 2009). Therefore, supplementation of Primalac™ was found to attenuate

colonization by pathogenic bacteria such as *Salmonella* (Grimes *et al.*, 2008) and decrease oocysts proliferation (Dalloul *et al.*, 2003). Primalac™ can also contribute to physical barrier function by stimulating the humoral immune response (Talebi *et al.*, 2008) and improving competitive exclusion effects (Chichlowski *et al.*, 2007b).

1.9 COMMUNICATION BETWEEN PROBIOTIC BACTERIA AND IMMUNE SYSTEM

1.9.1 Brief Review of Immune System and Immune Response

Host defense against foreign challenge is elicited by the immune system, which consists of the innate and the adaptive immune system. Cells responsible for innate immunity provide the first line of host defenses (Hay *et al.*, 2002). They include monocytes/macrophages, dendritic cells (DCs), natural killer (NK), and neutrophils (heterophiles in avian). These are the body's sentinels, able to detect danger and signal to other cells like T cells and B cells, by the synthesis of molecules, such as NO, chemokines,

Table 1.3 Toll-like receptors (Doan *et al.*, 2007)

	Ligand(s)
TLR 1	multiple triacyl lipopeptides
TLR 2	multiple glycolipids multiple lipopeptides multiple lipoproteins lipoteichoic acid HSP70 zymosan Numerous others
TLR 3	double-stranded RNA, poly I:C
TLR 4	lipopolysaccharide several heat shock proteins fibrinogen heparan sulfate fragments hyaluronic acid fragments Numerous others
TLR 5	flagellin
TLR 6	multiple diacyl lipopeptides
TLR 7	imidazoquinoline loxoribine (a guanosine analogue) bropirimine single-stranded RNA
TLR 8	small synthetic compounds; single-stranded RNA
TLR 9	unmethylated CpG DNA
TLR 10	unknown
TLR 11	Profilin

and cytokines. Innate immunity is fast, non-specific, and does not possess immunological

memory (Klein, 1982). It also plays an important role in acquired immunity by the process of antigen (Ag) presentation to T cells and through the synthesis of cytokines, which play an important role in the orientation of the specific immune response. Thus, innate immunity is the first to intervene following exposure to an Ag.

Macrophage and DCs are able to recognize “danger” via receptors, termed pattern recognition receptors (PRRs). Toll-like receptors (TLRs) and nucleotide-binding domain-like receptors (NLRs), which are present in the cytosol (Inohara *et al.*, 2005; Kim *et al.*, 2008), are the two most important families of Toll-like receptors respond to several bacterial components (Vasselon and Detmers, 2002; Underhill and Ozinsky, 2002). To date, at least 11 TLRs have been identified (Table 1.4), which are usually associated with cell membrane (Rakoff-Nahoum *et al.*, 2004). In addition to macrophages and DCs, mucosal epithelial cells also express TLR2 and TLR4 (Cario *et al.*, 2000). Structural components from bacteria, like non-methylated CpG (Bauer *et al.*, 2001), lipoteicoid acid (LTA), and peptidoglycan (PGN) (Schwandner *et al.*, 1999) have been demonstrated to serve as ligands for TLRs and NLRs and detection of the presence of a pathogen by one of these receptors initiates the immune response (Neish, 2002).

Following initial detection of an infection, resident innate immune cells in the tissue begin to produce chemokines, cytokines, and anti-microbial compounds (Klein, 1982). This recruits more innate immune cells, primarily neutrophils and other phagocytic cells to the affected area (Gyetko *et al.*, 2000). Collectively the innate immune cells induce localized inflammation and cytotoxic compounds to nonspecifically destroy the pathogen and damaged cells. At the same time other innate immune cells are homing to secondary immune tissues to

present the foreign antigens to the adaptive immune cells to identify T cells (cellular immunity) and B cells (humoral immunity) with antigen receptors specific for the infecting pathogen (Medzhitov and Janeway, 1997). Once identified, these antigen specific T and B cells proliferate and migrate to the site of infection to participate in the clearance of the infection.

Table 1.4 Cells primarily involved in immune function

Immune Cells		Functions
Innate Immune cells	Dendritic cells	Also known as interdigitating reticular cells found in T-cell areas of lymphoid tissues. They have a branched or dendritic morphology and are the most potent stimulators of T-cell responses.
	Neutrophil (Heterophil)	The major class of white blood cell in peripheral blood. They are phagocytes and have an important role in engulfing and killing extracellular pathogens.
	Nature killer cells (NK cells)	A large granular, non-T/B lymphocytes. They kill certain tumor cells. NK cells are important in innate immunity to viruses and other intracellular pathogens, as well as in antibody-dependent cell-mediated cytotoxicity.
	Cytotoxic T cells	Are also known as killer cells or CD8+ T-cells. They are capable of inducing the death of infected somatic or tumor cells, kill cells that are infected with viruses (or other pathogens), or are otherwise damaged or dysfunctional.
	Helper T cell (CD4 ⁺)	A sub-group of that play an important role in establishing and maximizing the capabilities of the immune system. These cells are unusual in that they have no cytotoxic or phagocytic activity.
Adaptive Immune cells	Th0 cell	Naïve T cells that have differentiated in bone marrow, and successfully undergone the positive and negative processes of central selection in the thymus.
	Th1 cell	Mainly involved in activating macrophages, and are sometimes called inflammatory CD4 T cells.
	Th2 cell	Mainly involved in stimulating B cells to produce antibody, and are often called helper CD4 T cells.
	T _{reg}	A specialized subpopulation of T cells that act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens.
	B1 cells	Also known as CD5 B cells, are a class of atypical, self-renewing B cells found mainly in the peritoneal. They have a far less diverse repertoire of receptors.
	B2 cells	Also known as conventional B cells, which are generated in the bone marrow, emerging to populate the blood and lymphoid tissues.

The specific subtypes of adaptive immune cells that respond and by extension of the type

adaptive response mounted is largely determined by the types of cytokines produced by the innate immune cells at the beginning of the response (Medzhitov and Janeway, 1997). Adaptive immunity can be sub-divided into two major types, cell-mediated immunity which targets intracellular pathogens and humoral immunity which targets extracellular pathogens (Janeway *et al.*, 2001). Cell-mediated immune responses involve the destruction of infected cells by cytotoxic T cells, or the destruction of intracellular pathogens by macrophages. The humoral immune response involves the production of antigen-specific antibodies by B cells to neutralize and/or opsonize extracellular pathogens.

The specific function of the cytotoxic T cell and B cell responses can be influenced by the helper T cells. There are several recognized subtypes of helper T cells (Table 1.4), primarily defined by the type of cytokines they produce. Th1 cells mainly secrete interferon- γ (IFN- γ) and IL-2. Th1 can induce a weak synthesis of Abs by B cells and are recruited more in the event of a cell-mediated response. In contrast, Th2 cells induce synthesis of cytokines IL-4, IL-5, IL-10, and IL-13, which have anti-inflammatory properties. They induce a large production of Abs by B cells. Indeed, the transition from Th0 (naïve T cell) to Th1 or Th2 depends on environmental factors, among which the innate immune cells play a considerable role through synthesis of some cytokines (Janeway *et al.*, 2001). It has been found that IL-12 and IFN- γ are critical for the development of Th1 cells involved in cell-mediated immunity (Manetti, 1993). In contrast, IL-4 and IL-10 are important for development of Th2 cells that are involved in humoral immunity (Hsieh *et al.*, 1993). The balance of the two cell populations is believed to be important for the maintenance of homeostasis in the host.

In fact, B cell activities can be regulated in a T-independent manner. The subset of B

cells, B1 cells, is involved in T-independent immune response. B1 cells recognize common bacterial antigens such as phosphorylcholine as well as self-antigens, such as membrane proteins (Kantor and Herzenberg, 1993). B1 cells play an important role in innate immunity by secreting large amounts of natural antibodies of the IgM class, which can be produced without exposure to any environmental antigens or immunization (Fagarasan and Honjo, 2000). Activated B1 cells also make a unique contribution to the mucosal immune response. It was demonstrated that IgA produced against cell wall protein antigens of commensal bacteria is principally secreted by B1 cells (Ishida *et al.*, 1992).

1.9.2 Probiotic and Immunity

Probiotics have been reported to modulate the immune function of host animals in many studies (Jijon *et al.*, 2004; Erickson and Hubbard, 2000; Christensen *et al.*, 2002b). Such altered function can involve one or several components of an immune response (Figure 1.8). Although several *in vitro* and *in vivo* studies on probiotic effects on immunity have been reported, the specific mechanisms of the observed changes remain unclear. In this section, I am going to review briefly the previous studies that have focused on probiotic effects on humoral response, cell-mediated responses and nonspecific immunity.

It is well recognized that nature killer (NK) cells act as cytolytic effector cells of the innate immune system. The augmented NK activity of spleen cells (Matsuzaki and Chin, 2000) and the increased phagocytic activity of peripheral blood leukocytes (Schiffrin *et al.*, 1995) with the supplementation of probiotics, suggest an enhanced innate immune response. Interestingly, probiotic supplementation has been reported to be effective in restoring the age-related decline in lymphoid cell activity (Gill *et al.*, 2001). This suggests that probiotics may

offer benefit to hosts to combat some of the deleterious effects of immunosenescence on cellular immunity.

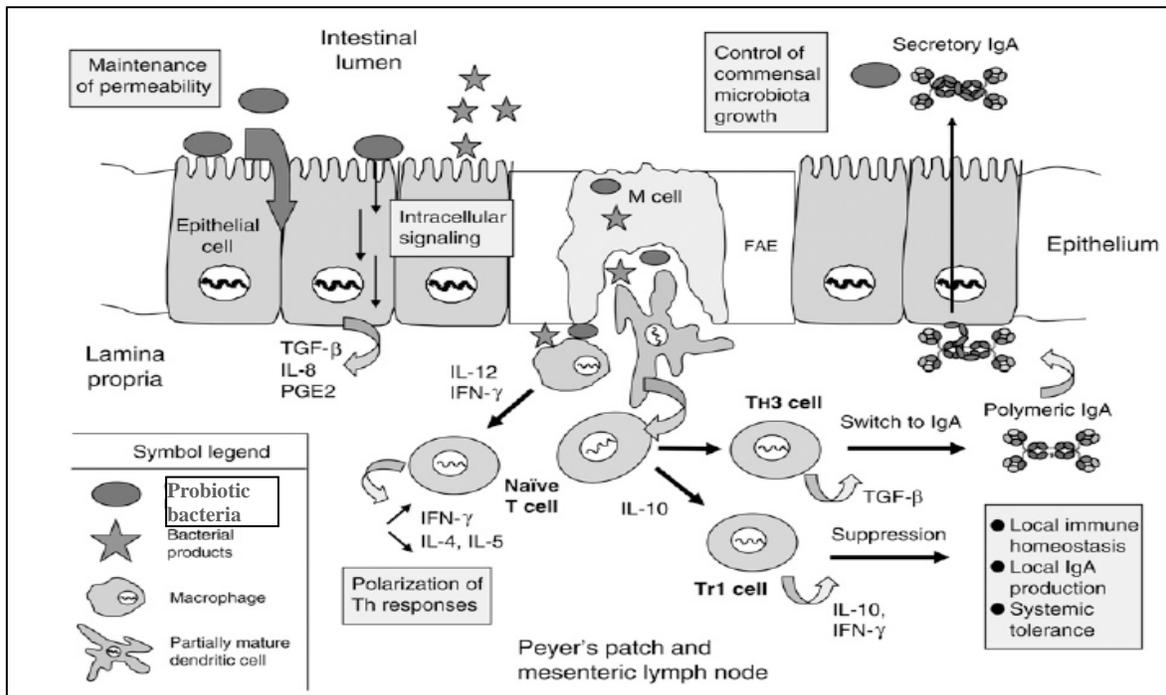


Figure 1.8 Schematic representation of the cross-talk of probiotic bacteria with GALT. Colonization of probiotic bacteria may affect innate and adaptive immunity by activating production of cytokines by epithelial cells or monocytes/macrophages. Consequently, this can not only allows IgA antibody-mediated mucosal response to take place, but help to maintain the homeostasis of gut by affecting the mucosal immune system toward a noninflammatory, tolerogenic pattern that takes place through the induction of regulatory T cells. (Figure modified and redrawn from reference (Corthesy *et al.*, 2007).

Additionally, the administered probiotic bacteria in GI tract can be sensed, via PRRs, by the DCs (Rescigno *et al.*, 2001), “M” cells (Neutra *et al.*, 1996), or epithelial cells (Cario *et al.*, 2000), and lead to activation of immune cells, and increased expression of their pathogen pattern receptors (Matsuzaki and Chin, 2000; Podolsky, 2002). The increased expression of these receptors facilitates the uptake of antigens by APCs, thus influencing the adaptive response (Mansour *et al.*, 2002). What’s more, probiotics have been found to influence Th1 and Th2 balance via the modulation of cytokine production. In some studies, administration

of probiotics was observed to increase IgG production (Yasui *et al.*, 1999) and upregulate IL-10 expression (He *et al.*, 2002). It suggests that administration of probiotics can shift the Th1/Th2 response balance to Th2. Contrary to these observations, the intake of probiotic bacteria has also been found to increase expression of pro-inflammatory Th1 cytokines like TNF- α and IFN- γ in mice and human subjects (Das, 2002; Perdigon *et al.*, 2002; Maassen *et al.*, 2000). This indicates that different species of probiotic bacteria may have different stimulatory effects on the immune system (Christensen *et al.*, 2002) and the mechanisms have not been fully elucidated.

1.9.2.1 Probiotics and IL-10

IL-10 is produced by activated T cells, B cells, monocytes/macrophages, mast cells, and keratinocytes (Moore *et al.*, 1993). The predominate effect of IL-10 is to promote the development of the Th 2 response through direct effects on many cells type like T cells, B cells and antigen-presenting cells (Mocellin *et al.*, 2003). In particular, it inhibits the synthesis of pro-inflammatory cytokines including IL-1 α , TNF- α and IL-12 (Fiorentino, 1991; Aste-Amezaga *et al.*, 1998; Hart *et al.*, 2004). In addition, it is suggested that IL-10 can induce NK-cell activation and facilitate target-cell destruction in a dose-dependent manner (Zheng, 1996).

Oral administration of probiotics has been observed to be associated with an increased production of IL-10 from PPs and the spleen (Calcinaro *et al.*, 2005). The protective effects by a probiotic on recurrent colitis have been found to be dependent on increased IL-10 (Di Giacinto *et al.*, 2005). Co-culture of probiotics upregulated IL-10 by DCs (Hart *et al.*, 2004), and then either the activated DCs or the bacteria carried by DCs further influence cells in the

mesenteric lymph node to produce more IL-10 (O'Mahony *et al.*, 2006).

It was found that intestinal probiotic bacteria actually increased regulatory T cell (Treg) proliferation by modulating DCs function (Smits *et al.*, 2005). These Treg cells then produced more IL-10. The upregulated IL-10 may be attributed to the CpG motif in probiotics, because both probiotic DNA (Lammers *et al.*, 2003) and CpG motif have been observed to enhance IL-10 production. Furthermore, deficiency of TLR9, which recognizes the CpG motif, has been demonstrated to abolish the anti-inflammatory effects by probiotics in mice (Rachmilewitz *et al.*, 2004).

1.9.2.2 Probiotics and Antibody Response

Ig production is tightly regulated and requires the activation of B cells by both activated T cells and antigens. After activation, if activated B cells are also activated by specific cytokines like IL-4 and IL-10, they will undergo antibody class switching to produce IgG, IgA or IgE antibodies (Tangye *et al.*, 2002). Probiotics have been reported to enhance antibody production and promote antibody isotype switching (Vrese *et al.*, 2005; Haghghi *et al.*, 2006). Antigen presenting cells, like DCs and macrophages, which play an important role in humoral immune responses to activate CD4 T cells, may be involved in this effect of probiotics. Probiotics was found to enhance phagocytosis of APCs (Hatcher and Lambrecht, 1993) or to modify their surface receptor protein expression (Galdeano and Perdigon, 2006). These may quicken the process of antigen presentation or enhancement of the sensitivity to antigens. Probiotics have also been observed to change APCs' cytokines releasing profiles (Morita *et al.*, 2002), especially increasing IL-10 production, which may promote isotype switching and result in an increased IgG production (He *et al.*, 2004).

1.9.2.3 Probiotics and sIgA

Mucosal epithelial surfaces, such as the respiratory system and GI tract, are sites at which the host encounters a large variety of microorganisms derived from the environment, and through which the pathogens invade the host and colonize to initiate infection (McGee *et al.*, 1983; McNabb and Tomasi, 1981). sIgA is the most abundantly produced Ig at the surface of mucous membranes in animals and contributes to specific immunity against invading pathogenic microorganisms (Corthesy and Kraehenbuhl, 1999), playing an essential role in intestinal defense. Binding of sIgA on the organism's surface may prevent attachment by direct blocking or induction of a conformational change (Lachmann, 1985; Dimmock, 1984). In vitro studies have demonstrated that purified sIgA antibodies can prevent the attachment of bacteria to mucosal surfaces (Williams and Gibbons, 1972; Svanborg-Eden and Svennerholm, 1978). Studies from activation-induced cytosine deaminase deficient animals (AID), who cannot undergo class switching to IgA, have shown that these mice display lymphoid hyperplasia of the gut and an altered microbiota, thereby causing the persistent expansion of segmented filamentous bacteria throughout the small intestine (Inagaki *et al.*, 1996).

The crucial role of sIgA in maintaining bacterial homeostasis is further reflected by its effects on innate immunity. Recently, IgA responses were shown to be involved in maintaining host–bacterial mutualism by limiting innate immune responses to a specific gut symbiont (Peterson *et al.*, 2007). Many studies have observed that supplementation of probiotics results in induction of mucosal sIgA responses (Rautava *et al.*, 2006; Chen and Ouyang, 2007), possibly through a T-independent pathway (Macpherson *et al.*, 2000). This

may allow the host to respond to fluctuations in commensal bacteria without eliciting a deleterious response and thus contribute to mucosal homeostasis (Macpherson and Uhr, 2004).

1.9.2.4 Probiotics Effects on Gastrointestinal Inflammation

Immunogenic inflammation arises when an antigen binds to an antibody or leukocyte receptor to trigger an inflammatory cascade (Meggs, 1995). The lumen is constantly full of antigens which can induce inflammation. This highlights the complexity of regulating homeostasis in the gut as such alleviate inflammation which can negatively affect the digestive and absorptive function of the gut (Miller *et al.*, 1993; McKay, 2001).

To induce inflammation in the intestine, the antigens must circumvent the intestinal barrier. Therefore, gastrointestinal inflammation is frequently accompanied by an “imbalance” in the intestinal microflora (Videla *et al.*, 1994). It was confirmed by some reports that the pathogenesis of Crohn’s disease (CD) and ulcerative colitis (UC), together referred to as IBD, is related to enteric microbiota (Duchmann *et al.*, 1995). Therefore, the alteration of the microbiota by the introduction of probiotic bacteria may result in clinical improvement of the condition (Isolauri *et al.*, 2002). Probiotics have been shown to reverse some of the immunological disturbances characteristic of Crohn's disease (Malin *et al.*, 1996). Similar effects were found to both acute and chronic intestinal inflammation in two different murine models of colitis (Schultz *et al.*, 2004). Probiotic may alleviate the intestinal inflammation by modulation of the cytokine response. The probiotic bacteria were found to increase the production of the anti-inflammatory cytokine IL-10 (O'Mahony *et al.*, 2001) while down-regulate the production of TNF- α from normal and inflamed mucosa (Borruel *et*

al., 2001; Borruel *et al.*, 2003). In addition, probiotics have been reported to stabilize gastrointestinal barrier function to counteract the inflammatory process (Rosenfeldt *et al.*, 2004) by decreasing the intestinal permeability (Mangell *et al.*, 2002). Probiotics may also reduce inflammation by enhancing the degradation of food antigens and altering their immunogenicity (Pessi *et al.*, 1998). Another explanation for the gut stabilizing effect could be improvement of the intestinal immunological barrier, particularly intestinal IgA responses. Supplementation of probiotics is always found to enhance sIgA production in the intestine (Viljanen *et al.*, 200; Rautava *et al.*, 2006).

1.10 PROBIOTICS AND ENERGY METABOLISM

Interaction between intestinal microflora and the intestinal epithelium has been increasingly recognized to play an important role in host nutrition, especially energy metabolism of host animals (Pridmore *et al.*, 2004). Intestinal microbial can produce energy from material in the digesta particularly carbohydrate, that are otherwise non-digestible by the host (Savage, 1986). As mentioned above, *Bacteroides thetaiotaomicron*, has been described to restore the fucosylation pathway in germfree animals (Bry *et al.*, 1996). This suggests commensal bacteria can affect the carbohydrate metabolism of the host.

The colonization activates a large number of genes in intestinal epithelial cells, some of which play important roles in digestive function (Hooper *et al.*, 2001). A large number of bacteria in the GI tract have been identified to have amylase secretory activity which was very efficient in the degradation of starch (Macfarlane and Englyst, 1986). As expected, the supplementation of probiotic bacteria was reported to increase the production of SCVFA (Sakata *et al.*, 1999), which may stimulate the proliferation of intestinal epithelial cells

(Sakata, 2007). Gut microbiota are thought to be an important environmental factor affecting energy metabolism. When no dietary energy is available during a fasting state, the presence of gut microflora could benefit the broilers by reducing energy losses whereas when dietary energy supplied in adequate amounts, the efficiency of energy utilization was reduced by the presence of the gut microflora (Muramatsu *et al.*, 2007). This observation may help to explain why the colonization of GF mice was found to increase the rate of whole body O₂ consumption (Backhed *et al.*, 2004). In the same study, hepatic triglyceride production, as well as the body fat content was also found to be increased by the introduction of commensal bacteria. The increased fat content may be attributed to the increased adipose tissue lipoprotein lipase (LPL) activity by suppression of the expression of intestinal fasting-induced adipocyte factor (Fiaf). In another study, obese mice were found to have a different gut microflora (Ley *et al.*, 2005). Therefore, the authors suggested that intentional manipulation of intestinal microbial community consortia may be useful for regulating energy balance in obesity. Additionally, as discussed above, GI microflora/probiotic may affect the immune functions of the host animal. Immune activity requires significant energy (Demas *et al.*, 1997; Martin II *et al.*, 2003). Therefore, GI microflora/probiotic may also affect host energy metabolism indirectly. The anti-inflammatory property of commensal bacteria may contribute to the energy metabolism as well (Nusrat *et al.*, 2001).

