ABSTRACT

CHICHLOWSKI, MACIEJ. Effect of probiotic consortium on level and mechanism of intestine function. (Under the direction of Warren James Croom, Jr.).

A probiotic consortium or direct-fed microbial (DFM) is a live-microbial food supplement that improves health and performance, in broilers and other animals. They may have potential as an alternative to supplemental feed antibiotics use within the poultry industry to prevent enteric disease. Mechanisms by which DFM operate, collectively called "competitive exclusion", includes spatial exclusion, micro-environmental alterations, production of antimicrobial substances and epithelial barrier integrity. The majority of recent research suggests the likelihood of a small but additive series of beneficial changes from the animal's exposure to DFM. The interactions between intestinal microbiota, the gut epithelium, and the immune system are important in the competitive exclusion process.

Data presented in this study suggest that DFM increase metabolic efficiency via changes in intestinal physiology and metabolism, especially when compared to the traditional feed supplement prophylactic, salinomycin. Also, the effects of DFM on ileal glucose and proline absorption and their relationship to gastrointestinal energy expenditures were examined. Increases in the efficiency of nutrient absorption and decreases in intestinal fermentation with DFM may contribute to previously observed decreases in energy expenditures; however, these contributions are relatively minor, indicating that other physiological mechanisms are involved. The effects of DFM on intestinal histomorphometrics and micro-architecture were examined. Since DFM colonization can prevent attachment of the pathogens to the gastrointestinal epithelium,

spatial relationships between the gastrointestinal bacteria and gastrointestinal epithelium, described in this study, confirm previous assumptions about the ability of DFM to physically exclude the colonization of bacterial pathogens, preventing enteric disease. In summary, it is likely that the beneficial effects of DFM are the result of the summation of a complex, multi-variate series of alterations in gut microbial and whole body metabolism.

EFFECT OF PROBIOTIC CONSORTIUM ON LEVEL AND MECHANISM OF INTESTINE FUNCTION

by

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To my family

BIOGRAPHY

Maciej Chichlowski was born in Poznan, Poland in 1978. Since the early childhood he was interested in the life sciences and decided to pursue a career in Physiology. He received his B.S. Degree in Animal Science at University of Agriculture in Poznan, and a Diploma in Food Chain Management at Dronten Professional University of Agriculture in The Netherlands. In 2003 he graduated with M.S. degree in Nutritional Physiology as well as a Graduate Certificate in Food Safety from North Dakota State University. He began pursuing his Ph.D. degree in Physiology in January 2004. His academic research has focused on the effects of probiotics on whole-body and intestinal energetics, as well as intestinal absorptive and immunological functions. Maciej's professional experiences include a broad spectrum of laboratory techniques and assays, experience in teaching several academic courses, as well as publishing and presenting scientific data. In his leisure time he enjoys rock climbing and back packing.

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Lastly, I want to emphasize how fortunate I feel being able to pursue a doctoral degree. Even though there are many hardships on our way to scientific career, it feels great to Learn, Know and Understand.

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CHAPTER 1: LITERATURE REVIEW

METABOLIC AND PHYSIOLOGICAL IMPACT OF PROBIOTICS OR DIRECT-FED-MICROBIALS ON POULTRY[1]

[1] M. Chichlowski, J. Croom, G. Havenstein, A. R. Bird, and M. D. Koci. *International Journal of Poultry Science.* Submitted.

ABSTRACT

The poultry industry is facing a forthcoming ban of antibiotic feed additives, and there is a growing interest in finding valuable alternatives to the prevention of disease and growth enhancement supplements. The effects of probiotic or direct fed microbials (DFM) on gut health and performance in poultry as well as other species are presented. The interactions between intestinal microbiota, the gut epithelium, and the immune system are important in the competitive exclusion process. The mechanisms by which probiotics operate include spatial exclusion, micro-environmental alterations, production of antimicrobial substances and epithelial barrier integrity. The majority of recent research in this field suggests the likelihood of a small but additive series of beneficial changes from the animal's exposure to probiotics. Further investigations are required to fully characterize the effects and sustained outcomes of probiotic and DFM treatments in poultry.

Key words: direct fed microbial, probiotic, poultry, and competitive exclusion

INTRODUCTION

The metabolic activity and energy requirements of the intestinal microbiota is comparable to that which takes place in the liver, the most metabolically active organ (Isolauri et al., 2004). In vertebrates, there are more microbial cells within the gastrointestinal (GI) tract than within the body-proper (Hove et al., 1999; Mai, 2004). The microorganisms most commonly observed are bacteria and yeast.

There are two populations of microorganisms that are found within the GI tract of poultry. The first, the autochthonous bacteria, colonize the gut by inoculation resulting from normal feeding activities of the bird (Gusils et al., 1999). The second, allocthonous bacteria, are exogenous in nature and are introduced into the GI tract through the feed or drinking water as direct fed microbials (DFM) or probiotics (Bird et al., 2002; Fooks and Gibson, 2002; Patterson and Burkholder, 2003). Modern nutritionists use the terms probiotics and DFM interchangeably, but currently the term probiotic is most often used. So, for the purposes of this review, probiotic or probiotics will be used to denote the use of both probiotics and direct fed microbials.

A large body of data in the literature now indicates that allocthonous bacteria introduced via probiotics can prevent infection and colonization of the GI tract by opportunistic pathogens (Lin, 2003; Netherwood et al., 1999; Ouwehand and Vesterlund, 2003). Introduction of such probiotics is believed to prevent or attenuate clinical and sub clinical enteric pathogens in poultry, thereby, resulting in enhanced growth and performance. This property has resulted in a widespread interest in the poultry industry in the use of probiotics as alternative to the prophylactic use of antibiotics for the prevention of disease

amongst poultry flocks (Patterson and Burkholder, 2003). This interest is because of growing concerns about antibiotic resistance, and bans or potential bans on antibiotic usage in poultry and other animal productions systems in Europe and the US (Klose et al., 2006; Patterson and Burkholder, 2003).

Unfortunately, the mode of action of probiotics is poorly understood. Many papers have been published on individual physiological actions of specific consortia of probiotic organisms, but to date, however, poultry scientists have failed to develop a comprehensive and integrated model of how probiotics work within the body of the bird. Such information is essential for the development of more efficacious probiotics. Once these mechanisms are elucidated, it may be possible to use modern molecular biological techniques to develop more efficacious and useful probiotics.

This review will briefly describe traditional definitions of probiotics and summarize what is currently understood about their colonization in the GI tract, their metabolism and their mechanisms of action in altering host animal health and performance as well as their potential for the prevention of diseased states in poultry flocks.

What are probiotics and how can they impact poultry production systems?

Probiotics are "live microbial feed supplements, which beneficially affect the host animal by improving its intestinal microbial balance" (Fuller, 1989) or "a live microbial feed that is beneficial to health" (Salminen et al., 1998). They may contain only one, or several (a consortium) different bacterial species. The mechanisms of action of different bacterial strains in a probiotic consortium may differ (Davis and Anderson, 2002; Mai, 2004). Additionally, different subtypes within the same species may have different biological effects; isolates within the same species can be unique, and may have differing areas of adherence, specific immunological effects, and other biological actions (Isolauri et al., 2004). Hence, probiotics containing similar species of bacteria may, in fact, differ in efficacy.

Although many articles in the current scientific and popular literature refer to the "beneficial effects" of probiotics, these articles are often vague as to exactly what benefits are conferred upon the animal by the probiotic (Fooks and Gibson, 2002; Netherwood et al., 1999; Patterson and Burkholder, 2003). In poultry production systems, benefits and efficacy can be easily defined. Any feed supplement or therapy that enhances poultry health and performance as measured by enhanced animal health, growth and/or feed efficiency can be defined as beneficial or efficacious. In the case of probiotics, the preponderance of literature suggests that these production endpoints are beneficially impacted by the ability of consortia to alter the immune or metabolic status of the animal.

What are the factors associated with colonization?

Successful probiotic colonization depends on the survival and stability of the probiotic strain, specificity of the strain relative to host, dose and frequency of administration, health and nutritional status of the host, effect of age, stress and genetics of the host. (Bomba et al., 2002). In general, probiotic bacteria are anaerobes or facultative anaerobes (Isolauri et al., 2004). In poultry, probiotic organism colonization, as measured by colony forming units (CFUs), increases as you go from the beak distally to the colon (Bouzaine et al., 2005).

The crop, proventriculus and gizzard have very low anaerobic bacterial numbers due to the presence of the O_2 consumed with the feed as well as the low environmental pH that is associated with the secretion of HCl within the proventriculus (Mason et al., 2005). The small intestine has large bacterial numbers consisting of facultative anaerobes such as *Lactobacilli*, *Streptococci*, and *Enterobacteria* as well as anaerobes such as *Bifidobacterium* spp., *Bacteroides* spp., and *Clostridia* spp. at levels of ~ 10^4 to10^8 CFU/ml (Salanitro et al., 1978). The most heavily colonized regions of the GI tract are the colon and cecum with colonization of 10^{10} to 10^{13} CFU/ml (Simon et al., 2004).

Autocthonous and allocthonous (probiotic) bacteria are present in three different major microenvironments within the GI tract, namely in the intestines, cecum and colon (Rastall, 2004). These first areas of the gut where gut microbes are found are in the digesta, which is created by the consumption of a rich milieu of feed nutrients and water. The digesta is an ideal environmental niche for many bacteria to flourish. Probiotic bacteria can be found attached to individual feed particles such as starch granules (Figure 1A and 1B). Other bacteria are not associated with the feed particles, but simply exist within the water matrix of

the digesta. The second area of the gut where microbes are found is within the mucous blanket that covers the epithelial lining of the GI tract including the intestinal villi (Figure 1C and 1D). The mucous not only serves as an environment within which these microbes exist, but also serves as a source of nutrients for bacteria (Gaskins, 2003). Finally, bacteria can also exist on the surface of epithelial cells or they can actually be attached to epithelial cells (Figures 2A and 2B). Figure 4 depicts *lactobacillus* occupying the surface area above the opening to a goblet cell on the ileal villus of a chick at d 21. Figure 5 depicts a cluster of segmented fusiform bacteria embedded into the cytoplasm of enterocytes of the ileum (Heczko et al., 2000; Klaasen et al., 1992). The significance of the occurrence of bacteria in relation to the GI architecture and its biological activity has not been fully established (Yamauchi and Snel, 2000).

The ability of many strains of probiotic bacteria to physically adhere to portions of the various GI microenvironments speaks to their ability to enhance bird enteric health (Jacobsen et al., 1999). Attachment is considered a very first step in the colonization of the host mucosal surfaces. That permits them to resist peristalsis and removal from the gut. However, adherent probiotic bacteria usually do not colonize the intestinal mucosa for long periods, and they are normally eliminated after a few days when the host stops ingesting them (Marteau et al., 2004).

It has been suggested that *Lactobacilli* can colonize the nonsecretory gastric epithelium by attaching to epithelial cells and can continuously inoculate gastric contents and the lower regions of the intestinal tract (Rojas and Conway, 1996). It has also been demonstrated that higher percentage of hydrophobic bacteria adhere to intestinal epithelial

cells than do hydrophilic strains (Wadstrom et al., 1987). The highest adhesion values were obtained at pH 7 (Gusils et al., 1999).

Lactobacilli, whether shed from epithelial surfaces or multiplying in ingested food, permeate all regions of the digestive tract in poultry (Gusils et al., 1999). However, very few studies have investigated adhesion and colonization, because of the complexity of the intestinal mucosa and the extensive interaction between cell types within the GI tract (Sarem-Damerdji et al., 1995). Generally, *Lactobacilli* 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).

Since it is very difficult to study bacterial adhesion *in vivo*, most experiments use *in vitro* models. Microbial Adhesion to Solvents (MATS) is a technique that have been used to investigate bacterial cell affinities for polar and non-polar solvents (Kankaanpaa et al., 2004). Non-polar solvents have been used to estimate the hydrophobic properties, while polar solvents have been used to help estimate Lewis acid/base properties (Briandet et al., 1999). The low affinities of *Lactobacilli* for non-polar solvents indicate that these bacteria possess a hydrophilic rather than hydrophobic cellular surface (Kankaanpaa et al., 2004). When those microorganisms were cultured with free polyunsaturated fatty acids (PUFA), hydrophobicity was diminished.

Huang and Adams (2003) used human intestinal epithelial cell line to study probiotic bacterial adhesion. This cell line is a model for investigating such mechanisms, because it spontaneously differentiates under standard *in vitro* culture conditions, and the differentiated cells then express characteristics of mature enterocytes. This study utilized *Lactobacillus acidophilus* and *Bifidobacterium lactis*, as positive and negative controls, respectively, while

testing adhesion of propionibacteria strains. In that experiment large numbers of *L. acidophilus* were observed that were adhering to the surface of the human enterocyte cell line by scanning electron microscopy (SEM); while very few *B. lactis* were observed on the surface. Additionally, polyunsaturated fatty acids (PUFA) were reported to alter bacterial adhesion sites on Caco-2 cells (Bomba et al., 2002). That study suggests that dietary PUFA affects the attachment sites for the GI microbiota, possibly by modifying the composition of fatty acids in the intestinal wall. The stimulatory effect of PUFA upon adhesion of *Lactobacilli* may be useful for enhancing the effectiveness of probiotics in inhibiting digestive tract pathogen colonization (Bomba et al., 2002).

It has also been observed that not all strains of *Lactobacillus* adhere to enterocytes, indicating that this property is strain specific (Servin and Coconnier, 2003). Also, a dose-dependent inhibition of adherence of Enterotoxigenic *E.coli*, Enteropathogenic *E.coli* (EPEC), and *S. typhimurium* to Caco-2 cells by strains of *Bifidobacteria* and *Lactobacillus* has been reported (Servin and Coconnier, 2003). *Lactobacillus animalis* has been demonstrated to inhibit growth of *Salmonella* strains, produce antimicrobial substances, and to inhibit *in vitro* adhesion (Gusils et al., 1999).

What is known about the metabolism of the major probiotic organisms within the GI tract?

As mentioned above, probiotic organisms can be divided into two general groups based on their tolerance to O_2, *i.e.* anaerobes and facultative anaerobes. The facultative anaerobe genera, *Bifidobacterium* and *Lactobacillus*, which are used in the manufacture of feed products, are frequently included in probiotic bacteria consortia (Lan et al., 2005).

They reduce the redox potential in the gut and render the environment suitable for obligate anaerobes (Fooks and Gibson, 2002).

Obligate anaerobic bacteria are those species capable of anaerobic fermentation only (Wagner and Cerniglia, 2005). Anaerobic fermentation is the metabolic processes performed by microorganisms that transform substrates, mainly carbohydrates and proteins, to provide metabolites. These metabolites act as electron acceptors via substrate level phosphorylation as opposed to O_2, which is the major ultimate electron acceptor in aerobic respiration (Fooks and Gibson, 2002; Jozefiak et al., 2004). Anaerobic fermentation results in the production of microbial metabolic end-products such as lactate, succinate, acetate, propionate, and butyrate (short chain volatile fatty acids; VFA), H_2, CO_2, CH_4 as well as bacterial biomass (Cummings and Macfarlane, 1997). Most of the VFA formed by intestinal bacteria are absorbed and metabolized by the bird, thereby contributing to the host energy requirements (Cummings and Macfarlane, 1997). Some bacterial metabolites that result from the breakdown and fermentation of proteins, such as ammonia, phenols and amines are toxic (Macfarlane and Cummings, 1999).

Bifidobacterium

Bifidobacteria are believed to be a major component of the microbial barrier to pathogenic bacterial infection (Gibson and Roberfroid, 1995). They are gram-positive, non-spore forming rod or club-shaped bacteria, with distinct cellular bifurcations. *Bifidobacteria* make a significant contribution to carbohydrate fermentation in the colon. Hexoses are fermented via fructose-6-phosphate, by fructose-6-phosphate phosphoketolase (de Vries and

Stouthammer, 1968). The principal end products of fermentation are acetate and lactate, which are produced in a 3:2 ratio (Fooks and Gibson, 2002).

Bifidobacteria produce a wide-range of antimicrobial agents that are effective against both gram-positive and gram–negative organisms. These agents might include antimicrobial peptides, such as defensins, cathelicidins, and lyzozyme (Dommett et al., 2005). In addition to producing antimicrobial agents, *Bifidobacteria* competitively exclude pathogens by competing for epithelial and mucosal binding sites and nutrients.

Bifidobacteria have been reported to alter fecal bacterial enzyme activities, reduce antibiotic induced side effects, inhibit mammary and liver tumors, and they have been reported to, in conjunction with oligofructose, reduce 1,2-dimethylhydrazine, reduce induced colonic carcinogenesis in mice (Marteau, 2000). Oral supplementation of *Bifidobacterium lactis* to elderly subjects increased the production of total, helper $CD4^+$, and activated $CD25^+$ T lymphocytes and natural killer cells (NK); and it increased the phagocytic activity of mononuclear and polymorphonuclear phagocytes and the tumoricidal activity of NK (Gill et al., 2001).

<u>*Lactobacilli*</u>

Lactobacilli are gram-positive, non-spore forming rods, usually non-motile, that are catalase negative, and do not reduce nitrate (Fooks and Gibson, 2002). They are generally accepted as safe to ingest, and have together with *Bifidobacterium*, therefore been granted GRAS status (Salminen et al., 1998). *Lactobacilli* are involved in both homo- and heterofermentation. Homolactic fermentation involves splitting of hexoses into C_3 moieties using fructose-1,6-bisphosphate via the glycolytic pathway. This process yields two

pyruvates molecules which are then converted into lactate. Two moles of ATP are generated per mole of glucose with this type of fermentation. Heterolactic fermentation acts via the pentose phosphate pathway, to produce lactate, CO_2 and ethanol, generating one mol of ATP per mol of glucose. Phosphoketolase is the key enzyme involved in this process (Fooks and Gibson, 2002). *Lactobacilli*, besides producing lactic acid, which is deleterious to many micro-organisms, are also capable of producing antibacterial proteins and bacteriocins (Marteau et al., 2004). Of these, some bacteriocins display a wide antibacterial spectrum against gram-positive bacteria.

What mechanisms of actions have been postulated for how probiotics enhance poultry health and productivity?

Numerous health benefits have been ascribed to probiotics when used in poultry and other species of animals, including man. Although the mechanisms of action associated with the beneficial effects of probiotics are still unclear and likely multifaceted (Bird et al., 2002; Fooks and Gibson, 2002), the single most frequently used term in describing their effects on enteric health is "competitive exclusion" (Edens et al., 1997). This term was originally used to describe the physical blocking of opportunistic pathogen colonization by the colonization of organisms in the probiotic consortia (Klose et al., 2006) (Figure 3A-3D). Others and we speculate that beside competitive exclusion there exist a number of mechanisms the increase *both* enteric and whole-bird health (Bauer et al., 2006; Chichlowski et al., 2006a; Chichlowski et al., 2006b; Hugo et al., 2006). Virtually all actions of probiotics, both physical and chemotaxic, attenuate or eliminate the ability of pathogens to foster diseased

states in the GI tract and other body tissues. Actions of probiotics include alterations in the microbial microenvironments, alteration of the host animal's metabolism, modifications of the host's immune system, improvement in feed digestion and absorption, and the production of antimicrobial compounds (Chichlowski et al., 2006a; Cummings and Macfarlane, 1997; Galdeano and Perdigon, 2006; Ichikawa et al., 1999; Mead, 1989). Potential benefits from the ingestion of probiotics include: 1) the production of β-galactosidase which improves tolerance to lactose (Savaiano et al., 1984); 2) treatment and reduction of intestinal infections (Colombel et al., 1987); 3) the suppression of cancer (Kinouchi et al., 1998; Reddy, 1998); 4) a decrease in the incidence of coronary heart disease (Schaafsma et al., 1998), and others.

What are components of competitive exclusion?

Physical interference

One of the first mechanisms that was proposed as a beneficial action of probiotic bacteria was their ability to physically colonize environmental niches within the intestinal tract, especially in the lower intestinal tract, which is favored by enteric pathogens (Mercenier et al., 2003). Colonization by probiotic organisms may physically exclude colonization by pathogens. For example, colonic crypts and intestinal villi crypts are the favorite sites colonized by *Salmonella* spp. (Garriga et al., 1998; Mare et al., 2006; Servin and Coconnier, 2003). Additionally, work in our laboratory suggests that probiotics may also selectively colonize areas around the opening to villus goblet cells (Chichlowski et al., in preparation).

Lactobacilli, whether attached to epithelial surfaces or multiplying in ingested food, permeate all regions of the digestive tract in poultry (Gusils et al., 1999). Very few studies have investigated adhesion and colonization, because of the complexity of the intestinal mucosa and the extensive interactions that occur between cell types (Sarem-Damerdji et al., 1995). It has been proposed that *Lactobacilli* adhere to the epithelial surfaces by interactions occurring between specific molecules on the bacterial cells and with molecules on the GI surface of the host (Gusils et al., 1999). Henriksson et al. (1991) propose that *Lactobacilli* adhere to the stomach epithelial cells through proteinaceus components located on the bacterial surface. Recently, it was suggested that lactic acid bacteria display various surface determinants, and that these are involved in their interaction with intestinal epithelial cells. Those determinants include passive forces, electrostatic interactions, hydrophobic forces, steric forces, lipoteichoic acids, and specific structures such as external appendages covered by lectins (Servin and Coconnier, 2003).

Competition for binding sites

Probiotics exclude the colonization of pathogens by preventing their adhesion to the epithelium (Kohler et al., 2003). The ability of a probiotic strain to adhere to mucus and epithelial cell surfaces is one of the main selection criteria for a candidate probiotic (Kankaanpaa et al., 2004). Gastrointestinal microbial colonization is related to the bacterium's intestinal ability to adhere and is initiated from the initial ingestion of microbes at the day of age. Attachment is considered to be the very first step in the colonization of host's mucosal surfaces. The binding of bacteria to the mucus layer prevents the bacterial

removal by intestinal peristalsis and is a prerequisite for adhesion to the enterocyte surface (Erickson et al., 1992).