1.11 SAFETY OF PROBIOTIC PRODUCTS

With the increased use of probiotics in humans and animals, more and more concerns have been paid to the safety of these bacterial products. Theoretically, there are some adverse risks that have been raised to be possibly associated with the use of probiotics (Boyle *et al.*,

2006; Ishibashi and Yamazaki, 2001). These possible risks include potential for transmigration, pathogenicity, toxicity, and antibiotic-resistance transfer. Furthermore, it is not known whether probiotic alteration of the innate or systemic immune system can cause autoimmune diseases in animals or humans genetically predisposed to such diseases (W. J. Croom, personal communication). As defined, probiotics should bring beneficial effects to host animals. They should not be pathogenic and should not post a risk to the host. For instance, *Enterococci*, in which some virulence factors have been identified (Kayser, 2003), are currently ranked second/third in frequency of bacteria isolated from hospitalized patients. Therefore we should be very careful when we select this species of bacteria as a candidate of probiotic bacteria to ensure no virulence genes are present. Another important issue is antibiotic resistance. As discussed above, it has become a very serious public health concern in the world. Probiotic production has been thought to be a potential alternative to the use of antibiotics (Reid and Burton, 2002; Alvarez-Olmos and Oberhelman, 2001). Therefore, acquired resistance to antimicrobial compounds used in clinical applications must be avoided in probiotic bacteria. Even though such resistance would not have direct effects on the health of the host, such resistance genes can serve as genetic reservoirs for other potentially pathogenic bacteria. To avoid potential risks that may be introduced to host animals from probiotics, safety evaluation of probiotic products is necessary. Therefore, we need to identify the possible risks specifically associated with each probiotic strain or consortia on a strain-by-strain basis. Although there is no common worldwide safety assessment standard with legal status for dietary supplements containing probiotics, the European Food Safety

Authority has launched a system for a common approach to safety of bacterial dietary supplements (Feedap, 2005).

1.12 PREBIOTICS AND SYNBIOTICS

Prebiotics are defined as “non digestible dietary ingredients that beneficially affect the host by selectively stimulating the growth and /or activity of one or a limited number of bacteria in the colon, thus improving host health” (Gibson and Roberfroid, 1995). This concept was first introduced in 1995 as an alternative approach for gut microbiota modulation (Gibson and Roberfroid, 1995). At the present time, all prebiotics described are short-chain carbohydrates with a degree of polymerisation of two to sixty monosaccharide units such as inulin and oligofructose. They alter the composition, or balance of the microbiota, both in the lumen and on the mucosal surface, to one in which *Bifidobacteria* and *Lactobacilli* are found in larger numbers (Gibson and Roberfroid, 1995; Williams *et al.*, 1994). This putative “healthier flora”, should provide increased resistance to gut infections and may also have immunomodulatory properties (Furrie *et al.*, 2005; Bakker-Zierikzee *et al.*, 2006). Prebiotics also act as carbon and energy sources for bacteria growing in the large bowel where they are fermented to SCVFA which can serve as energy sources for the gut and other body tissues (Younes *et al.*, 1995).

The term *synbiotic* is used when a product contains both probiotics and prebiotics (Roberfroid, 1998). Appropriate prebiotics would overcome survivability issues of probiotics during storage and gastrointestinal passage. The basis for efficacious symbiotic combinations has not been defined. It was suggested that a synbiotics might be more active than either a probiotic or prebiotic alone (Roberfroid, 1998). This idea was supported by Rowland *et al.*

(1998), who showed that administration of the prebiotic inulin with the probiotic *Bifidobacterium longum* to rats resulted in additive effects, with a more potent inhibition of AOM-induced ACF than administration of either inulin or *B. longum* separately (Rowland *et al.*, 1998).

1.13 CONCLUSION

Since Dr. Metchnikoff first reported the beneficial effect of probiotic bacteria about a century ago, probiotics have been shown to have very promising effects in promoting resistance to diseases, reducing allergic responses, alleviating gut disorders like IBD, and boosting immunity (Haenel and Bendig, 1975; Mitsuoka, 1982). Regulation of the composition and localization of microbial communities in the GI tract is a multi-factorial process in which any or all of these numerous forces may come into play (Savage, 1977). That's why, even though there are considerable data demonstrating beneficial effects in different animal species by using a variety of probiotic bacteria, the mechanisms are still unclear. It is important to use well defined strains of probiotic bacteria and appropriate biomarkers to evaluate their effects on a specific animal model. Primalac™ is a probiotic product with defined bacteria consortium, which has been reported to modulate GI tract structure and energy metabolism in broiler chicken in this laboratory (Chichlowski *et al.*, 2007b; Chichlowski *et al.*, 2007a; Croom *et al.*, 2009). To better understand how this specific probiotic model interacts with GI mucosa and whether it can further modulate immune function and performance, my research project was designed to assess its effects on GI development, energy, immunity, as well as GI gene expression in broiler chicken. The results of this research may improve our understanding about the mechanisms of probiotic functions.

They may also indicate additional areas for probiotic application and improve the efficacy of probiotics in both human and animals.

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CHAPTER II

DIRECT FED MICROBIAL SUPPLEMENTATION REPARTITIONS HOST ENERGY TO THE IMMUNE SYSTEM¹

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Journal of Nutrition. Submitted.

2.1 ABSTRACT

Direct fed microbials and probiotics have been used for decades to promote health; however, their mechanisms of action are still poorly understood. Previous studies have demonstrated reduced ileal respiration without a change in whole-body energy expenditure following supplementation and suggesting energy repartitioning. The current studies were conducted to further investigate the effects of direct fed microbials on energy metabolism in different tissues. One day old broiler chickens were randomly assigned to 2 diet groups: standard starter diet (CON) and CON plus direct fed microbial (DFMD; Primalac[™]; 0.3% w/w). Body weight, feed consumption, whole-body energy expenditure, organ mass, tissue respiration rates and peripheral blood mononuclear cell (PBMC) ATP levels were measured to assess energy metabolism. No difference ($P>0.1$) in whole body energy expenditure or bodyweight gain was observed; however, DFMD fed animals demonstrated decreased ileal O_2 respiration ($P<0.05$). In contrast, the respiration rate of the thymus was increased significantly ($P<0.05$). DFMD fed animals also demonstrated increased ATP levels and ATP turnover in PBMC ($p<0.01$). To determine if the increased energy consumption by immune cells corresponds with an altered immune response, animals were immunized and assayed for differences in their humoral response. DFM treated animals demonstrated a faster rate of antigen specific IgG production and an increase in total sIgA. Collectively, these data suggest DFM supplementation results in increased energy partitioning to the immune system and an enhanced immune response.

Key Words: direct fed microbials, probiotics, systemic immune function, energy metabolism, broiler

2.2 INTRODUCTION

Direct fed microbials (DFM) and probiotics have been used in animal and human nutrition as a mean of promoting health (Rao *et al.*, 2009). In spite of their widespread use, across multiple species, our understanding of how they impart beneficial effects on the host remains inconclusive. In animal agriculture, there are numerous anecdotal reports from the field suggesting DFMs are capable of enhancing animal performance (Nahashon *et al.*, 1994, Haddadin *et al.*, 1996). These observations have lead to two main hypotheses. One suggests that DFMs aid in the control of pathogens through competitive exclusion (O'Brien *et al.*, 2001) and/or through stimulating the immune system (Galdeano and Perdigon, 2006; Prescott *et al.*, 2005). The second suggests DFMs promote digestion (Champ *et al.*, 1981) and gut function (Chichlowski *et al.*, 2007b; Madsen *et al.*, 2001).

Previous studies in our laboratory have investigated the effects of DFM supplementation on animal and intestinal energy consumption using the rapidly growing broiler chick model. No difference in growth, feed conversion, or whole animal energy consumption were noted (Chichlowski *et al.*, 2007a); however, a decrease in energy consumption by the small intestine together with a transient increase in gut length was observed (Chichlowski *et al.*, 2007a and unpublished observation). This suggested direct fed microbial supplementation results in repartitioning of energy amongst the tissues of the body.

The current study was designed to further characterize the effects of DFM on animal energy consumption and to identify tissues whose respiration rates may be changed following supplementation. The data demonstrate DFM supplementation results in the repartitioning of energy within the host leading to increased consumption by the immune system.

2.3 MATERIALS AND METHODS

Animals and Diet. One hundred and ninety two 1d-old male broiler chicks were randomly assigned to one of two dietary treatments: with six pens per treatment and 16 animals per pen. One treatment group was fed a standard control starter diet (CON, Table 2.2.1). The second treatment group was fed CON supplemented with 0.3% direct fed microbial (DFMD, Primalac™, Star Labs Inc., Clarksdale, MO) for four weeks. Lyophilized DFM pre-mix was added to feed prior to each experiment, and samples of the premix (data not shown), DFMD, and CON were collected to confirm the presence of viable organisms (Table 2.1).

Twelve animals from each diet group were immunized intravenously with 7% sheep red blood cells (SRBC) in phosphate buffer saline (PBS; pH 7.4) at 7d and re-immunized at 14d and 21d. Serum samples were collected at 7, 10, 14, 16, 21, 23, and 28 d of age.

Animals in both groups were fed their respective diets *ad libitum* for the duration of the experiment and housed, maintained, and euthanized under an approved protocol from the Institutional Animal Care and Use Committee at North Carolina State University.

Bacteriology. The numbers of viable *Lactobacillus*, *Bifidobacteria*, and *Enterococcus* species in 1 g of Primalac premix, mixed diets, or cecal contents was determined using selective media. Samples of pre-mix and mixed diets were incubated at room temp for 18 h 1g/100 ml in PBS with 0.1% Tween 20 (PBS-Tween buffer) prior to plating, per manufacturer's instructions. Cecal contents from both diet groups were collected at 7 and 14 d of age and stored at -80° C until cultured, at which time 1g was suspended in 100 ml PBS-Tween buffer, subjected to serial 10-fold dilutions, and 500 µl of each dilution was plated onto selective media (*Lactobacillus* = MRS agar, *Bifidobacteria* = BS-LV agar, and *Enterococcus* =

Enterococcus agar). Enterococcus agar plates were incubated aerobically, and MRS agar and BS-LV agar were incubated anaerobically for 48 h at 42° C. Colony counts were obtained and expressed as a log₁₀ of the colony-forming units (CFUs)/g sample. Direct fed microbial fed animals were found to contain approximately 10 × more bacteria in the cecum as compared to controls (Table 2.2).

Whole Body O₂ Consumption. Whole body O₂ consumption was conducted as previously described (Chichlowski *et al.*, 2007a). Six animals per treatment were individually analyzed at 3, 8, 15, and 22 d of age using an O₂-ECO system (Columbus Instruments International, Columbus, OH). Each animal was allowed a 15 min adjustment period in the chamber with an air flow rate of 2.5, 3, 3 or 3.5 L/min for 3, 8, 15 or 22 d of age animals, respectively. O₂ consumption and CO₂ expiration was measured during 3 consecutive 60s periods over a total of 12 min. The body weight of each bird was measured immediately after the repeated measurements of gas exchange and the mean O₂ consumption and CO₂ expiration were expressed as μmol/min per gram of body weight.

Tissue O₂ Consumption. Six animals per treatment were euthanized at 8, 15, and 22 d of age, and samples of ileum, thymus, bursa of Fabricius, spleen, liver, and muscle were collected for estimation of tissue O₂ consumption. Sections from each organ were divided into two 40-mg pieces and placed in separate respiration chambers (YSI, Yellow Springs, OH) equipped with an O₂ electrode in 4 ml of M199 media at 37°C with constant stirring, as previously described (11). Tissue O₂ consumption was expressed as μmol O₂/min/mg.

Isolation of Peripheral Blood Mononuclear Cells (PBMC). Whole peripheral blood was collected from four animals per diet group at 8, 10, 14, 16, and 23 d of age in heparinized

syringes and cells separated by a Ficoll–Hypaque gradient (specific density 1.077 g/ml). Density separated cells were then washed twice with ice-cold PBS, enumerated using a hemocytometer and the cell concentration adjusted to 10^6 cells/ml.

Analysis of PBMC ATP. Cellular ATP levels were measured in white 96-well plates using the CellTiter Glo luminescence ATP assay kit (Promega, Madison, Wisconsin, USA). One hundred μ l of PBMC (10^5 cells per well) were seeded in six replicate wells, lysed and ATP concentration detected as relative light units (RLU) using a luminometer (Fluoroskan FL, Thermo Scientific, Hudson, NH). To assay for differences in the rate of ATP depletion, PBMC were seeded in white 96-well plates at 10^5 cells/well and incubated with the proton ionophore 2, 4-dinitrophenol (DNP, 5 mM), the ATP synthase inhibitor oligomycin (5 μ g/ml), or vehicle only (ethanol) at 42° C for 0 and 15 min. The DNP or oligomycin specific change in ATP was calculated as the change in RLU over time with inhibitor (DNP or oligomycin_{t0} – DNP or oligomycin_{t15}) minus the spontaneous change in RLU (Vehicle_{t0} - Vehicle_{t15}).

Anti-SRBC ELISA Test. Soluble SRBC antigen was prepared as previously described (12). Each well of a 96-well plate was coated with two μ g of SRBC protein (NUNC MaxiSorp™ High Protein-Binding Capacity ELISA plates) in carbonate buffer (pH 9.6) and incubated overnight at 4° C. Serum samples from immunized and control animals, from both dietary treatments, were diluted 1:50 in sample diluent (50 mM Tris buffered saline, pH 8.0, 1% BSA; 0.05% Tween 20) and incubated for 1 h at room temperature. Anti-SRBC IgM and IgG were detected using goat-anti-chicken antibody (Ab) conjugated to horse radish peroxidase (HRP), incubated for 1 h at room temperature followed by 15 min incubation with substrate (Bethyl Laboratory, Inc., Montgomery, TX).

Secretary IgA ELISA. Jejunal tissue samples were collected at 7, 10, 14, 16, 21, and 28 d of age by luminal washing whole jejunum (Elson et al, 1984), four times, with 1 ml of flushing solution (PBS, pH 7.4 containing 0.1 mg/ml soybean trypsin inhibitor, 0.064 mM EDTA and 1 mM phenylmethanesulphonylfluoride (PMSF, Thermo Scientific, Rockford, IL). After collection, solid material was removed by centrifugation (10 min at $650 \times g$), the supernatant was mixed with sodium azide (final concentration 1% w/v) and PMSF (final concentration 1 mM), and further clarified by centrifugation at $27,000 \times g$ at 4°C for 20 min (13). Total protein concentration was determined for each intestinal wash sample (BCA, Thermo Scientific, Rockford, IL), analyzed for IgA using a chicken total IgA specific ELISA (Bethyl Laboratory, Inc.) and expressed on an equivalent protein basis.

Statistical Analysis. Results are expressed as means \pm SD. The data were analyzed using a one-way ANOVA (Statistix 9 Analytical Software, Tallahassee, FL). The data from each room were analyzed independently. The effects of feed DFM on whole body and organ O_2 consumption, PBMC ATP level, relative organ weights, and antibody production were determined. Birds within a pen were used as the experimental unit. $P \leq 0.05$ was considered significant.

2.4 RESULTS AND DISCUSSION

Direct fed microbial supplementation increases energy consumption by immune tissue

Previous studies in our laboratory have suggested direct fed microbial supplementation induces repartitioning of energy in the animal without affecting total body energy expenditures (Chichlowski *et al.*, 2007 a). These previous studies were conducted after 21 d of supplementation (Primalac[™]). To determine if supplementation resulted in a transient

change in energy expenditures, in the present study we estimated differences in energy expenditures at various time points over a 28-d period. The results of these experiments supported our previous observations that direct fed microbial supplementation has no effect on whole body energy consumption (Figure 2.1). Additionally, DFMD fed animals were not found to have any difference in total feed intake, weight gain, or feed conversion (data not shown).

Although DFM bacteria have been reported to salvage energy from nutrients (Savage, 1986), in our case, it is obviously that the energy was not used for weight gain. What's more, DFM has been found to decrease ileal energy consumption by Chichlowski *et al.*, (2007a). Collectively, these data suggest DFM (Primalac™) supplementation result in a repartitioning of energy within the tissues of the animal. To better understand these changes and identify other organ systems which may be affected, sections of ileum, thymus, bursa of Fabricius, spleen, liver, and muscle were collected at 8, 15, and 22 d of age and analyzed for difference in respiration rates between DFMD and CON animals. Interestingly only the ileum and thymus demonstrated a significant difference in O₂ consumption between DFMD and CON fed animals. While the respiration of the ileum was found to decrease, the thymus of DFMD fed animal increased O₂ consumption by 35% and 16% at 8 and 15 d of age, respectively (Table 2.3). Even though the other organs did not demonstrate a difference in energy consumption per unit of mass, the adjusted weight (mg/g BW) of the bursa of Fabricius from DFMD fed animals was found to be heavier than CON animals at 8 and 15 d of age (data not shown). Collectively, these data suggest direct fed microbial supplementation affects the

distribution of energy within the animal in a way which does not affect the total amount of whole body energy consumed.

DFM increased ATP content and turnover of PBM

The increase in thymus respiration of animals fed direct fed microbial supplemented diets suggested an increase in energy consumption by immune cells; however, the differences measured (Table 2.3) may have resulted from increased respiration in the structural (non-immune) cells of the thymus. To determine if supplementation affected the energy consumed by immune cells, we assayed for differences in the amount of ATP. PBMC isolated from DFMD fed animals were found to have an increased ATP level as compared to CON fed animals (Figure 2.2). These data suggest the PBMC of DFMD fed animals may be using more energy and thus require more ATP/cell to meet energy demands. If this is true then we would expect the ATP consumption, over time, to be greater in PBMC from DFMD fed animals. To assay for differences in the rate of ATP utilization, PBMC were treated with DNP or oligomycin to prevent ATP synthesis and the difference in ATP utilization determined. The results of these experiments demonstrated a greater change in RLUs in both DNP and oligomycin treated PBMCs from DFMD fed animals as compared to CON animals, suggesting the cells from DFMD fed animals consume more energy (Figure 2.2B).

Enhanced immune response following direct fed microbial supplementation

In the initial estimates of energy utilization in whole-body, organ tissue and PBMC described above, the effects of direct fed microbials on energy consumption by the immune system were observed in animals which had not been given an immunologic challenge. Hence, the increased energy consumption was described in animals whose immune system

was presumably not activated. To determine if increases in energy consumption by the immune system, is associated with increased immune function, animals were immunized with SRBCs and assayed for differences in the kinetics of their Ab responses. Animals fed DFMD were found to produce anti-SRBC IgG within 7 days of immunization while the animals fed CON had no detectable Ab until 14 days post immunization (Figure 2.3). The increased anti-SRBC IgG of DFMD fed animals over CON animals suggest direct fed microbial supplementation resulted in a faster systemic immune response against SRBC.

To assess if direct fed microbial supplementation mediated effects on the immune response were also associated with changes in mucosal Ab responses, the total amount of jejunal secretory IgA (sIgA) was compared between treatment groups. The results of these experiments demonstrate that the immunized DFMD fed animals have a higher concentration of total sIgA with the most pronounced differences between 10 and 16 d of age (Figure 2.4), similar to that observed with antigen specific serum IgG. Immunization did lead to increases in total sIgA in both diet groups as compared to unimmunized animals, however anti-SRBC specific sIgA was not detectable (data not shown). Collectively these results suggest the effects of direct fed microbial supplementation on the host immune response influences both the systemic and mucosal antibody response.

Immune function and energy repartitioning

The observation that DFMD fed animals had a more rapid serum IgG response to SRBC antigen than that of CON suggests a faster response by activated T helper cells, which are required to drive Ab isotype switching by B cells. This period of increased Ab response was concomitant with increased thymus respiration as well as increased total sIgA in the

immunized DFMD fed animals. Using germ-free mice, commensal bacteria has been demonstrated to play an vital role in both cellular and physical maturation of developing immune system (Mazmanian *et al.*, 2005). Enormous evidences now are available to show that supplementation of DFM can help host animals to combat against pathogens by either stimulation of innate immunity (Matsuzaki and Chin, 2000; Schiffrin *et al.*, 1995) or adaptive immune function (Yasui *et al.*, 1999). It is possible that supplementation of DFM stimulate the development of immune system especially thymus and bursa such that more mature immune system can response more quickly and powerfully when encountering antigens. Interestingly, the increase in total sIgA was only detected in the immunized animals. This suggests that while the immune system of the DFMD fed animals consume more energy this is not activating an immune response to the direct fed microbial organisms, but rather priming the immune system to be more reactive to a subsequent challenge.

The present study was designed to better understand the biological significance of direct fed microbial-mediated changes in intestinal energy expenditures. If the small intestine is consuming less energy and whole-animal energy consumption remains unchanged, how is the conserved intestinal energy repartitioned? These data suggest the energy is repartitioned, at least in part, to the immune system, and is associated with changes in the immune response. The results of the present study suggest energy repartitioning may play a key role in these direct fed microbial-mediated increases immune function; however the mechanism by which direct fed microbial supplementation results in alterations in immune function are still unclear.

2.5 ACKNOWLEDGMENTS

Koci, Croom, and Qiu designed the research; Qiu, Ali, Jiang, and Chaing conducted the research; Qui analyzed data and performed statistical analysis; Qui, Croom, and Koci wrote the paper. Koci had primary responsibility for final content. All authors read and approved the final manuscript. We are grateful to Dr. Hassan in Department of Microbiology (North Carolina State University) for his kind help on my bacteria culture. This work was supported in part by Star Labs Inc., Clarksdale, MO.

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2.7 TABLES AND FIGURES

Table 2.1 Experimental Diet¹

Ingredient	CON	DFMD
Corn 8.5%	51.66 %	51.66 %
Soybean meal	37.96 %	37.96 %
Poultry Fat	6 %	6 %
Dicalcium phosphorus	2.09 %	2.09 %
Limestone Fine	1.02 %	1.02 %
Salt (Plain)	0.5 %	0.5 %
Choline Chloride 60	0.2 %	0.2 %
PX NCSU BR Mineral ²	0.2 %	0.2 %
DL-methionine	0.164 %	0.164 %
Selenium premix NCSU (0.02%)	0.1 %	0.1 %
L-Threonine	0.056 %	0.056 %
PX NCSU BR Vitamin ³	0.05 %	0.05 %
<i>Lactobacilli</i> ⁴	7.10±0.02 ⁴	7.91±0.02 ⁴
<i>Bifidobacteria</i> ⁴	7.07±0.14 ⁴	7.89±0.03 ⁴
<i>Enterococcus</i> ⁴	7.20±0.03 ⁴	7.45±0.17 ⁴

¹ Analysis result (%): protein 23.8; Calcium 0.79; Phosphorus 0.88%;

² Containing the following (% mineral mix): manganese (Mn), 6.0; zinc (Zn), 6.0; iron (Fe), 4.00; copper (Cu), 0.5; iodine (I), 0.125; cobalt (Co), 0.05.

³ Containing the following (per kg vitamin mix): vitamin A, 13,227,600 IU; vitamin D3, 3,968,280 IU; vitamin E, 66,138 IU; vitamin B12, 40 mg; riboflavin, 13,228 mg; niacin, 110,230 mg; d-pantothenic acid, 22,046 mg; vitamin K3, 968 mg; folic acid, 2,205 mg; vitamin B6, 7,937; thiamine, 3,968 mg; d-biotin, 254 mg.

⁴ Log₍₁₀₎ colony forming units (cfu)/g

Table 2.2 Bacteria isolation in cecum following DFM supplementation¹

		Cecum ¹	
		Day 7	Day 14
<i>Lactobacilli</i>	CON	8.75±0.08	8.38±0.02
	DFMD	9.83±0.03	9.11±0.04
<i>Bifidobacteria</i>	CON	8.78±0.01	8.12±0.11
	DFMD	9.27±0.10	9.33±0.14
<i>Enterococcus</i>	CON	8.37±0.01	ND ²
	DFMD	9.14±0.49	ND

¹ Data are presented as means ± SD, n = 3. Log₍₁₀₎ cfu/g.