The exact mechanism by which probiotic bacteria prevent the attachment and colonization of pathogens can vary from organism to organism. *Lactobacillus plantarum* inhibits pathogen adhesion without competing for binding sites. This probiotic bacterium induces the transcription and excretion of the mucins MUC2 and MUC3 from goblet cells and thereby inhibits the adherence of EPEC to the intestinal surface (Mack et al., 1999). Another example is a *Lactobacillus* spp. which directly inhibit the attachment of *Salmonella*, *E.Coli* and other food borne pathogens (Duggan et al., 2002). *Lactobacilli* have also been reported to suppress the growth of *Shigella flexneri*, *Salmonella typhimurium*, *Clostridium difficile* and other pathogens (Forestier et al., 2001; Pochapin, 2000). The exact mechanisms of this inhibition are unknown.

Micro-environmental alterations

Probiotics can alter the microenvironments of the GI tract via a very complex and interdependent mechanism that is based on digesta substrate utilization (Cummings and Macfarlane, 1997; Delzenne and Williams, 2002). These alterations often result in changes in the physical environment of the gut in such a manner that opportunistic pathogens cannot compete as well as changes in GI epithelial function and metabolism. Probiotic organisms compete with pathogens for nutrients thus preventing them from acquiring energy to effectively compete in the gut environment (Fooks and Gibson, 2002). The collateral effect of this competition for nutrients is that probiotics produce a variety of organic acids end products such a as volatile fatty acids (VFA) and lactic acid as a part of their metabolism of

nutrients in the gut digesta (Mead, 1989). These weak organic acids lower the pH of the gut environment below that essential for the survival of such pathogenic bacteria as *E. coli* and *Salmonella* (Isolauri et al., 2004).

Volatile fatty acids serve as energy yielding substrates to the host animal, in addition to inhibiting the growth of pathogenic bacteria. Volatile fatty acids can affect colonic epithelial cell transport, colonocyte metabolism, growth and differentiation, hepatic control of lipid and carbohydrates, and they provide energy to the muscle, kidney, heart and brain (Gibson, 1999). Volatile fatty acids are rapidly absorbed from the small intestinal tract and colon, stimulate electrolyte and water absorption within the intestinal tract and have a major effect on the growth of epithelial cells. The colonic epithelium derives 60-70% of its energy from bacterial fermentation (Cummings and Macfarlane, 1997).

Production of antimicrobial substances

Probiotic organisms are known to produce a class of small, antimicrobial molecules that are collectively known as *bacteriocins* (Marteau et al., 2004). These bacteriocins can kill pathogenic bacteria or impede their colonization (Marteau et al., 2004). They are proteins, or protein complexes, produced by certain strains of bacteria, which can have antagonistic actions against species related to the producer bacterium. Other, non-bacteriocin compounds which inhibit the growth of pathogens are also produced by probiotic bacteria (Meghrous et al., 1990). The polyamine derivate piperidine, which is also produced by intestinal microflora as the result of amino acid degradation, has been shown to inhibit the binding and internalization of *Salmonella* and *Shigella* to intestinal epithelial cells *in vitro* (Kohler et al., 2002).

Do probiotics have an effect on maintenance of epithelial barrier integrity?

One of the major secondary functions of the GI tract is to act as a protective barrier, which shields the body from organisms and substances that do not serve as nutrients (Bar-Shira and Friedman, 2006). There are two major mechanisms which epithelial barrier maintains integrity. The mucous barrier or "blanket" (Figure 1D), which is composed of a complex mixture of proteins and carbohydrates and the tight junctions or *zona occludens* (tight junctions) via which the intestinal epithelial cells attach to one another to form an unbroken, contiguous biological barrier. Mechanisms of probiotic action are closely related to both of them.

Mucous layer

A relatively thick layer of mucus that is secreted by goblet cells in the gut wall covers the epithelial cells in the small intestine. This mucus consists of mucin, many small associated proteins, glycoproteins, lipids, and glycolipids (Gusils et al., 2003). It also contains soluble receptors that recognize specific adhesion proteins (Metcalfe et al., 1991). Both pathogens and probiotic bacteria adhere to specific glycoconjugates on the microvilli in the small intestinal tract.

Access to the Epithelium

Non-nutrient substances and microorganisms can enter the body through the GI tract in one of several ways; including transepithelial transit, paracellular transit and through the "M" cells that make up about 1% of the total cell population in the intestinal tract (Macpherson and Harris, 2004; Marteau et al., 2004). Normally, these mechanisms function

effectively in preventing the entrance of antigens and pathogens that could prove harmful to the bird into the blood stream. Indeed, the function of "M" cells themselves may be important in acclimatizing the animal's immune system to non life-threatening antigens (Pohlmeyer et al., 2005). Furthermore, Tyrer et al. (2006) suggested that pathogen-associated molecular pattern interactions with pattern recognition receptors are key factors in "M" cell recognition of intestinal antigens for mucosal immune priming.

Tight Junctions

The development of intestinal barrier function is determined by the assembly of tight junction and adherent junction proteins (Kohler et al., 2003). That is also the most vulnerable point of intestinal bacteria penetration, however, there is a very scarce data regarding effects of probiotic on tight junction structure. Tight junctions can be viewed by electron microscopy as a series of discrete contacts between plasma membranes of adjoining cells containing a multiprotein complex that affiliates with the underlying actin cytoskeleton (Nusrat et al., 2001). The tight junction and so-called adherens junction are known as the *apical junction complex*. The adherens junction in the intestine has been documented to be a target for infectious diseases (Barton et al., 2001). Furthermore, infectious agents induce inflammatory response associated with an influx of leukocytes and the release of cytokines such as interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α, not identified in birds yet), both of which are known to modify tight junction structure and paracellular permeability across epithelial cells (Nusrat et al., 2001).

PUFA and probiotics

It is possible that consumption of specific PUFA may enhance the intestinal barrier (Bomba et al., 2002). Claudins and occludin have been identified as tight junctions specific integral membrane proteins (Furuse et al., 1998), which are regulated by immune mediators and whose expression restricts paracellular transport of macromolecules. Occludin is approximately 65kDa and is attached to the cytoskeleton by another group of proteins including cingulin and 7H6 (Jiang et al., 1998). Occludin, which plays a major role in tight junctions, was up regulated by γ-linolenic acid (GLA; 18:3, ω-6) and by eicosapentaenoic acid (EPA; 20:5, ω-3) but was down regulated by arachidonic acid (AA; 20:4, ω-4) and linoleic acid (LA; 18:2, ω-6; (Jiang et al., 1998).

The extra cellular domain of occludin binds to another occludin molecule on an adjacent cell and thus forms a tight junction (Furuse et al., 1996). Pathogens aim to destroy the integrity of the epithelial barrier to gain easy access to the gut interstitium, which allows further access to the blood stream and systemic spreading of the organism (Kohler et al., 2003). Probiotics are known to enhance the epithelial barrier. Madsen et al. (2001) have shown that a commercial mixture of various *Bifidobacterium* and *Lactobacilli* strains can enhance the epithelial barrier in IL-10 knock-out mice.

Nutrient transport

The trophic effects of probiotics include increases in the specific and total activities of the brush-border membrane enzymes in the jejunal mucosa of growing rats (Marteau et al., 2004). After oral treatment of rats with *S. boulardii*, there was a marked stimulation of sodium dependent D-glucose uptake into the brush border membrane vesicles with a

corresponding increase of the sodium D-glucose cotransporter-1, SGLT-1 (Buts et al., 2002). It has also been also reported that the oral administration of *Lactobacillus casei* increased the crypt cell production rate of the jejunum, ileum, cecum and distal colon in rats (Ichikawa et al., 1999). Additionally, Chichlowski et al. (2006b) have reported that probiotic consortium increases passive absorption of glucose in the chicken ileum.

What is the effect of probiotic on maintenance and enhancement of intestinal immune function?

Introduction to mucosal immunity

The intestinal immune system has several unique elements to maintain the equilibrium with bacterial flora and the rest of the organism (Mowat, 2003). There is a constant interaction among different cell types, including members of the innate and the adaptive immune systems, and bacteria in the gut lumen, epithelium or lamina propria (Eberl, 2005). Furthermore, the epithelial lining is an important permeability barrier between the external environment and the internal body environment (Nusrat et al., 2001). The dendritic cells sample bacterial antigen, and migrate to the T-cell zones within the epithelium (Macpherson and Uhr, 2004). Presentation of microbial antigen to immune response cells can be to both $CD4^+$ helper cells and to $CD8^+$ cytotoxic/suppressor cells. Then, adaptive immune response can be induced, that can lead to effector T-cells and immunoglobulin A (IgA) production (Niess et al., 2005). Probiotics may reach the inductive mucosal immune system through several routes including the specialized M cells near the Peyers patches and dendritic cells of the small intestinal tract (Marteau et al., 2004). Mucosal epithelium

contains at least two types of immunocompetent cells, the intraepithelial lymphocytes and the intestinal epithelial cells (Donnet-Hughes et al., 2001). Also, epithelial cells express many important immune molecules and a wide range of cytokines, which modulate their interaction with T lymphocytes (Strober, 1998). The number of Peyer's patches (lymphoid aggregates) and Ig A producing cells increase in the presence of the intestinal microbiota (Isolauri et al., 2004) which constructs an immunological barrier of the gut mucosa. It has been suggested that surface Ig A attached to the mucosal membrane may limit or inhibit the adherence of enteropathogens that are invading the GI tract (Perdigon et al., 1990). Furthermore, the intestinal microbiota provide an important stimulus for the maturation of the immune system (Isolauri et al., 2004).

Th1 and Th2 responses

The cytokines released by intestinal immune cells in the presence of bacteria, may indicate either a Th1 immunogenic response or a Th2 humoral response (Gonnella et al., 1998). Th1 cell-driven responses are exemplified by a delayed-type hypersensitivity reaction, together with regulation of phagocytes. More specifically, Th1 cells secrete interferon-γ (IFN-γ), tumor necrosis factor (TNF; not identified in chickens yet), IL (interleukin)-2, IL-12 and are central to the development of cellular immunity against intracellular pathogens. Th2 cells promote Ig E production and eosinophil activity (Donnet-Hughes et al., 2001). Th2 cells produce IL-4, IL-5 and IL-13 and promote allergic inflammation (Avery et al., 2004; Das, 2002). This is especially true for IL-4 and IL-5, which induce the production of Ig E and IgG1 by B lymphocytes (Romagnani, 1997). The differentiation of naïve Th cells into Th1 and Th2 is influenced by cytokines that are present

during and after antigen presentation. These naïve Th cells produce all of these cytokines and are considered the precursor of the Th1 and Th2.

Establishment and maintenance of intestinal tolerance is mostly dependent on suppressive cytokines such as IL-10 and transforming growth factor-β (TGF-β) produced by regulatory T cells and T helper cells characteristic of the intestinal immune system (Izcue et al., 2006; Pessi et al., 2000). Probiotics seem to shift the Th1/Th2 response balance toward Th1 (Das, 2002), they also augment the production of TGF-β, IL-10 and Ig A (Gaskins, 1997). For example, the expression of the key proinflammatory cytokine (Th2), IL-8 was inhibited by a probiotic consortium (Vidal et al., 2002). Furthermore, *Bifidobacteria* isolated from infants suffering from allergic diseases induced the secretion of TNF-α, IL-1β, IL-6 and IL-12 by macrophages *in vitro*, while B*ifidobacteria* from healthy infants stimulated the secretion of IL-10 (He et al., 2002). Also, non-pathogenic enteric microbes, like *Bifidobacteria*, exert an immunosuppressive effect on intestinal epithelial cells by inhibition of the transcription factor NF-κB pathway (Neish et al., 2000). The tolerogenic effects of the gut microbiota may partially be mediated by generation of regulatory T cells (Singh et al., 2001), however, for immune regulation; specific adherence properties may be required (O'Hara et al., 2006).

Cytokines produced by epithelial cells transmit information on the relative state of intestinal health to intra-epithelial T lymphocytes and immune cells in the underlying lamina propria (Gaskins, 2003). Cytokines play an important role in these mucosal humoral and cell-mediated responses (Peters et al., 2005). $CD4^+$ T helper cells exert their effect through the secretion of pro-inflammatory (IL-1β, IL-6) or immunomodulatory (IL-10) cytokines (Strober, 1998).

In one study, *Lactobacillus casei* fed mice have shown an increase in IL-10, while a significant induction of IL-2 and IL-12 was observed in mice fed *L. acidophilus* (Perdigon et al., 2002). These effects were dose dependent. It has also been reported that a commercial probiotic consortium, containing strains of *Lactobacilli* and *Bifidobacterium* could enhance the epithelial barrier in IL-10 knock-out mice which serve as a model for Inflammatory Bowel Disease (Kohler et al., 2003). Madsen and co-workers (Madsen et al., 2001) have also demonstrated that the production of proinflammatory cytokine secretion is down-regulated by attenuation of the NF-kB pathway.

Signaling pathways

Probiotics may have the ability to directly influence the inflammatory response elicited by pathogens by downregulating a specific signaling pathways (Kohler et al., 2003). There are several pathways proposed for activation of immune response by gut microflora or when cells are infected by a variety of pathogens, including MAP kinase and NF-κB pathways. Also, several enteric organisms have been shown to inhibit these pathways, and thus the subsequent induction of inflammatory mediators (Neish et al., 2000). Active or acute inflammation of the intestinal epithelium triggered by the bacterial antigens is associated with the migration of polymorphonuclear leukocytes from the microvasculature into the lumen (Nusrat et al., 2001). The primary polymorphonuclear leukocyte in poultry is heterophil, the avian equivalent to mammalian neutrophil (Kogut et al., 2006). These cells are involved in the phagocytosis and killing of invading microbes. Heterophils activate MAPK signaling cascade leading to the upregulation of pro-inflammatory gene expression (Kogut et al., 2005).

Adenosine, in particular, seems to play a central role in mediating neutrophil-epithelial crosstalk in most species. Adenosine is generated from neutrophil-derived 5'AMP through protein kinase A (PKA) and it stimulates trans-epithelial chloride flux, up-regulates CD73 (which possesses the enzymatic activity of 5'nucleotidase), and induces IL-6 secretion by epithelial cells, which in turn activate neutrophils to modulate inflammation (Nusrat et al., 2001). However, it has been demonstrated that *Salmonella typhimurium* activates a protein kinase C (PKC)-dependent signal transduction pathway, which is independent of NF-κB, and influences transepithelial neutrophil movement (Kohler et al., 2003). The PKC is a cellular receptor for the second lipid messenger diacylglycerol (DAG) and is therefore a crucial element in signal transduction pathways. In addition, the cyclic-AMP (cAMP) production activated by microflora, can also have a profound effect on pro and anti-inflammatory cytokines production, which increases IL-10 production by monocytes, increases IL-8 production by mast cells, and increases IL-6 production by astrocytes (Singh et al., 2006). Further, an inflammatory state can modify tight junctions and paracellular permeability across the intestinal epithelium (Shen and Turner, 2006).

Clearly, all DFM do not share the same immunomodulating properties, and can even have opposite effects on some parameters (Marteau et al., 2004). Mercenier et al. (2003) reported large variation in the ability of different *Lactobacillus* strains to induce pro- and anti-inflammatory cytokines. Colonization of the small intestine with commensal organisms may result in altered sensitivity to various luminal stimuli. These considerations may be particularly important during animal growth, given the energetic costs likely associated with intestinal secretory responses (Gaskins, 2003).

It has been shown that probiotic bacteria may cause the intestinal epithelial cells to limit immune activation by inhibiting the ubiquitination and degradation of the IκB signaling pathway (Neish et al., 2000). Ubiquitination is a common covalent modification of cellular proteins that serves a number of regulatory functions, often targeting modified proteins for the control of cellular degradation. The results lead to a significant reduction in the amount of IL-8 that is secreted from the intestinal epithelial cells. Also, the lipoteichoic acid from *Lactobacillus johnsonii* and
L. acidophilus has been shown to downregulate the inflammatory response in epithelial cells to lipopolysaccharide and enteric bacteria.

Toll-like receptors

The host's innate immune response distinguishes signals from pathogens and commensals via Toll-Like Receptors (TLR) (Marteau et al., 2004). TLRs are the highly conserved receptors existing in most animal cells and are important initiators of innate immunity (Doyle and O'Neill, 2006). The TLRs mediate mechanisms of intestinal epithelial tolerance versus intolerance (Cario and Podolsky, 2005). The immune cells express multiple TLRs in order to recognize the specific microbial environment involved, and trigger an appropriate adaptive response as well as to induce antimicrobial effector pathways, which eventually leads to efficient elimination of host-threatening pathogens (Kobayashi et al., 2002). Immunomodulation may be due to an increased transport of antigens across the mucosal barrier (via increased intestinal permeability) or to an up-regulation of antigen presenting molecules and co-stimulatory molecules on immune cells (Marteau et al., 2004).

CONCLUSIONS

It is likely that the beneficial effects of probiotics are the result of the summation of a complex, multi-variate series of alterations in gut microbial and whole body metabolism. Those alterations might include whole body and tissue oxygen consumption, absorption of nutrients, production of cytokines, as well as configurations and intestine histomorphometry. Data described above demonstrate also that some probiotic species can communicate with the epithelium and immune system, modulating tissue physiology and response to host's infection. Overall, probiotic products are putative alternative to growth promoting antibiotics. However, more research is necessary to define mechanisms of action of probiotics.

FIGURES

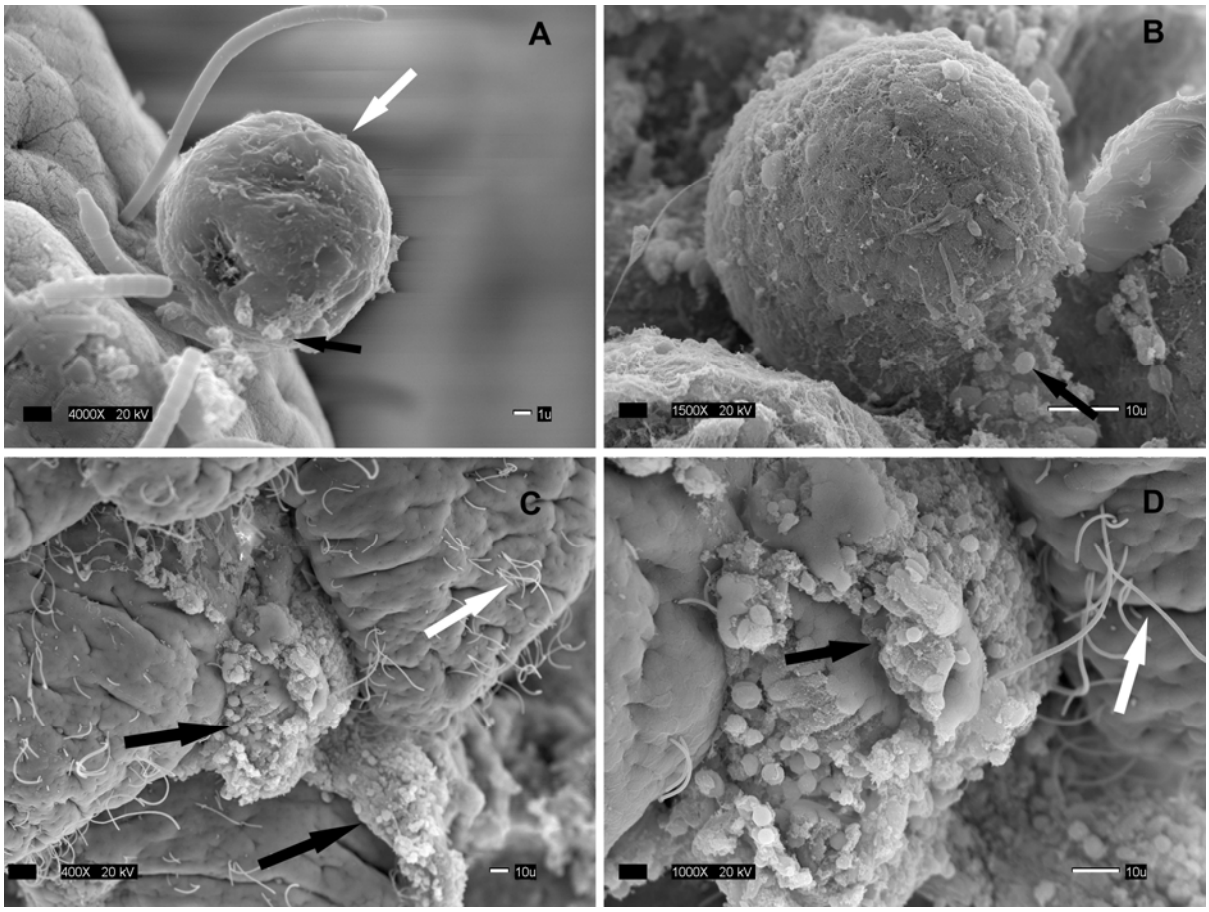

Figure 1. SEM micrograph of chicken ileal mucosa at d 21 after hatch. Tissues were fixed in 1%OsO_4 and examined using JEOL 5900LV microscope at 20kV. A, B: several microorganisms (black arrows) are visible attached to starch granules (white arrow) in the ileal lumen; C, D: mucous blanket seen between ileal villi (black arrows), several SFB are also noticeable (white arrows)