² Not determined

Table 2.3 Tissue respiratory rate (μ mol/g/min) ¹

		CON	DFMD	P
Thymus	day8	1.172 ^b ±0.047	1.579 ^a ±0.094	0.034
	day15	1.651 ^b ±0.086	1.923 ^a ±0.224	0.028
	day22	1.781 ±0.057	1.781 ±0.061	0.289
Ileum	day8	2.432±0.218	1.601±0.128	0.076
	day15	2.6071 ^a ±0.302	1.823 ^b ±0.393	0.028
	day22	2.514 ^a ±0.255	2.128 ^b ±0.206	0.021

¹Data are presented as means \pm SD, n=6. Means in a row with superscripts without a common letter differ, $P < 0.05$.

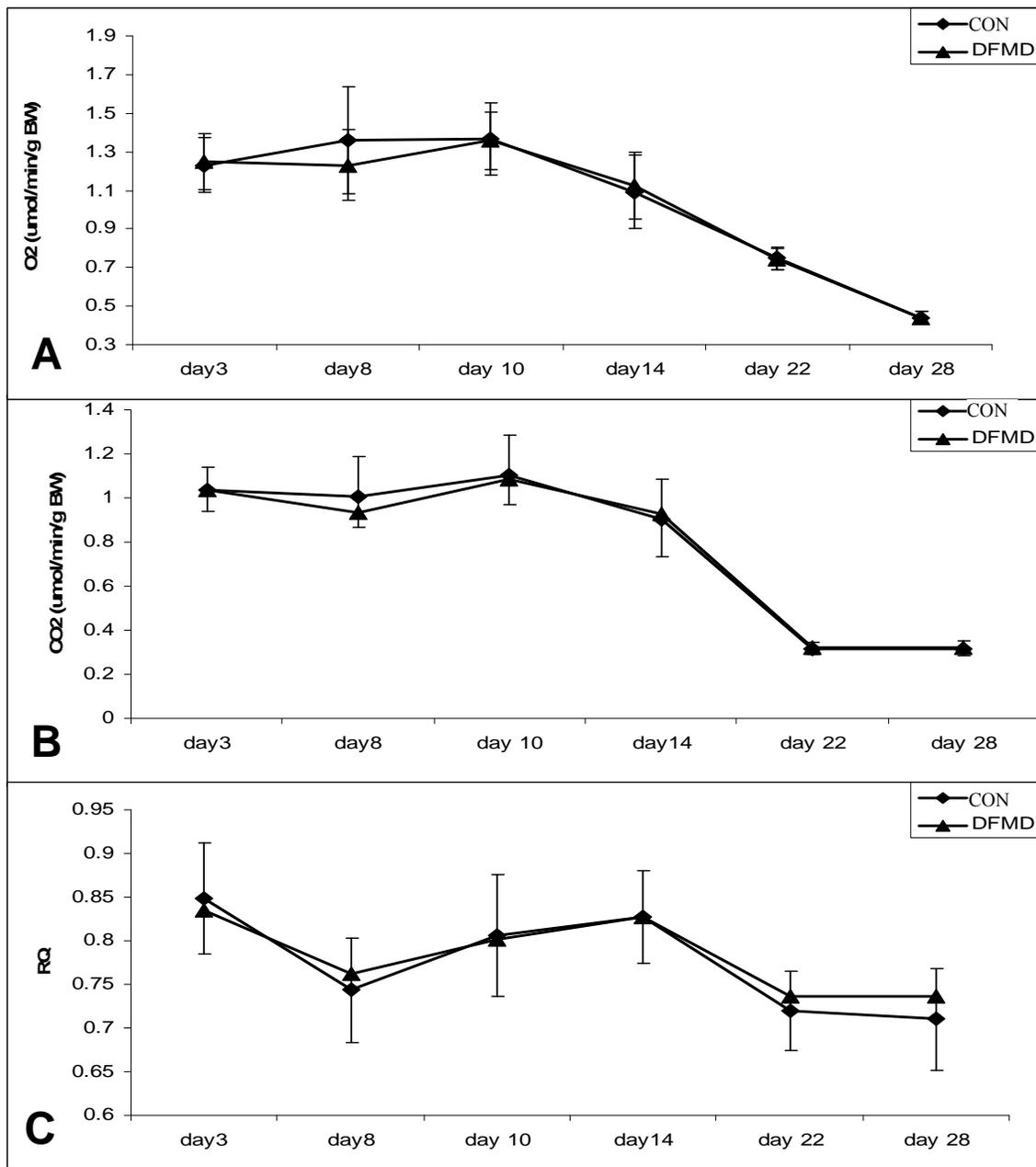


Figure 2.1 DFM effects on whole body energy consumption. DFM supplementation does not affect whole body O₂ consumption (A), CO₂ production (B), or respiratory quotient (RQ) = CO₂ production/O₂ consumption (C) as compared with control. Six animals/time point from each treatment were selected for analysis and data was adjusted based on metabolic bodyweight. Symbols represent the mean and error bars represent the standard deviation. Data presented are representative of 5 independent experiments.

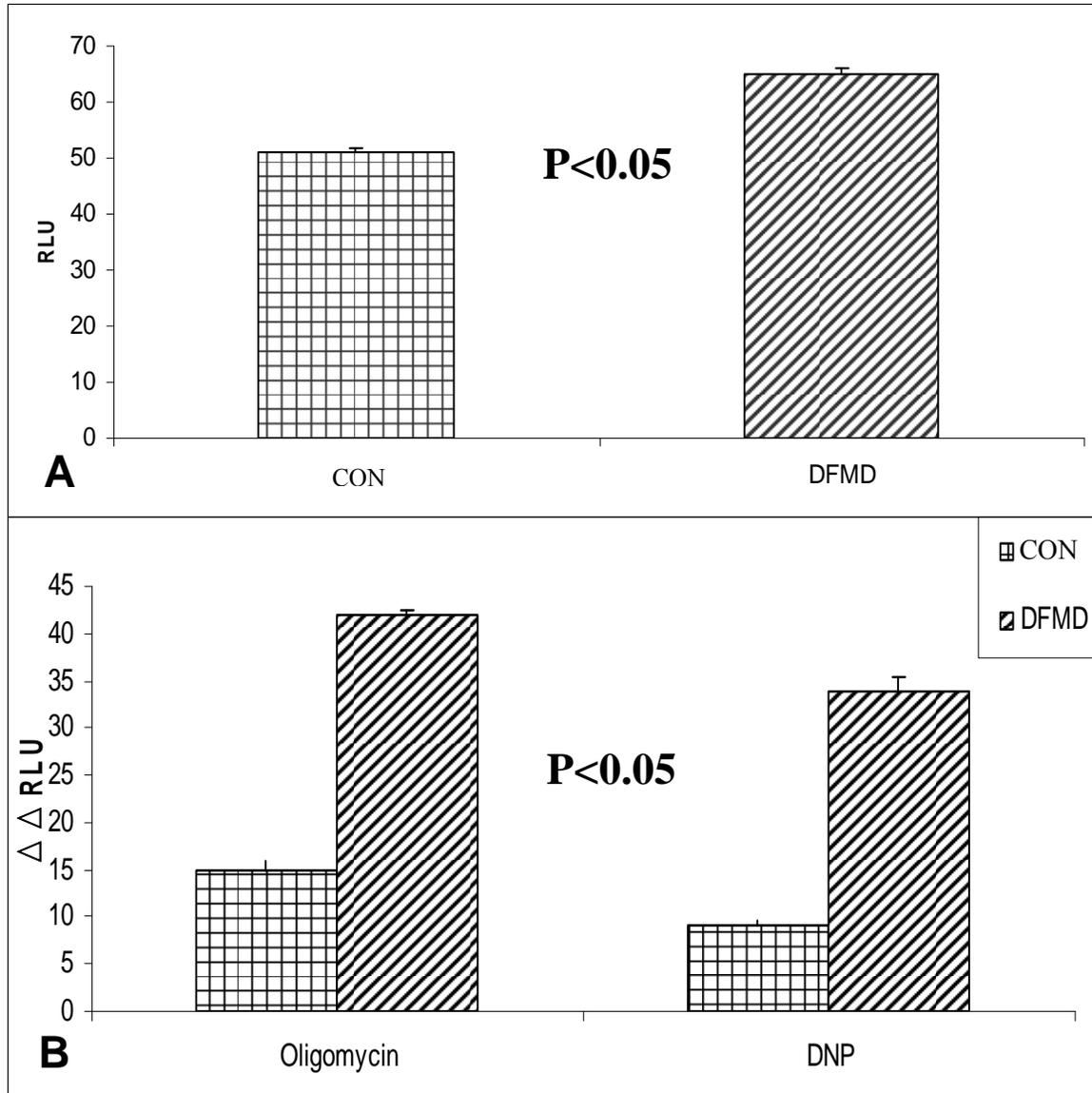


Figure 2.2 DFM effects on ATP content and turnover in PBMC. PBMC isolated from DFM supplemented animals use increased amounts of ATP/cell. Peripheral blood was collected from 4 CON and DFMD treated animals at day 16. Equal volume of 4 replicate samples were pooled, PBMC isolated, and 10^5 cells/well were assayed for the presence of ATP (A). To assay for differences in the rate of ATP turnover in each cell population, cells (10^5 /well) were treated with the ATPase inhibitor oligomycin ($5 \mu\text{g/ml}$), DNP ($5 \mu\text{M}$), or vehicle alone for 15 min. The specific effect of ATPase inhibition was calculated by $(\text{RLU}_{\text{drug}_{t0}} - \text{RLU}_{\text{drug}_{t15}}) - (\text{RLU}_{\text{vehicle}_{t0}} - \text{RLU}_{\text{vehicle}_{t15}})$ for each PBMC source and inhibitor, and expressed as $\Delta\Delta$ RLU (B). Bars represent the mean RLU of 6 replicate wells. Error bars represent the standard deviation of the mean. Data presented are representative of 5 independent experiments.

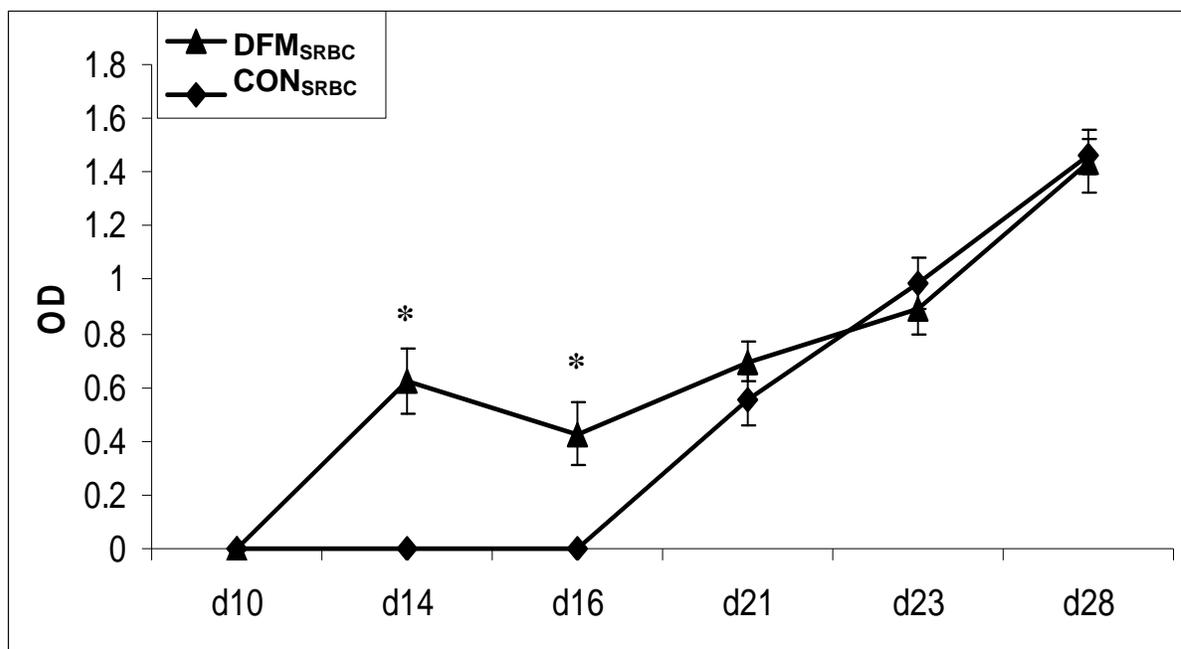


Figure 2.3 Animals fed DFM supplemented diet have increased antigen specific serum IgG response. Animals were vaccinated with SRBC at day 7 and boosted at 14 and 21 d of age. Serum samples were collected from 6 animals/group at 7, 14, and 21 d of age and analyzed for the presence of anti-SRBC IgG by ELISA. Symbols represent the mean O.D. of replicate samples. Error bars represent the standard deviation of the mean. * denotes significant difference between CON_{SRBC} and DFM_{SRBC} ($p < 0.05$). Data presented are representative of 3 independent experiments.

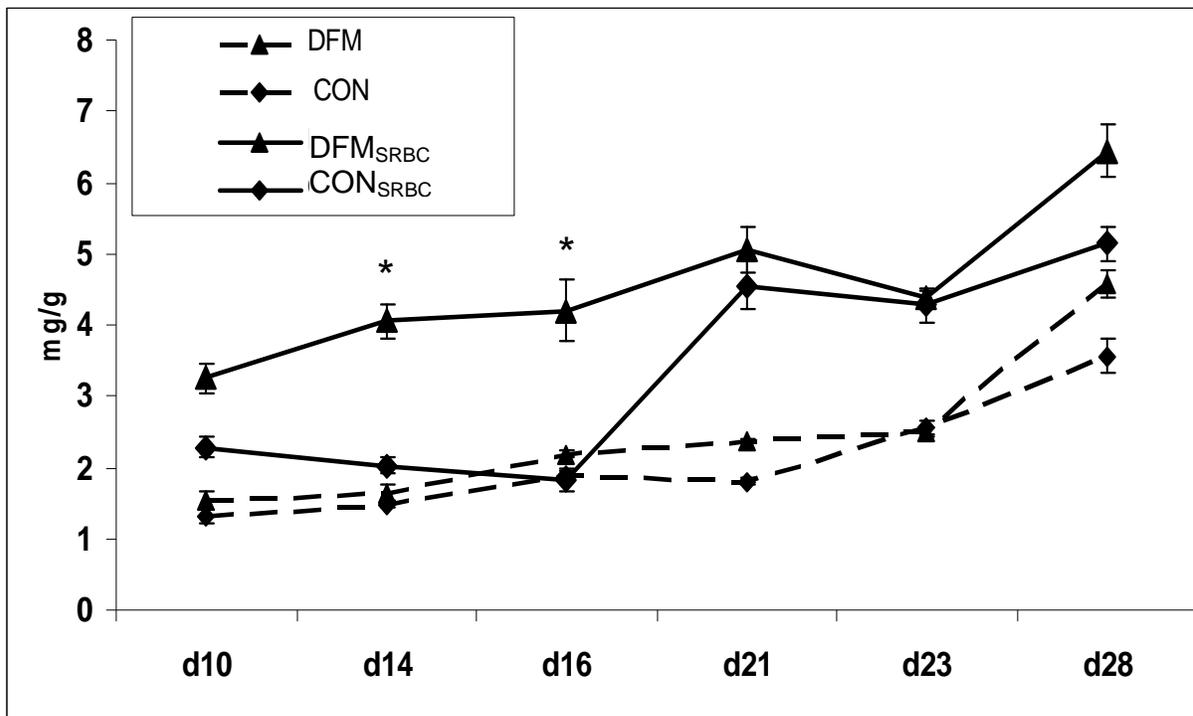


Figure 2.4 Animals fed DFM supplemented diets have increased total sIgA production. Sections of jejunum were washed and samples were collected at 7, 10, 14, 16, 21, and 28 d of age. Total sIgA was detected using ELISA, quantitated using a standard curve, and normalized to total protein content of the sample. Data are presented as mean mg of sIgA/g to total protein of replicate samples. Error bars represent the standard deviation of the mean. CON and DFMD represent samples from unimmunized animals. CON_{SRBC} and DFMD_{SRBC} represent samples from immunized animals. * denotes significant difference between CON_{SRBC} and DFMD_{SRBC} ($p < 0.05$). Data presented are representative of 3 independent experiments.

CHAPTER III

DIRECT FED MICROBIAL SUPPLEMENTATION ALTERS GASTROINTESTINAL TRACT HISTOLOGY AND ULTRASTRUCTURE IN GROWING BROILER CHICKEN¹

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Will be submitted to *Journal of Applied Microbiology*

3.1 ABSTRACT

Direct fed microbials (DFMs) have been proposed to be a potent regulator of gastrointestinal (GI) function and may serve to prevent colonization by opportunistic pathogens. Previous studies suggest DFM supplementation can affect the intestinal structural integrity. This study was conducted to further investigate spatial characteristics of DFM colonization in the GI tract and its effects on development of the GI tract using the rapidly growing chicken as a model. Animals were randomly assigned to 2 diet groups: control standard starter diet (CON) and control diet supplemented with a commercial DFM, Primalac™ (DFMD). Small intestinal (SI) length and weight were measured at different ages. Ileum was sampled for histomorphometric measurement. Crop, esophagus, ileum and cecum were sampled for scanning and transmission electron microscopy. Supplementation of DFM increased bacteria colonies in both crop and cecum. It was also associated with increased SI growth ($P<0.01$), ileal villi height (VH), mid-width, and perimeter ($P<0.01$) while crypt depth (CD) was reduced ($P<0.01$). The increased VH:CD suggests increased enterocyte turnover. More mucous was observed throughout the GI tract with DFM supplementation which may be due to increased number of goblet cells. As compared with control, DFM-treated animals were observed to have a reduction in the number of incidences of epithelial erosion on the surfaces of the crop, esophagus and cecum. These data suggest that DFM supplementation promotes the integrity of the GI epithelium.

Keywords: direct fed microbial, probiotic, GI tract histology and ultrastructure, broiler

3.2 INTRODUCTION

It is generally accepted that the gastrointestinal (GI) microflora have a role in maintaining animal health (Filho-Lima *et al.*, 2000). Commensal bacteria have been shown to affect the development of the intestinal mucosa (Bry *et al.*, 1996). As compared with control animals, intestinal epithelial cells in germ-free animals were found to have altered patterns of microvilli formation and decreased rates of epithelial cell turnover (Abrams *et al.*, 1963). Gastrointestinal microflora can also affect intestinal permeability (Beaver and Wostmann, 1962; Heyman *et al.*, 1987), the rate of nutrient absorption (Mehrazar *et al.*, 1993), and alleviate the severity of enteric pathogens such as rotavirus (Heyman *et al.*, 1987).

Direct fed microbials (DFM), also referred to as probiotics, are live microbial supplements, which are intentionally administered to animals and human to beneficially improve intestinal function by affecting the composition of the GI microflora (Fuller, 1989). Administration of DFM has been demonstrated to alleviate inflammatory bowel disease (Shanahan, 2001) and improve animal health and growth (Endo and Nakano, 1999; Jukna *et al.*, 2005; Kabir *et al.*, 2004). The mechanisms of action, however, remain unclear. Supplementation with DFM have been reported to increase intestinal epithelial barrier function (Isolauri *et al.*, 1993; Madsen *et al.*, 2001b; Mangell *et al.*, 2002; Rosenfeldt *et al.*, 2004). DFM administration to interleukin 10 (IL-10) knockout mice normalized colonic water and electrolyte permeability along with a reduction in the severity of colitis (Madsen *et al.*, 2001a). Furthermore, the synthetic bacterial peptidoglycan, Pam₃CysSK4, can induce a greater impermeability of tight junction associated zonula-occludins-1 via activation of PKC (Cario *et al.*, 2004). Collectively, these studies suggest that the mechanisms by which DFM

beneficially affect host animals could involve improvement in mucosal development and intestinal barrier function (Madsen *et al.*, 2001b).

Previous observations in our laboratory have suggested improved development of intestinal mucosa with the supplementation of the DFM, Primalac™ (Chichlowski *et al.*, 2007). This study was designed to further investigate the effects of DFM supplementation on the development of different parts of the GI tract. Of special interest was the crop and cecum, which are two important sites of bacterial fermentation in chickens, as well as the ileum which is one of the important sites for nutrient absorption (Whittow, 2000). Scanning and transmission electron microscopy were used to visualize and compare differences, between control and DFM fed animals, in regards to the bacterial colonization and attachment of GI microorganisms. Histomorphometry was used to measure possible treatment differences in GI tract surface area and structure differences.

3.3 MATERIAL AND METHODS

Animals and Diet

One day old broiler chicks were randomly assigned to one of two dietary treatments: the controls were fed a standard starter diet (CON, 23% crude protein,) and the treatment group was fed a starter diet supplemented with 0.3 % of the DFM Primalac™ (DFMD, Star Labs Inc., Clarksdale, MO) as previously described (Qiu *et al.*, 2009). Both diets were fed *ad libitum* for three weeks. The lyophilized DFM pre-mix was added to the feed prior to the experiment, and a sample of the premix and final Con and DFM diets were collected to confirm the presence of viable organisms. Each member of the Primalac™ consortium was confirmed to contain the same number of viable organisms as indicated on the product label

(10^8 CFU/g). DFM diet was also found to contain approximately 10-fold more *Lactobacillus*, *Bifidobacterium* and *Enterococcus* as compared to Control. Additionally, digesta from the cecum was cultured and DFM was found to have 10^1 more *Lactobacillus*, *Bifidobacterium* and *Enterococcus* than CON (Qiu *et al.*, 2009)

Animals in both groups were housed, maintained, and euthanized under an approved protocol from the Institutional Animal Care and Use Committee at North Carolina State University.

Sample Collection

At 1, 7, 14, and 21 d of age, twelve animals, from each treatment, with similar bodyweight were euthanized for tissue and digesta sampling after fasting overnight (12 h). The whole GI tract from esophagus to colon was dissected for measurement and sampling. The length and weight of the small intestine tract (Mesentery removed) were measured on d 7, 14, and 21. Ileum was sampled (5 cm from the ileo-cecal colonic junction) for histomorphometry at d 7 and 14. Segments from the midpoint of the esophagus, and ileum, and ventral cecum were sampled for SEM and TEM at d 7.

Histomorphometry

Ileal segments from d 7 and 14 were fixed in 10% neutral buffered formalin solution for 24 h and embedded in paraffin wax. The tissue was cut and stained with haematoxylin and eosin, and examined by light microscopy. A computerized microscopic image analyzer (Southern Micro Instruments, Atlanta, GA) was used to estimate the histomorphometric parameters including enterocytes height at mid-villus, villus height, width, and perimeter length as well as crypt depth and external muscle layer thickness (Fan *et al.*, 1997). The

criterion for selection of histological sections for examination was based on the presence of an intact lamina propria, and villi were chosen that were perpendicularly sectioned through the midline axis.

Scanning Electron Microscopy (SEM)

Ten to twelve 1-mm³ tissue pieces from crop, esophagus, ileum and cecum (d7) were excised, rinsed with cold phosphate buffered saline (PBS, 0.1M, pH 7.4) and fixed in a mixture of 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for at least 24 d at 4°C. Tissue specimens were rinsed in cold PBS and post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 20 min. The specimens were rinsed in cold PBS as before, dehydrated in a graded series of ethanol solutions (30, 50 70, 95 and 100% - 20 min to 1 d each), and then critical-point dried in liquid carbon dioxide (Samdri-795, Tousimis Research Corporation, Rockville MD). The dried specimens were secured to stubs with silver paint and coated with gold/palladium (Hummer 6.2, Anatech U.S.A, Union City CA). Specimens were examined with a JEOL 5900LV scanning electron microscope (JEOL U.S.A, Peabody MA) at an accelerating voltage of 15kV and at viewed at magnifications between 150X to 20,000X.