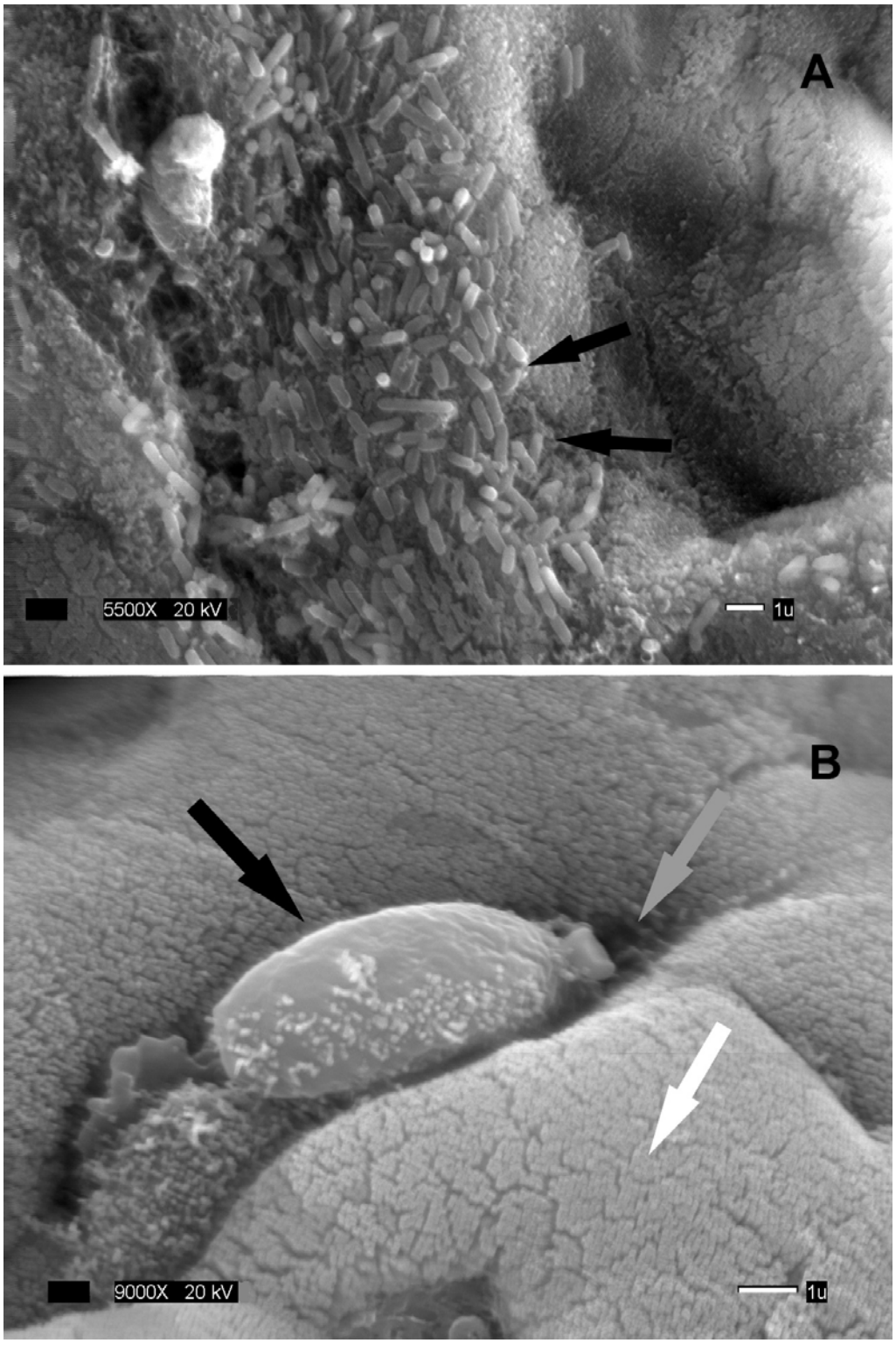

Figure 2. SEM micrograph of chicken ileal mucosa at d 21 after hatch. Tissues were fixed in 1% OsO_4 and examined using JEOL 5900LV microscope at 20kV. A: SEM micrograph of cecal epithelium, multiple microbobes are visible and are attached to the surface of the enterocyte microvilli (black arrows); B: microorganism (black arrow) seen near a goblet cell (gray arrow) in the chicken ileum; white arrow indicates epithelial brush border

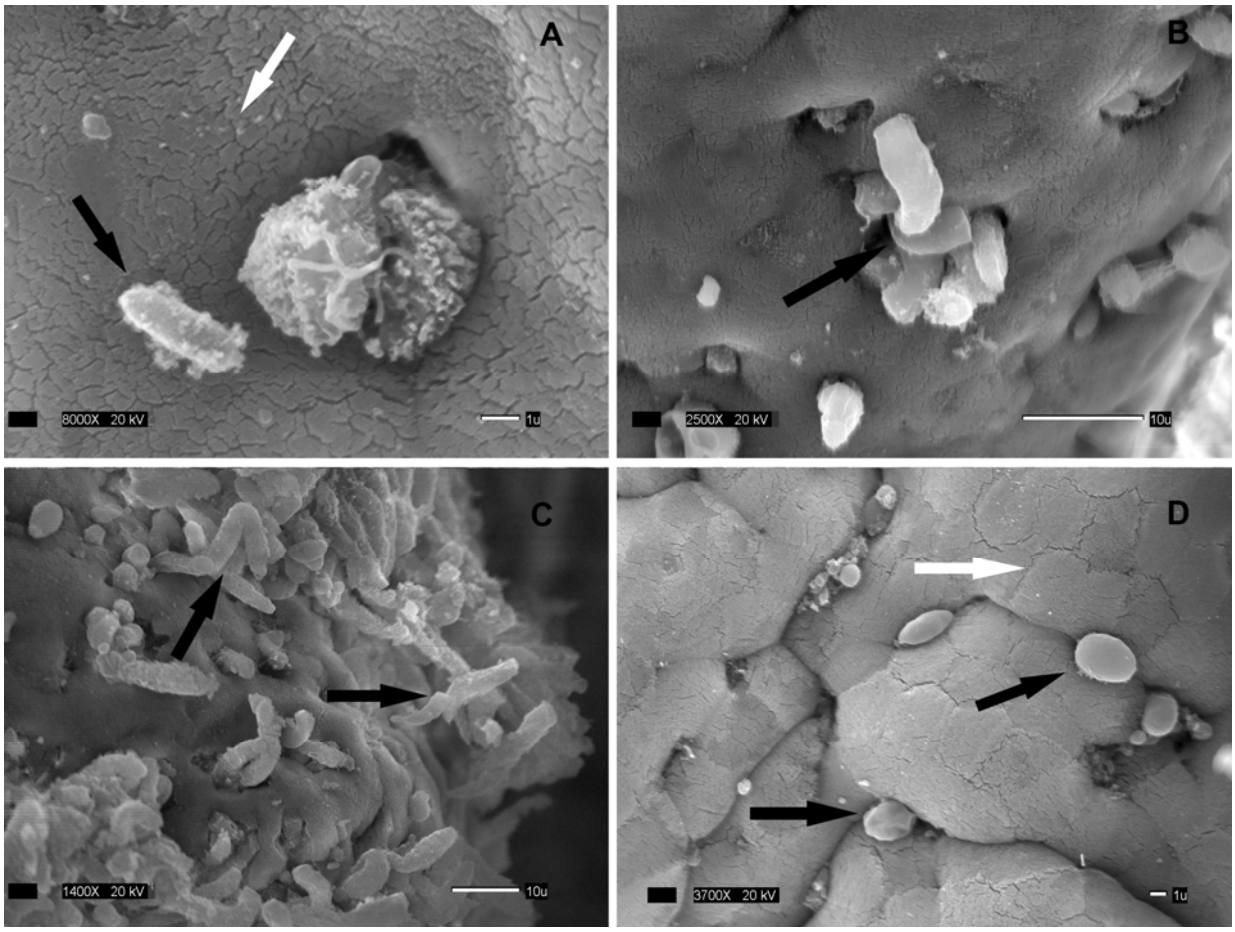

Figure 3. SEM micrograph of chicken ileal mucosa at d 21 after hatch. Tissues were fixed in 1%OsO_4 and examined using JEOL 5900LV microscope at 20kV. A, B and C: various probiotic organisms attached to epithelial tissue in the chicken cecum (black arrows); white arrow indicates epithelial brush border; D: ileal microvilli surface with several organisms (black arrows) attached in the transversal furrows of the villus, white arrow shows enterocyte border in the mid villus

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CHAPTER 2

DIRECT-FED MICROBIAL AND SALINOMYCIN MODULATE WHOLE BODY AND INTESTINAL OXYGEN CONSUMPTION AND INTESTINAL MUCOSAL CYTOKINE PRODUCTION IN THE BROILER CHICK[1]

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ABSTRACT

A direct-fed microbial (DFM) is a live-microbial food supplement that improves health and performance, in broilers and other animals. No studies have described their effects on whole-animal or intestinal metabolism. Previous research has shown that intestinal inflammation is an energy consuming process. This study investigated whole-body O_2 consumption, intestinal O_2 consumption and intestinal inflammation state through mucosal cytokine production on broiler chicks fed the DFM, PrimaLac®. One hundred and twenty, 1-d-old broiler chicks were randomly assigned to one of three experimental diets: standard starter diet (control; CON); standard starter diet + salinomycin (SAL), and standard starter diet + PrimaLac® (DFM). Birds were housed in two separate rooms, the CON and SAL treatments in one room and the DFM in another. Water and feed were provided *ad libitum* and BW and feed intakes recorded. Intact ileal and cecal samples were collected on d 19, 20, and 21 after whole-body oxygen measurement using indirect calorimetry. The O_2 uptake of ileal tissue was measured using an *in vitro* O_2 monitor. Analysis of immune status of chicken broilers was measured by the relative differences in mRNA of both pro- and anti-inflammatory cytokines: IL-1beta, IL-6, and IL-10 using Real-Time RT-PCR. Broilers exhibited a 6-16% decrease in whole-body energy expenditures and a 47% decrease ($P<0.05$) in ileal energy expenditures. The RT-PCR data demonstrated that this DFM consortium numerically altered both pro- and anti-inflammatory cytokines within the ileum of 19 d post-hatch broilers. These data suggest that direct-fed microbials like PrimaLac® increase metabolic efficiency via changes in intestinal physiology and metabolism.

Key words: broilers, direct fed microbials, body energetics, cytokines

INTRODUCTION

There is an increasing interest in finding alternatives to antibiotics in poultry production because of growing concerns about antibiotic resistance, the ban on antibiotics usage in Europe, and the potential for a ban in US (Patterson and Burkholder, 2003). One putative choice could be direct fed microbials (DFM), also referred to as probiotics, which are "live microbial feed supplements, that beneficially affect the host animal by improving its intestinal health" (Fuller, 1989). The DFMs can be composed of one or several different species of micro-organisms including bacteria and yeast (Patterson and Burkholder, 2003). The DFM colonization characteristics of bacterial species can differ (Isolauri et al., 2004). Additionally, different strains of the same species of DFM can have unique biological activity, such as different sites of adhesion, specific immunological effects, and fermentation characteristics (Isolauri et al., 2004). Despite advances in microbial molecular biology and the availability of significant amounts of genome sequences for most commensal intestinal bacteria, the current understanding of the biological actions of DFM microflora is incomplete (Mai, 2004). This is especially true of the effects of DFM on whole body and organ tissue energy expenditures. The gastrointestinal tract (GI) of most animals has been estimated to consume 25% of total energy needs (Cant et al., 1996). The immune system has been estimated to account for approximately 1% to 3% of the basal metabolic rate in healthy vertebrates (Romanyukha et al., 2006). In contrast, immunologically challenged vertebrates can have resting metabolic rates that are increased from 8% to 27% (Martin et al., 2003), which might suggest that immunological challenge increases energy consumption.

Interaction between DFM bacteria and the intestinal epithelium is referred to as "cross-talk", and is currently the object of intensive investigation (Kohler et al., 2003). The beneficial effects of DFM bacteria are dependent on the interaction with the innate immune system and possibly modulation of adaptive immunity (Kohler et al., 2003; Tien et al., 2006). Commensal bacteria have the ability to suppress inflammatory responses by inhibiting specific intracellular signal transduction pathways (Nusrat et al., 2001). Enteric commensal bacteria could be signaling epithelial cells to dampen host inflammatory responses via direct communication with cells of the innate intestinal immune system as well as enterocytes (Gaskins, 2003; Peters et al., 2005). It is well-established that most immuno-inflammatory effector genes, including IL-8, IL-6, and others, are controlled at the transcriptional level (Nusrat et al., 2001).

To our knowledge, there have been no reports in the literature concerning the effects of DFM or prophylactic ionophore salinomycin (SAL) on whole-body or tissue energy utilization in any species. This study was designed to estimate changes in whole body energy expenditures in chicks supplemented with a DFM. Additionally, since the gastrointestinal tract and the immune system require large amounts of energy, of special interest was the effect of the DFM on intestinal energy consumption as well as its effect on concomitant changes in the innate immune system of the broiler chick's intestinal tract.

MATERIALS AND METHODS

Experimental Design

One hundred twenty (Trial 1) and sixty (Trial 2) 1-d-old broiler chicks were placed on a standard corn-soybean meal diet (17.08 % CP, 2.4 % fat, and 2830 kcal ME/kg). All of the broilers were housed, maintained and euthanized under an approved protocol from the Institutional Animal Care and Use Committee (IACUC) at North Carolina State University. The objective of Trial 1 was to determine whether DFM and salinomycin supplementation affects whole-body or intestinal tissue respiration. In Trial 2, the objective was to measure treatment differences in intestinal length, weight, as well as serosal and mucosal dry matter and organ weight. Additionally, intact ileal cytokine expression was measured.

A completely randomized design was used for the two trials. Chicks from each treatment were randomly selected for experimental measurements, so the average age of the chicks was 21 d at the time of measurements. Chicks were assigned to one of following treatments: CON (no additives,), SAL (salinomycin, 50ppm of feed) and a DFM consortium (Primalac®, Clarksdale, MO; 0.3% of a diet). Primalac® was added as a lyophylyzed mix containing 1 x 10^8 CFU/g* of *Lactobacillus casei*, *L. acidophilus*, *Bifidobacterium thermophilium*, and *Enterococcus faecium*. This level of DFM supplementation was chosen to ensure thorough colonization in the intestines of treated chicks. Salinomycin was chosen as our negative control because of its widespread use in the poultry industry as a coccidiostat, and its antimicrobial properties against gram-negative organisms (Duffy et al., 2005). Feed intake was also recorded throughout all the study.

Chicks were placed after hatch in Petersime batteries; CON and SAL were housed in batteries in a separate room from DFM group with single pass air. In order to prevent cross-

* As reported by Star Labs Inc.

contamination, access to birds was restricted to essential personnel and all personnel were required to enter the control room prior to entering the DFM room, and were not allowed to re-enter the control room without showering and changing clothes. Chickens were fed the respected treatment diets for 21 d, and fasted for 12h prior to sample collection on d 21.

Individual bird data were regarded as the experimental units. The data from each trial were analyzed using a one way ANOVA statistical program, STATISTIX® 8 (Analytical Software, Tallahassee, FL). Fisher's Least Significant Difference was used to test differences between means only when the ANOVA indicated significance at $P \leq 0.05$ (Motulsky, 2005).

Sample collection

On d 21 Trial 1, whole-body O_2 consumption was measured after a 12 h feed deprivation period. Thereafter, the chicks were euthanized by cervical dislocation and ileal and cecal tissue samples were collected for O_2 consumption analysis. All tissue sampling was completed within 15 min after euthanasia. Ileal samples were obtained 2 cm above ileo-cecal colonic junction. In Trial 2, birds were fasted for 12 hours, weighed and euthanized by cervical dislocation. The abdominal cavity was exposed and two sections, one at the gizzard-duodenal junction, the other at the end of the colon, were made to excise the small and large intestine, cecum and colon. Liver, crop, gizzard, bursa of Fabricius and pancreas were also removed and weighed. All organ weights were expressed per g of fasted BW. After blotting dry, the total GI tract was weighed and its unstretched length was measured. The weight and length of the doudenum (pyloric sphincter to bile duct), jejunum (bile duct to yolk stalk), ileum (yolk stalk to ileo-cecal junction), cecum, and colon were also recorded. Portions of

each intestinal segment were rinsed in ice-cold 0.9% NaCl (wt/vol), blotted dry, weighed, and the mucosa was gently removed by scrapping with the edge of a glass microscope slide. The remaining muscularis externa and serosa were weighed and the weight of the mucosa was calculated by difference. The amount of dry matter in the intestinal mucosa, serosa or intact tissues was determined by drying at 80°C in a forced air oven for 48 h. Approximately 100 mg of the ileal tissue collected was placed in "RNA later" (Ambion, Austin, TX) for subsequent analysis of cytokine mRNA.

Whole-Body Oxygen Consumption

Whole-body O_2 consumption was measured using an O_2-ECO (Columbus Instruments Int., Columbus, OH) indirect calorimeter. Birds were placed in measurement chambers with airflow of 4.0 L/min. Oxygen and CO_2 measurements were initiated after the bird was in the measuring chamber for 20 min to allow for behavioral adjustment. Whole-body O_2 consumption and CO_2 expiration were measured for 3 consecutive 60 sec periods, and the mean value was calculated. The BW of each bird was measured immediately after the repeated measurements of gas exchange. Whole-body O_2 consumption and CO_2 expiration were expressed as μmol O_2 or CO_2/min.

Ileal and cecal oxygen consumption

Each ileal and cecal sample was longitudinally cut and divided into two 20- to 40-mg pieces. The O_2 consumption rates of intact ileal and cecal tissue were monitored in constantly stirred buffer containing 11g M199 (Sigma Chemical Co., St. Louis, MO), 5.96g HEPES, and 0.36g $NaHCO_3$ in 1 L of deionized water at 37°C using an incubation chamber (YSI,

Yellow Springs, Ohio) fitted with an O_2 electrode as previously described (Fan et al., 1997). Tissue O_2 consumption was expressed as nmol O_2/min/mg.

Real time RT-PCR

Real time RT-PCR was used to assess the level of gene expression for three cytokines. Relative levels of interleukin 1-β (IL-1β) and IL-6 (both pro-inflammatory cytokines) and IL-10 (an anti-inflammatory cytokine) were determined as indicators of general mucosal innate immune activity. Total RNA was isolated from 100 mg of intact ileal tissue by phase separation, and treated with DNase I (Promega Corporation, Madison, WI). Samples were incubated at 37°C for 30 min with DNase I. The DNA-free total RNA was quantitated and an equal mass from each sample (1 μg) was used to generate a cDNA library using random primers (5 μl of M-MLV 5X Reaction Buffer, conc. 200u/μl; 1.25μl of Random Primers Mix, conc. 500μg/ml; 0.625μl of Recombinant RNasin® Ribonuclease Inhibitor, conc. 40u/μl; Nuclease-Free Water to final volume 25μl; Promega Corporation, Madison, WI). This mixture was then incubated for 60 min at 37°C. The relative level of each of the above genes was assessed in individual reactions using gene specific primers and dual labeled probes, IL-1β fwd (GCTCTACATGTCGTGTGTGATGAG), IL-1β probe (FAM-CCACACTGCAGCTGGAGGAAGCC-BHQ), IL-1β rev (TGTCGATGTCCCGCATGA), IL-6 fwd (GCTCGCCGGCTTCGA), IL-6 probe (FAM-AGGAGAAATGCCTGACGAAGCTCTCCA-BHQ), IL-6 rev (GGTAGGTCTGAAAGGCGAACAG), IL-10 fwd (CATGCTGCTGGGCCTGAA), IL-10 probe (FAM- CGACGATGCGGCGCTGTCA-BHQ), IL-10 rev (CGTCTCCTTGATCTGCTTGATG) (Rothwell et al., 2004). A 5μl quantity of cDNA

template was used for a reaction mix (qPCR Core kit; Eurogentec, San Diego, CA). Expression of each gene was normalized within each treatment group by also measuring the expression of the housekeeping gene: 28s fwd (GGCGAAGCCAGAGGAAACT), 28s probe (FAM- AGGACCGCTACGGACCTCCACCA-TAMRA), 28s rev (GACGACCGATTTGCACGTC; Integrated DNA Technologies, Coralville, IA) (Rothwell et al., 2004). The reaction was carried out using a BioRad iCycler. The cycle profile was as following: one cycle of 95°C for 10 min, and 45 cycles of 94°C for 30 sec (step 1) and 59°C for 1 min (step 2). The cycle threshold (C_t), a cycle at which the change in the reporter dye passes a significant threshold, was calculated for each reaction, and changes in expression of the different cytokines are reported using the ΔC_t [$\Delta C_t = (C_{t\ (\text{gene of interest treatment n})} - C_{t\ (\text{28s treatment n})})$]. Positive ΔC_t results reflect a decrease in gene expression relative to the control population and negative ΔC_t results reflect an increase in gene expression relative to the controls.

RESULTS

Body weight gain and feed intake in CON, SAL, and DFM groups were calculated in Trial 1 at 18 d of age (Table 1). The SAL treatment had significantly lower BW, BW gain, feed intake and feed conversion compared to the CON and DFM treatments ($P = 0.0001$, 0.0001, 0.015, and 0.0001, respectively). There were no significant differences in BW gain or feed intake between the CON and DFM treated birds.

Cecal, colonic, ileal and jejunal mucosal and serosal DM contents were not greatly affected by the treatment, with the exception of the ileal mucosa from the DFM birds, which had a lower DM compared to CON and SAL treated birds ($P < 0.02$; Table 2). Differences between treatments in weight of intestinal segments adjusted for fasted BW (mg/g FBW) were significant only in the jejunum, where DFM treated birds had the lowest weight ($P < 0.001$; Table 3). No differences were observed between CON and DFM treated birds, but SAL birds had longer intestinal segments, total intestinal length, cecum and colon when adjusted for BW ($P < 0.05$; Table 3) than those two treatments. Salinomycin decreased intestinal segment density (mg/cm) compared to CON and DFM. The DFM decreased jejunal and density in comparison to CON group ($P < 0.001$).

The DFM treatment decreased liver weight adjusted for fasting BW compared to the CON birds ($P = 0.04$), while the proventriculus was smaller in the CON group ($P = 0.004$). There were no significant differences between treatments in bursa, pancreas, or gizzard weight.

Whole-body O_2 consumption and CO_2 production values are listed in Table 4. Data presented herein are in the range of those reported previously (Fan et al., 1997). Whole-body O_2 consumption adjusted for FBW was 16% less ($P < 0.05$) in the DFM group, than in the SAL treatment. The DFM treated birds had the lowest numerical adjusted whole-body O_2 consumption of all treatments, although this difference was statistically significant ($P < 0.05$) only in comparison with the SAL treatment. There were no differences in adjusted whole-body CO_2 expiration amongst treatments. There were also no differences in respiratory quotient (CO_2 production/O_2 consumption) between the treatments.

Total ileal O_2 consumption was lower in the DFM group than in the SAL-treated birds ($P = 0.01$;Table 5, Figure 1). Although there was a numerical decrease with DFM, no statistically significant differences were noted between the DFM and CON groups. Non-significant ($P > 0.05$) numerical decreases were noted for total cecal O_2 consumption when the DFM birds were compared with the CON and SAL birds.

Interleukin mRNA measured in this experiment were selected based on their possible overall indication of an inflammatory state in the chicken gut (Isolauri et al., 2001). The DFM treatment provided lower expressions than estimated for IL 1β and IL 6 (Figure 2), both pro-inflammatory cytokines, in the chicken ileum, than the CON and SAL diets; but the differences observed were not significant. IL 10 mRNA, an anti-inflammatory cytokine, was elevated in the DFM group in comparison with the rest of the treatments.