Transmission Electron Microscopy (TEM)

The preparation of samples (esophagus, crop, ileum and cecum at d 7) for TEM followed the same protocol as for the SEM samples up to dehydration in 100% ethanol. The samples were then embedded in Spurr low-viscosity resin, sectioned with an LKB NOVA Ultramicrotome (Leica Microsystems, Inc., Bannockburn IL), stained with uranyl acetate and lead citrate, and examined with a Philips transmission electron microscope (FEI Co.,

Hillsboro OR) at an accelerating voltage of 80 kV. Images were viewed at magnifications between 6000X to 60000X and were captured with Kodak 4489 film, processed with Kodak D-19 developer and subsequently scanned to digital form with an Epson 4870 (Epson America Inc., Long Beach CA) flatbed scanner at 1200dpi.

Calculations and Statistical analyses

Small intestinal length and weight (adjusted by body weight), as well as ileal histomorphometric data from different age of animals were analyzed using analysis of variance (2×2 ANOVA) with treatment and age as the main effects. After overall significance was determined for a parameter ($P<0.05$) differences between means were determined using Tukey's Honestly Significant Difference (Statistix 9; Analytical Software, Tallahassee, FL).

3.4 RESULTS

Small Intestinal Development

Previous studies in our laboratory have suggested DFM supplementation affects the growth and development the small intestine (Chichlowski *et al.*, 2007). To further characterize DFM-mediated changes to the GI tract, the whole small intestine was excised and analyzed for differences in length relative to body weight. DFM was found to have significant effect on the length of both duodenum and whole small intestine ($P<0.05$). Interestingly, the effects were primarily observed in young age animals, including jejunum and ileum (Table 3.1), but appear to be transient with no significant difference in any section of the small intestine at the end of the time course. Increases in small intestinal length were also observed, regardless of treatment, with age ($P<0.01$).

Histomorphometry

The ileum is a major site for nutrient absorption and its structure and function can be influenced more easily by microbial activity (Cho, 2008; Sartor, 2008). To further investigate DFM effects on intestinal development, ileal samples from 7 and 14 day-old animals were analyzed for differences in histomorphometry. Supplementation with DFM increased ileal villi height and villus width ($P < 0.01$, Figure 3.1A). Consequently, the planar perimeter of villi was higher in DFM-treated animals than in CON ($P < 0.01$). It was also found that DFM-treated animals have a decreased crypt depth ($P < 0.01$) and an increase in enterocyte length ($P < 0.05$). Therefore, DFM-treated animals tend to have a greater villus height /crypt depth ratio at both 7 and 14 d of age (Table 3.2). More goblet cells were found on the ileal villi in DFMD-fed animals, suggesting an increased stimulation of mucous secretion (Figure 3.1E).

Scanning and Transmission Electron Microscopy

Ileum.

To further examine the histological changes mediated by DFM supplementation, ileal tissues were analyzed for ultrastructural changes by electron microscopy. The ileal villi, as observed by SEM, were found to have a more flattened and leaf-like shape in DFMD fed animals as compared to CON (Figure 3.2). In addition, more mucous was observed in the DFM fed animals (Figure 3.2) which is consistent with the increased number of goblet cells found under light microscopy (Figure 3.1). Closer examination of the ileum demonstrated that the microvilli of DFMD fed animals appear to be thinner, longer and more densely arranged (Figure 3.3).

Cecum

Previous studies in our laboratory have demonstrated DFM supplementation affected both the microflora and fermentation products found in the cecum (Croom *et al.*, 2009; Qiu *et al.*, 2009). To further determine if these changes also have effects on the ultrastructure of the cecum, cecal tissues were analyzed by SEM which demonstrated an increased amount of mucus covering the epithelium in DFMD fed animals (Figure 3.4 A and B). Closer analysis found that more idiopathic, nodular lesions and ulcerations were noticed on the cecal surface of CON (Figure 3.5). And these ulcerations and nodules may be the initial stage of erosion widespread on the cecal epithelial surface CON (Figure 3.5B).

Analysis of the cecum by TEM demonstrated an increase in the number of goblet cells with more mucus vacuoles in DFMD fed animals, which appear closer to the apical surface than CON (Figure 3.4 C and D). Supplementation of DFM increased the bacterial number in the cecum and these bacteria were usually associated with mucous (Figure 3.4 E and F).

Esophagus and Crop

Most studies investigating the effects of DFM on animal and human health have focused on the post-gastric small intestine and colon (Alander *et al.*, 1999; Pronio *et al.*, 2008; Rafter, 2003). Given the observations above, we wondered whether oral administration of DFM may also affect the histology and ultrastructure of the pre-gastric regions of the GI tract. To begin to address this, the esophagus and crop were analyzed and found to be covered with a stratified squamous epithelium layer, on which there are numerous microfolds (Kessel and Kardon, 1979; Figure 3.6 and 3.7). As compared with CON, the crop surfaces in DFMD fed animals showed a more continuous and intact array of microfolds (Figure 3.6C and D).

Interestingly, erosion on the esophagus and crop surface, caused by unknown factors, were observed in CON, however, these putative lesions were less apparent in animals supplemented with DFM (Figure 3.7).

Supplementation with DFM resulted in increased numbers of rod-shaped bacteria on the surface of both the crop (Figure 3.8A and B) and esophagus (Figure 3.8C and D). The bacteria were found attached to the crop surface by filamentous structures (Figure 3.8 F). These filamentous structures were also observed between bacteria (Figure 3.8G). In addition, bacteria were also observed to be attached to feed particles in the crop of DFM-treated animals (Figure 3.8 E).

3.5 DISCUSSION

Commensal bacteria have been demonstrated to play a central role in the intestine for proper development and function of the gut, as well as overall health (Haenel and Bendig, 1975; Helgeland *et al.*, 1996; Mitsuoka, 1982). These observations became the primary justifications for the use of DFM as a means of maintaining gut health and promoting well-being. A lot of studies have observed that DFM supplementation can positively affect growth, and development as well as health outcomes in various host species (Pascual *et al.*, 1999; Vanderhoof and Young, 1998; Zani *et al.*, 1998). In spite of this, our understanding of the mechanisms by which DFM organisms deliver their affects is still limited.

As defined, DFMs should have the ability to survive or proliferate in sufficient numbers in the GI tract so they can exert a biological effect (Schrezenmeir and de Vrese, 2001). This means any successful DFM should lead to increased numbers of bacterial species in the gut. Using SEM and TEM, more bacteria were observed in the esophagus, crop and cecum in

DFMD fed animals as compared with CON (Figure 3.4 and 3.8). While we cannot determine if the increased bacteria observed are DFM organisms, they do have morphology consistent with the DFM species and these results are also consistent with previous studies, which demonstrated increased numbers of bacteria in the ileum, cecum, and colon after supplementation of DFM (Chichlowski *et al.*, 2007; Qiu *et al.*, 2009). It should be noted that there was little difference in the colony forming units between the CON and DFMD diets, however, DFM supplementation brought about a 10-fold increase in the numbers of viable *Lactobacilli* and *Bifidobacteria* in the cecum (Qiu *et al.*, 2009). This suggests that the increased organisms observed are either the DFM organisms themselves colonizing the gut and/or endogenous bacteria that may be stimulated to proliferate by treatment with DFM.

The increased bacterial numbers were associated with structural changes to the GI tract. Previous studies demonstrated DFM treatment resulted in changes in the small and large intestine at 21d (Chichlowski *et al.*, 2007), suggesting DFM may affect the growth and development of the GI. Gross examination of the small intestine over the course of 21 d in the current study support this hypothesis. DFM demonstrated a stimulatory effect on small intestine length (Table 3.1). Given the known association between commensal bacteria and the development of the gut (Hooper and Gordon, 2001; Macpherson and Harris, 2004), these data suggest the increased bacteria by DFM may help promote growth and differentiation of gut in the developing animal.

Further analysis showed DFM treatment also resulted in changes to the ileal villi and microvilli. DFM fed animals demonstrated longer and wider ileal villi with greater perimeter of cross section (Table 3.2). The microvilli tend to be longer, thinner but more compacted

(Figure 3.3). It has been estimated that villi can increase the area of intestine by 25 folds while microvilli by 60 folds (Strocchi and Levitt, 1993). These observations, combination with small intestine length data, suggest more surface area for nutrient absorption in intestine by supplementation of DFM. This may, in part, explain the mechanisms by which this DFM resulted in improved animal performance in previous studies (Grimes *et al.*, 2008; Nayeopor *et al.*, 2007; Talebi *et al.*, 2008). Increased bacteria have also been reported to stimulate the production of short chain volatile fatty acids (SCVFA), which can stimulate epithelial proliferation (Sakata, 2007). And the stimulated capillary network development in villi by DFM may further support this effect (Stappenbeck *et al.*, 2002). It has been reported that longer villi were induced by increased cell mitosis with the use of DFM (Samanya and Yamauchi, 2002). This is consistent with our observations in present study that the villi height to crypt depth ratio was increased by DFM supplementation (Table 3.2), indicating an enhanced epithelial cell turnover.

Microscopic analysis of the ileal villi also suggest DFM treatment results in more goblet cells in both ileum (Figure 3.1E) and cecum (Figure 3.4D). The primary function of goblet cells is the production and release of mucus (Strous and Dekker, 1992). An increase in goblet cells would suggest increased secretion of mucus, which was observed in both the ileum and cecum from DFMD fed animals (Figure 3.2B and Figure 3.4B). This is consistent with previous observation in this laboratory (Chichlowski *et al.*, 2007).

Few studies have examined the colonization and role of commensal bacteria/DFMs in the upper GI tract. The results of the current study suggest DFM treatment also has remarkable effects on the structure and integrity of this region of the GI tract, specifically the esophagus

and crop. Both sections were found to have a surface covered with squamous epithelial cells, on which there are numerous microfold structures. The function of the microfolds is unknown and there are no previous reports that have described these features in chickens. They may serve to increase epithelia surface area. More microfolds were found in the crop of DFM fed animals, which appear more continuous (Figure 3.6D and Figure 3.7B). Between the microfolds, numerous erosions were noted in the crop of CON fed animals (Figure 3.7A). The cause of these apparent defects is unknown. Another pattern of erosions were also observed spreading in the esophagus of CON fed animals (Figure 3.7C). Interestingly the surface of the DFMD fed crop and esophagus were found to have considerably fewer sites of erosion. Recent studies examining human biopsies of the distal esophagus suggest conditions such as esophagitis and intestinal metaplasia are associated with major changes in the flora of the esophagus (Yang *et al.*, 2009). Data in present study suggest that DFMs may have therapeutic potential in the treatment of pathologies of the pre-gastric GI tract such as reflux disorders.

Evidences of DFM-mediated prevention of submicroscopic epithelial defects were also observed in the cecum. The defects in the epithelium on the CON ceca appear as nodular lesion extruding out of the surface (Figure 3.5A) and irregular erosion of the micovilli (Figure 3.5B). It is unclear if these two different defects are related, however, some nodules were found with impaired microvilli (Figure 3.5 B, with circle), suggesting that these nodular lesions may be the initial stage of erosions noted on the cecal surface of CON (Figure 3.5B). As compared, these sites were not apparent in the DFMD fed cecal tissues.

Many factors may induce disruption of GI epithelial integrity, including pathogens (Godinez *et al.*, 2008), inflammatory cytokines (Halpern *et al.*, 2006; Markel *et al.*, 2006), and physiological factors (Allen and Flemstrom, 2005). Current data suggest that DFM bacteria may form a layer on the surface of GI tract, excluding pathogenic microbes and therefore promoting gut development by preventing damage from invading species (Jarry *et al.*, 1996; Plaisancie *et al.*, 1998; Resta-Lenert and Barrett, 2006). This exclusion could also involve in the increased mucus production or release by DFM supplementation (Shimotoyodome *et al.*, 2000). This increased mucus may provide a barrier protecting the underlying mucosal epithelium from damaging agents and/or act as lubricants to attenuate the shear forces as food transits down the digesta tract (Cone, 2009). In addition, acidic mucins have been implicated in the protection of the mucosa from pathogens (Brockhausen, 2003; Karlsson *et al.*, 2000). Alternatively, the DFM-mediated changes to the gut may be related to direct interactions between these bacterial species and the host. Stimulation of the host tissue through Toll-like receptors or other innate immune receptors may result in the production of cytokines and growth factors which stimulate the growth and replication of epithelial cells (Rakoff-Nahoum *et al.*, 2004). Finally, the DFM bacteria may be aiding in digestion and nutrient absorption such that there is more energy available to the cells of the gut and resulting in more cell division. Food particles sampled from the crop of DFM supplemented animals were found to be fixed with more rod-shape bacteria (similar in shape to some species of *Lactobacilli*; Figure 3.8E), supporting the involvement of DFM in food digestion (Sakata *et al.*, 1999). Furthermore, previous study also suggests DFM may stimulate the absorption of SCVFA by GI tract (Croom *et al.*, 2009). Future studies are necessary to

determine the participation of each of these pathways in the promotion of intestinal growth and development by DFMs.

This is the first study, of which we are aware, to describe DFM-mediated histological and ultrastructural changes in both pre- and post-gastric sections of the GI tract. Supplementation of DFM has demonstrated a protective effect on GI epithelia, especially to esophagus and crop, and a stimulatory effect on the growth of small intestine as well as ileal villi and microvilli. Changes in intestinal morphology as observed in this study may be a key factor affecting animal gut health and function, which may further affect animal performance and well-being.

3.6 ACKNOWLEDGEMENTS

We are grateful to the technicians of the Histopathology Laboratory, Department of Population Health and Pathobiology, College of Veterinary Medicine (North Carolina State University) for their kind cooperation in sample preparation. This work was supported in part by Star Labs Inc., Clarksdale, MO.

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3.8 TABLES AND FIGURES

Table 3.1 DFM effects on small intestinal development (length/bodyweight, cm/g)¹

		Duodenum	Jejunum	Ileum	Small Intestine
D7	CON	0.068±0.010 ^{bc}	0.235±0.029 ^a	0.203±0.032 ^a	0.505±0.066 ^a
	DFMD	0.094±0.026 ^a	0.232±0.047 ^a	0.190±0.034 ^a	0.516±0.090 ^a
D10	CON	0.078±0.015 ^{ab}	0.133±0.006 ^c	0.108±0.017 ^c	0.319±0.048 ^c
	DFM	0.085±0.019 ^{ab}	0.161±0.013 ^b	0.130±0.028 ^b	0.375±0.058 ^b
D14	CON	0.041±0.012 ^{de}	0.091±0.012 ^d	0.087±0.018 ^{cd}	0.220±0.031 ^d
	DFM	0.041±0.013 ^{de}	0.104±0.015 ^d	0.099±0.016 ^c	0.244±0.033 ^d
D16	CON	0.039±0.007 ^{def}	0.087±0.008 ^d	0.068±0.012 ^d	0.194±0.019 ^e
	DFMD	0.048±0.025 ^{cd}	0.077±0.026 ^e	0.074±0.014 ^d	0.199±0.030 ^e
D21	CON	0.022±0.003 ^f	0.039±0.003 ^f	0.034±0.003 ^e	0.094±0.004 ^f
	DFMD	0.022±0.002 ^{ef}	0.040±0.004 ^f	0.035±0.003 ^e	0.097±0.030 ^f
	DFMD	0.0044	0.0656	0.1277	0.0110
P	Age	<0.01	<0.01	<0.01	<0.01
	DFMD*Age	0.0727	0.0012	0.0323	0.0398

¹Data are presented as means ± SD, n=12. Means in a column with superscripts without a common letter differ, $P < 0.05$.

Table 3.2 DFM effects on ileal villi development¹

	D 7		D14		Significance <i>P</i>		
	CON	DFMD	CON	DFMD	DFMD	age	DFMD*age
VH ²	468.3±24.0 ^c	536.6±15.6 ^{ab}	524.4±50.1 ^b	568.8±49.7 ^a	0.0000	0.0000	0.0034
VW ²	73.8±7.64 ^{bc}	77.4±8.41 ^b	71.73±7.96 ^c	82.9±5.29 ^a	0.0000	0.074	0.0001
VP ³	35.65±4.58 ^b	37.29±5.93 ^b	35.57±2.33 ^b	41.46±5.29 ^a	0.0000	0.0003	0.0009
CD ²	81.6±5.4 ^c	66.7±7.9 ^d	100.8±17.5 ^a	94.5±12.7 ^b	0.0000	0.0000	0.0004
MT ²	104.6±17.8 ^b	104.1±19.8 ^b	112.9±14.1 ^a	117.0±16.2 ^a	0.3360	0.0000	0.2061
EL ²	33.29±3.40 ^a	33.51±3.03 ^a	31.32±3.69 ^b	33.47±3.80 ^a	0.0002	0.0016	0.0023
VH:CD	5.74	8.04	5.19	6.02	-	-	-

¹Data are presented as means ± SD, n=60. Means in a row with superscripts without a common letter differ, *P* < 0.05.

² VH: villi height; VW: mid-width of villi; CD: crypt depth; MT: muscle thickness; EL: enterocyte length. Unit: μm.

³ VP: perimeter of cross section of villi. Unit: ×1000μm²

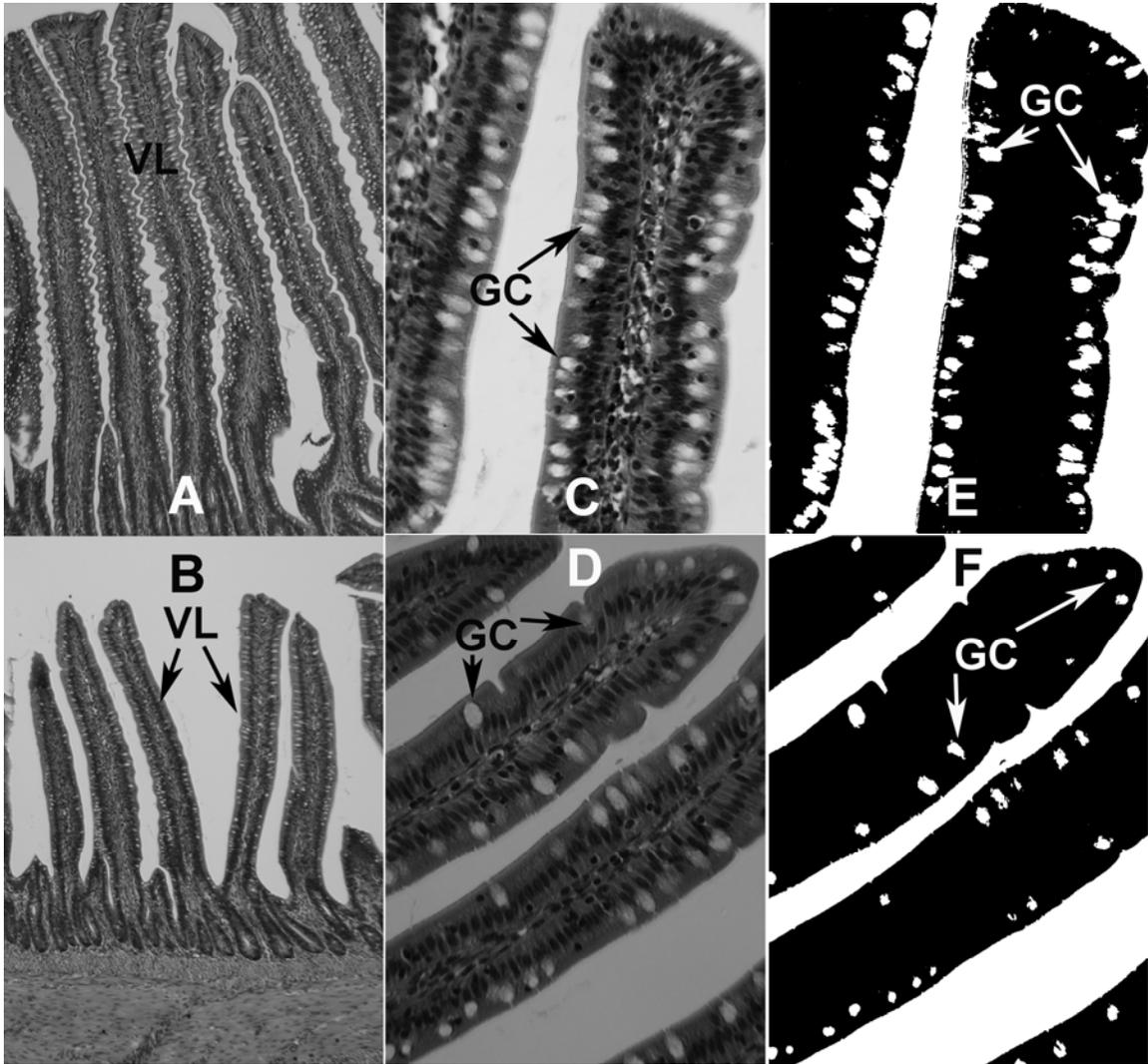


Figure 3.1 DFMD supplementation affects villi morphology. Ileum sections were collected at day 7 from DFMD fed (A, C, and E) or CON fed (B, D, and F) fed animals and H&E stained sections were analyzed by light microscopy at 100 \times (A and B) and 400 \times (C-F) for differences in villi (VL) length and mid-villi width and numbers of goblet cells (GC). E and F are high contrast images of panels C and D to enhance visualization of goblet cells. DFMD fed animals show longer and wider villi than CON. It also demonstrated to stimulate goblet cells on ileal villi. Micrographs shown are representative of 60 total fields per treatment.

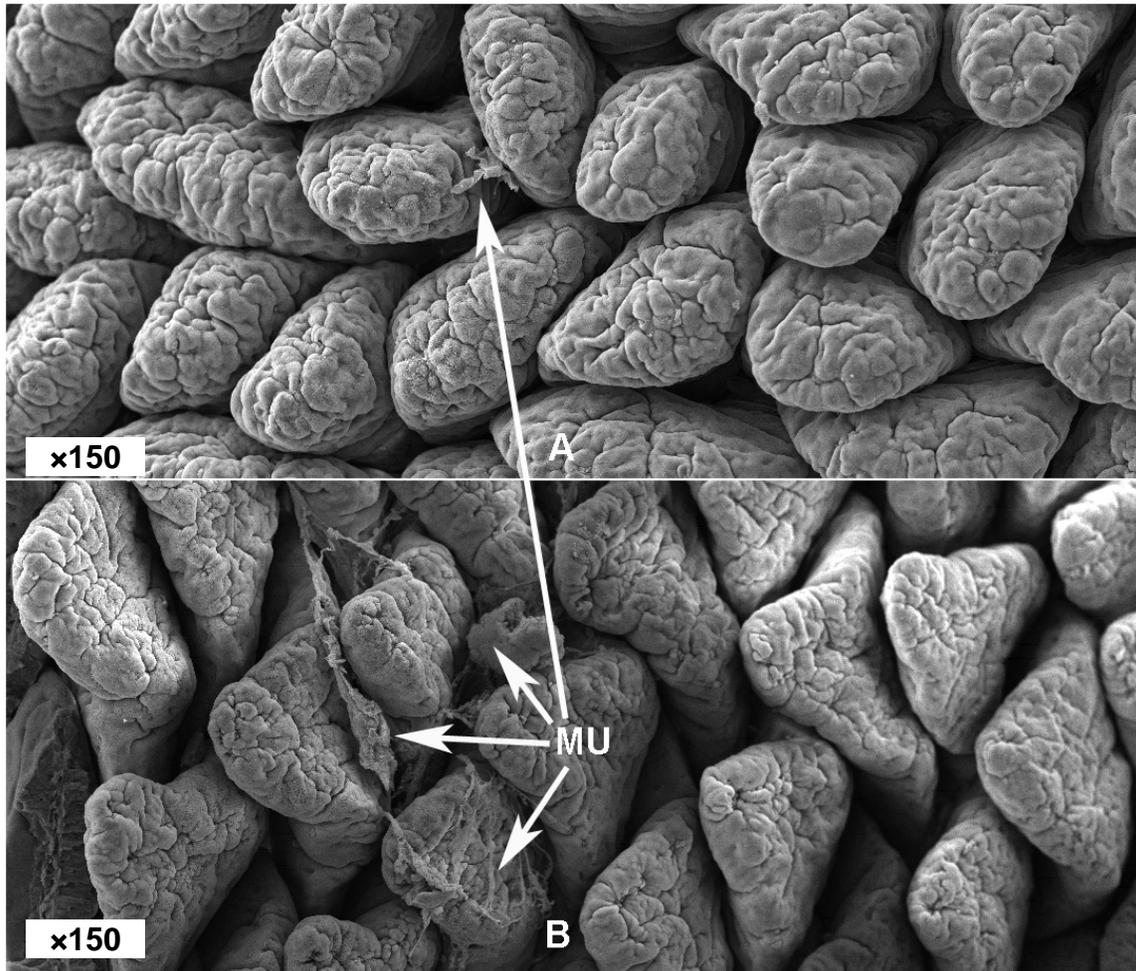


Figure 3.2 Villi of DFM treated animals have altered shape. Ileum sections were collected at day 7 from CON (**A**) and DFMD (**B**) fed animals and analyzed by SEM for changes in structure and the presence of mucous (MU). DFM supplemented animals showed thinner and more leaf-shaped villi (**B**). More mucous was noticed surrounding villi from DFM animals. Images are shown at a magnification of $\times 150$. Micrographs shown are representative of 6 total fields per treatment.

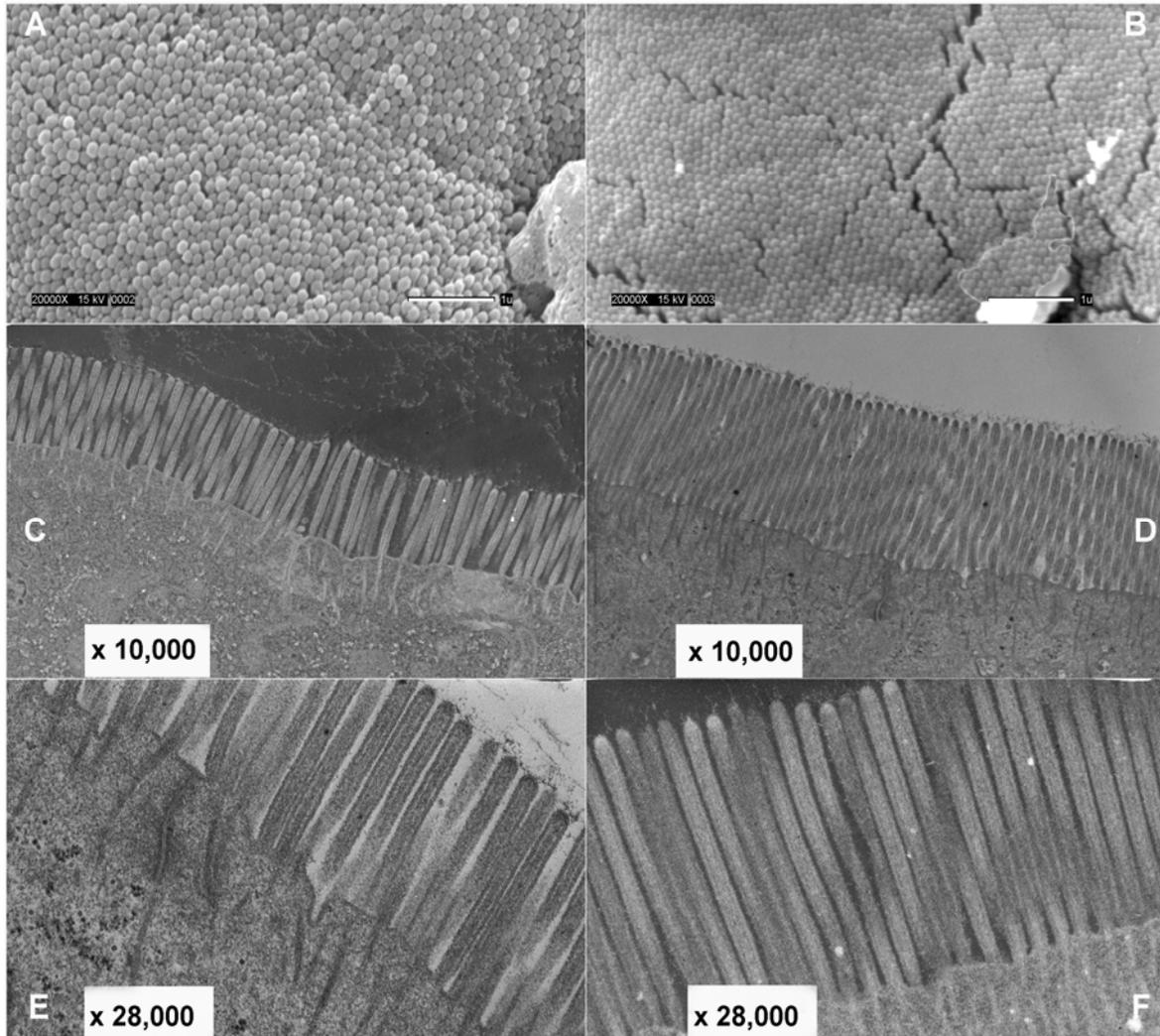


Figure 3.3 Supplementation of DFM affects ultrastructure of ileal villi. Ileum sections were collected at day 7 from CON (**A**, **C**, and **E**) and DFMD (**B**, **D**, and **F**) fed animals and analyzed by SEM and TEM for changes in ultrastructure changes in microvilli. The microvilli from DFM-treated animals are much thinner, longer, and more compacted than CON.

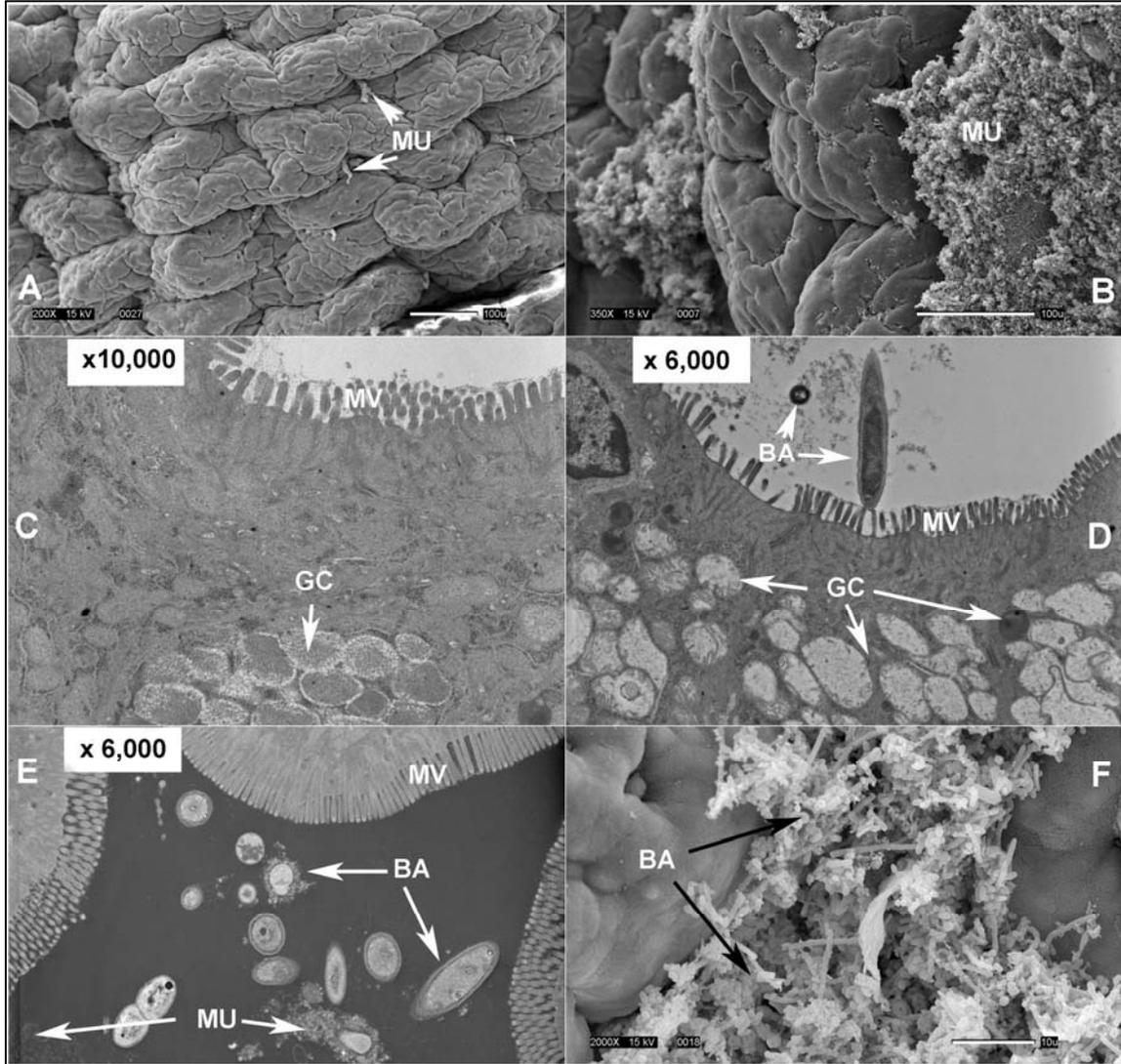


Figure 3.4 Changes of the ultrastructure and bacterial colony in the cecum by DFM supplementation. Cecum tissues were collected at day 7 and analyzed by SEM and TEM for changes on goblet cell number, mucous secretion and bacteria. SEM demonstrated increased mucous (MU) surrounding cecal villi from DFM supplemented animal (**B**) over CON (**A**). As compared to CON (**C**), more goblet cells (GC) were observed in DFM-treated cecum (**D**) which appears closer to the apical surface. Higher-magnification of SEM and TEM, demonstrated the MU from DFM-treated cecum (**E** and **F**) contain bacteria (BA) with different morphologies. Note MV: microvilli.

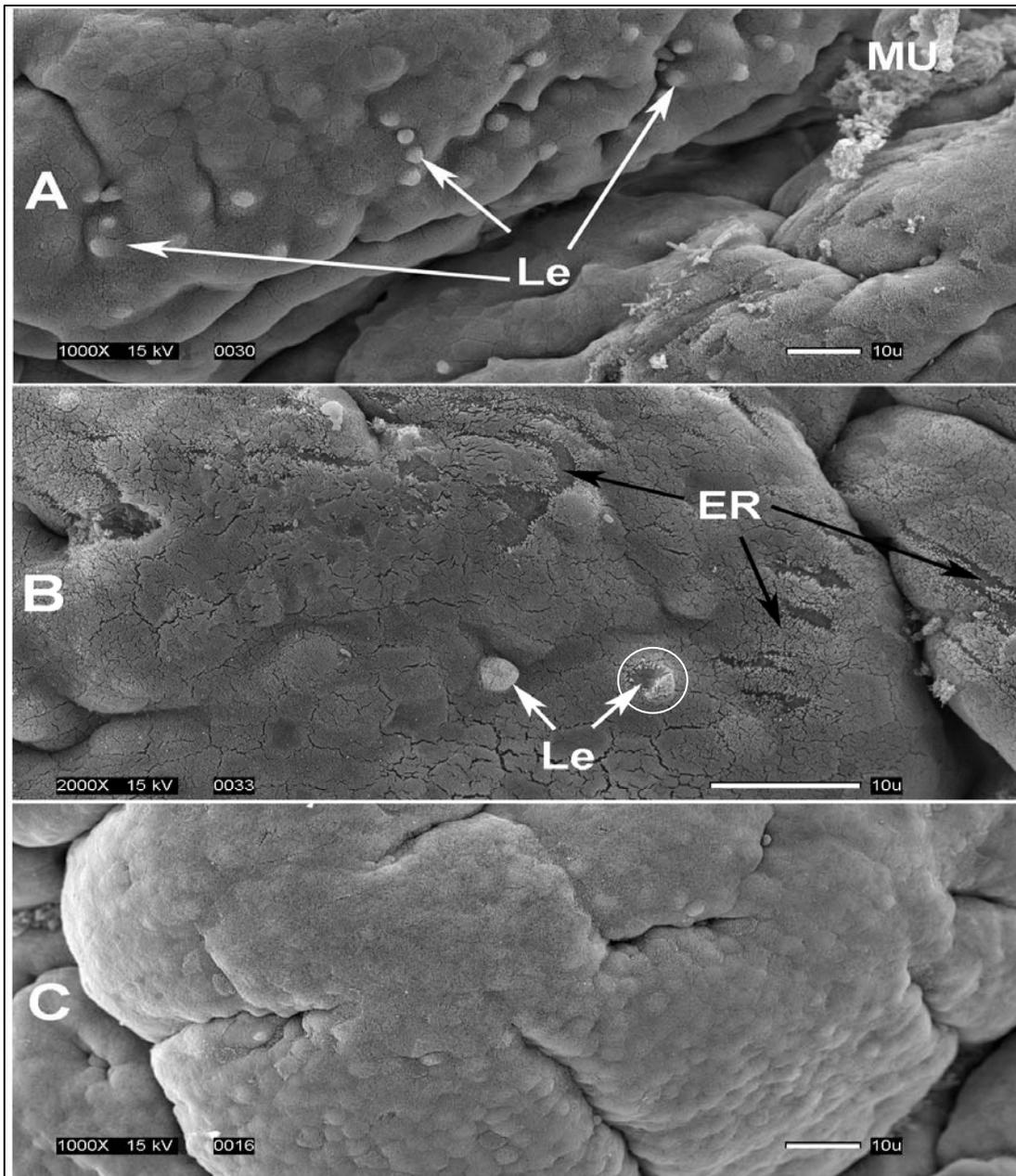


Figure 3.5 DFM supplementation affected the appearance of the epithelial surface of the cecum. Cecum tissues were collected at day 7 and analyzed by SEM for changes on the structure of cecal epithelial surface. **A.** SEM of cecum from CON animals demonstrated numerous nodular lesions (Le, white arrow). **B.** Higher magnification of these features demonstrated both nodular lesion and erosion (ER, black arrow) on the surface of cecum from CON animals. Some of these lesions appear to have lost microvilli (circle), indicating it may be the initial stage of epithelial erosion. **C.** SEM examination of the cecal surface from DFMD fed animals. Note MU: mucous.

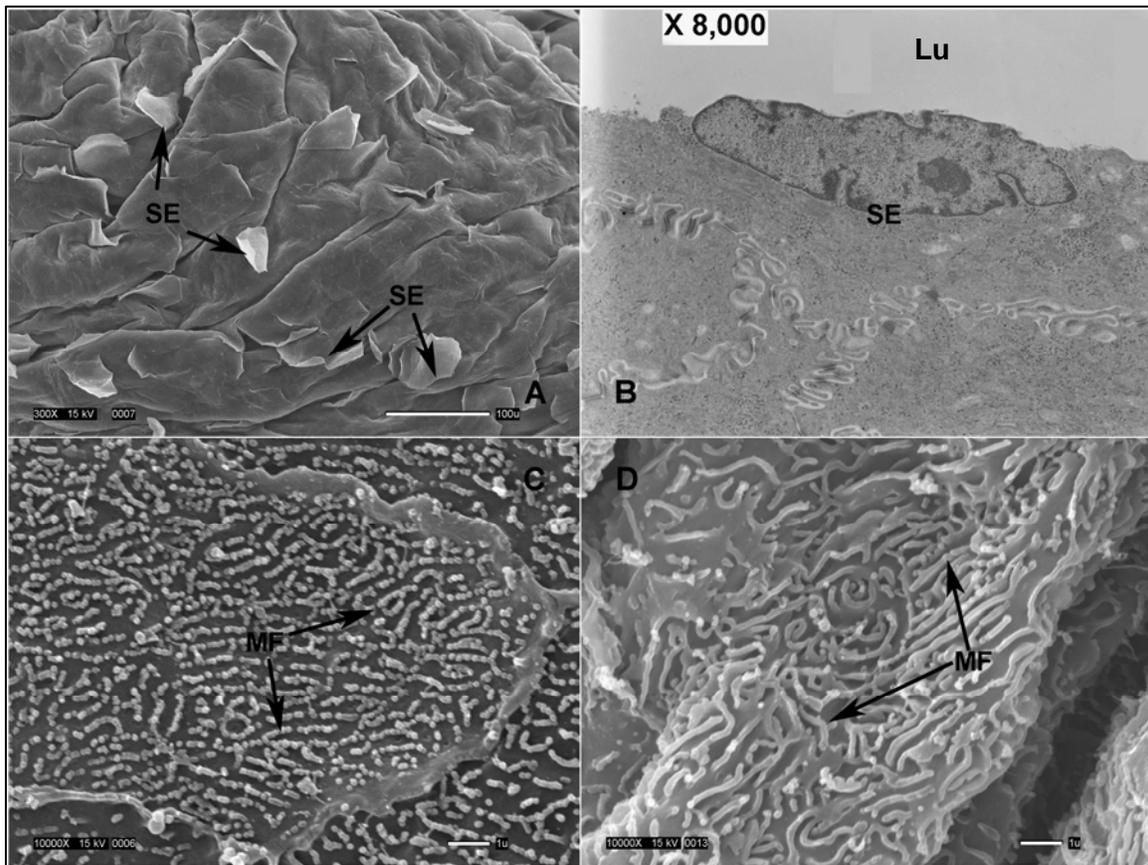


Figure 3.6 Electron microscopic visualization of the crop epithelium. Crop tissues from animals in both groups were collected at day 7 and analyzed by SEM and TEM for the structure of epithelium. SEM (A) and TEM (B) illustrate the surface and structure of chicken crop is covered with stratified squamous epithelia cells (SE). Analysis of the crop by SEM under higher magnification (C and D) demonstrates the surface of these cells contain numerous microfold (MF) structures. C. SEM showing a discontinuous microfolds (MF) on the crop surface of CON animals. D. SEM showing a more continuous and intact array of microfolds on the crop surface of DFM supplemented animals. Note: Lu: Lumen.

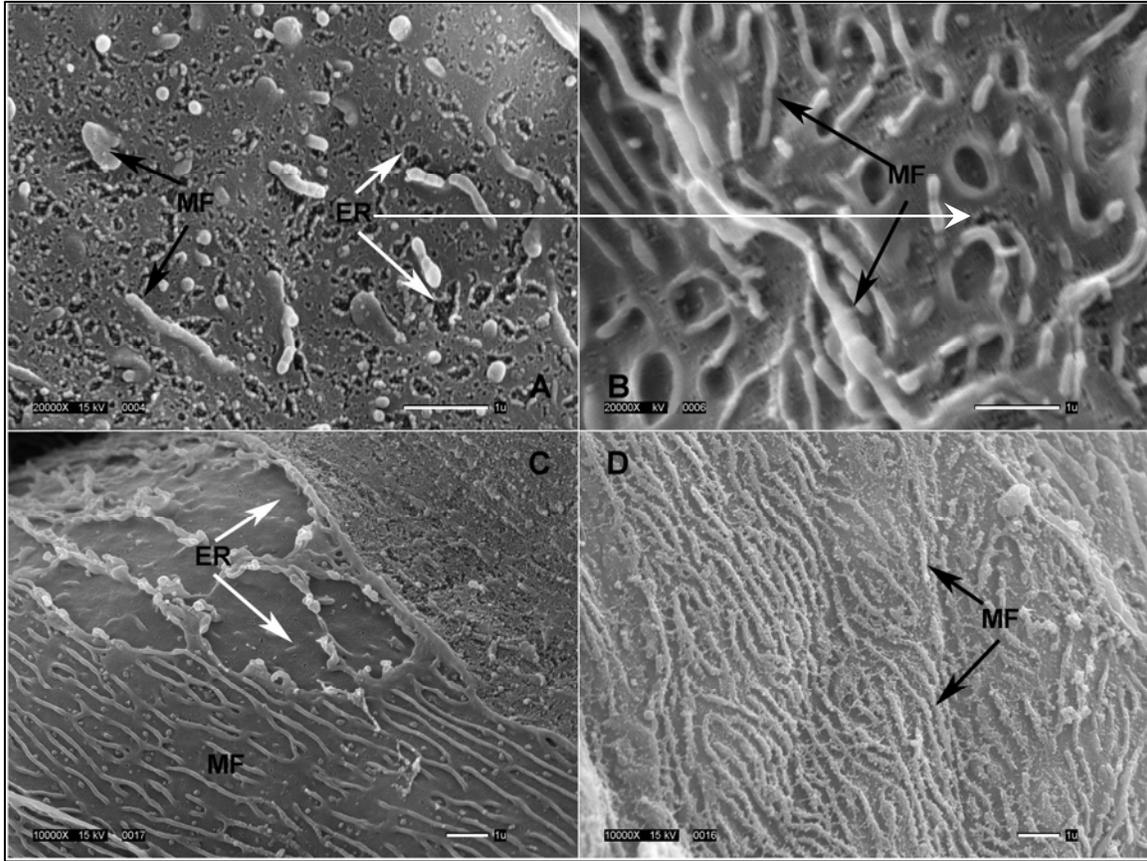


Figure 3.7 Reduced evidence of epithelial erosion of the crop and esophagus following DFM supplementation. Crop and Esophagus tissues from animals in both groups were collected at day 7 and analyzed by SEM for changes of the epithelial surface. On the surface of crop (A) and esophagus (C) from CON, different pattern erosions (ER, white arrow) were observed. Supplementation of DFM reduced number of erosion on both crop (B) and esophagus (D) surface.