DISCUSSION

To our knowledge, this is the first study to describe a link between the feeding of DFM or salinomycin with changes in whole-body O_2 consumption and with changes in the innate immune system of the gut. Feeding of the DFM, PrimaLac®**,** decreased whole-body O_2 consumption by 17% when compared with the feeding of SAL. Marked differences were also noted in ileal O_2 consumption from the feeding of the DFM as compared with the feeding of SAL, where the O_2 consumption rate was 47% less in the DFM than in the SAL fed birds. It should also be noted that differences in a broad array of parameters including BW gain and feed intake were adversely affected by the feeding of salinomycin in the

present study. This is puzzling, given the common use of salinomycin as an anticoccidial treatment within the poultry industry (Scalzo et al., 2004), and the fact that the 50 ppm level utilized is well below the 80-160 ppm concentration for which salinomycin toxicity has been reported in chickens (Keshavarz and McDougald, 1982). However, there have been reports of salinomycin intoxication in turkeys at the level of 60 ppm of the feed (Van Assen, 2006). Salinomycin exerts its action by its ability to insert itself into membranes thereby increasing the intracellular flux of K^+ and other cations such as Na^+ (Mitani et al., 1976). Ionophores, like salinomycin, do not discriminate between bacterial and mammalian membranes to affect cellular ion transport capacity (Butaye et al., 2003). In the mature bird there are more microbial cells in the gut than avian cells within the body (Lin, 2003). In the present study, birds on all treatments were kept under very clean conditions within properly maintained brooder batteries in well-ventilated bird rooms with filtered air. Under these conditions, the intestinal allocthonous bacteria load for SAL treated birds may have been reduced such that excess salinomycin may have been available for binding directly to the intestinal enterocytes. Salinomycin is known to bind to cellular and mitochondrial membranes, ultimately increasing intramitochondrial K^+, disrupting cytoplasmic and mitochondrial redox potential and oxidative phosphorylation (Mitani et al., 1976; Williams, 2005). This may explain the increase in ileal O_2 consumption and subsequent increased whole body O_2 consumption in the SAL birds in comparison with the DFM birds. Fan et al. (2003) reported that another polyether ionophore, laidlomycin propionate, might have adverse effects on enterocyte function. As a result of this phenomenon, it is possible that the SAL treatment resulted in an induced salinomycin toxic state as opposed to the more natural physiological states represented by the O_2 consumption in the CON and DFM birds. Histological and scanning

electron microscopy studies in this laboratory showing unusual changes in intestinal architecture as well as a paucity of bacterial colonization in all segments of the lower intestinal tract of SAL birds support this interpretation (Chichlowski et al., in preparation).

Furthermore, changes in relative organ weights support a change in intestinal function rather than in intestinal mass as an explanation for the decreased whole-body and ileal O_2 consumption from the DFM compared to the SAL treatment. In general, all adjusted intestinal segment weights (Table 3) were the same for the SAL and DFM treatments. The SAL intestinal lengths were, however, longer than in both the CON and DFM birds (Table 3). This resulted in a uniformly lower intestinal density (mg/cm) for SAL treated as compared to CON and DFM treated birds. In addition, no significant changes were noted in intestinal serosal and mucosal DM percentage between treatments. Our laboratory has generally noted a positive relationship between tissue mass and DM and O_2. The lighter yet more metabolically active intestinal tissue observed with SAL in the present study, despite greatly increased O_2 consumption, suggests fundamental changes in intracellular metabolism of intestinal tissues of SAL treated birds. It should be cautioned, however, that the O_2 consumption of duodenal and jejunal segments were not measured. Hence regardless of the compelling nature of this explanation, final explanations await measurements of the effects of SAL and DFM on all intestinal tract segments.

It is of interest to note that no statistically significant differences between the CON and DFM treatments were found for whole-body O_2 consumption rates despite a previous report that this DFM increased growth and feed efficiency in chickens (Davis and Anderson, 2002). The present study found that the DFM decreased adjusted liver weight by 15%. Similar decreases in the liver weight of birds treated with this DFM, Primalac®, have been

reported previously (Mohan, 1991). The gastrointestinal tract and the liver account for 42% of total body energy expenditures (McBride and Early, 1989). No consistent differences between the CON and DFM treatments were noted in adjusted gastrointestinal weight and length in the present study (Table 3). The decreased liver weight that was observed with the DFM treatment in this study may explain, in part, the non-significant, numerically lower whole-body O_2 consumption rates.

Another significant observation that was noted in the present study was a detectable alteration in the expression of cytokines in the innate intestinal immune system. The cytokines IL-1β, IL-6 and IL-10 all showed changes in their relative expression rates in whole ileal tissue depending on the dietary treatment. The IL-1β and IL-6 decreased in DFM birds in comparison to those measured in CON and SAL birds, while the IL-10 rate was higher. It is agreement with other studies, where the expression of IL-10 was higher in the DFM birds than in the other treatments, while IL-1β and IL-6 were lower (Nusrat et al., 2001). Both IL-1β and IL-6 are known pro-inflammatory cytokines (Peters et al., 2005; Strober, 1998), while IL-10 is a known anti-inflammatory cytokine. It is possible, therefore, that DFM might block pathways within the intestinal tract involved in the synthesis of pro-inflammatory cytokines. *Lactobacillus casei* has been reported to down-regulate pro-inflammatory cytokines, chemokines, and adherence molecules in cultured *Shigella*-infected human intestinal cells (Tien et al., 2006). Similarly, *L. acidophilus* down regulates the inflammatory response in epithelial cells to lipopolysaccharides and pathogenic enteric bacteria (Kohler et al., 2003; Langhout et al., 1999).

Inflammation is known to increase energy expenditures in animals (Isolauri et al., 2001; Lochmiller and Deerenberg, 2000). Indeed, as stated by Lochmiller and Deerenberg

(2000), “in general, stimulation of a host’s immune system equates to proportional declines in growth as endogenous strategies of resource allocation shift towards survival and away from nonessential processes such as growth”. It is possible that the action of DFM in the present study indicates a potential decrease in intestinal inflammation and the amount of energy partitioned to the immune system. Hence DFM may lower the maintenance costs of the broiler leaving more energy available for growth. This preliminary experiment suggests alterations in trends; however, methodology employed has not come to qualititative statistical analysis. Future experiments are currently planned.

This study demonstrates the potential effects of SAL and DFM on intestinal and whole-body metabolism. The mechanisms are not clearly understood; however, alterations in intestinal and liver function as well as in intestinal immune function may explain, in part, this phenomenon. In the present study, the biosecurity protocol involved with the housing of the birds (meaning almost sterile conditions) may have caused an induced case of salinomycin toxicity. It is not clear whether all DFM or probiotic consortia will evoke similar changes in energy consumption or immune function. Allocthonous bacteria, even strains within the same species, have been shown to vary in their ability to colonize sections of the gastrointestinal tract as well as in their ability to affect change in the intestinal immune system (Garriga et al., 1998). The beneficial effects on growth and feed conversion reported in many studies using DFM are likely due to a complex and highly integrated cascade of alterations in the physiological mechanisms of the bird. More studies on the effects of DFM and probiotics on physiological, biochemical and immune processes are needed to develop a more dynamic understanding of the beneficial actions of DFM and probiotic bacteria in the intestinal tract and their subsequent effects on whole-body metabolism.

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TABLES AND FIGURES

Table 1. Body Weight (BW) and feed intake of 18 d old broiler chickens[1]

	Diet[2]			Sig.
	CON	**SAL**	**DFM**	
BW [g][3]	468.3 ± 11.2[a]	274.0 ± 11.2[b]	438.5 ± 11.2[a]	0.000
BW gain [g]	423.1 ± 11.1[a]	234.9 ± 10.9[b]	399.7 ± 10.9[a]	0.000
Feed intake [g] total per pen[4]	7885.5 ± 242.4[a]	6432.8 ± 242.4[b]	7727.8 ± 242.4[a]	0.015
Feed conversion[5]	0.56 ± 0.017[a]	0.39 ± 0.017[b]	0.51 ± 0.017[a]	0.0001

[a,b]Means in rows lacking a common superscript are significantly different ($P \leq 0.05$), as a result of LSD mean comparison

[1] n= 117 (3 missing observations)

[2] CON = no additives, SAL = Salinomycin (50 ppm), DFM = Direct-Fed Microbial (Primalac®)

[3] Least Square Means ± SEM

[4]10 birds per pen

[5]Feed conversion = average BW gain/average FI per bird

Table 2. Dry matter (DM) of serosa and mucosa in ileum, jejunum and intact colon and cecum in 3 wk old broiler chickens[1]

	Diet[2]			Sig.
	CON	**SAL**	**DFM**	
DM content%				
Jejunal serosa[3]	26.72 ± 0.53	27.01 ± 0.74	28.14 ± 0.74	0.30
Jejunal mucosa	23.96 ± 1.08	20.28 ± 1.53	24.59 ± 1.62	0.10
Ileal serosa	30.37 ± 0.80	30.44 ± 1.13	30.46 ± 1.14	0.99
Ileal mucosa[4]	21.08 ± 0.65[a]	18.67 ± 0.91[ab]	17.85 ± 0.97[b]	0.02
Intact cecum	22.51 ± 0.69	23.94 ± 0.98	24.82 ± 0.99	0.15
Intact colon	19.46 ± 0.37	19.51 ± 0.53	19.31 ± 0.53	0.96

[a,b]Means in rows lacking a common superscript are significantly different ($P \leq 0.05$), as a result of LSD mean comparison

[1] n= 35 (1 missing observation)

[2] CON = no additives, SAL = Salinomycin (50ppm), DFM = Direct-Fed Microbial (Primalac®)

[3] Least Square Means ± SEM

[4] Means with different superscripts differ significantly ($P < 0.05$)

Table 3. Adjusted weight of intestinal segments in 3 wk old broiler chickens[1]

	Diet[2]			Sig.
	CON	SAL	DFM	
Adjusted weight, mg/g FBW[3]:				
Duodenum[4]	7.21 ± 0.32	7.56 ± 0.45	7.22 ± 0.45	0.808
Jejunum	16.16 ± 0.48^{a}	14.33 ± 0.68^{ab}	12.86 ± 0.68^{b}	0.001
Ileum	12.294 ± 0.50	10.69 ± 0.71	11.88 ± 0.71	0.201
Cecum	5.88 ± 0.40	5.28 ± 0.56	5.69 ± 0.57	0.70
Colon	1.08 ± 0.13^{b}	1.81 ± 0.18^{a}	1.76 ± 0.18^{a}	0.002
Total weight	42.63 ± 1.16	39.72 ± 1.65	39.39 ± 1.65	0.188
Adjusted length, mm/g FBW:				
Duodenum	0.32 ± 0.02^{b}	0.50 ± 0.02^{a}	0.33 ± 0.02^{b}	0.001
Jejunum	0.78 ± 0.03^{b}	1.187 ± 0.05^{a}	0.77 ± 0.05^{b}	0.001
Ileum	0.76 ± 0.03^{b}	1.05 ± 0.04^{a}	0.80 ± 0.04^{b}	0.001
Cecum	0.19 ± 0.01^{b}	0.28 ± 0.01^{a}	0.19 ± 0.01^{b}	0.001
Colon	0.08 ± 0.01^{b}	0.12 ± 0.01^{a}	0.09 ± 0.01^{ab}	0.005
Total length	2.13 ± 0.08^{b}	3.14 ± 0.11^{a}	2.19 ± 0.11^{b}	0.001
Density mg/cm:				
Duodenum	228.85 ± 9.30^{a}	150.42 ± 12.79^{b}	219.99 ± 12.79^{a}	0.001
Jejunum	204.60 ± 6.10^{a}	123.74 ± 8.38^{c}	170.30 ± 8.38^{b}	0.001
Ileum	165.05 ± 5.78^{a}	104.23 ± 7.95^{b}	149.14 ± 7.95^{a}	0.001
Cecum	321.12 ± 14.96^{a}	190.07 ± 20.55^{b}	300.53 ± 20.55^{a}	0.001
Colon	122.91 ± 14.83^{b}	156.40 ± 20.39^{ab}	196.82 ± 20.39^{a}	0.0213
Total	201.14 ± 5.13^{a}	128.26 ± 7.06^{b}	181.41 ± 7.06^{a}	0.001
Adjusted weight, mg/g FBW:				
Proventriculus	4.66 ± 0.16^{b}	5.69 ± 0.23^{a}	4.90 ± 0.23^{ab}	0.0036
Gizzard	28.63 ± 0.79	30.24 ± 1.12	26.80 ± 1.12	0.1108
Bursa	2.10 ± 0.28	2.15 ± 0.41	2.12 ± 0.39	0.9949
Liver	26.15 ± 0.87^{a}	23.81 ± 1.23^{ab}	22.28 ± 1.23^{b}	0.0392
Pancreas	2.46 ± 0.18	3.21 ± 0.25	2.92 ± 0.25	0.0553

$^{a-c}$Means in rows lacking a common superscript are significantly different ($P \leq 0.05$), as a result of LSD mean comparison

[1] n= 35 (1 missing observation)

[2] CON = no additives, SAL = Salinomycin (50 ppm), DFM = Direct-Fed Microbial (Primalac®)

[3]FBW = Feed-Deprived Body Weight

[4] Least Square Means ± SEM

Table 4. Whole- body oxygen consumption and CO_2 production in 3 wk old broiler chickens[1]

	Diet[2]			SEM[3]	Sig.
	CON	**SAL**	**DFM**		
BW [g]	521[a]	304.9[c]	478.04[b]	11.15	0.001
RQ[4]	0.85	0.81	0.82	0.02	0.48
Whole-body O_2 consumption, μmol O_2/min	632.6[a]	412.06[b]	574.4[a]	30.84	0.001
Adjusted whole-body O_2 consumption, μmol O_2/min/g BW	1.24[ab]	1.39[a]	1.17[b]	0.06	0.027
Whole-body CO_2 production, μmol CO_2/min	544.3[a]	337.9[b]	484[a]	33.1	0.001
Adjusted whole-body CO_2 production, μmol CO_2/min/g BW	1.05	1.15	1.002	0.07	0.29

[a-c]Means in rows lacking a common superscript are significantly different ($P \leq 0.05$), as a result of LSD mean comparison

[1] n= 118

[2] CON = no additives, SAL = Salinomycin (50 ppm), DFM = Direct-Fed Microbial (Primalac®)

[3] Standard Error of the Mean

[4]Respiratory Quotient = CO_2 production/O_2 consumption

Table 5. Ileal and Cecal Oxygen consumption in 3 wk old broiler chickens[1]

	Diet[2]			Sig.
	CON	SAL	DFM	
Ileal O_2 consumption, nmol O_2/min/mg[3]	1.89 ± 0.29[ab]	2.23 ± 0.29[a]	0.99 ± 0.29[b]	0.012
Cecal O_2 consumption, nmol O_2/min/mg	2.23 ± 0.41	2.708 ± 0.41	1.725 ± 0.40	0.232

[a,b]Means in rows lacking a common superscript are significantly different ($P \leq 0.05$), as a result of LSD mean comparison

[1] n= 52 (2 missing observations)

[2] CON = no additives, SAL = Salinomycin (50 ppm), DFM = Direct-Fed Microbial (Primalac®)

[3] Least Square Means ± SEM

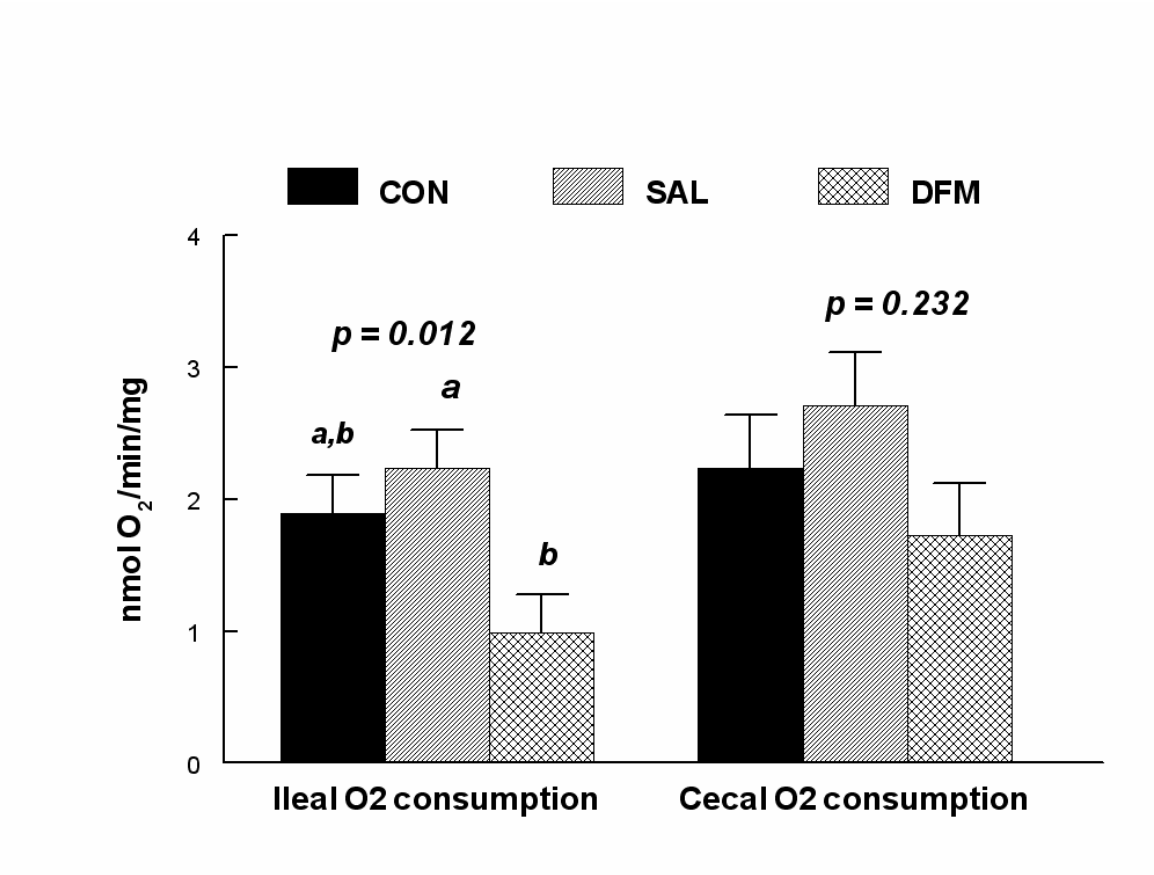
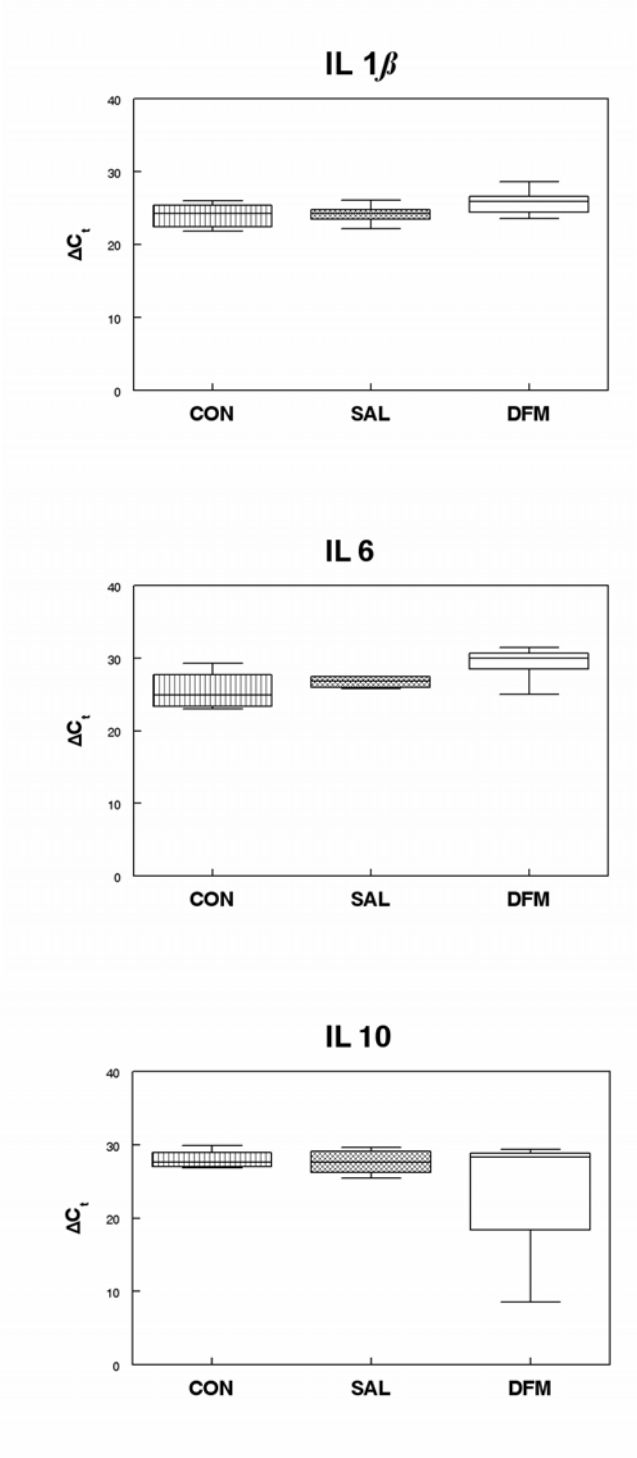


Figure 1. Ileal and cecal O_2 consumption in 3 wk old broiler chickens. Values are means ± SEM; n = 52. CON = no additives; SAL = salinomycin (50ppm); DFM = Direct-Fed Microbial (Primalac®). Means with different superscript differ significantly ($P < 0.05$).

Figure 2. Whisker plot of cytokine production in the broiler chicken ileum; n=18. CON = no additives; SAL = salinomycin (50ppm); DFM = Direct-Fed Microbial (Primalac®). Cytokines expressed (IL-1β, IL-6 and IL-10) were determined using real time RT-PCR, and presented as $\Delta C_t = (C_{t\ (\text{gene of interest treatment n})} - C_{t\ (\text{28s treatment n})})$, where increasing ΔC_t represents decreased gene expression. The box represents a percentile range; the median is marked as a vertical line inside the box and the lines outside the box extend to the highest and the lowest observations.