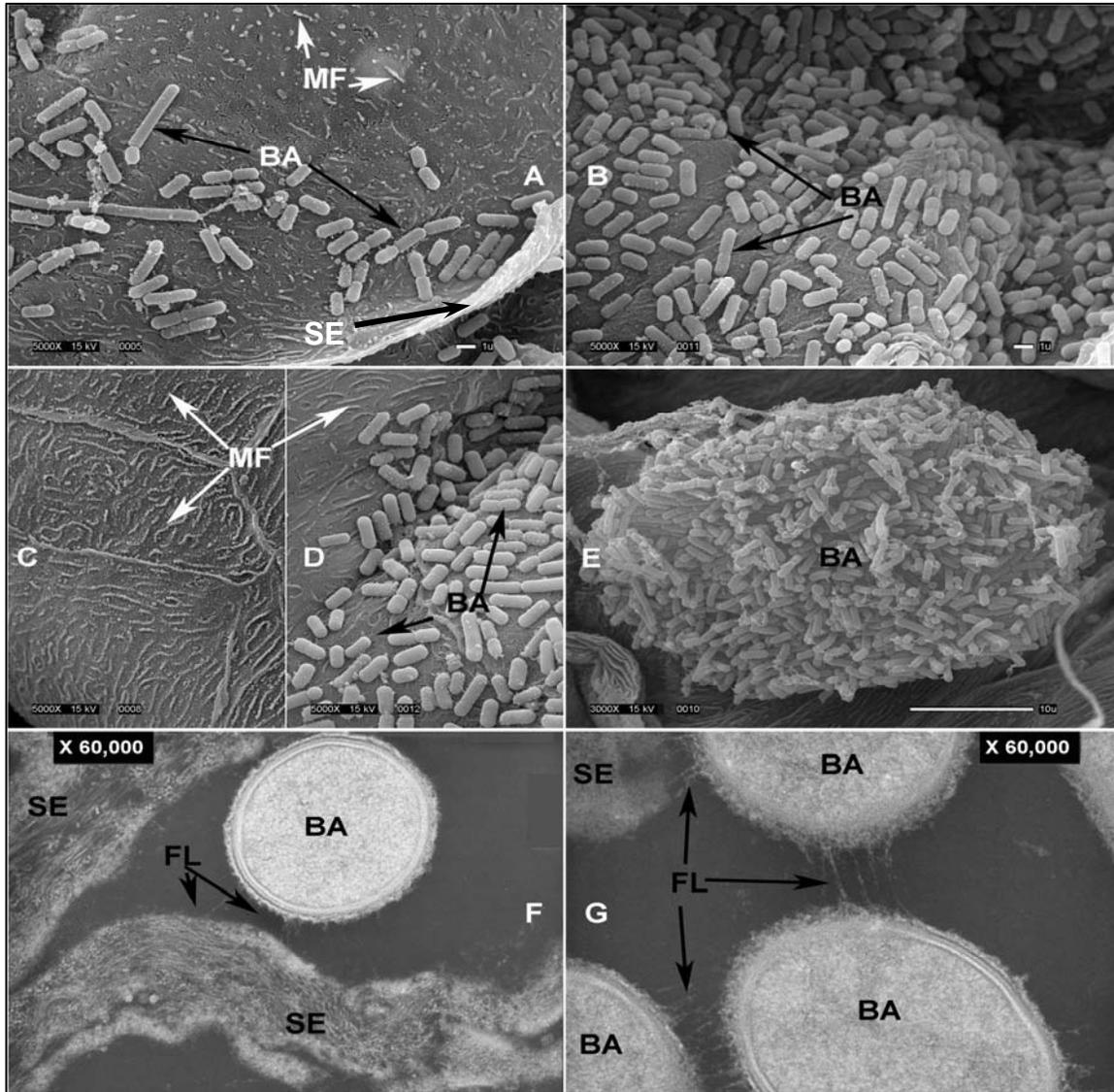


Figure 3.8 Supplementation of DFM increased bacterial colonies in both crop and esophagus of chicken. Crop and esophagus tissues from animals in both groups were collected at day 7 and screened by SEM for bacteria colonization. **A.** SEM showing bacteria (BA) on the crop surface of CON. **B.** SEM showing high density of bacteria on crop surface from DFM supplemented animals. **C:** SEM showing esophagus surface from CON. **D:** SEM showing many bacteria found on the esophagus surface from DFM supplemented animals. **E:** Food particle isolated from crop of DFM supplemented animals was found to contain many rod-shaped bacteria. **F.** TEM showing the bacteria in crop from DFM supplemented animals sending out filamentous (FL) structure to stratified epithelia (SE). **G:** TEM showing the bacteria in crop from DFM supplemented animals sending out FL to other bacteria. Note: microfold (MF).

CHAPTER IV

DIRECT FED MICROBIAL SUPPLEMENTATION DIFFERENTIALLY AFFECTS ILEAL AND CECAL GENE EXPRESSION¹

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Will be submitted to *Journal of Poultry Science*.

4.1 ABSTRACT

Direct fed microbial (DFM) supplementation has been used extensively as a means to promote health. Previous studies in our laboratory have demonstrated treatment with DFM can augment the immune response, repartition energy within the host, and alter the histology of the intestinal tract; however the mechanism(s) by which DFM influence the host is still unclear. To characterize the communications between these organisms and the host, we assayed for differences in host gene expression in the ileum and cecum following DFM treatment. One d-old broiler chicks were randomly assigned to one of two dietary treatments: control starter diet (CON) and DFMD diet (CON supplemented with 0.3% Primalac™). At d14, six birds from each group were euthanized and ileal and ceca were sampled for gene expression analysis by focused oligo-nucleotide array. Expression analysis identified 29 genes were differentially regulated in the ileum and 20 genes were found to be changed in the cecum following DFM supplementation. Interestingly there were only 2 genes (c-Myc and IL-12R β 2) identified in both sections of the intestine. In fact IL-12R β 2 was found downregulated in the ileum but was upregulated in the cecum following DFM. Expression profile data was further assessed using the Metcore program to identify signaling pathways affected by DFM supplementation. In both ileum and cecum, the IL-27 signaling pathway was identified; however, it is affected differently between these two intestinal sections. The present study demonstrated DFM supplementation alters the expression of host genes in the intestine, however, the specific genes affected and whether their expression increases or decreases is specific to the region of the intestine being examined. Further studies are needed

to determine the full biological significance of DFM-mediated changes in gastrointestinal gene expression.

Keywords: Direct fed microbial, probiotic, focused microarray, gene expression, broiler chicken

4.2 INTRODUCTION

Gastrointestinal (GI) microflora has been suggested to play an important role in maintaining animal health (Filho-Lima *et al.*, 2000). The composition of GI microflora is central to normal immune development (Umesaki and Setoyama, 2000). It was also found to contribute to the development of the intestinal architecture (Hooper and Gordon, 2001), protect from infection (Baba *et al.*, 1991; Corrier *et al.*, 1991), as well as affect energy metabolism (Bry *et al.*, 1996). Direct fed microbial (DFM) are deliberately administered to animal and human diets to enhance these beneficial effects caused by GI microbial consortia (Rao *et al.*, 2009). Our laboratory has used the DFM, Primalac™, as an experimental model and have shown that DFM supplementation can affect immune function, and energy metabolism (Qiu *et al.*, 2009a), as well as the development of GI tract of the host broiler chicks (Chichlowski *et al.*, 2007b; Qiu *et al.*, 2009b).

Supplementation of DFM bacteria has been demonstrated to increase bacteria number in GI tract (Qiu *et al.*, 2009 a, b), which can be detected by pattern recognition receptors (Gordon, 2002), like Toll-like receptors (TLRs) expressed on the surface of most antigen presenting cells as well as mucosal epithelial cells (Cario *et al.*, 2000). Structural components from bacteria, like non-methylated CpG and peptidoglycan, have been demonstrated to serve as ligands for TLRs (Bauer *et al.*, 2001; Schwandner *et al.*, 1999), initiating the immune response (Neish, 2002). Furthermore, activation of TLRs has been conceivably demonstrated to induce intestinal distinct tissue protection and repair response (Rakoff-Nahoum *et al.*, 2004). Collectively, these evidences suggest that it is possible that DFM exerts its functions via regulation of some key genes that may relate to some pathways modulating both immune

function, GI histology and energy metabolism. The objective of the current study is to identify patterns of gene expression underlying the effects by supplementation with DFM.

4.3 MATERIAL AND METHODS

Animals and Diet

One day-old broiler chicks were randomly assigned to one of two dietary treatments: control and DFM. One treatment group was fed a standard control starter diet (Con). The second treatment group was fed a starter diet supplemented with 0.3% DFM Primalac™ (Star Labs Inc., Clarksdale, MO) for two weeks. Lyophilized DFM pre-mix was confirmed to contain the number of viable organisms indicated on the product label by plate counts before it was added to feed prior to experiment. A sample of DFM diet was also collected to confirm the presence of viable organisms. DFM diet was also found to contain more *Lactobacillus*, *Bifidobacterium* and *Enterococcus* as compared to the control diet (Qiu *et al.*, 2009).

Animals in both groups were fed their respective diets *ad libitum* for the duration of the experiment and housed, maintained, and euthanized under an approved protocol from the Institutional Animal Care and Use Committee at North Carolina State University.

Samples Collection

At d14, six animals with similar body weights were selected from each treatment group, euthanized, and a 2 cm section of ileum (mid-point) and cecum (sac bottom) were excised, rinsed with cold phosphate buffered saline (PBS, pH=7.4), dissected longitudinally along the mesenteric line, and immediately immersed in RNA Later (Ambion, Foster City, CA), and subsequently stored at -80 °C until processed for RNA extraction.

RNA Extraction and Quality Control

The tubes containing tissue samples were thawed and 50 mg of tissue was weighted and placed in 1 ml of TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) with 0.3 ml of 0.5 mm glass beads. Tissues were disrupted using a Mini-Beadbeater (Biospec, Inc., Bartlesville, OK) at 4,800 oscillations/min for 30 s, a total of 3 times, with samples placed on ice for 1min between cycles to avoid sample overheating. Following disruption, samples were centrifuged 4 min at $1,000 \times g$ at $4^\circ C$ and the supernatants were transferred to a new tube and RNA isolated by mixing samples with equal volume of phenol/chloroform/ isoamyl alcohol 125:24:1), phases separated by centrifugation ($12,000 \times g$ for 20 min).

The resulting aqueous phase was transferred to a new tube and mixed with an equal volume of chloroform/isoamyl alcohol (24:1), phases separated by centrifugation ($12,000 \times g$ for 10 min at $4^\circ C$). The aqueous phase was transferred to a new tube, mixed with 500 ul isopropanol + 312 ul 4M LiCl, placed at $-20^\circ C$ for 2.5 hr then the RNA was precipitated by centrifugation ($12,000 \times g$ for 20 min at $4^\circ C$). The RNA pellet was washed with 1ml of 75% ethanol. RNA was air-dried and then re-hydrated with 30 μ l DEPC-treated RNase free H_2O ($65^\circ C$). Samples were then treated with DNase per the manufacturer's instructions (Ambion, Foster City, CA). DNA free RNA samples were quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) and RNA integrity was verified by electrophoresis on 1.5% agarose gel.

Oligonucleotide Array

Microarrays were manufactured at North Carolina State University as described by (Druyan et al., 2008). Seventy oligonucleotides (oligos) were designed for 514 unique gene sequences (See Appendix 1) selected from the chicken and turkey genome using OligoWiz (www.cbs.dtu.dk/services/OligoWiz/), and manufactured by Operon Biotechnologies Inc. (Germantown, MD). Oligos were printed (spotted) on UltraGAPS Amino-Silane Coated Slides (Corning Inc., Acton, MA) using a VersArray Chipwriter Compact Arrayer (Bio-Rad Inc., Waterloo, Ontario, Canada). Each gene was spotted a total of 12 times per array (technical replicates), dried for 24 h, and then cross-linked to the slides using a CL-1000 UV cross-linker (UVP Inc., Upland, CA) set to $6,000 \times 100 \mu\text{J}/\text{cm}^2$.

cDNA Synthesis and Labeling

Briefly, 10 μg of RNA from each sample was prepared in 2 tubes (5 $\mu\text{g}/\text{tube}$) and incubated with random primer and oligo (dT) at 70°C for 10 min, chilled on ice for 2 min, and cDNA synthesized using reverse transcriptase and aminoallyl-dNTP mix and incubated for 60 min at 42° C following the manufacturer's instructions (Promega, Madison, WI). Following cDNA synthesis, all reaction volume was added directly to either Cy3-NHS or Cy5-NHS ester (GE Healthcare, Piscataway, NJ). Samples were column purified to remove unincorporated nucleotides and dye, CyDye-labeled cDNA was quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE), and the frequency of incorporation (FOI) determined for each sample with each dye according to the equation: FOI = pmol of dye incorporated \times 324.5/ng of cDNA. Only those samples with the FOI of 12 - 35 for Cy3 and 12 - 40 for Cy5 were used for hybridization.

Hybridization and Image Acquisition

Thirty pmol of the Cy3- and Cy5-labeled cDNA probes were dried and resuspended in the hybridization solution (Corning Inc., Corning, NY), applied to an array slide covered, with a precleaned glass coverslip (Lifterslip, Portsmouth, NH), and hybridized for 16 h at 42° C. Microarray slides were scanned on a ScanArray GX PLUS Microarray Scanner (PerkinElmer Life and Analytical Sciences, Shelton, CT). For each tissue and each treatment there were 8 individually labeled cDNAs (Fig 1).

Array Data Processing and Statistical Analysis

Microarray data files were generated by extracting the intensity raw data for each slide and dye combination by using ScanAlyze Software (Stanford University, Stanford, CA). Intensity data files were joined, transformed to a \log_2 and analyzed in JMP Genomics (SAS Institute Inc., Cary, NC). Data normalization was performed by using locally weighted regression and smoothing, first within array and then across all arrays. Evaluation of the normalization was monitored by distribution analysis of the transformed data. The resulting normalized \log^2 intensities were analyzed by using a mixed-model ANOVA (Wolfinger et al., 2001), with channel, treatment, and channel \times treatment as fixed effects, and array as a random effect.

Pathway and Process Analysis

Biological processes were identified by using the approach of statistical overrepresentation in the Metacore database (GeneGo Inc., St. Joseph, MI), a Web-based application for identification of gene ontology processes associated with genes with significant different in expression by the treatment, and rank these genes according to their *P*-value (Nikolsky et al.,

2005). The list of differentially expressed genes was uploaded to Metacore for analysis and construction of predicted biological network(s).

4.4 RESULTS

Effects of DFM Supplementation on Gene Expression in the Ileum.

To understand how DFM supplementation results in alternations in intestinal ultrastructure (Qiu *et al.*, 2009b), intestinal energy consumption, and immune responses (Qiu *et al.*, 2009a), it is important to characterize how DFM supplementation may affect gene transcription in the gastrointestinal tract. Analysis of changes in gene expression between CON and DFMD fed animals using a focused oligo-nucleotide array demonstrated a total of 29 genes out of approximately 514 total oligo-nucleotide probes were differentially expressed in the ileum ($P < 0.05$), with the majority of them being down regulated following DFM supplementation (Figure 4.2 and Table 4.1). These genes primarily belong to the following families: morphology, immune function, energy metabolism, and antioxidation.

Effect of DFM Supplementation on Gene Expression in the Cecum

The cecum is the major site for bacterial fermentation in chicken (Whittow, 2000). Supplementation of DFM was found to affect the cecal epithelial histology (Qiu *et al.*, 2009b) and may alter the function of cecum (Croom *et al.*, 2009). Cecal samples were examined by focused microarray to illustrate the mechanisms for these changes and to determine if these changes relate to the humoral immune function changed by DFM supplementation simultaneously. Results showed a total of 20 genes out of approximately 514 were significantly changed ($P < 0.05$) in the cecum by the supplementation of DFM with most associated with immune function (Figure 4.3 and Table 4.2).

Pathway Analysis of Ileal Gene Expression

To further understand how these changes in expression may interrelate and to identify putative signaling pathways involved in the host response to DFM supplementation, both ileum and cecum microarray data were analyzed for gene ontology. These results identified the IL-27 pathway to be the most likely pathway associated with DFM supplementation in both the ileum and cecum (Figure 4.4 and Figure 4.5).

4.5 DISCUSSION

We have previously demonstrated that oral supplementation of DFM can affect gut structure, energy usage of various tissues, and the kinetics of the immune response (Chichlowski *et al.*, 2007b; Qiu *et al.*, 2009 a, b). It is currently unclear if these diverse phenotypic changes are the result of multiple, independent effects of DFM supplementation on different systems or if these effects are interrelated. These systemic changes in the host, however, do suggest the DFM microbes communicate with the host epithelium influencing its function and gene expression. To begin to understand the molecular mechanisms which may be involved in DFM-mediated changes in the gut, we assayed both ileum and cecum tissues for differences in gene expression using a focus oligo nucleotide array.

Expression analysis demonstrated only 2 genes changed are in common between the ileum and cecum by DFM supplementation, and only one (c-Myc) was affected in the same direction. These differences likely reflect the very different function and environment of the ileum and cecum. The ileum is one of the major sites for nutrient absorption as such the ileum must maintain a delicate balance between absorption capacity and inflammation (Johnson, 2006). Alternatively, the cecum is the primary site for bacterial fermentation in

chicken and has the highest level of bacteria as compared to other regions of the intestine (Whittow, 2000). These differences suggest the ileum would likely need to suppress pro-inflammatory responses in an effort to preserve its absorption function, while the ceca would inherently be more activated in immune response to control its microflora community.

It is interesting to note that IL-27 signaling pathway was predicted by Metcore system (GeneGo Inc., St. Joseph, MI) as the most likely pathway affected by supplementation of DFM in both ileal and cecal tissues (Figure 4.2 and 4.4). IL-27 is a novel member of the IL-6/IL-12 family and its principal function *in vivo* is to limit the intensity and duration of innate and adaptive immune responses (Hunter, 2005), and has been reported to increase in expression following stimulation by Gram-positive bacteria (Veckman *et al.*, 2004). IL-27 was found to affect Th1 cell proliferation by regulating IL-12 receptor β 2 (IL-12RB2) expression (Pflanz *et al.*, 2002). IL-27 can also affect CD4-T cell proliferation via the regulation of gene expression of either IL-2 (Villarino *et al.*, 2006) or c-Myc (Kiuchi *et al.*, 1999). Additionally, IL-10 was found to contribute to IL-27 signaling (Awasthi *et al.*, 2007; Stumhofer *et al.*, 2007).

In ileum, depending on the gene panel, it seems that supplementation of DFM can inhibit Th1 cell differentiation by regulating IL-10, NF- κ B, c-Myc and IL-12 receptor (Figure 4.2). Specifically IL-10 has been reported to inhibit the synthesis of pro-inflammatory cytokines including IL-12 and TNF- α (Aste-Amezaga *et al.*, 1998; Fiorentino, 1991; Hart *et al.*, 2004; Heyen *et al.*, 2000; Romano *et al.*, 1996). Hence, upregulation of IL-10 can promote the development of Th 2 response (antibody production) through direct effects on T and B cells (Mocellin *et al.*, 2003). As a result, Th2 cells may be stimulated by DFM supplementation in

ileum, which is consistent with our previous studies that found IL-10 expression was up regulated in the ileum (Chichlowski *et al.*, 2007a) and DFMD fed animals had a more rapid humoral antibody response as compared to CON (Qiu *et al.*, 2009a). The cecum, however, appears to have a different response. Supplementation of DFM increased the expression of IL-12 receptor and IL-2, which may result in Th1 cell proliferation and differentiation. Th1 response may bring about inflammation, which may enhance the oxidative stress of the tissue (Mezzano *et al.*, 2001). This is consistent with our previous observation that we found cecal malondialdehyde concentrations were increased by 124% after supplementation of DFM (Qiu *et al.*, 2007).

The current study demonstrated supplementation with DFM resulted in changes in host gene expression in both the ileum and cecum, however, few genes were identified in both sections. In spite of this, in both ileum and cecum, pathway analysis identified IL-27 as the most likely associated with the current data set. Examination of how the changes in expression in the ileum and cecum further suggest DFM supplementation has differential effects on gene expression in different regions of the intestine. Additional experiments are required to understand the biological significance of changes in IL-27 in both ileum and cecum and to better understand the mechanisms by which DFM organisms communicate with the host cells.

4.6 ACKNOWLEDGEMENTS

We are grateful to Dr. Ashwell's lab group in Department of Poultry Science (North Carolina State University) for their kind cooperation in Microarray sample preparation and data collection. This work was supported in part by Star Labs Inc., Clarksdale, MO.

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4.8 TABLES AND FIGURES

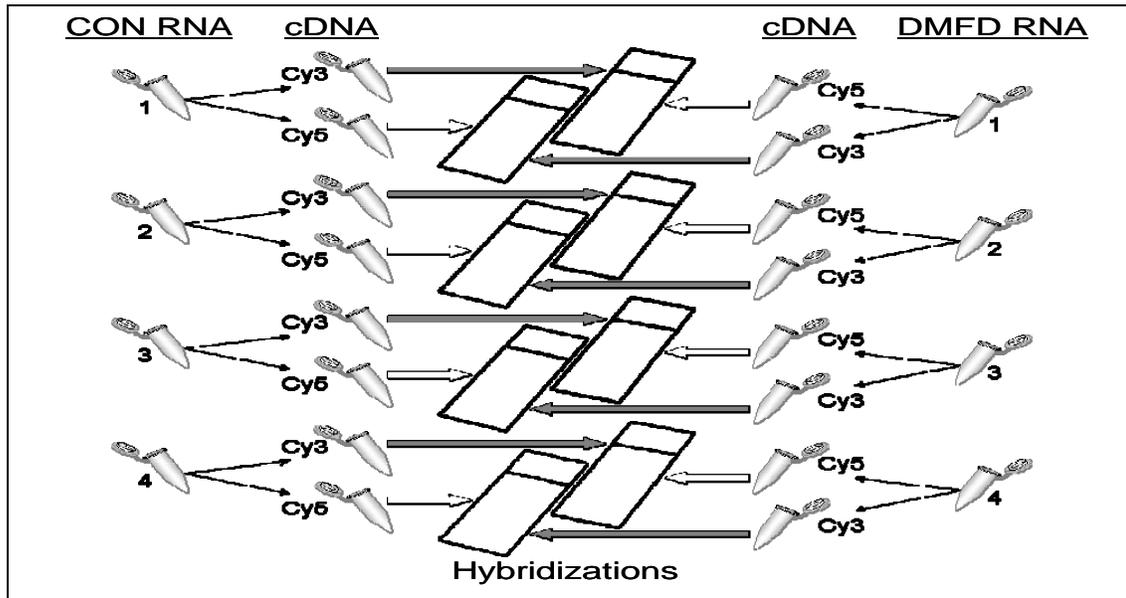


Figure 4.1 Array hybridization scheme. RNA was isolated from 4 animals/treatment. Each RNA was transcribed into 2 cDNA (Cy3- and Cy5-labeled). Labeled cDNAs were paired (CON and DFMD) and hybridized to oligo nucleotide arrays on glass slides (Cy3, shaded arrow and Cy5 open arrow) with a total of 8 independent measurements.

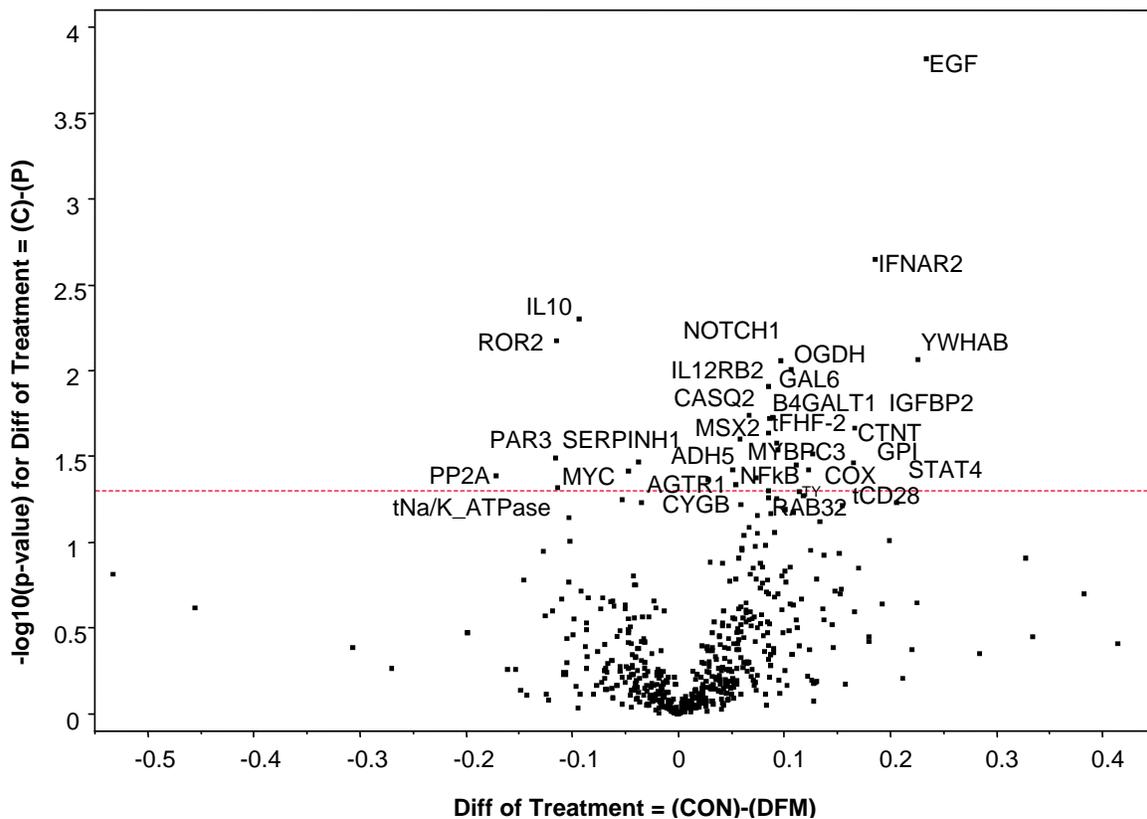


Figure 4.2. DFM supplementation affects gene expression in the ileum. Volcano plot of significance level (P-value) versus difference in expression level. Each square represents one of 514 genes in ileum, with the negative \log_{10} of the P-value from the gene model plotted against the difference between least squares means for the indicated effect. The horizontal dashed line represents the testwise threshold of $P=0.05$.

Table 4.1 Ileal genes identified as differentially expressed from as compared with CON

Gene symbol	Fold change	P-value	Protein Function
CASQ2	0.9545	0.0183	Calcium binding protein that stores calcium for muscle function
B4GALT1	0.9419	0.0188	The cell surface form functions as a recognition molecule during a variety of cell to cell and cell to matrix interactions.
OGDH	0.9291	0.0099	Participate in the citric acid cycle.
IL12RB2	0.9429	0.0123	A subunit of the interleukin 12 receptor complex. Plays a role in Th1 cell differentiation
FHF-2	0.9425	0.0232	Stimulate growth or differentiation of cells of mesodermal or origin
MSX2	0.9369	0.0290	May also have a role in promoting cell growth under certain conditions and may be an important target for the RAS signaling pathways
COX	0.9185	0.0379	The terminal component of the mitochondrial respiratory chain, catalyzes the electron transfer from reduced cytochrome c to oxygen
GPI	0.9376	0.0263	Multifunctional phosphoglucose isomerase proteins involved in energy pathways
GAL6	0.9405	0.0187	Beta-defensins gallinacin-6. An antimicrobial peptides implicated in the resistance of epithelial surfaces to microbial colonization.
AGTR1	0.9805	0.0430	A potent vasopressor hormone and a primary regulator of aldosterone secretion
ADH5	0.9651	0.0378	Metabolize a wide variety of substrates including lipid peroxidation product. Exhibits high activity for oxidation of S-hydroxymethyl-glutathione
CTNT	0.8914	0.0217	Troponin T type 2 cardiac. regulates muscle contraction in response to alterations in intracellular calcium ion concentration
PP2A	1.1262	0.0408	Protein phosphatase 2. targets of PP2A are proteins of oncogenic signaling cascades, such as Raf, MEK (Mitogen-activated protein kinase kinase), and AKT.
MYBPC3	0.9603	0.0252	Myosin binding protein C, cardiac.
CYGB	0.9632	0.0464	Cytoglobin may facilitate diffusion of oxygen through tissues, scavenge nitric oxide or other reactive oxygen species, or serve a protective function during oxidative stress
MYC	1.0332	0.0383	Plays a role in cell cycle progression, apoptosis and cellular transformation. It functions as a transcription factor that regulates transcription of specific target genes.
SERPINH1	1.0260	0.0340	Serine peptidase inhibitor. heat shock protein 47. May be involved in the maturation of collagen molecules.

Table 4.1 Continued

NOTCH1	0.9353	0.0088	Share structural characteristics including an extracellular domain consisting of multiple epidermal growth factor-like (EGF) repeats, regulates interactions between physically adjacent cells
CD28	0.9158	0.0307	Expressed on T cells that provide co-stimulatory signals, which are required for T cell activation.
IL10	1.0668	0.0050	An anti-inflammatory cytokine.
ROR2	1.0831	0.0067	May be involved in the early formation of the chondrocytes and may be required for cartilage and growth plate development.
IFNAR2	0.8795	0.0022	Phosphorylate several proteins, including STAT1 and STAT2
EGF	0.8504	0.0001	EGF results in cellular proliferation, differentiation, and survival
YWHAB	0.8550	0.0087	It may play a role in linking mitogenic signaling and the cell cycle machinery
PAR3	1.0834	0.0321	Adapter protein involved in asymmetrical cell division and cell polarization processes.
IGFBP2	0.9257	0.0354	Prolong the half-life of the IGFs
Na/K_ATPase	1.0817	0.0482	Sodium pump
STAT4	0.8672	0.0345	Signal transducer and activator of transcription 4. This protein is essential for mediating responses to IL12 in lymphocyte, and regulating the differentiation of T helper cells.
NFKB2	0.8917	0.0488	Nuclear factor NF-kappa-B p100 subunit, plays a key role in regulating the immune response to infection.

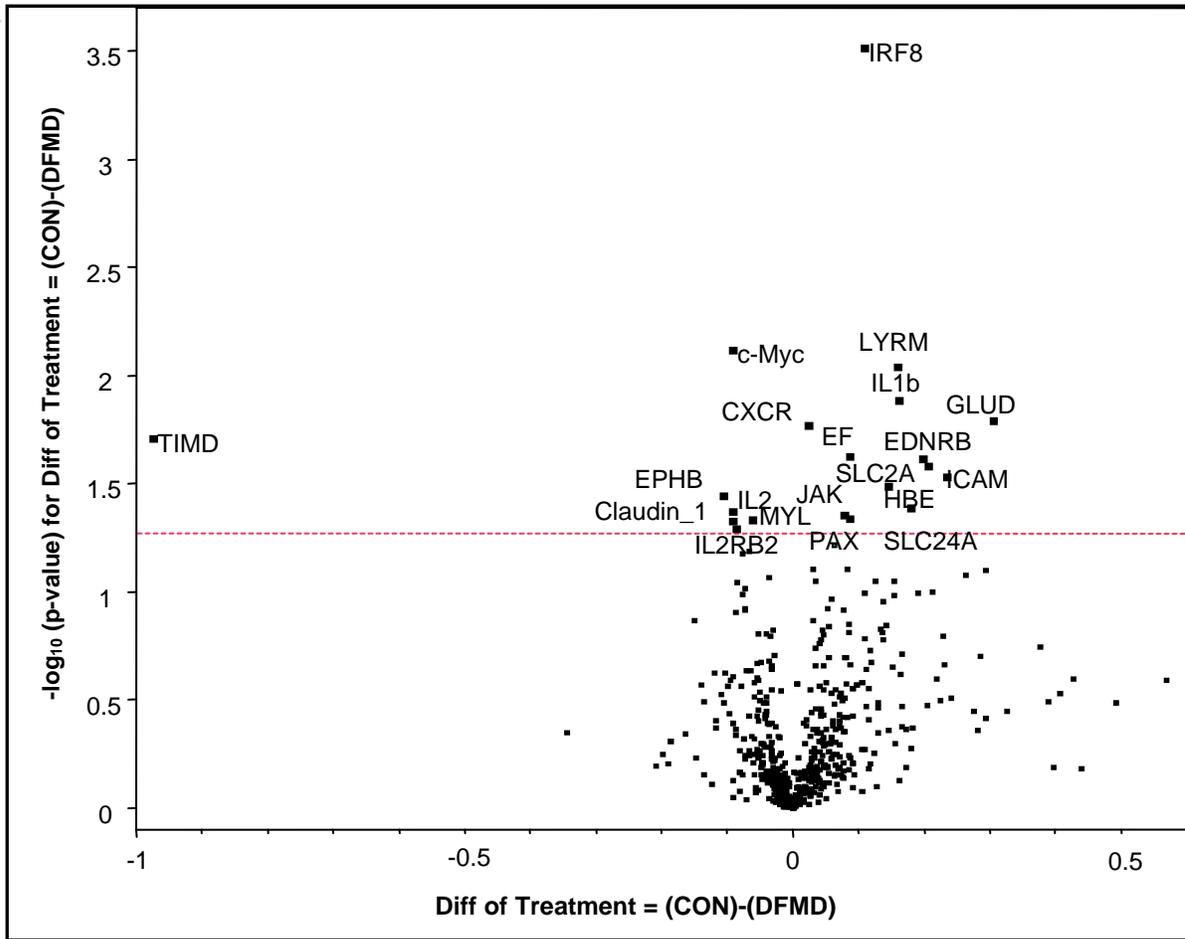


Figure 4.3 DFM supplementation affects gene expression in the cecum. Volcano plot of significance level (P-value) versus difference in expression level. Each square represents one of 514 genes in ileum, with the negative \log_{10} of the P-value from the gene model plotted against the difference between least squares means for the indicated effect. The horizontal dashed line represents the testwise threshold of $P=0.05$.

Table 4.2 Cecal genes identified as differentially expressed as compared with CON

Gene symbol	Fold change	P-value	Gene description/function
SLC2A1	0.8658	0.0264	Solute carrier family 2. A major glucose transporter in the mammalian blood-brain barrier.
SLC24A5	0.8818	0.0411	Sodium/potassium/calcium exchanger. It is also a gene that causes skin pigmentation variation
HBE1	0.9038	0.0326	A beta-type chain of early embryonic hemoglobin.
EDNRB2	0.8716	0.0244	G-protein coupled receptor, activates a phosphatidylinositol-calcium second messenger system.
LYRM5	0.8947	0.0092	Growth hormone-inducible soluble protein.
GLUD1	0.8091	0.0162	L-glutamate dehydrogenase. Has a central role in nitrogen metabolism.
IL2	1.0642	0.0426	A leukocytotropic hormone that is instrumental in the body's natural response to microbial infection and in discriminating between foreign (non-self) and self.
cMyc	1.0651	0.0076	Encodes for a transcription factor that is believed to regulate expression of 15% of all genes
IL12RB2	1.0667	0.0490	Receptor for IL-12. Promotes the proliferation of T-cells and NK cells. Induces the promotion of T-cells towards the Th1 phenotype by strongly enhancing IFN-gamma production.
MYLK	1.0424	0.0468	Implicated in smooth muscle contraction. Regulation of endothelial and vascular permeability.
EPHB2	1.0748	0.0363	Critical participant in signaling sequences resulting in fibroblast apoptosis.
CXCR4	0.9830	0.0171	Acts as a tumor suppressor
Claudin_13	1.0646	0.0471	Chemokine receptor 4. Transduces a signal by increasing the intracellular calcium ions level. Plays an essential role in vascularization of the gastrointestinal tract
EF2	0.9406	0.0237	Plays a major role in tight junction-specific obliteration of the intercellular space.
PAX7	0.9405	0.0461	Are a set of proteins that facilitate the events of translational elongation, the steps in protein synthesis from the formation of the first peptide bond to the formation of the last one.
IRF8	0.9265	0.0003	A member of the paired box (PAX) family of transcription factors. Probable transcription factor. May have a role in myogenesis. speculated to involve tumor suppression
IL1b	0.8934	0.0132	Interferon regulatory factor 8. Regulate expression of genes stimulated by type I IFNs, namely IFN- α and IFN- β . Plays a negative regulatory role in cells
ICAM 1	0.8487	0.0295	An important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.
JAK2	0.9464	0.0445	A ligand for the leukocyte adhesion protein. Can be induced by IL-1 and TNF α and is expressed by the vascular endothelium, macrophages, and lymphocytes.
TIMD4	1.9647	0.0196	A protein tyrosine kinase involved in a specific subset of cytokine receptor signaling pathways.is required for responses to IFN- γ . involved in IL-3 and probably interleukin-23 signal transduction
			Homologue to T-cell immunoglobulin and mucin domain containing 4

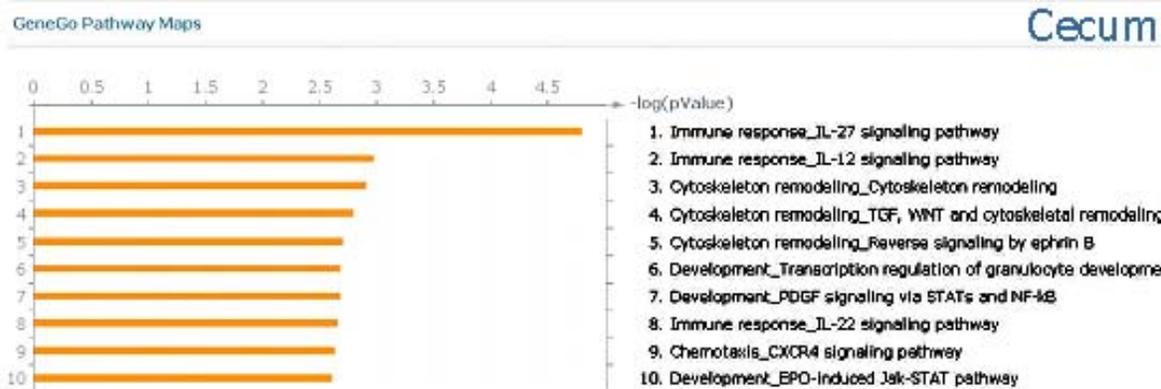
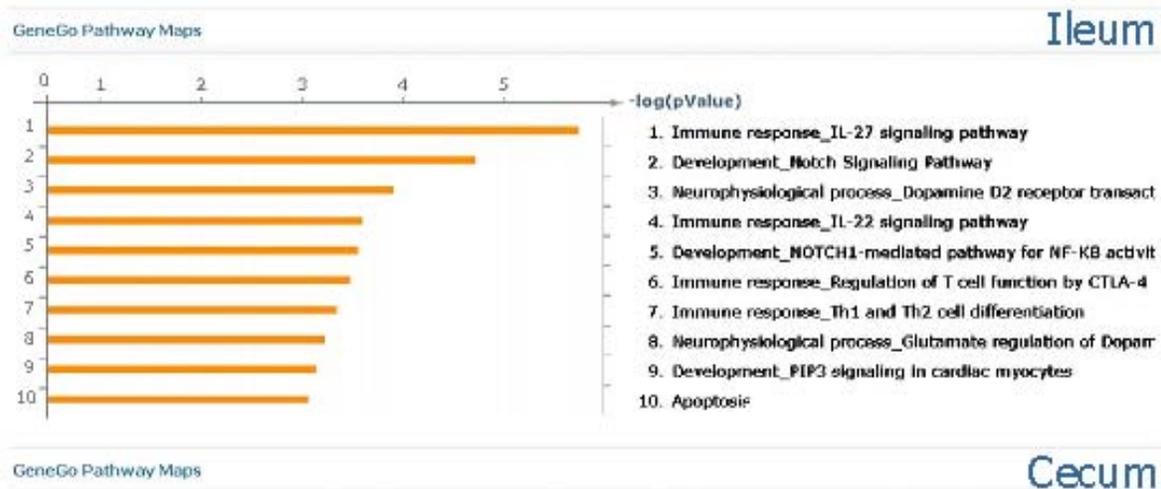


Figure 4.4 List of gene pathways with highest significance identified by Metacore program. Microarray results of either ileum or cecum, were submitted to the Metcore program for pathway analysis. Using the shortest path algorithms, the Metcore program will predict several pathways that may be associated with the data depending on the genes that have been changed significantly (Table 4.1 and 4.2). The pathways predicted are listed by ranking of the significance like the figure from 1 to 10.

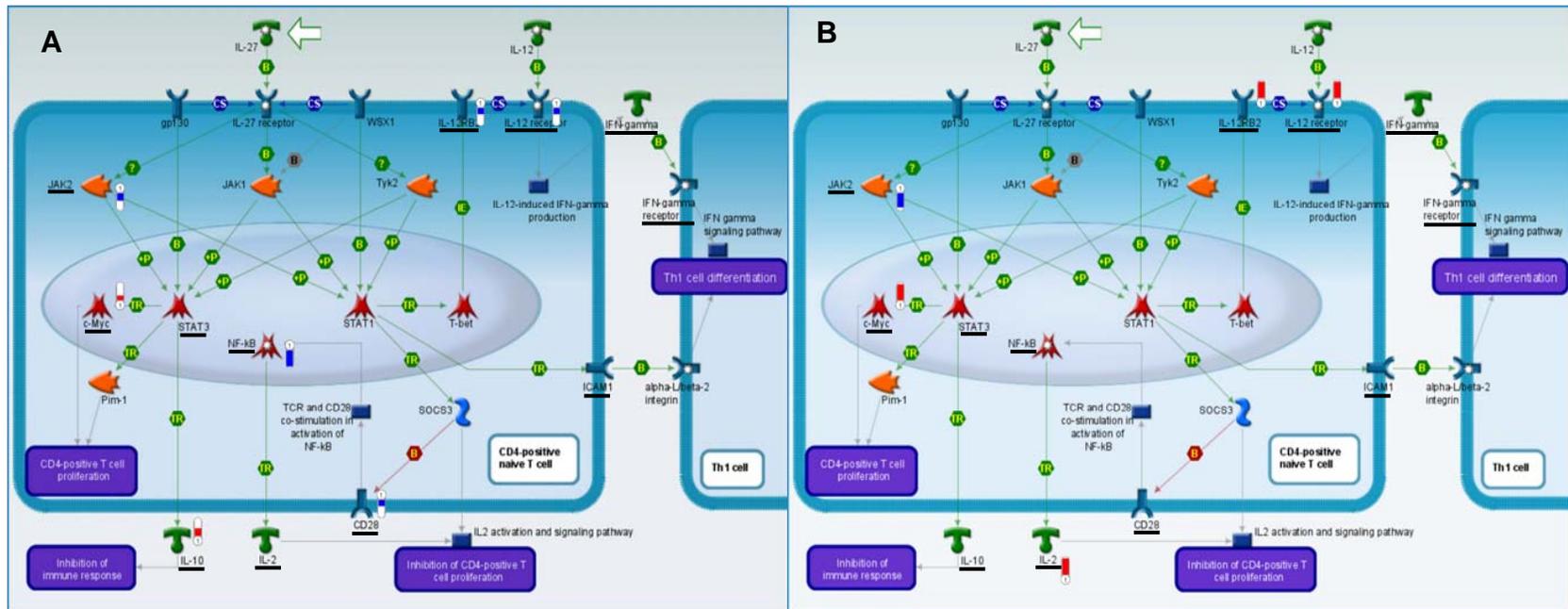


Figure 4.5 Diagram of IL-27 signaling pathway. Biological network analysis using the shortest path algorithm (GeneGo Inc., St. Joseph, MI) predicted the genes identified in the focused array analysis of ileum (A) and cecum (B) to have the highest association with the IL-27 signaling pathway. Genes involved in this pathway way are depicted. Underlined genes denote those represented on the focused array. Those with colored bars next to the protein symbol denotes genes who's expression was found to be significantly altered by DFM supplementation. Red bars indicate increased expression with DFM. Blue bars indicate decreased expression with DFM. Lines between members of the pathway indicate interactions of activation, induction, modification, or direct binding. Green line means positive regulation, red negative regulation and grey unspecific. Green hexagons denote specific interactions between pathway members: binding (B), transcription regulation (TR), influence on expression (IE), phosphorylation (P+), compound subunit (CS). A complete legend for this figure is provided in Appendix II.

APPENDICES

Appendix I Genes Printed on Focused Microarray

Gene_Symbol	TC	Gene
14_3_3	TC229596	UP 1433Z_CHICK (Q5ZKC9) 14-3-3 protein zeta, complete
AAK1		
ACAA	TC187430	acetyl-CoA acyltransferase
ACACA	TC194518	acetyl-CoA carboxylase
ACADS	TC212313	butyryl-CoA dehydrogenase
ACE	TC211222	ACE
ACLY	TC187511	citrate lyase
ACSS2	TC198192	acetate CoA ligase
ACTA2	TC196698	ACTA2
ACTB	TC234083	Beta-actin
ACTB	TC253132	Cytoskeletal beta actin
Activin_A	TC278956	GB AAC59738.1 1155283 GGU42377 inhibin/activin beta A-subunit {Gallus gallus} (exp=-1; wgp=0; cg=0), complete
Activin_AR	TC228307	UP Q90669_CHICK (Q90669) Activin receptor IIA, complete
Activin_R	TC228579	UP Q90ZK6_CHICK (Q90ZK6) Activin receptor I precursor, complete
ADH5	TC187356	formaldehyde dehydrogenase
AGTR1	TC197933	AGTR1
AK1	TC187936	adenylate kinase
ALDH2	TC208477	aldehyde dehydrogenase
ALDOB	TC187124	fructose biphosphate aldolase B
ALDOC	TC207134	fructose-biphosphate aldolase
AMY2A	TC186705	alpha-amylase
ANG1	TC194431	Ang1
ANG2B	TC214356	Ang2B
ANKRD1		ANKRD1
ANPEP	TC214367	aminopeptidase Ey
AP1	TC231264	UP JUN_CHICK (P18870) Transcription factor AP-1 (Proto-oncogene c-jun), complete
AP2B1		
APLP2	TC186185	APLP2
AR	TC217958	AR
ARNTL	TC214418	ARNTL
ARNTL2		ARNTL2
ARPC1B		ARPC1B
astrovirus2		
AZIN1	TC189780	AZIN1
b_Catenin	TC250950	UP O42486_CHICK (O42486) Beta catenin, partial (25%)
B4GALT1		
B4GALT1_bd		
BACT		BACT

Appendix I Continued

Bcl_2	TC250722	UP BCLX_CHICK (Q07816) Apoptosis regulator Bcl-X (Bcl-2-like 1 protein), complete
BMP4		
BMPER		BMPER
BMPR1A		
BMPR1B		
BPEF1	TC202805	protein phosphatase 1
Bu-1b	TC264669	Bu-1b
CAB2		CAB2
CACNA2D1		
CAMK2G	TC188444	CaM kinase II gamma
CASQ2		CASQ2
CAT	TC212504	carnitine acetylase
CCL16	TC240827	Chemokine K203 precursor
CCL2	TC289521	Small inducible cytokine A2
CCL5	TC198289	Rantes
CCR	TC228810	CCR
CCR5	TC287964	CCR5
CCR9	TC288248	CCR9
CD28		
CD36	TC228996	RF NP_001025902.1 71897003 NM_001030731 CD36 antigen {Gallus gallus} (exp=-1; wgp=0; cg=0), complete
CD3D	TC251657	CD3 glycoprotein
CD4		
CD8A		
CD8B		
CD9		
cdc42	TC273657	similar to UP BORG1_MOUSE (Q8JZX9) Cdc42 effector protein 2 (Binder of Rho GTPases 1), partial (68%)
CDK1	TC230512	UP CDC2_CHICK (P13863) Cell division control protein 2 homolog (p34 protein kinase) (Cyclin-dependent kinase 1) (CDK1), complete
CDK2	TC265133	similar to GB AAH34717.1 21961207 BC034717 CDK2AP1 protein {Homo sapiens} (exp=-1; wgp=0; cg=0), complete
chDNA	chDNA	CHICK DNA
CHP1	TC279522	Gal-1 alpha
CHP2	TC274133	Gal-1 alpha
Cingulin	TC234787	similar to UP Q52LR0_HUMAN (Q52LR0) Cingulin-like 1, partial (20%)
CKM	TC186914	creatine kinase
Claudin_1	TC228768	RF NP_001013629.1 61889131 NM_001013611 claudin 1 {Gallus gallus} (exp=-1; wgp=0; cg=0), complete
Claudin_10	TC290376	similar to GB CAI16403.1 55959060 AL357061 claudin 10 {Homo sapiens} (exp=0; wgp=1; cg=0), partial (82%)
Claudin_11	TC294790	similar to UP CLD11_MACFA (Q4R3L1) Claudin-11, complete
Claudin_12	TC299923	similar to UP CLD12_HUMAN (P56749) Claudin-12, partial (54%)