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CHAPTER 3

DIRECT-FED MICROBIAL AND SALINOMYCIN SUPPLEMENTATION AND GASTROINTESTINAL FUNCTION IN CHICKEN BROILERS[1]

[1] M. Chichlowski, J. Croom, M. A. Froetschel, B. W. McBride, L. R. Daniel, R.Qiu, G. Davis, and M.D. Koci. *Journal of Poultry Science.* Submitted.

ABSTRACT

Direct fed microbials are a putative alternative to the feeding of low levels of antibiotics in poultry production. Previous studies with a DFM Primalac® have demonstrated decreases in whole body and ileal energy expenditures in broilers. We suggest that those changes might be related to whole body and tissue metabolism, including nutrient transport in the gastrointestinal (GI) tract. The study herein examined the effects of feeding a DFM and salinomycin on ileal glucose and proline absorption and their relationships to GI energy expenditures. GI fluid digesta fermentation products also were measured. In Trial 1, broilers (n = 36) were fed a standard starter diet (CON), Con + DFM, (PrimaLac® 0.3% w/w), or Con + Salinomycin (SAL; 0.05% w/w) from hatch to 3 wk of age. On d 21, birds were euthanized, ileal tissue was dissected and glucose and proline uptake were estimated. In adjacent tissue, total O_2 (TO_2) and Na/K ATPase-sensitive O_2 consumption ($ATPO_2$) were estimated. Trial 2 was similarly designed except jejunal, ileal and cecal fluids were collected for short chain volatile fatty acids (VFA) and L/D lactate isomer analysis (n=54). None of the treatments changed active ileal glucose or proline uptake. Passive and total glucose and proline uptakes were increased ($P < 0.07$) by the DFM compared to SAL. TO_2 and $ATPO_2$ did not change with treatment. However, the percentage of total ileal cell O_2 consumption attributed to $ATPO_2$ increased (44%; $P < 0.04$) with SAL. Apparent energetic efficiency of total glucose and proline uptakes were increased ($P < 0.10$) by the DFM in comparison to the CON. L-lactate and total lactate concentrations in GI fluid decreased ($P < 0.01$) with the DFM feeding in comparison to CON and SAL treatments, and d-lactate increased ($P < 0.04$) in comparison to CON. Overall the ratio of L/D lactate did not change amongst treatments. Total cecal VFA concentration was lower ($P < 0.003$) with DFM feeding than in the CON.

Total VFA concentrations in jejunum and ileum were not different between treatments. Increases in the efficiency of nutrient absorption and decreases in intestinal fermentation associated with the DFM feeding may contribute to previously observed decreases in energy expenditures; however, these contributions are relatively minor, indicating that other physiological mechanisms are involved.

Key words: direct fed microbial, broiler, intestinal function and fermentation

INTRODUCTION

Direct fed microbials (DFM) are microorganisms that contribute to intestinal microbial balance (Davis and Anderson, 2002). The use of DFM is considered to be a potential alternative to the feeding of antibiotic growth promoters in poultry production (Patterson and Burkholder, 2003). Mechanisms of DFM action include inhibition of pathogen growth in the gastrointestinal (GI) tract, competitive exclusion, alterations in immune response, and others (Fuller, 1989; Galdeano and Perdigon, 2006; McCracken and Gaskins, 1999; Simon and Jadamus, 2002; Vaughan and Mollet, 1999). The present study was designed to provide data on physical actions of DFMs and their influence on intestinal function. Previous studies in this laboratory have demonstrated that the supplementation of the diet with a DFM or probiotic decreased whole-body and intestinal energy expenditures in broiler chicks as well as decreased the expression of certain cytokines in comparison to those in salinomycin treated group (Chichlowski et al., 2006). Their data suggested that a DFM could affect bird metabolism and immune function; however, those changes do not seem to fully explain the overall effects of the DFM. Others have reported various changes in both intestinal and whole-animal physiological response to DFM (Fooks and Gibson, 2002; Tucker and Taylor-Pickard, 2004) including intestinal microbial fermentation (Hutt et al., 2006; Marounek et al., 1999) and intestinal transport of nutrients (Hooper et al., 2001). It is likely that the beneficial effects of DFM are the result of the summation of a complex, multi-variate series of alterations in gut microbial and whole body metabolism.

Hutt and co-workers (Hutt et al., 2006) reported that several probiotic bacteria act antagonistically against pathogenic bacteria through fermentation and anti-oxidative activity. It has been previously demonstrated that the concentration of anaerobic probiotics increases

from the proximal to the distal parts of the gastrointestinal tract with the cecum having the highest concentration (Cummings and Macfarlane, 1997). The principal end products of anaerobic fermentation within the gastrointestinal tract are the short chain volatile fatty acids (VFA), acetate, propionate and butyrate (Fooks et al., 1999).

Butyrate and lactate have been identified as fermentation end-products which have specific effects on intestinal function. Butyrate is a major energy source for enterocytes and colonocytes (Wong et al., 2006). Additionally, increased absorption of butyrate from the gastrointestinal tract may influence lipid metabolism in the liver (Wong et al., 2006). Other fermentation end-products include the VFA valerate and caproate as well as the branched-chain fatty acids isobutyrate, 2-methyl-butyrate and isovalerate (Fooks et al., 1999). Ethanol and the organic acids, lactate, succinate and formate have also been reported (Fooks et al., 1999). Lactic acid, the predominate fermentation end-product of *Lactobacilli* spp. can be further metabolized by microbes in the intestinal tract as well as absorbed and metabolized by the broiler (Fooks et al., 1999). The increase in lactate and VFA concentrations causes the gastrointestinal pH to drop preventing the colonization of acid sensitive pathogens (Nemcova, 1997).

In addition to gastrointestinal bacteriostatic effects, lactate production by DFM within the gastrointestinal tract may alter whole animal metabolism (Mirdamadi et al., 2002). Generally, the L-isomer of lactic acid is that considered available for metabolism by vertebrate tissues (Ewaschuk et al., 2005). An increasing body of evidence is accumulating, however, that suggests that the D-isomer of lactate may also play a role in metabolism (Ewaschuk et al., 2005). The exact mechanisms and metabolic importance of this function of D-lactate is still the subject of intensive investigation.

While a normal population of DFM can protect chickens against bacterial infection (Dalloul et al., 2005), they also contribute significantly to nutrient digestion and absorption (Lan et al., 2004). Both *Lactobacilli* sp. and *Bifidobacteria* sp. are saccharolytic (Fooks and Gibson, 2002); significant bacterial dietary nutrient conversion by majority of gastrointestinal bacteria occurs in the small intestinal tract (Tucker and Taylor-Pickard, 2004). It has been reported that gastrointestinal microflora may affect glucose transport via alterations in the enterocyte Na-dependent SGLT-1 glucose transporter (Hooper et al., 2001). There is a paucity of data regarding the influence of DFM on nutrient transport in the chicken intestine. After oral treatment of rats with *S. Boulardii*, there was a marked stimulation of Na-dependent D-glucose uptake into jejunal enterocyte's brush border membrane vesicles with a corresponding increase in the membrane density of the SGLT-1 Na-dependent glucose transporter (Marteau et al., 2004).

This study was conducted to further elucidate possible mechanisms of a DFM consortium, Primalac®, on the intestinal function of chicks, and specifically on intestinal absorption. Of special interest was relating possible changes in microbial fermentation and ileal glucose absorption to decreases in whole body and intestinal energy expenditures in the broiler chicks when compared with the same effects in chicks supplemented with salinomycin (Chichlowski et al., 2006).

MATERIALS AND METHODS

Experimental Design

Fifty-four (Trial 1) and thirty-six (Trial 2) one-day old broiler chickens were placed on a standard corn-soybean meal broiler diet (17.08 % CP, 2.4 % fat, and 2830 kcal ME/kg). Chicks were assigned to one of following treatments: CON (no additives,), SAL (Salinomycin, 50ppm of feed), and DFM (0.3%, Primalac®). DFM chickens were supplemented with a consortium of *L. Casei*, *L. Acidophilus*, *B. Thermophilium*, and *Enterococcus faecium* (PRIMALAC®, Clarksdale, MO).

A completely randomized design was used for the two trials. Chicks from each treatment were randomly selected for experimental measurements, so the average age of the chicks was 21 d at the time of measurements. The individual bird measurements provided the experimental units. The data from each trial were statistically analyzed using the ANOVA procedure of STATISTIX®8 (Tallahassee, FL). Each bird's BW was used as a covariate for all intestinal transport analysis. Fisher's LSD test was used to test the significance of differences between the treatment means if overall significance was $P < 0.05$. All calculations on molar percentages of VFA were performed on *Arcsine* transformations. Table values consist of the least square means.

Animal care and biosecurity were as previously described (Chichlowski et al., 2006). All experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at North Carolina State University.

Sample collection and analyses

On day 21, birds in Trials 1 and 2 were killed by cervical dislocation after 12-h feed deprivation period (Trial 2). In Trial 1, jejunal, ileal and cecal digesta samples were collected, frozen in liquid N_2 and stored at -20°C until analyses for VFA and D/L lactate could be performed. Jejunal, ileal and cecal digesta fluid was collected by centrifuging thawed digesta from the respective sites at 13000 x g for 10 minutes. Twenty μl of digesta fluid was mixed with 5 µl of 25% metaphosphoric acid. The samples were then stored until VFA and lactate analysis could be conducted. In Trial 2, ileal tissue samples were immediately used for nutrient uptake and whole-tissue O_2 consumption analyses.

Digesta liquid concentrations of acetate, propionate, isobutyrate, butyrate, isovalerate and valerate were determined using a Schimadzu GC 15 gas-liquid chromatograph fitted with a 0.6' stainless column with SP 1200/ chromabsorb W packing. Analysis was modified from one described by Croom and co-workers (Croom et al., 1981). Injected volumes of digesta fluid were 2 μL for cecum, and 3 μL for ileum and jejunum. The flame ionization detector was set at 360°C. The analysis was conducted via programmed changes in column temperature. The column conditions for the cecum digesta were set initially at 130°C, and than increased by 5°C/min to 160°C. They were then increased by 15°C/min to 190°C. For the ileum and jejunum samples, the final temperature was 160°C. The concentration of fatty acids in the digesta samples was expressed as mM per L (Hass et al., 1999). Short chain volatile fatty acids concentrations were calculated against an external standard using a (Shimadzu, Columbia, Maryland) computerized integrator. Molar percentage of single VFA was calculated based on the proportion of the concentration of a particular VFA to the sum of all VFAs, and is reported for a selected sample multiplied by 100%.

The concentration of L-lactate in the digesta fluid was determined with an L-Lactate Analyzer (YSI 2300 Stat Plus, Yellow Springs, Ohio), whilst D-lactate concentrations were estimated using a colorimetric assay (Megazyme, Megazyme International, Ireland LTD Bray, CO).

In trial 2, ileal and cecal samples of each bird were longitudinally cut and divided into ten (ileum) and two (cecum) 20 to 40 mg pieces. Active, passive and total ileal uptake of glucose and proline were estimated using 3H-3-O-methyl-D-Glucose and ^{14}C-proline as described by others (Bird et al., 1994; Fan et al., 1997). Whole ileal glucose and proline flux was calculated by multiplying the transport value (ηmoles/minute/gram of intact ileum) by total weight of the ileum.

The O_2 consumption rates of intact ileal and cecal tissue were estimated using an incubation chamber (YSI, Yellow Springs, OH) fitted with an O_2 electrode as described by Fan and co-workers (Fan et al., 1997). The O_2 consumption rates of intact ileal tissue attributable to Na^+/K^+ ATPase and cytoplasmic protein synthesis were measured by the difference in O_2 consumption in the absence and presence of ouabain (2.0 μM; Fisher Scientific Co., Pittsburgh, PA). The percentage of ouabain sensitive O_2 consumption rate was expressed as the O_2 consumption rate of intact ileal or cecal tissue in the presence of ouabain divided by the O_2 consumption rate of the same tissue in the absence of ouabain and then multiplied by 100.

The apparent energetic efficiency (APEE) of both ileal glucose and proline uptake, a scalar of the relative energetic efficiency of the transport of these nutrients (Croom et al., 1998; Fan et al., 1996) was calculated according to Bird et al. (Bird et al., 1994) and expressed as ηmoles ATP expended/ηmole of glucose or proline uptake.

Whole tissue protein and serosal and mucosal DNA was determined for all tissue after homogenizing 200 mg of intact tissue (serosa and mucosa) for 30 s in ice-cold buffer (2 ml, pH 7.4, 10 mM Tris, 1 mM EDTA and 1 M NaCl). The DNA content of the intestinal homogenate was estimated using a TD-360 Mini-Fluorometer (Turner Designs, Sunnyvale, CA), which utilizes calf thymus DNA as a standard. Total protein was determined by measuring the absorbance of bicinchoninic acid complex with Cu^{+} at 550 nm using bovine serum albumin as a standard (Smith et al., 1985).

RESULTS

Treatment had only minor effects on the protein and DNA composition of the lower GI tract (Table 1). The duodenum of DFM treated birds had dramatically higher protein ($P < 0.001$) and DNA concentrations ($P < 0.001$) than in the CON and SAL treated birds. This resulted in a significant ($P = 0.03$) 25-84% decrease in the protein/DNA ratio. Changes were also noted in ileal tissue composition; however, they were not large enough to result in significantly different protein/DNA ratio. Cecal protein concentrations decreased ($P < 0.03$; Table 1) and DNA concentrations increased ($P < 0.04$) compared concentrations in the ceca of CON and SAL treated animals.

Table 2, 3, and 4 provide the least square means for the jejunal, ileal, and cecal fluid VFA analysis, respectively. In the jejunal fluid, while propionate was only slightly above the levels of detection, there were no differences between treatments in molar concentrations of VFA. The molar percentage of acetate was lower ($P < 0.05$) in SAL treated than in either the

CON or DFM treated animals, while the isobutyrate concentration was the highest in the SAL treatment (Table 2). Ileal fluid concentrations or molar percentages of VFA did not differ significantly among the three treatments ($P > 0.05$; Table 3), except for the propionate ($P = 0.08$). Cecal fluid total VFA concentration was significantly reduced by the SAL and DFM treatments by as much as 42% in comparison with the CON treatment ($P = 0.003$; Table 4). Cecal acetate concentrations decreased as much as 46% ($P = 0.003$; Table 4) in the SAL and DFM birds in comparison to the levels found in the CON birds. No changes in the molar percentages of VFA in the cecal fluid were noted with any treatment.

Total and isomer lactic acid concentrations of jejunal, ileal, and cecal fluid are presented in Table 5. In general, total digesta fluid lactic acid (L + D isomers) was 50-79% higher in the jejunum or ileum than in the cecum. Across treatments the concentration of digesta fluid L-lactic acid was as much as 58% lower in the jejunum of DFM treated broilers than it was in the CON and SAL treated broilers ($P = 0.001$; Table 5), while it was as much as 37% greater in cecal fluid. D-lactic acid was as much as 218% higher ($P < 0.001$; Table 5) in the jejunal fluid of DFM birds than it was in the CON and SAL treated birds. This resulted in a significant 79% decrease ($P < 0.03$; Table 5) in the L/D isomer ratio in the jejunal fluid of DFM treated birds as compared with the levels in other treatments. The only difference in the molar percentage of L or D digesta fluid lactic acid occurred in the jejunum where the percentage of L-lactic acid molar percent decreased from a range of 94-96% in the CON and SAL birds to 82% ($P = 0.001$; Table 5) in the DFM treated birds.

Table 6 lists uptake rates for glucose and proline in the ileum. Table 7 contains the estimated total, active and passive glucose and proline across the entire ileum. The SAL treatment decreased both glucose and proline transport rates ($P = 0.02$, Table 6) in

comparison to those observed in the CON and DFM treatments. Treatment had no effect on the Na-dependent active transport rate of nutrients. The SAL treatment decreased estimated active and passive glucose and proline flux across the entire ileum ($P < 0.05$; Table 7). Hence, the decrease in active and passive glucose and proline transport rates resulted in a significant decrease in the estimated total glucose and proline flux across the entire ileum ($P < 0.001$; Table 7).

The feeding of SAL decreased ileal non-ouabain sensitive O_2 consumption rates to below the level of detection for the measurement system used ($P < 0.01$; Table 8). No differences were noted in non-ouabain sensitive O_2 consumption rates between the CON and DFM treatments. Ouabain sensitive respiration increased (43-55%; $P < 0.05$) as a percentage of total ileal O_2 consumption ($P < 0.05$; Table 8), while the percentage of non-ouabain sensitive O_2 consumption decreased dramatically (79-84%; $P < 0.05$) with the feeding of SAL in comparison to that observed following the feeding of the CON and DFM supplemented diets.

The statistically significant decrease in total ileal passive glucose and proline flux (Table 7) and the numerical trends for decreased O_2 with the CON and SAL diets resulted in trends for increased APEE of total ileal glucose and proline uptake ($P = 0.06$ and $P = 0.10$, respectively; Figure 1). No statistical difference or numerical trends were noted for the APEE of ileal active glucose and proline transport (data not shown).

DISCUSSION

The objective of this study was to elucidate the mechanisms responsible for the changes previously reported in whole-body and ileal energy expenditures due to the supplementation with the direct fed microbial, Primalac®, in comparison with the feeding of the antibiotic salinomycin (Chichlowski et al., 2006). While no single set of measured parameters explain the phenomenon, small and potentially important changes were noted in a variety of variables.

With the exception of the duodenum and cecum, no large changes were noted in the protein and DNA concentrations due to treatment (Table 1); however, in the cecum those changes were not reflected in the protein/DNA ratio. Although this might suggest that gastrointestinal cellular turnover plays a minor role in energetic alterations at the tissue level, the dramatic decreases in duodenum protein/DNA ratio may suggest that this portion of the intestinal tract may be undergoing hypertrophy. This could have a major impact on gastrointestinal and whole-body metabolism because the duodenum contains a very high percentage of enteroendocrine cells which secrete in a paracrine and endocrine fashion a wide variety of regulatory peptides and hormones (Bird et al., 1996).

Short chain volatile fatty acid concentrations, whether expressed as concentrations per unit of digesta fluid or wet contents, ultimately reflect the equilibrium between the rate of fermentation and the rate of uptake or metabolism of VFA. In pigs and chickens they are unusually hard to assay because of the physicochemical properties of the digesta (Clayton and Blake, 2005). In the present study, the amounts of feed consumed by birds on each treatment could affect VFA concentrations since feed acts as substrate for gastrointestinal bacteria. This is especially true for the SAL treated birds, because birds on that treatment

exhibited a dramatic decrease in feed intake (data not shown). Hence, VFA concentrations can be used as relative indicators of gastrointestinal fermentation status. The molar percentage of VFA produced is more indicative of the type of fermentation in the gastrointestinal tract. Indeed, type of feed as well as the addition of such additives as ionophore antibiotics can influence molar percentage of VFA (Marounek et al., 1999).

In this present study, treatment had little effect on the actual concentrations of VFA in the jejunum and ileum (Tables 2 and 3). This may be due to the fact that fermentation activity is low at both sites. Comparison of total VFA concentrations in the jejunum and ileum with that of the cecum (Table 4) supports this conclusion. Small statistically significant changes were observed in individual VFA concentrations and molar percents; however, the changes did not fit a particular pattern that one might associate with the treatments. For instance, the ionophore antibiotic salinomycin have been shown to increase both the total concentration and molar percentage of propionate in *in vitro* cultures from chicken ceca (Marounek et al., 1996; Marounek et al., 1999). This was not observed in the present study. Additionally, fermentation studies with human digesta have shown that *L. acidophilus* and *B. bifidum* do not influence the production and concentrations of VFA (Hove et al., 1999).

The molar percentages of cecal VFA observed in the present study were similar to that reported by (Marounek et al., 1999) in cultured cecal digesta. Total cecal VFA concentration decreased following the feeding of the DFM by 31% ($P < 0.01$; Table 4) in comparison to the level observed in the CON fed birds. This is surprising, since the cecum is the largest site of fermentation in the broiler digestive tract (Marounek and Rada, 1998) and its fermentation has been shown to increase production of propionate in chicken digesta

treated with lasalocid and salinomycin (Marounek and Rada, 1998; Marounek et al., 1996). It is likely the decrease in cecal fluid VFA concentration with DFM as compared to CON is due to decreased nutrient flow resulting from more efficient passive absorption of nutrients in the intestines. This is puzzling since other researchers (Nisbet et al., 1996) demonstrated an increase in cecal propionic acid production with a bacterial competitive exclusion culture fed to chicks, and a concomitant decrease in cecal *Salmonella* colonization. The reason for such low propionate concentration with the DFM treatment is unclear.

Total lactic acid concentrations were highest in the jejunal and ileal fluids and lowest in the cecal fluid (Table 5). L-lactic acid concentrations followed the same pattern while D lactic acid concentrations were higher in the cecum. Dramatic differences in the concentrations of L and D lactate as well as the L/D ratio occurred in the jejunum between the CON and DFM groups. L-lactic acid concentration decreased 142% in the DFM birds in comparison to what was observed with the CON birds ($P < 0.001$; Table 5). Conversely, the d isomer of lactic acid increased 218% ($P < 0.001$; Table 5) in the jejunal digesta fluid of DFM birds. This resulted in a 70% decrease in the l/D lactic acid ratio in jejunal fluid ($P = 0.02$). No significant changes were noted in lactic acid isomers in the ileal and cecal fluids ($P > 0.05$).

The biological significance of the shifts in jejunal lactic acid isomer concentrations in the jejunum with DFM is not clear. Different strains of *Lactobacilli* produce varying amounts of D and L isomers of lactic acid (Mirdomadi et al., 2002). It is possible that the jejunum provided an environment for a strain of bacteria that favored production of D lactic acid. This may account for the drop in L-lactic concentration since both isomers are believed to be absorbed by the same H^+-linked monocarboxylate transporter (Garcia et al., 1994), thus

allowing more competition for binding sites by D-lactic acid. Alternatively, the drop in L lactic acid from the DFM treatment may indicate an increased total absorption of L-lactic acid, which could benefit the bird by providing substrates for gluconeogenesis (Ewaschuk et al., 2005). It also is surprising that the cecal fluid concentrations of total lactic acid were the lowest among all analyzed sites, despite the fact that the cecum has a much higher concentration of lactic acid producing bacteria than the jejunum or ileum; $10^7 – 10^{10}$ versus $10^{11} – 10^{12}$ CFU/g of digesta (Simon et al., 2004). This may be in part due to the fact that both L- and D-lactic acid can be fermented by enteric bacteria into various short chain volatile fatty acids (Hungate, 1966). This may explain the increased concentration of VFA in cecal fluid compared to jejunal and ileal fluid.