Appendix I Continued

Claudin_13	TC268889	similar to UP CLD13_MOUSE (Q9Z0S4) Claudin-13, partial (7%)
Claudin_19	TC244511	UP Q6E5S5_FUGRU (Q6E5S5) Claudin 19, partial (5%)
Claudin_2	TC277831	similar to UP CLD2_HUMAN (P57739) Claudin-2 (SP82), partial (32%)
Claudin_22	TC253767	similar to UP CLD22_MOUSE (Q9D7U6) Claudin-22, partial (7%)
Claudin_24	TC260399	similar to UP CLP24_HUMAN (Q9BSN7) Claudin-like protein 24, partial (30%)
Claudin_3	TC229685	UP Q98SR2_CHICK (Q98SR2) Claudin-3, complete
Claudin_4	TC299414	claudin 4 [Gallus gallus]
Claudin_5	TC256697	UP Q98SR1_CHICK (Q98SR1) Claudin-5, complete
CMF1	TC214693	CMF1
c-Myc	TC250935	UP Q7T235_9GALL (Q7T235) C-myc (Fragment), partial (91%)
CNDP2	TC243402	CNDP dipeptidase 2 (metallopeptidase M20 family) {Gallus gallus} (exp=-1; wgp=0; cg=0), complete
COL14A1		
COL18A1		
COL4A4		
COUP-TFII		COUP-TFII
COX		cox
CPT1A	TC210710	carnitine O-palmitoyltransferase
CRP		
CS	TC213364	ATP citrate synthase
CTNT		cTnT
CXCR1	TC230774	Putative CXCR1 isoform I and II
CXCR4	TC230261	CXCR4
Cyclin_D1	TC236886	UP CCND1_CHICK (P55169) G1/S-specific cyclin-D1, partial (77%)
CYGB	TC226344	CYGB
DBH	TC225040	Dopamine beta-hydroxylase
DCN	TC209608	DCN
DEFB1		
DES	TC206985	Desmin
DIO1	TC217180	thyroxine deiodinase I
DIO2		DIO2
E_cadherin	TC228059	UP CADH1_CHICK (P08641) Epithelial-cadherin precursor (E-cadherin) (Cadherin-1) (Liver cell adhesion molecule) (L-CAM), complete
E2F	TC229068	UP E2F1_CHICK (Q90977) Transcription factor E2F1 (E2F-1), complete
ECE1	TC192318	ECE1
EDN1		
EDN3		
EDNRA		
EDNRB		
EDNRB2	TC196241	EDNRB2
EF2	EF2	EF2

Appendix I Continued

EFNB2	TC218455	EFNB2
EGF		
EGFR		
EGR1		EGR1
EHHADH	TC200175	enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase
EKL	TC285720	similar to (Q9XYW2)
ELN	TC193337	Elastin
EN1		
ENG	TC210338	Endoglin
ENO1		ENO1
EPAS1	TC214190	EPAS1
EPHB2		
ERBB2		
ERBB3		
ERBB4		
ERK1	TC298015	similar to UP M3K1_HUMAN (Q13233) Mitogen-activated protein kinase kinase kinase 1 (MAPK/ERK kinase kinase 1) (MEK kinase 1) (MEKK 1) (Fragment) , partial (20%)
ERK2	TC230693	UP Q8UWG6_CHICK (Q8UWG6) Extracellular signal-regulated kinase 2, complete
ETBR-LP-2		
F_actin	TC228202	UP ACTN1_CHICK (P05094) Alpha-actinin-1 (Alpha-actinin cytoskeletal isoform) (Non-muscle alpha-actinin-1) (F-actin cross linking protein), complete
Fascin	TC294610	homologue to UP Q6EE45_SCYCA (Q6EE45) Fascin (Fragment), partial (18%)
FasL	TC234344	FasL
FBP1	TC188815	fructose biphosphatase (F1,6 BPase)
FGF	TC297224	UP Q07659_CHICK (Q07659) Fibroblast growth factor, complete
FGF1		
FGF2		
FGF3		
FGFR	TC247312	homologue to UP Q90330_COTCO (Q90330) Fibroblast growth factor receptor precursor, partial (38%)
FGFR1		
FGFR2		
FGFR3		
FH	TC210057	fumarate hydratase
FIGF		FIGF
FIH1	TC189896	Fih1
FLK1		Flk1
FLT1		
FN1		
FOXD3		

Appendix I Continued

FSTL1		FSTL1
G6PC	TC188827	Glucose-6-phosphatase (G-6-Pase)
GAB3	TC190340	aconitate hydratase
GAL10		
GAL11		
Gal-2	TC286576	Gal-2
GAL6		
GAL7		
GAL8		
GAL9		
GALK2		
GALT	TC214310	galactose-1-phosphate uridyltransferase
GAPDH	TC207111	glyceraldehyde 3-phosphate dehydrogenase
GATA4	TC206873	GATA-4
GBE1	TC191346	glycogen branching enzyme
GCK	TC201283	glucokinase
GCRG	TC193574	glucagon receptor
GDF10		
Gene_Symbol	TC	gene
GH	TC207821	growth hormone
GHR	TC193508	growth hormone receptor
GHRH	TC215002	growth hormone-releasing hormone (GHRH)
GHRHR	TC221240	GHRHR
Glucoc.R	TC228807	UPIQ38HX7_CHICK (Q38HX7) Glucocorticoid receptor, complete
GLUD1	TC193299	glutamate dehydrogenase
GLUL	TC210122	glutamine synthetase
GM-CSF	TC219402	GM-CSF
GNA11		
GNAQ		
GPD2	TC189614	glycerol-3-phosphate dehydrogenase
GPI		GPI
GSK3B	TC210716	glycogen synthase kinase-3 beta
GYS1	TC195364	glycogen synthase
HA		HA
HADH	TC208805	3-hydroxyacyl-CoA dehydrogenase
HAGH	TC209686	hydroxyacylglutathione hydrolase
HAND1	TC221573	HAND1
HAND2	TC193975	HAND2
HB rho	TC187403	HB rho
HBA2	TC207660	HB alphaA
HBB	TC207666	HBB
HBD	TC186781	HB alphaD
HBE1	TC209414	HBE

Appendix I Continued

HBZ		HBZ
HDAC	TC233366	GB AAH31055.2 71051977 BC031055 HDAC2 protein {Homo sapiens} (exp=-1; wgp=0; cg=0), partial (48%)
HDAC1		
HDGF	TC215499	Hepatoma-derived growth factor
Hemoglob	TC199407	Probable hemoglobin and hemoglobin-haptoglobin binding protein 3 precursor
HIF1A	TC196652	HIF1A
HK1	TC186925	hexokinase 1
HK2	TC192763	HK2
HMGR	TC208609	3-hydroxy-3-methylglutaryl-CoA reductase
HMOX1	TC209954	HMOX1
HNF4a	TC229472	UP HNF1A_CHICK (Q90867) Hepatocyte nuclear factor 1-alpha (HNF-1A) (HNF-1-alpha), complete
HOXA13	TC198810	HOXA13
HOXA3		HOXA3
HSPE1	TC196302	HSPE1
HYOU1	TC194969	HYOU1
IBSP		
IBV_3a		
ICAM 1	TC270183	weakly similar to UP Q5NKV7_HUMAN (Q5NKV7) Intercellular adhesion molecule 1 (Fragment), partial (4%)
Id_1	TC243460	RF NP_001029043.1 76563913 NM_001033871 zinc finger protein 289, ID1 regulated {Gallus gallus} (exp=-1; wgp=0; cg=0), partial (60%)
ID2		
IDH2	TC210085	isocitrate dehydrogenase (NADP+)
IDH3A	TC209183	isocitrate dehydrogenase (NAD+)
IER5	TC216296	IER5
IFN	TC195766	IFN?
IFN2	TC264863	Interferon type B precursor
IFNAA	TC258323	Interferon alpha-A precursor
IFNAR2	TC229540	IFNAR2
IFNG	TC230252	Interferon gamma precursor (IFN-gamma)
IFNGR2	TC288597	IFNGR2
IGF1	TC236322	Insulin-like growth factor I precursor (IGF-I) (Somatomedin)
IGF1R		
IGF2		
IGF2BP3		IGF2BP3
IGFBP1	TC216581	IGFBP1
IGFBP2	TC214722	insulin-like growtg factor 2 receptor (ILGF-2 receptor)
IL1	TC212665	IL1
IL10	TC197579	IL10
IL10RA	TC259509	IL10RA
IL11RA	TC242575	IL11RA

Appendix I Continued

IL12RB2	TC296939	Interleukin 12 receptor beta 2
IL13	TC221524	IL13
IL13RA2	TC272839	IL13RA2
IL15	TC214674	IL15
IL17R	TC228787	IL17R
IL18	TC210696	IL18
IL1b	TC230691	UP O73909_CHICK (O73909) Interleukin-1beta, complete
IL1R1	TC232347	IL1R1
IL2	TC214019	IL2
IL2RA	TC230927	IL2RA
IL3	TC221532	IL3
IL4	TC196102	IL4
IL5	TC200395	IL5
IL6	TC194010	IL6
IL7	TC239714	Interleukin-7
IL8	TC230515	IL-8
INSR	TC210106	isulin receptor
IRF1	TC188846	IRF1
IRF10	TC216539	IRF10
IRF2	TC210368	IRF2
IRF3	TC192542	IRF3
IRF4	TC211361	IRF4
IRF5		
IRF6	TC186941	IRF6
IRF7		
IRF8		
IRX1		IRX1
IRX2		IRX2
IRX4	TC196427	IRX4
ITGAV		
ITGB1		
Jak	TC195947	Jak
JAK2		
JAK3		
JAM	TC252052	weakly similar to UP JAM3_MOUSE (Q9D8B7) Junctional adhesion molecule C precursor (JAM-C) (Junctional adhesion molecule 3) (JAM-3) (JAM-2), partial (80%)
JNK1	TC302117	homologue to UP MK08_MOUSE (Q91Y86) Mitogen-activated protein kinase 8 (Stress-activated protein kinase JNK1) (c-Jun N-terminal kinase 1) , partial (33%)
JNK2	TC229898	homologue to UP MK09_HUMAN (P45984) Mitogen-activated protein kinase 9 (Stress-activated protein kinase JNK2) (c-Jun N-terminal kinase 2) (JNK-55) , complete
KCNA3		KCNA3

Appendix I Continued

KCND3		KCND3
KCNG2	TC218406	KCNG2
KIF3A	TC188064	KIF3A
KIT		
KRT1		
KRT10		
LBR	TC192784	LBR
LDHA	TC207931	L-lactate dehydrogenase
LDHD	TC196585	D-lactate dehydrogenase
LEF1		
LITAF	TC243080	Lipopolysaccharide-induced tumor necrosis factor-alpha factor homolog
LPL	TC213413	lipoprotein lipase (LPL)
LYRM5	TC188929	growth hormone-inducible soluble protein
MAG1	TC277598	homologue to UP MAGI2_HUMAN (Q86UL8) Membrane-associated guanylate kinase, WW and PDZ domain-containing protein 2 (Membrane-associated guanylate kinase inverted 2) (MAGI-2) (Atrophin-1-interacting protein 1) (Atrophin-1-interacting protein A), partial (25)
MAP3K2	TC275325	similar to UP Q6P084_HUMAN (Q6P084) MAP3K2 protein (Fragment), partial (28%)
MAPK9	TC246235	JNK2 beta2 protein kinase
MDH1	TC198849	malate dehydrogenase
MDH1	TC198849	malate dehydrogenase
ME1	TC211999	malic enzyme
MEK	TC267333	homologue to UP Q92961_HUMAN (Q92961) MAP kinase kinase MEK5b (Mitogen-activated protein kinase kinase 5, isoform A) (MAP2K5 protein), partial (81%)
MGAM	TC192437	maltase-glucoamylase
MGP		
MINPP1		
MITF		
MLANA		
MLC2A	TC207977	MLC2a
MLCK	TC230202	A clone isolated from a chicken gizzard cDNA library which has high homology with the 3'-end sequence of the chicken gizzard myosin light chain kinase (MLCK) cDNA; The 5'-leader sequence derives from the intronic sequence of the MLCK gene
MMP10		
MMP13		
MMP2	TC228355	UP MMP2_CHICK (Q90611) 72 kDa type IV collagenase precursor (72 kDa gelatinase) (Matrix metalloproteinase-2) (MMP-2) (Gelatinase A) , complete
MMP9		
MSX1		
MSX2		

Appendix I Continued

MTPN	TC188631	MTPN
MUC		
MX	TC195106	MX
MYBPC3	TC191332	MYBPC3
MYC		
MYD88		
MYH7		MYH7
MYH7B		MYH7B
MYL4		MYL4
MYL9	TC210862	MYL9
MYLK		
NA		NA
Na_K ATPase	TC229436	PRF 1908226A 444792 1908226A Na/K ATPase:SUBUNIT=beta1. {Gallus gallus} (exp=-1; wgp=-1; cg=-1), complete
NCAM	TC233888	GB AAB59957.1 459710 CHKNCAM16 neural cell adhesion molecule {Gallus gallus} (exp=-1; wgp=0; cg=0), complete
Newcastle		
NFKB2	TC288734	Nuclear factor NF-kappa-B p100
NKX25		NKX2-5
NOS2A	TC209493	NOS2A
NOS3	TC217613	eNOS
NOTCH1		
NP		NP
NP959225		NP959225
NPPA	TC208792	ANF / ANP
NR2F2		NR2F2
NS		NS
NT5C3	TC213495	NT5C3
NUDT7	TC206940	acetyl CoA hydrolase
OA1		
OAS*A	TC273089	2' 5'-oligo adenylate synthetase A
OCA2		
Occludin	TC229388	UP OCLN_CHICK (Q91049) Occludin, complete
OGDH	TC188867	oxoglutarate dehydrogenase
p21Cip1	TC229967	UP Q8AYE7_CHICK (Q8AYE7) Cdk inhibitor CIP1, complete
p27Kip1	TC240308	RF NP_989587.1 45383622 NM_204256 cyclin-dependent kinase inhibitor 1B (p27, Kip1) {Gallus gallus} (exp=-1; wgp=0; cg=0), complete
P2RX1	TC198738	ATP-gated ion channel receptor
p38 MAPK	TC269647	homologue to GB AAB40118.1 1772646 HSU66243 p38gamma MAP Kinase {Homo sapiens} (exp=-1; wgp=0; cg=0), partial (16%)
p53	TC229905	UP P53_CHICK (P10360) Cellular tumor antigen p53 (Tumor suppressor p53), complete
PA		PA

Appendix I Continued

PAR3	TC228103	homologue to UP Q5VWV0_HUMAN (Q5VWV0) Par-3 partitioning defective 3 homolog (C.elegans), partial (69%)
PAX3		
PAX6		
PAX7		
PB1		PB1
PB2		PB2
PC	TC194407	pyruvate carboxylase
PCDH1		
PCK1	TC188637	phosphoenolpyruvate carboxykinase (PEPCK)
PDGFA		
PDGFRA		PDGFRA
PDHB	TC187369	pyruvate dehydrogenase
PDK1		PDK1
PFKFB3	TC210127	phosphofructokinase (PFK-1)
PFKM	TC213033	6-phosphofructokinase (PFK-1)
PGD	TC208369	phosphogluconate dehydrogenase
PGGT1B		
PGGT1B_bd		
PGM1	TC189600	phosphoglucomutase
PHEX		
PHF14	TC211891	PHF14
PHF20L1		PHF20L1
PHKB	TC192696	phosphorylase kinase B alpha regulatory chain
PI3K	TC255915	homologue to UP PK3C3_HUMAN (Q8NEB9) Phosphatidylinositol 3-kinase catalytic subunit type 3 (PtdIns-3-kinase type 3) (PI3-kinase type 3) (PI3K type 3) (Phosphoinositide-3-kinase class 3) (Phosphatidylinositol 3-kinase p100 subunit) , partial (42%)
PKM2	TC207878	pyruvate kinase
PLB	TC190986	PLB
PMEL17		
PP2A	TC228177	UP 2ABD_CHICK (Q5ZII5) Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B delta isoform (PP2A, subunit B, B-delta isoform) (PP2A, subunit B, B55-delta isoform) (PP2A, subunit B, PR55-delta isoform) (PP2A, subunit B, R2-delta isoform), com
PRKAG2	TC210077	PRKAG2
PRKCE		
PTK2		PTK2
PYGL	TC209295	glycogen phosphorylase
PYGM	TC223014	glycogen phosphorylase (muscle)
Q4JIM4	TC229568	Q4JIM4
Q5ZKW4	TC228700	Q5ZKW4
RAB1A	TC249692	UP Q5U0I6_HUMAN (Q5U0I6) RAB1A, member RAS oncogene family (RAB1A protein), complete

Appendix I Continued

RAB27A		
RAB32		
RAB38		
Rac1	TC262469	UP O93466_CHICK (O93466) GTPase cRac1B, complete
RAP1GDS1	TC205515	RAP1GDS1
RARB	TC198637	RARB
reovirusLambdaA		
reovirusM1		
reovirusS3		
Rho_A	TC232117	UP O93467_CHICK (O93467) GTPase cRhoA (GTP-binding protein) (RhoA GTPase), complete
RHOB		
RNASEL	TC228391	RNaseL
ROR2	TC202032	insulin receptor tyrosine kinase
RUNX2		
SCARB1		
sclerostin		
SDHB	TC225543	succinate dehydrogenase
sEH		sEH
Selectin	TC273197	weakly similar to UP Q7Z614_HUMAN (Q7Z614) Selectin ligand interactor cytoplasmic-1, partial (35%)
SERCA2	TC189944	SERCA2
SERPINH1		
SHH		
SI	TC207102	Sucrase-isomaltase intestinal
SLC15A1	TC214754	peptide transporter
SLC24A5		
SLC2A1	TC191959	SLC2A1
SLC45A2		
SLC5A1	TC216375	sodium/glucose cotransporter 1
SLC9A3		
SLC9A8		
SM22	TC189636	SM22
Smad2	TC265847	homologue to UP Q1W668_BOVIN (Q1W668) SMAD2, partial (38%)
Smad5	TC250721	UP Q56I99_CHICK (Q56I99) SMAD5, complete
SNAI1		
SOD1		SOD1
SOD2		SOD2
SOX10		
SOX18		
SOX2		
SOX4	TC194994	SOX4
SOX5		

Appendix I Continued

SOX9		
SPARC	TC187113	SPARC
STAT2	TC299780	Signal transducer and activator of transcription 2
STAT3	TC227805	Signal transducer and activator of transcription 3
STAT4	TC227960	signal transducer and activator of transcription 4
STAT5	TC260683	Signal transducer and activator of transcription 5
SUCLG1	TC208213	succinate CoA ligase
TALDO1	TC188910	transaldolase
t-aminopep		
t-beta_defensin		
TBX20	TC197879	TBX20
TBX5	TC218562	TBX5
tCD28		
tCD4		
tCD8		
tCD9		
t-c-Fos		
tCRP		
tDNA	tDNA	TURKEY DNA
tFHF-2		
TFIP11		
tGAPDH		
TGF_a	TC230695	UP Q6J1M9_CHICK (Q6J1M9) Transforming growth factor alpha, complete
TGF_aR	TC229132	UP Q90836_CHICK (Q90836) EGF/TGF-alpha receptor precursor, complete
TGF_b1	TC240643	UP Q9R231_RAT (Q9R231) Transforming growth factor beta-1 (Fragment), partial (52%)
TGF_b1R	TC301273	UP Q9YH45_CHICK (Q9YH45) Type I TGF B receptor, complete
TGF_b2	TC230416	UP TGFB2_CHICK (P30371) Transforming growth factor beta-2 precursor (TGF-beta-2), complete
TGFB2		
TGFB3		
TGFBR1		TGFBR1
TGFBR2		
t-GHRL		
TH	TC199532	TH
t-HGFA		
THRA	TC197415	THRA
THRB1	TC196862	THRB-1
tIFN		
tIFNg		
tIGF1		
t-IGF-II		

Appendix I Continued

tIL-10		
tIL-12		
tIL-13		
tIL-18		
tIL-1b		
tIL-2		
tIL-8		
TIMD4	TC301924	homologue to T-cell immunoglobulin and mucin domain containing 4 {Gallus gallus} (exp=-1; wgp=0; cg=0), partial (66%)
tiNOS		
TKT	TC189690	transketolase
TLR1	TC228249	TLR1
TLR2 -2	TC218153	TLR2 -2
TLR2-1	TC216531	TLR2-1
TLR3	TC262334	Toll-like receptor 3
TLR4	TC201541	TLR4
TLR5	TC228744	TLR5
TLR7	TC229100	TLR7
tMx		
tNa/K_ATPase		
TNC	TC227712	Tenascin precursor
TNF_R	TC228488	UP Q805B0_CHICK (Q805B0) Tumor necrosis factor receptor-II, complete
tNHE1		
TNN2		TNN2
TNNC1	TC225202	pCTnC1
TNS1		TNS1
TOLLIP		
TOP2B	TC189578	TOP2B
t-SGLT-1		
tTHP1		
tTHP2		
TUFT1		
tVIL1		
tVIP		
TWIST1		
TWIST2		
TYR	TC219401	tyrosinase precursor
TYRP1		
TYRP2		
UCP3	TC195226	Mitochondrial uncoupling protein
UGDH	TC208909	UDP-glucose 6-dehydrogenase
UGP2	TC216336	UTP-glucose-1-phosphate urydyltransferase

Appendix I Continued

VCAM1		
VDR		
VEGF		VEGF
VEGF_A	TC246964	UP VEGFA_CHICK (P67964) Vascular endothelial growth factor A precursor (VEGF-A) (Vascular permeability factor) (VPF), complete
VEGFC		
VIL1		
VIP		
XIN		XIN
XPNPEP1	TC212978	aminopeptidase
YWHAB		YWHAB
ZC3H15	TC224514	LEREPO4 protein - similar
ZO1	TC276238	homologue to UP ZO1_MOUSE (P39447) Tight junction protein ZO-1 (Zonula occludens 1 protein) (Zona occludens 1 protein) (Tight junction protein 1), partial (19%)
ZO3	TC260524	similar to GB AAC72274.1 3851202 AC005954 ZO-3 {Homo sapiens} (exp=0; wgp=1; cg=0), partial (13%)
ZYX	TC209233	ZYX

Appendix II Complete Legend for Metcore Pathway