Neither active nor total glucose uptake rates were affected by treatments (Table 6). However, passive glucose and proline uptake rates decreased dramatically following the feeding of SAL in comparison to those observed following the feeding of the CON and DFM diets ($P < 0.03$). As a result, estimates of the active, passive and total glucose and proline uptakes for the entire ileum were much lower for SAL fed than for the CON and DFM fed birds ($P < 0.05$; Table 7).

This dramatic decrease in ileal nutrient absorption following the consumption of SAL is likely due to an induced level of salinomycin in the SAL treated broilers as described by Chichlowski et al. (in preparation). This hypothesis is supported by the observations in the present study that non-ouabain sensitive respiration rate for the SAL treatment (cytoplasmic and mitochondrial energy requiring processes) were below the sensitivity of the assays used in the present study to detect ($P < 0.01$; Table 8). This was reflected in the fact that the percentage of respiration due to non-ouabain sensitive processes was only 8% in the

SAL treated group in comparison to 54% and 44% for CON and DFM treatments, respectively ($P < 0.05$; Table 8).

The APEE for total ileal glucose and proline was calculated from total ileal transport in Table 7 and from the intact ileal tissue respiration rates in Table 8. Although not significant, there were strong trends for increased efficiency of absorption for both SAL and DFM compared to CON ($P < 0.06$ and $P < 0.10$, respectively; Figure 1). This increase in efficiency is likely due to the substantial increases in passive absorption of both nutrients. The nutrient absorptive rates of the duodenum and jejunum were not measured in the present study. It is possible that in broilers increased passive absorption of nutrients will increase the efficiency of feed utilization for growth, because no energy is required in these transport processes. Indeed, nectavore birds absorb more energy via passive than active processes, because of their high rates of basal metabolic rates (McWhorter et al., 2006).

While the present study demonstrated changes in fermentation and function of the lower gastrointestinal tract of broilers treated with DFM or SAL, no complete paradigm emerged from the data that fully explained the decreased whole-body and ileal O_2 consumption noted previously with these treatments by Chichlowski et al. (2006). It is likely that the beneficial effects of direct fed microbials are the result of a complex, multi-variate series of alterations in gut microbial and whole body metabolism. The present study suggests the likelihood of a small but additive series of beneficial changes with direct fed microbials. More detailed studies will be needed to explain this exceedingly complex phenomenon.

ACKNOWLEDGEMENTS

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TABLES AND FIGURES:

Table 1. Total duodenal, jejunal, ileal, cecal, and colonal protein and DNA contents in chicken at 21 d of age[1]

	Diet[2]			SEM[3]	Sig.
	CON	SAL	DFM		
Protein, mg/g					
Duodenum	8.55[b]	12.45[b]	25.72[a]	1.26	0.001
Jejunum	18.91	23.17	22.75	1.65	0.15
Ileum	23.20[b]	26.31[ab]	30.91[a]	2.07	0.04
Cecum	45.88[a]	40.76[ab]	36.48[b]	2.32	0.025
Colon	23.96	25.99	28.53	2.44	0.426
DNA, mg/g					
Duodenum	4.78[b]	5.99[b]	39.29[a]	2.33	0.001
Jejunum	3.063[b]	2.528[b]	6.83[a]	0.90	0.003
Ileum	19.43	22.28	20.08	3.32	0.813
Cecum	6.31[ab]	4.95[b]	14.78[a]	2.81	0.039
Colon	2.89	2.42	5.69	2.04	0.483
Protein/DNA ratio					
Duodenum	4.56[a]	3.13[ab]	0.68[b]	1.01	0.034
Jejunum	11.62	17.12	5.43	3.91	0.123
Ileum	2.068	1.81	2.75	0.71	0.63
Cecum	16.91	28.83	11.88	6.18	0.154
Colon	19.73	29.56	33.19	12.69	0.742

[a,b]Means in rows lacking a common superscript are significantly different ($P \leq 0.05$), as a result of LSD mean comparison

[1] n= 36

[2] CON = no additives, SAL = Salinomycin (50 ppm), DFM = Direct-Fed Microbial (Primalac®)

[3] Standard Error of the Mean

Table 2. Jejunal fluid VFA concentrations [mM] and molar percentages at 21 d of age [1]

	Diet[2]			Sig.
	CON	SAL	DFM	
Concentration:	----------mM----------			
Acetate[3]	26.53 ± 3.74	26.47 ± 6.48	27.28 ± 5.01	0.99
Propionate	3.52 ± 0.79	4.67 ± 0.79	4.80 ± 0.79	0.49
Isobutyrate	0.66 ± 0.14	0.89 ± 0.14	0.91 ± 0.23	0.43
Butyrate	2.09 ± 0.51	1.81 ± 0.47	3.09 ± 0.54	0.19
Isovalerate	1.63 ± 0.20[b]	2.25 ± 0.21[a]	2.27 ± 0.23[a]	0.06
Valerate	0.35 ± 0.08	0.23 ± 0.20	0.30 ± 0.20	0.86
Total	17.96 ± 3.71	10.54 ± 3.95	15.14 ± 4.09	0.39
Molar:	----------%----------			
Acetate	87.85 ± 1.88[a]	76.80 ± 3.26[b]	82.38 ± 2.53[a,b]	0.02
Propionate	10.47 ± 1.79	13.98 ± 1.79	11.99 ± 1.79	0.43
Isobutyrate	4.47 ± 2.87[b]	16.81 ± 2.87[a]	3.0 ± 4.69[b]	0.01
Butyrate	38.33 ± 7.66	36.69 ± 6.99	52.60 ± 8.07	0.29
Isovalerate	25.38 ± 6.39	42.67 ± 6.60	36.07 ± 7.09	0.17
Valerate	7.531 ± 4.14	12.70 ± 10.96	0.88 ± 10.96	0.75

[a,b]Means in rows lacking a common superscript are significantly different ($P \leq 0.05$), as a result of LSD mean comparison

[1] n= 17 (Acetate), 9 (Propionate), 19 (Isobutyrate), 31 (Butyrate), 44 (Isovalerate), 9 (Valerate), 46 (Total)

[2] CON = no additives, SAL = Salinomycin (50 ppm), DFM = Direct-Fed Microbial (Primalac®)

[3] Least Square Means ± SEM

Table 3. Ileal fluid VFA concentrations [mM] and molar percentages at 21 d of age [1]

	Diet[2]			Sig.
	CON	SAL	DFM	
Concentration:	----------mM----------			
Acetate[3]	35.48 ± 6.39	20.36 ± 7.14	17.44 ± 8.24	0.19
Propionate	4.18 ± 1.13	4.42 ± 0.98	4.87 ± 1.13	0.91
Isobutyrate	0.51 ± 0.11	0.64 ± 0.12	0.62 ± 0.14	0.71
Butyrate	1.96 ± 0.57	1.92 ± 0.59	2.15 ± 0.52	0.94
Isovalerate	1.19 ± 0.28	1.71 ± 0.29	1.39 ± 0.28	0.44
Valerate	0.31 ± 0.09	0.47 ± 0.12	0.37 ± 0.12	0.61
Total	17.86 ± 4.11	10.26 ± 3.96	8.31 ± 3.96	0.22
Molar:	----------%----------			
Acetate	82.48 ± 4.41	77.80 ± 4.92	84.41 ± 5.68	0.66
Propionate	14.34 ± 11.72[b]	18.39 ± 10.15[b]	54.67 ± 11.72[a]	0.08
Isobutyrate	13.06 ± 8.68	25.99 ± 9.28	11.67 ± 10.98	0.51
Butyrate	31.97 ± 8.04	37.33 ± 8.43	41.35 ± 7.39	0.69
Isovalerate	28.29 ± 6.66	37.47 ± 6.96	29.45 ± 6.66	0.59
Valerate	6.97 ± 2.83	11.94 ± 3.74	8.37 ± 3.74	0.58

[a,b]Means in rows lacking a common superscript are significantly different ($P \leq 0.05$), as a result of LSD mean comparison

[1] n= 12 (Acetate), 10 (Propionate), 20 (Isobutyrate), 34 (Butyrate), 35 (Isovalerate), 15 (Valerate), 41 (Total)

[2] CON = no additives, SAL = Salinomycin (50 ppm), DFM = Direct-Fed Microbial (Primalac®)

[3] Least Square Means ± SEM

Table 4. Cecal fluid VFA concentrations [mM] and molar percentages at 21 d of age [1]

	Diet[2]			Sig.
	CON	SAL	DFM	
Concentration:	----------mM----------			
Acetate[3]	74.95 ± 7.22[a]	40.44 ± 6.14[b]	53.68 ± 6.96[b]	0.003
Propionate	7.95 ± 0.92	6.67 ± 0.79	6.34 ± 0.89	0.42
Isobutyrate	0.73 ± 0.10	0.52 ± 0.08	0.45 ± 0.09	0.12
Butyrate	16.08 ± 1.99[a]	9.56 ± 1.69[b]	9.00 ± 1.91[b]	0.022
Isovalerate	0.43 ± 0.06	0.33 ± 0.06	0.28 ± 0.06	0.30
Valerate	1.03 ± 0.12	0.77 ± 0.10	0.63 ± 0.12	0.09
Total	101.18 ± 9.05[a]	58.28 ± 7.69[b]	70.39 ± 8.72[b]	0.003
Molar:	----------%----------			
Acetate	60.15 ± 4.97	49.63 ± 4.23	57.00 ± 4.79	0.25
Propionate	15.96 ± 2.32	21.41 ± 1.97	18.68 ± 2.23	0.20
Isobutyrate	4.69 ± 0.50	5.79 ± 0.43	4.44 ± 0.49	0.09
Butyrate	22.67 ± 2.22	25.57 ± 1.89	21.43 ± 2.15	0.33
Isovalerate	3.59 ± 0.41	4.21 ± 0.35	3.38 ± 0.39	0.26
Valerate	5.64 ± 0.54	7.09 ± 0.46	5.66 ± 0.51	0.06
A/P ratio	3.88 ± 0.36	2.86 ± 0.30	3.38 ± 0.36	0.10

[a,b]Means in rows lacking a common superscript are significantly different ($P \leq 0.05$), as a result of LSD mean comparison

[1] n= 45 (9 missing observations)

[2] CON = no additives, SAL = Salinomycin (50 ppm), DFM = Direct-Fed Microbial (Primalac®)

[3] Least Square Means ± SEM

Table 5. Lactic acid concentration in 21 d old chicken[1]

	Diet[2]			Sig.
	CON	SAL	DFM	
L-lactic acid:	----------mM----------			
Jejunum[3]	10.99 ± 1.37[a]	12.26 ± 1.42[a]	4.55 ± 1.18[b]	0.0001
Ileum	14.13 ± 2.46	17.90 ± 2.38	10.08 ± 2.72	0.10
Cecum	1.04 ± 0.12	0.89 ± 0.14	1.22 ± 0.12	0.25
D-lactic acid:	----------mM----------			
Jejunum	0.45 ± 0.29[b]	0.78 ± 0.35[b]	1.43 ± 0.29[a]	0.0002
Ileum	0.46 ± 0.44	1.36 ± 0.46	1.28 ± 0.49	0.31
Cecum	2.35 ± 0.57	2.94 ± 0.44	3.33 ± 0.51	0.46
Total lactic acid:	----------mM----------			
Jejunum	11.96 ± 1.67[a]	13.19 ± 2.22[a]	6.00 ± 1.67[b]	0.003
Ileum	14.40 ± 2.62[ab]	19.93 ± 2.70[a]	10.85 ± 3.31[b]	0.103
Cecum	2.47 ± 0.47	3.50 ± 0.55	3.04 ± 0.45	0.361
L/D ratio				
Jejunum	31.17 ± 8.19[ab]	43.98 ± 10.21[a]	9.13 ± 9.24[b]	0.023
Ileum	48.39 ± 15.15	32.21 ± 15.15	57.36 ± 16.93	0.53
Cecum	0.52 ± 0.45	0.30 ± 0.42	1.08 ± 0.43	0.42
L-lactic acid	----------%----------			
Jejunum	96.34 ± 6.95[a]	94.43 ± 9.27[a]	82.19 ± 6.95[b]	0.001
Ileum	97.39 ± 0.91	95.85 ± 0.94	97.31 ± 1.15	0.45
Cecum	59.31 ± 8.85	29.36 ± 10.33	57.43 ± 8.57	0.06
D-lactic acid	----------%----------			
Jejunum	4.21 ± 3.75	5.57 ± 4.67	22.95 ± 4.04	0.108
Ileum	4.21 ± 1.08	6.25 ± 1.08	3.36 ± 1.21	0.195
Cecum	67.72 ± 5.20	77.83 ± 4.94	68.14 ± 4.94	0.286

[a,b]Means in rows lacking a common superscript are significantly different ($P \leq 0.05$), as a result of LSD mean comparison

[1] n= 47 (7 missing observations)

[2] CON = no additives, SAL = Salinomycin (50 ppm), DFM = Direct-Fed Microbial (Primalac®)

[3] Least Square Means ± SEM

Table 6. Ileal glucose and proline transport in 21 d old chicken[1]

	Diet[2]			Sig.
	CON	SAL	DFM	
Ileal glucose uptake:				
Active[3,4,5]	128.06 ± 25.39	55.33 ± 28.36	101.13 ± 13.07	0.391
Passive	156.45 ± 20.50[ab]	121.55 ± 25.55[b]	179.57 ± 11.00[a]	0.023
Total	274.85 ± 29.10	168.78 ± 36.26	269.86 ± 15.62	0.075
Ileal proline uptake:				
Active	47.77 ± 19.06	36.21 ± 20.81	39.93 ± 11.99	0.87
Passive	166.98 ± 20.01[ab]	129.68 ± 24.93[b]	187.96 ± 10.74[a]	0.024
Total	217.03 ± 24.96	143.51 ± 31.09	222.87 ± 13.39	0.07

[a,b]Means in rows lacking a common superscript are significantly different ($P \leq 0.05$), as a result of LSD mean comparison

[1] n= 36

[2] CON = no additives, SAL = Salinomycin (50 ppm), DFM = Direct-Fed Microbial (Primalac®)

[3] Least Square Means ± SEM

[4] All transport values = ηmoles nutrient/minute/gram of intact ileum

[5] All calculations performed with BW [g] as a covariate

Table 7. Analysis of estimated total ileal glucose, proline and oxygen flux [1]

	Diet[2]			Sig.
	CON	SAL	DFM	
TGTI[3,4]	1928.0 ± 192.75[a]	310.8 ± 240.17[b]	1715.6 ± 103.44[a]	0.0004
TPTI	1527.7 ± 169.02[a]	302.6 ± 210.60[b]	1417.0 ± 90.71[a]	0.0008
AGTI	909.51 ± 168.46[a]	12.68 ± 188.11[b]	649.38 ± 86.67[a]	0.05
PGTI	1078.0 ± 130.94[a]	351.0 ± 163.15[b]	1132.6 ± 70.27[a]	0.0006
APTI	388.55 ± 98.84	70.75 ± 107.90	277.08 ± 62.16	0.272
PPTI	1136.9 ± 128.25[a]	380.5 ± 159.80[b]	1181.7 ± 68.83[a]	0.0004

Acronyms:
TGTI = Total Glucose for Total Ileum (nmol/min)
TPTI = Total Proline for Total Ileum (nmol/min)
AGTI = Active Glucose for Total Ileum (nmol/min)
PGTI = Passive Glucose for Total Ileum (nmol/min)
APTI = Active Proline for Total Ileum (nmol/min)
PPTI = Passive Proline for Total Ileum (nmol/min)

[a,b]Means in rows lacking a common superscript are significantly different ($P \leq 0.05$), as a result of LSD mean comparison
[1] n= 36
[2] CON = no additives, SAL = Salinomycin (50 ppm), DFM = Direct-Fed Microbial (Primalac®)
[3] Least Square Means ± SEM
[4] All calculations performed with BW [g] as a covariate

Table 8. Ileal oxygen consumption in 21 d old chicken[1]

	Diet[2]			Sig.
	CON	SAL	DFM	
Intact tissue, *µmol O_2/min/g*[3,4]	2.13 ± 0.39	0.62 ± 0.49	1.45 ± 0.20	0.16
Ouabain sensitive	1.06 ± 0.31	0.89 ± 0.39	0.88 ± 0.15	0.70
Non-ouabain	1.29 ± 0.24[a]	-0.58 ± 0.31[b]	0.87 ± 0.11[a]	0.006
Percentage:	----------**%**----------			
Ouabain sensitive	36.19 ± 7.92[b]	81.25 ± 10.03[a]	46.48 ± 3.85[b]	0.044
Non-ouabain	53.80 ± 7.92[a]	8.75 ± 10.03[b]	43.52 ± 3.85[a]	0.044

[a,b]Means in rows lacking a common superscript are significantly different ($P \leq 0.05$), as a result of LSD mean comparison

[1] n= 30

[2] CON = no additives, SAL = Salinomycin (50 ppm), DFM = Direct-Fed Microbial (Primalac®)

[3] Least Square Means ± SEM

[4] All calculations performed with BW [g] as a covariate

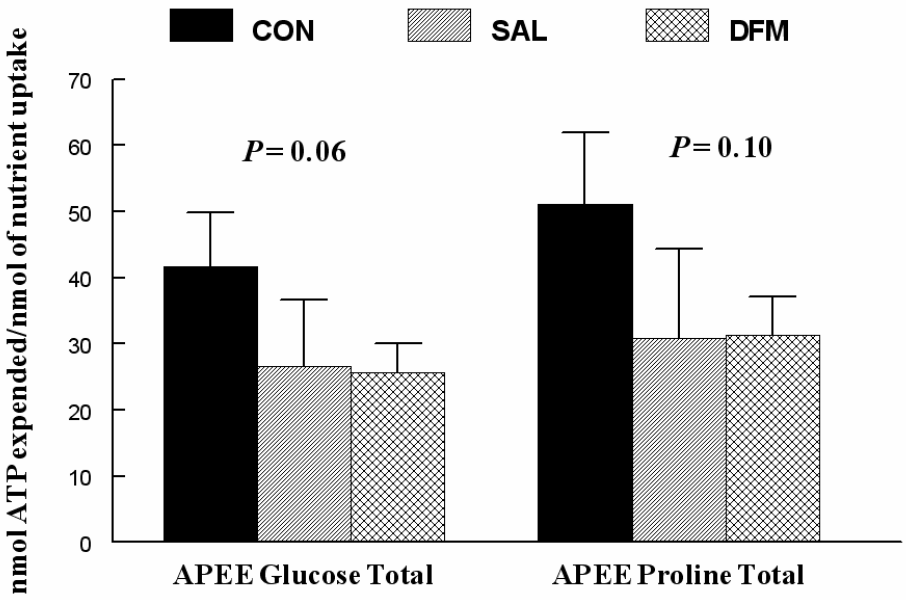

Figure 1. Apparent energetic efficiency (APEE) of total ileal glucose and proline uptake in response to DFM or SAL treatment in chicken broilers on d 21; n = 36, values are mean ± SEM

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CHAPTER 4

MICRO-ARCHITECTURE AND SPATIAL RELATIONSHIP BETWEEN BACTERIA AND ILEAL, CECAL AND COLONIC EPITHELIUM IN CHICKS FED A DIRECT-FED MICROBIAL AND SALINOMYCIN[1]

[1] M. Chichlowski, J. Croom, F. Edens, B. W. McBride, R. Qiu, C. C. Chiang, L. R. Daniel, G. W. Havenstein and M.D. Koci. *Journal of Poultry Science.* Submitted.

ABSTRACT

Direct fed microbials (DFM) could serves as a potential alternative to the feeding of antibiotics in poultry production. In this study, the effects of providing a DFM was compared with the feeding of Salinomycin on intestinal histomorphometrics and micro-architecture were examined. Broiler chicks (n=18 per treatment; Trials 1 and 2) were fed a standard starter diet (CON), CON + PrimaLac® (DFM; 0.3% w/w), and CON + Salinomycin (SAL; 50ppm) from hatch to 21d. The birds were euthanized on d 21, and the ileal, jejunal, cecal and colon tissues were dissected. Samples were examined by light microscopy (jejunum and ileum; Trial 1) and scanning electron microscopy (SEM; ileum, cecum and colon; Trial 2). Feeding of the DFM increased intestinal muscle thickness ($P < 0.05$) up to 33% in comparison to the CON treatment. DFM group had also increased villus height and perimeter ($P = 0.009$ and 0.003, respectively) in jejunum. Segmented filamentous-like bacteria (SFB) were less numerous in DFM treated chicks than in the CON chicks. Very few SFB were found in close proximity to other microbes in the ileum. The DFM chicks had a larger number of bacteria positioned over or near goblet cells and in inter-villi spaces. Bacteria in the colon were observed to be attached primarily around and within the villi crypts. Mucous thickness and the density of bacteria embedded in the mucus blanket appeared to be greater in DFM treated animals than in the CON in all intestinal segments. The birds fed SAL had fewer bacteria and enterocytes in the ileum than in the Con and DFM treated birds, and they had thicker and fewer microvilli. Since GI track colonization by the DFM organisms can prevent the attachment of pathogens to the epithelium, spatial relationships described in this study might speak to the functionality of DFM and probiotics

in preventing disease and altering intestinal energy consumption. It also supports previous observations that the feeding of salinomycin alters intestinal function.

Key words: chicken broilers, direct-fed microbial, scanning electron microscopy, histology

INTRODUCTION

The utilization of direct fed microbials (DFM) in animal feed is considered as a possible alternative to the low-level feeding of antibiotics (Hong et al., 2005). Previous studies in our laboratory have demonstrated that the use of a DFM, also referred to as probiotic, affects intestinal energy expenditures, and it alters intestinal fermentation and passive nutrient transport (Chichlowski et al., 2006b). Microbial colonization of the intestinal tract of chicks takes place soon after hatching, immediately after the animal starts to ingest food (Bird et al., 2002). The ability to adhere to the intestinal mucosa or to intestinal mucus is an important characteristics of any allocthonous bacteria and this ability varies between bacterial strains (Marteau et al., 2004). A DFM consortium of bacteria administered orally adheres to the host's intestinal epithelium, colonizes the digesta and mucous blanket, secretes antibiotics, and metabolites, releases metabolic enzymes, and modulates the host's immune system (Lin, 2003). The DFM bacteria that survive usually do not colonize the intestinal mucosa for long periods of time, and are generally eliminated within a few days of the cessation of their ingestion (Marteau et al., 2004). Furthermore, DFM organisms can compete for common binding sites with pathogens on the gastrointestinal (GI) surface (Fooks et al., 1999; Lin, 2003).

Lactobacilli and *Bifidobacteria* are the most frequently used DFM genera. *Lactobacilli* are gram-positive, non spore forming rods, usually non motile, and do not reduce nitrate, they can be divided into three distinct 16s rRNA groups (Fooks and Gibson, 2002). *Bifidobacteria* are also gram-positive, non-spore forming rods, with distinct cellular bifurcations with club-shaped morphologies. They make a significant contribution to carbohydrate fermentation in the colon (Fooks and Gibson, 2002). They possess

fructosylfructanosidase, which hydrolyzes the link between the fructose moieties of inulin and oligofructose (Fooks and Gibson, 2002). Both *Lactobacilli* and *Bifidobacteria* have been associated with beneficial effects for the host, such as promotion of gut maturation, gut integrity, antagonism against pathogens and immune modulation (Lan et al., 2005).

Furthermore, DFM bacteria are believed to have a number of effects on GI tract histology and ultrastructure (Awad et al., 2006), and on the regulation of mucous synthesis and secretion (Deplancke and Gaskins, 2001). Mucous is secreted by the goblet cells throughout the GI tract and forms an adherent gel on the mucosal surface (Sklan, 2004). The mucous layer acts as a barrier between the luminal contents and intestinal nutrient transporters, and it protects the mucosal surface from exogenous and endogenous luminal irritants, such as bile salts (Yagi et al., 1990). Additionally, several studies have shown that DFM may enhance the integrity of the tight-junctions between the intestinal epithelial cells during infections or inflammatory conditions (Montalto et al., 2004; Shen et al., 2006).

In the present experiment, histological and ultrastructural changes in intestinal architecture were analyzed as well as the spatial relationship between microorganisms and the epithelial cells lining the GI tract. In addition, because of its common usage as a coccidiostat antimicrobial in poultry production systems, the effects of feeding salinomycin on gut structure were also investigated in the present study. Salinomycin is an ionophore that alters the transport of ions across biological membranes (Augustine and Danforth, 1999). The data presented herein strongly suggest that the DFM organisms alter intestinal configuration and ultrastructure, as well as mucus production and the distribution of SFB. Furthermore, salinomycin seems to alter both the level of intestinal colonization by bacteria and the ultrastructure of the surface of intestinal epithelium.

MATERIALS AND METHODS

Experimental Design

Sixty (Trial 1) and eighty (Trial 2) one-day old broiler chickens were placed on a standard corn-soybean meal diet (17.08 % CP, 2.4 % fat, and 2830 kcal ME/kg). All birds were housed, maintained and euthanized according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at North Carolina State University. The objective of this trial was to quantitatively analyze the effects of feeding a DFM and SAL on intestinal histomorphometry. Ileal, cecal and colonic surface ultrastructure and their relationships with adherent bacteria were also examined using scanning electron microscopy (SEM).

A completely randomized design was used for both trials. Chicks from each treatment were randomly selected for experimental measurements, so the average age of the chicks was 21 d at the time of measurements. Chicks were assigned to one of following treatments: CON (no additives,), SAL (salinomycin, 50ppm of feed) and a DFM consortium (Primalac®, Clarksdale, MO; 0.3% of a diet). Primalac® was added as a lyophylyzed mix containing 1 x 10^8 CFU/g of *Lactobacillus casei*, *L. acidophilus*, *Bifidobacterium thermophilium*, and *Enterococcus faecium*. Salinomycin was chosen because of its widespread use as a coccidiostat within the poultry industry and its antimicrobial properties against gram-negative organisms (Duffy et al., 2005).

Chicks were placed at hatch in Petersime batteries; CON and SAL were housed in batteries in a separate room from the live organism DFM treatment with single pass air. In order to prevent cross-contamination between the CON and SAL chicks and the DFM

chicks, access to the bird rooms was restricted to essential personnel, with all procedures being performed on the CON and SAL room before entering the DFM room. Personnel were required to shower and change clothes before re-entering the CON room. Chickens were fed their respective treatment diets for 21 d. They were then taken off of feed for 12h prior to sample collection on d 21.

Individual bird measurements were regarded as the experimental unit. For the histomorphometric calculations, an average of ten measurements for each parameter from each bird were statistically analyzed as a one way ANOVA using the statistical program, STATISTIX® 8 (Analytical Software, Tallahassee, FL). Since sample sizes in these trials were small, Fisher's Least Significant Difference was used to test differences between means only when the ANOVA indicated significance at $p \leq 0.05$ (Motulsky, 2005).

Sample collection and analyses

Birds in Trials 1 and 2 were killed by cervical dislocation after a 12-h feed deprivation period. Immediately after euthanasia, the middle portion of the ileum, between Meckel's diverticulum and the ileo-cecal-colonic junction (Trial 1), and between the cecal and colonic junction (Trial 2), samples were collected from 18 birds (6 per treatment) and flushed with phosphate-buffered saline for scanning electron microscopy imaging (SEM).

In Trial 1, ileal and jejunal samples were collected and tissue samples were fixed in 10% Neutral Buffered Formalin for 24 hours. Trimmed cross sections placed in biopsy cassettes were rinsed in running tap water and processed into paraffin on a Sakura VIP Tissue processor. A routine over night process cycle was used. Tissues were embedded in paraffin and four 1-μm sections were cut on a Leica 2135 microtome and placed on slides.

Step sections at 200 μm intervals allowed for visualization of different sets of villi and crypts. Slides were stained with Hematoxylin and Eosin on a Sakura automatic stainer. A computerized microscopic image analyzer (Southern Micro Instruments, Atlanta, GA) was used to determine the histomorphometric parameters; villus height, villus width at its base, villus perimeter length, crypt depth, external muscle layer thickness, and height of enterocytes at mid villus, as previously described (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.

In trial 2, 10-12 1-mm pieces from each sample were fixed in a mixture of 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for SEM analysis. Tissue specimens were postfixed with 1% osmium tetroxide in ice-cold buffer for 20 min. The specimens were dehydrated in a graded series of ethanol solutions (30, 50 70, 90 and 100% - 20 min each), and were then subjected to critical-point drying (Samdri-795, Peabody, MS) using liquid carbon dioxide as the medium. The dried specimens were coated with gold/palladium (Anatech Hummer 6.2) and examined with a JEOL 5900 scanning electron microscope (Rockville, MD) at 20kV.

RESULTS

Histomorphometrics

The effects of dietary treatment on histomorphometric parameters are presented in Table 1. In the jejunum, the DFM treated broilers had increased villus height and perimeter

(P = 0.009 and 0.003, respectively) in comparison to the SAL fed chicks (Figure 1). Similarly, jejunal crypt depth and muscle thickness values were greater in the DFM treated (P = 0.01 and 0.01, respectively) than in the birds treated with SAL. The ilea of the DFM treated birds had a greater muscle thickness than did the ilea of the CON and SAL treated birds (P = 0.02; Figure 2). There were no significant differences between treatments for villus height:crypt depth ratio and mid villus enterocyte height in the ileum and jejunum of the three treatment groups. An increased density of goblet cells was also observed for the DFM treated birds in comparison to what was observed for the CON and SAL treated birds (data not shown).

Scanning Electron Microscopy

The villi observed in all treatments were as a leaf-like in appearance with a wide base (Figure 3A). Often the extrusion zones for dead enterocytes at the apex of the villi were wide and contained deep furrows and a large number of shedding cells. Occasionally, at the apex of the villus spaces (a few μm in width) were observed between the epithelial cells, as well as disintegrating cells. The microvilli at the brush border were well developed and densely packed (data not shown). They were approximately 2 μm long. The mucous blanket showed a pronounced tendency across all treatments to condense during dehydration and to separate from the tissue surface in specimens prepared for SEM. In most samples, the fibrous polysaccharide glycocalyx of the microvillous surface is easily observed.

The structure of the villi in the ileum of the 21 d old chicks appeared normal, when all dietary treatments were examined by SEM, with the exception of two surface features of the ileum. First, there was a lower density and consistency of mucus in DFM birds than in the

CON and SAL treated birds. Mucus on the intestinal surface of the samples collected from the DFM treated birds was more evenly distributed and thinner than in the CON birds (Figure 4A, 4B, 5A and 5B). The thickness of the mucous blanket observed in the ileal samples from the 21 d-old broiler chickens was estimated to be 10 and 15 μm for the DFM and CON groups, respectively (Figures 4A and 5A). The thicker mucous blanket in the ileum of CON birds was apparently able to withstand more damage during sample preparation, but was still seen as a discontinuous, balled, and rolled layered structure rather than as the continuous blanket, which normally covers tissue *in vivo* (Skrzypek et al., 2005). Although the mucous blanket was usually dehydrated to various extents in the samples from all of the treatment groups; bacteria could always be seen within the mucous layer. A higher bacterial density was apparent in the sample mucus layer of the CON birds in comparison with samples from the DFM birds (Figure 5A). Second, the ileal DFM samples had a lower density of segmented filamentous-like bacteria (SFB) in comparison to the samples from the CON birds.

In the present study, coccoid-like and bacillus-type bacteria were observed throughout the surface of the GI tract, in addition to the segmented filamentous-type bacteria (SFB; Figure 6D). Few SFB coexisted in close proximity to other microbial organisms (Figure 6A-6D). Rod-shaped bacteria (similar to *Lactobacilli* in morphology) were observed on the surface of the ileal, cecal, and colonic mucosa (Figures 5, 7C, 7D, 8C, and 8D). There was clearly a higher density of those microorganisms, however, in broiler chicks treated with the DFM (Figure 3B). Bacteria observed in the ileum of the DFM treated birds seemed to be associated with goblet cells compared to those observed in CON and SAL

groups (Figure 7C and 9). The largest populations of surface-associated bacteria were observed in the colon of DFM treated birds.

There were large differences that were noted between the treatments in bacterial colonization in the cecum and colon. The DFM diet increased overall bacterial colonization in those segments compared to other treatments (Figure 7A-7F and 8A-8F). The SEM imaging showed an increase in bacterial attachment to epithelial tissue with DFM supplementation in comparison with the bacterial attachment in the other treatments. The SAL treatment altered the cecal epithelium, with large areas that were smooth and denuded of mucus, as well as by lowering the number of goblet cells (Figure 5E and 5F). There were also fewer transversed furrows in the SAL treated birds in contrast to their abundance in the DFM treated group. Similar differences were noted in the colon. Bacterial colonization associated with the epithelial surface of the colon was very pronounced with DFM as was colonization of the mucous blanket. There were more goblet cells present in the colon of SAL treated birds than in cecum, however, most of the colonic goblet cells in the SAL treated birds were not associated with bacterial colonization in their proximal areas.

DISCUSSION

Histomorphometric analysis indicated that increased villus height and perimeter, as well as muscle thickness and crypt depth in the jejunum was associated with the feeding of the DFM in comparison with the feeding of SAL (Table 1). *Lactobacillus* treatment caused similar changes in poultry as previously described (Dobrogosz et al., 1991). Muscle thickness was also greater in the ileum of DFM fed than in SAL fed birds. Increases in the

villus height and the villus height/crypt depth ratio are directly correlated with increased epithelial cell turnover (Fan et al., 1997). In the present study, analysis of these parameters did not show any significant differences among the treatments. This suggests that the increases in villus height and perimeter that were observed with the DFM treatment are not associated with enterocyte turnover rates. That observation is in agreement with previous studies from this laboratory, which have shown no differences in protein/DNA ratio of the intact ileum and jejunum (Chichlowski et al., 2006b). Increased passive absorption of glucose and proline in 21 d old chicken broilers fed DFM diet was also observed in those previous studies. Adjustment in the absorptive area from the DFM treatment might be linked with increased passive absorption of nutrients. Skrzypek and co-workers (Skrzypek et al., 2005) used the term "indifferent absorptive area", which described potential absorptive ability not entirely utilized in the nutrient absorption of growing piglets. In their study, the shift was made from such state into "effective absorptive area" after the first feeding in synchrony with increases in the local circulation in the gut mucosa. It is possible that DFM treatment in our study triggered a similar shift, increasing the "effective absorptive area" via alterations in villus size.

The bacteria in the gastrointestinal tract are associated with the mucous layer, which is easily removed from the epithelial surface when tissue sections are processed for SEM. In conventional fixation methods, preserving the mucus layer is a challenge since in most cases it is washed away or dissolved more quickly than it can be stabilized. That was the case in most instances in the present study. Nevertheless, despite those challenges, some differences were noted in the mucous layers associated with the different dietary treatments (Figure 4A-H). Other investigators have reported this problem as a limiting factor in the interpretation of

mucosal integrity with SEM micrographs of intestinal samples (Allan-Wojtas et al., 1997). Extensive damage to the mucous layer during SEM preparation of samples was observed in this study, even so it is thought that *in vivo* this structure is likely continuous and covers the microvillous surface almost completely by 21 d (Allan-Wojtas et al., 1997). As the animal matures, the mucous layer thickens and more microbial flora colonizes within it (Rozee et al., 1982).

Upon examination of samples in the present trial, it was concluded that some microorganisms are firmly attached to the epithelial surface, especially in the DFM treatment. Infrequent occurrence in the ileum and colon suggest that the bacterial colonies might be easily dislodged during preparation of the specimens from those areas. Previous studies have not observed any structural elements, such as filaments, connecting bacteria to the epithelium or microbial penetration of the mucosa, with the exception of SFB in SEM imaging of intestinal villi tissue (Salanitro et al., 1974). Additionally, microbes are able to colonize the mucous matrix within which a number of different microbial flora exist. Because the mucous blanket is normally very thick, understanding its microbial population dynamics is of considerable importance. Contrary to previous reports, in the present experiment the majority of the observed bacteria were positioned on the tissue surface rather than imbedded in the mucous blanket (Rozee et al., 1982).

The number of goblet cells per villus increases as the villi grow, but the proportion of goblet cells to enterocytes remains constant with age (Tucker and Taylor-Pickard, 2004). The role of the mucous in absorption and protection against pathogens is not yet fully understood; however, Ikeda et al. (2002) reported that goblet cells may play an important role in epithelial cell repair following damage to the GI mucosa. In this experiment, there was an

apparent increase in goblet cells associated with the DFM treatment, as compared to SAL and CON treatments as observed through both the light and scanning electron microscopy (data not shown).

In the present experiment, the feeding of SAL greatly altered the epithelial surface in all sections analyzed. The effects of the ionophores on cell and tissue integrity are a result of physiological effects on the permeability of the cell membranes to the alkali metal cations; they can also physically change the intracellular osmolality (Zhu and McDougald, 1992). In previous studies, salinomycin markedly disrupted the integrity of merozoite membranes and caused cytoplasmic vacuolization (Augustine and Danforth, 1999). It is possible that in the present study, where chicks were not exposed to protozoa or bacterial pathogens, and where they were housed in clean conditions, that this ionophore could have blocked some of the microbial binding sites by becoming inserted into the host's intestinal epithelial membranes. The presence of salinomycin in the membranes of the intestinal epithelium would likely have a critical affect upon the epithelial water balance, thereby causing enterocyte damage. Indeed, besides a decreased level of bacterial colonization in the ileum and colon samples from the birds fed SAL, we also observed many areas with dehydrated and damaged tissue (Figure 6E, 6F, 8E, and 8F). Furthermore, in the cecum, where bacterial colonization is usually the greatest, the SAL treated birds had visible indentations and a lower number of goblet cells dispersed in their epithelial tissue. It is possible that decreased ileal glucose and proline absorption from the SAL treatment reported previously in this laboratory (Chichlowski et al., 2006b) is linked to the epithelial damage caused by the feeding of SAL.

The CON treated birds contained an abundance of segmented bacteria embedded in the epithelium within the ileum. To date, *segmented fusiform bacteria*, which are abundant

in the intestine of young animals, have not been cultured (Tucker and Taylor-Pickard, 2004). They are known to be nonpathogenic, gram-positive, anaerobic, spore-forming bacteria that inhabit the intestinal tract (Dewhirst et al., 1999; Yamauchi and Snel, 2000) as well as the respiratory tract (Jang and Hirsh, 1994). Yamauchi and Snel (2000) referred to these organisms as simply unclassified *segmented filamentous bacteria*, while other authors have used the description of *Fusiform EOS bacteria* (Dewhirst et al., 1999) or *Fusobacterium* (Omata, 1953). Thus far, the genus *Fusobacterium* currently includes 13 species (Citron, 2002). In this study we refer to these organisms as segmented filamentous-like bacteria or SFB. Their function as immune-stimulating agents has been reported (Meyerholz et al., 2002). Furthermore, *segmented fusiform bacteria* have been reported to have a potential antagonistic effect against GI bacterial pathogens (Heczko et al., 2000); and, they adhere to intestinal epithelial cells with holdfasts, and filaments which are usually found only at the ileal villus tip (Davis and Savage, 1974; Glick et al., 1978). The decreased density of SFB colonization following the feeding of the DFM in the present trial is not understood; however, it is possible that increased colonization with DFM organisms affected this reduction.

The most efficient DFM bacteria will likely be strains that are robust enough to survive the harsh physico-chemical conditions present in the GI tract (Fooks and Gibson, 2002). This includes gastric acid, bile secretions and competition with the resident microflora. Higher density of the microbial flora associated both with the epithelium (Figure 9) and mucous blanket in the birds fed the DFM in the present trial, suggest that the consortium of bacteria contained in the DFM product used in the present trial colonize effectively. The large complement of colonizing microorganisms in the DFM fed group,

may inhibit pathogens and other opportunistic microbiota from reaching and colonizing small and large intestinal tissue, as well as the cecum. We suggest that the presence of the DFM bacteria and a continuous and heavily colonized mucous blanket may preclude colonization by opportunistic potential pathogens, even when they are repeatedly introduced into the GI tract.

The action of antibiotics or bacterial toxins may distort the mucous barrier that facilitates the development and attachment of pathogens. It is possible that invading bacteria may depend on the alterations of the mucous layer and its association with the epithelial tissue. Also, changes in the properties of this barrier can alter absorption of both dietary and endogenous macromolecules and ions (Sklan, 2004). In previous studies, increased passive nutrient absorption was observed in the DFM birds in comparison o CON and SAL treated birds (Chichlowski et al., 2006b). Thus, there exists a potential connection between alterations in the appearance of the mucous layer in the present study and previous reports and nutrient transport. It is possible that the presence of the DFM organisms in the diet of broilers might facilitate nutrient transport in the GI tract that is not dependent on sodium transporters. Furthermore, it has previously been demonstrated that bacteria can up-regulate a complex of gene action in epithelial cells and by doing so dramatically influence the expression of a diverse array of epithelial products, thereby altering the biochemistry, physiology, and function of the intestinal barrier (Tucker and Taylor-Pickard, 2004). In previous studies in this laboratory, feeding of the DFM was potentially shown to affect the inflammatory state of the ileum via altered cytokine production (Chichlowski et al., 2006a). It should be mentioned that most of the differences between the DFM and CON dietary treatments in the present and previous trials from this laboratory , showed non-significant.

trends. Previous trials have consistently shown numerical decreases in whole body and tissue oxygen consumption, a decrease in passive nutrient absorption, and a lower expression of the pro-inflammatory cytokines in ileum from the DFM treatment in comparison with the CON treatment. It is possible that although those trends were not significant, that they all together form a complex and dynamic mechanism of DFM action. Nevertheless, further studies are needed to fully understand this phenomenon.

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TABLES AND FIGURES

Table 1. Histomorphometric analysis of ileal and jejunal tissue in chicken at d 21 of age, Trial 1[1]

	Diet[2]			Sig.
	CON	**SAL**	**DFM**	
Jejunum				
Villus, μm				
Height[3]	1181.8 ± 62.81[ab]	959.0 ± 62.81[b]	1266.5 ± 62.81[a]	0.009
Width[4]	132.02 ± 8.65	104.85 ± 8.65	129.20 ± 8.65	0.081
Perimeter	2627.4 ± 118.62[a]	2186.9 ± 118.62[b]	2888.0 ± 118.62[a]	0.003
Crypt depth, μm	185.91 ± 11.36[a,b]	145.21 ± 11.36[b]	200.09 ± 11.36[a]	0.01
Muscle thickness[5], μm	262.30 ± 22.60[ab]	200.35 ± 22.60[b]	317.57 ± 22.60[a]	0.008
Villus height:crypt depth ratio	6.5434 ± 0.39	6.8556 ± 0.39	6.6315 ± 0.39	0.85
Enterocyte height[6], μm	63.693 ± 4.37	50.557 ± 4.37	58.147 ± 4.36	0.14
Ileum				
Villus, μm				
Height	946.82 ± 46.5	830.42 ± 46.5	860.40 ± 51.0	0.22
Width	105.36 ± 7.45	107.65 ± 7.45	100.04 ± 8.16	0.79
Perimeter	2139.7 ± 106.77	1892.8 ± 106.77	1838.5 ± 116.96	0.15
Crypt depth, μm	175.10 ± 11.46	164.91 ± 11.46	179.25 ± 12.55	0.69
Muscle thickness, μm	252.29 ± 27.05[b]	253.86 ± 27.05[b]	363.10 ± 29.63[a]	0.024
Villus height:crypt depth ratio	5.7663 ± 0.45	5.4796 ± 0.45	4.8378 ± 0.49	0.39
Enterocyte height, μm	50.682 ± 6.98	60.681 ± 6.98	48.947 ± 7.65	0.48

[a,b]Means in rows lacking a common superscript are significantly different ($P \leq 0.05$)

[1] n= 18 (1 missing observation for ileum)

[2] CON = no additives, SAL = Salinomycin (50ppm), DFM = Direct-Fed Microbial (Primalac®)

[3] Least Square Means ± SEM

[4] Width at villus base

[5] Total thickness of muscularis externa

[6] Enterocyte height at mid villus

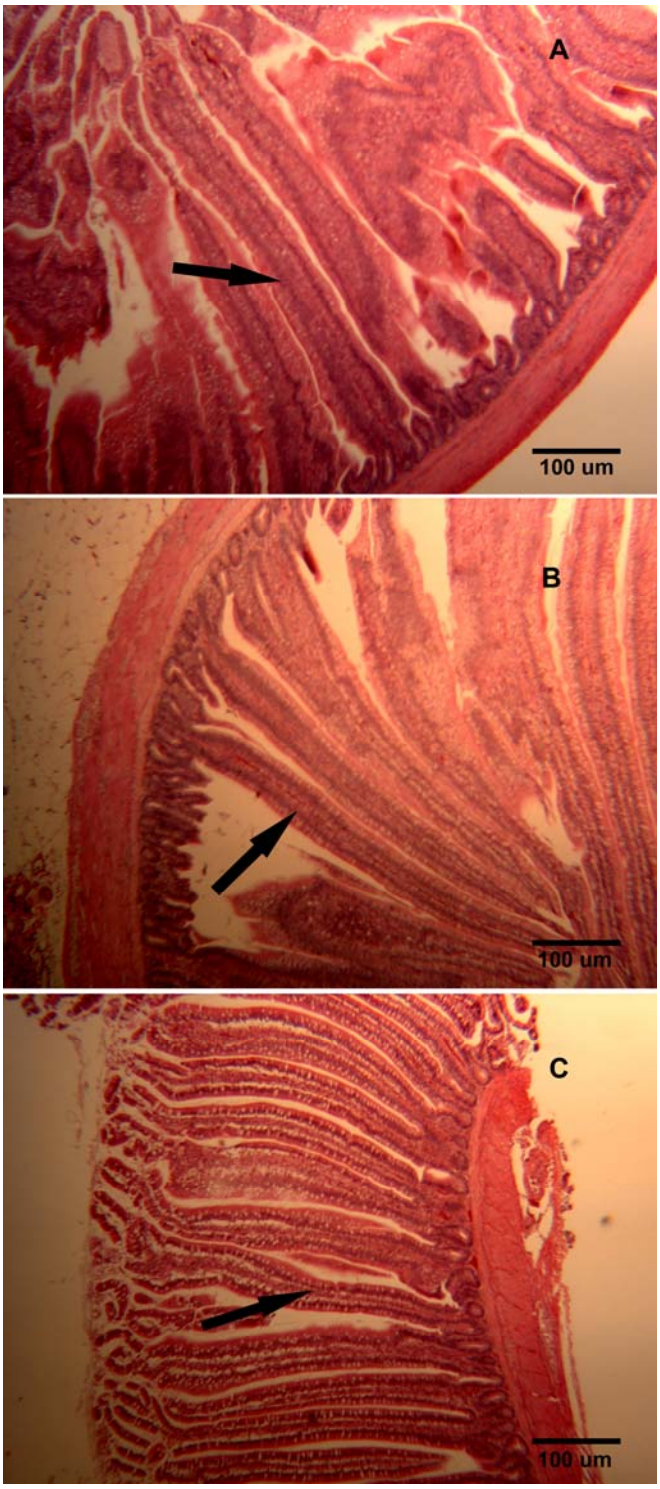

Figure 1. Histomorphometric analysis of the jejunum of a 3 wk old chicken. Villus height in DFM birds (B) was numerically higher than in the CON birds (A), and was significantly higher than in the SAL treated birds (C). Arrows indicate jejunal villi.

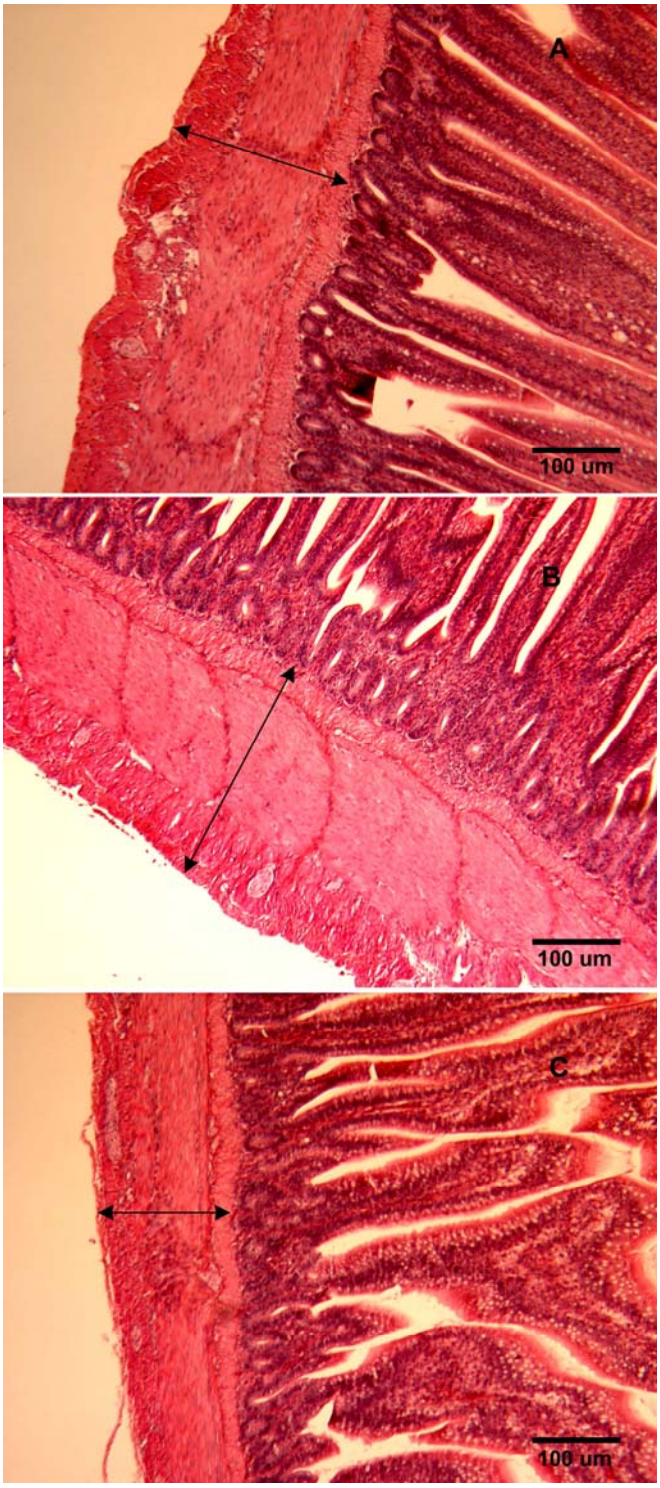

Figure 2. Histomorphometric analysis of the ilea from 3 wk old chickens. Muscle thickness in the DFM treatment was significantly increased in comparison with that observed CON and SAL treated chickens (A and C, respectively).

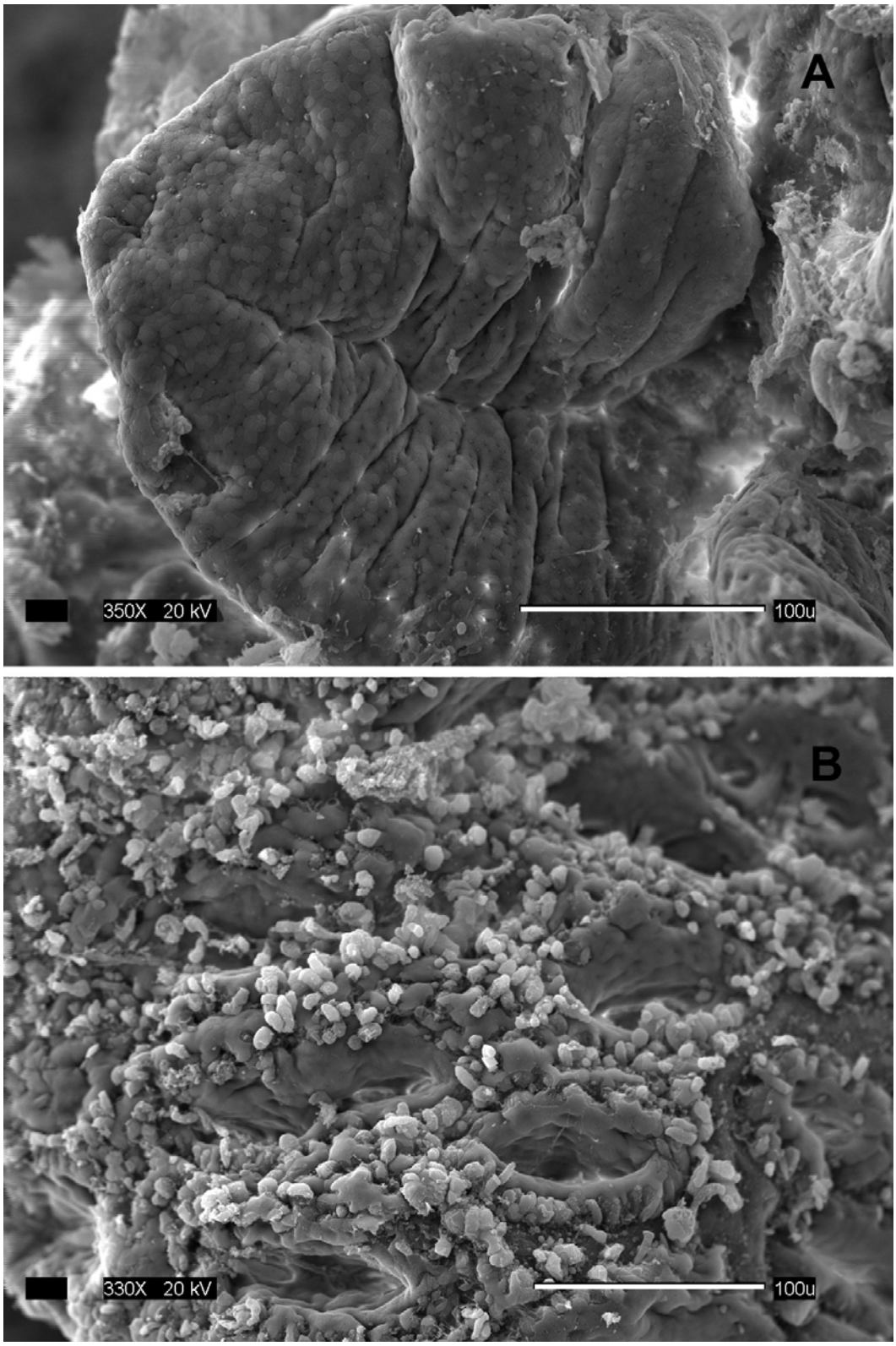

Figure 3. SEM micrographs of the colonic surface from a 21 d old chicken broiler. A: The CON sample has transverse furrows with very low bacterial colonization; B: The DFM sample has a high level of bacterial colonization with microorganisms of multiple-morphologies that are attached to the same tissue.

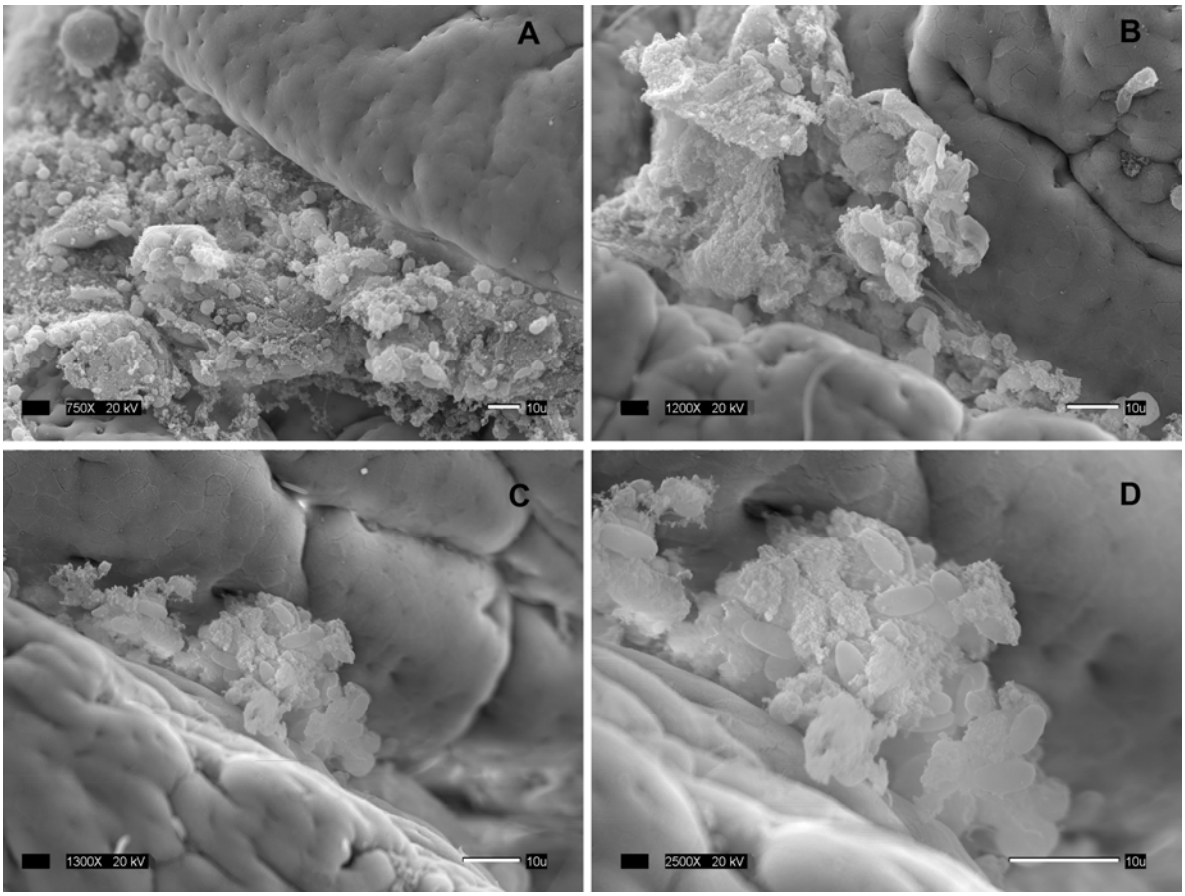

Figure 4. SEM micrographs of the ileal surface from a 21 d old chicken broiler fed DFM. A: The mucous layer has altered structure in comparison to the mucous from CON birds; B, C, D: The visible presence of microbial flora in the mucous blanket of DFM birds.

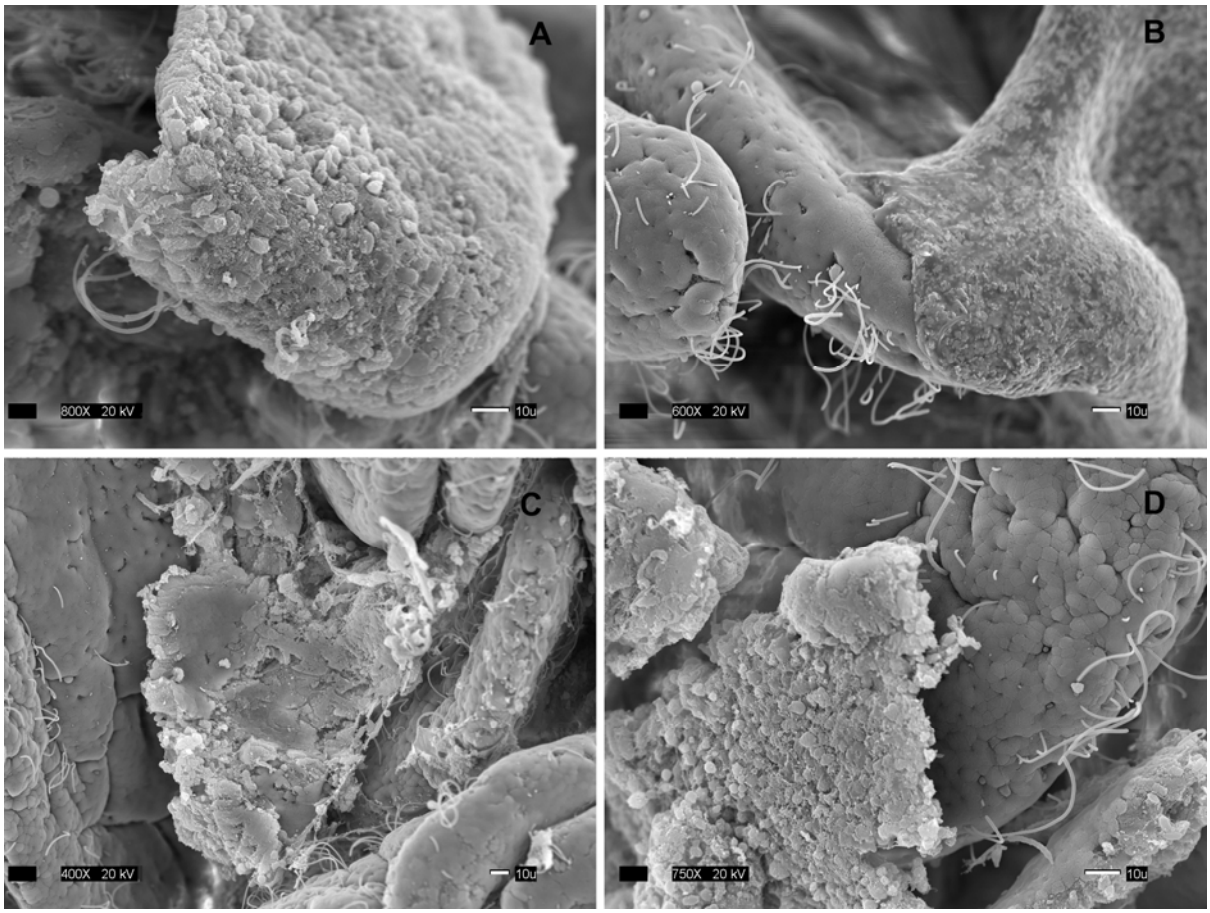

Figure 5. SEM micrographs of the ileal surface from a 21 d old chicken broiler fed the CON diet. A: thicker mucous layer in the CON birds than in the DFM birds; B: The visible attachment sites for SFB in close proximity to the mucous blanket; C: mucous layer present in between two separate villi; D: The mucous blanket at the mid villus.

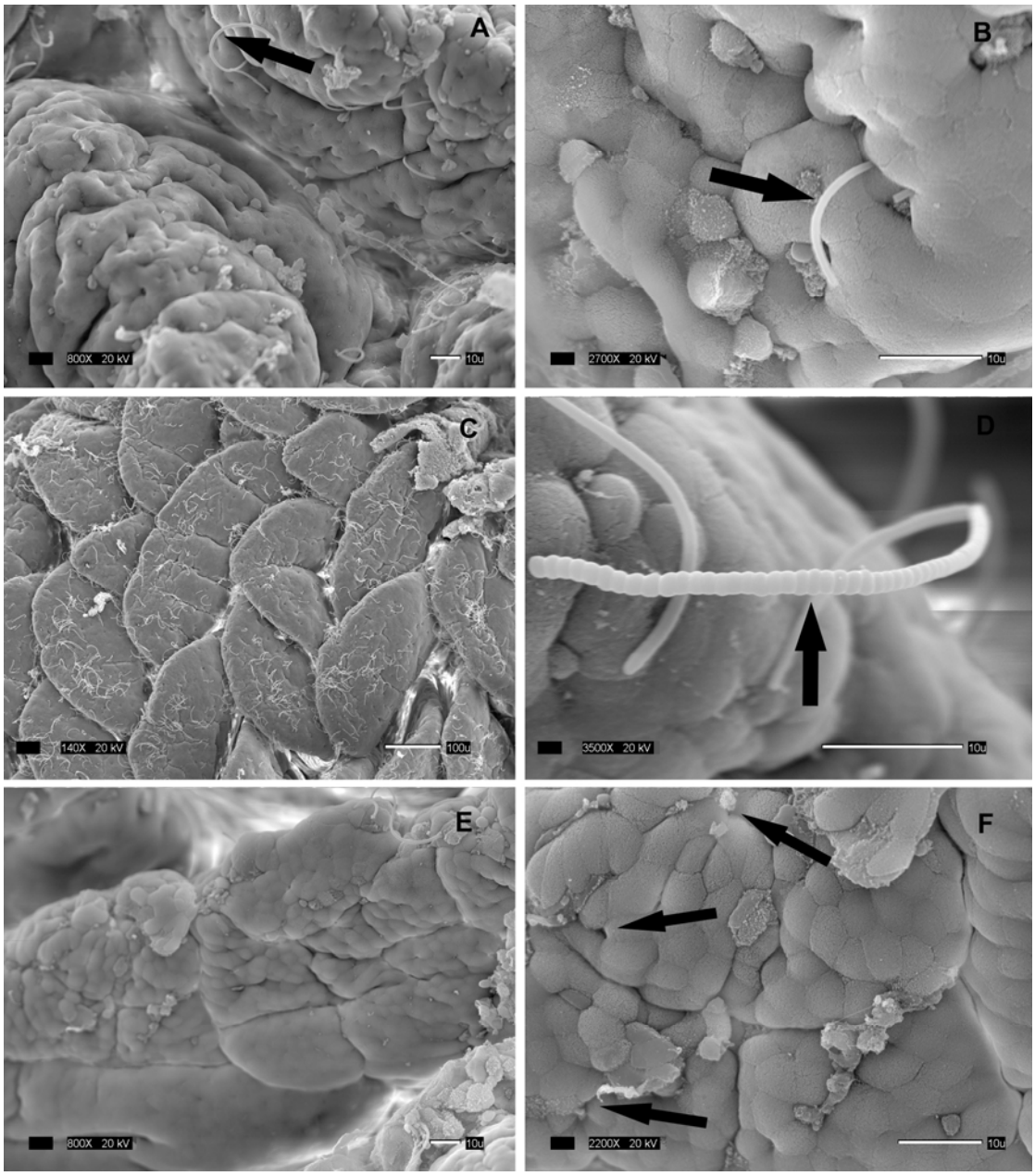

Figure 6. SEM micrographs of the ileal surface of a 21 d old chicken broiler. A: CON, the arrows indicate SFB colonization sites; B: CON, at a higher magnification, SFB are clearly associated with the goblet cells; C: DFM, the arrows indicate scattered SFB colonization; D: DFM, with a high magnification of SFB structure; E: SAL, no colonies of bacteria visible; F: SAL, higher magnification, several goblet cells, not associated with any bacterial colonies (arrows).

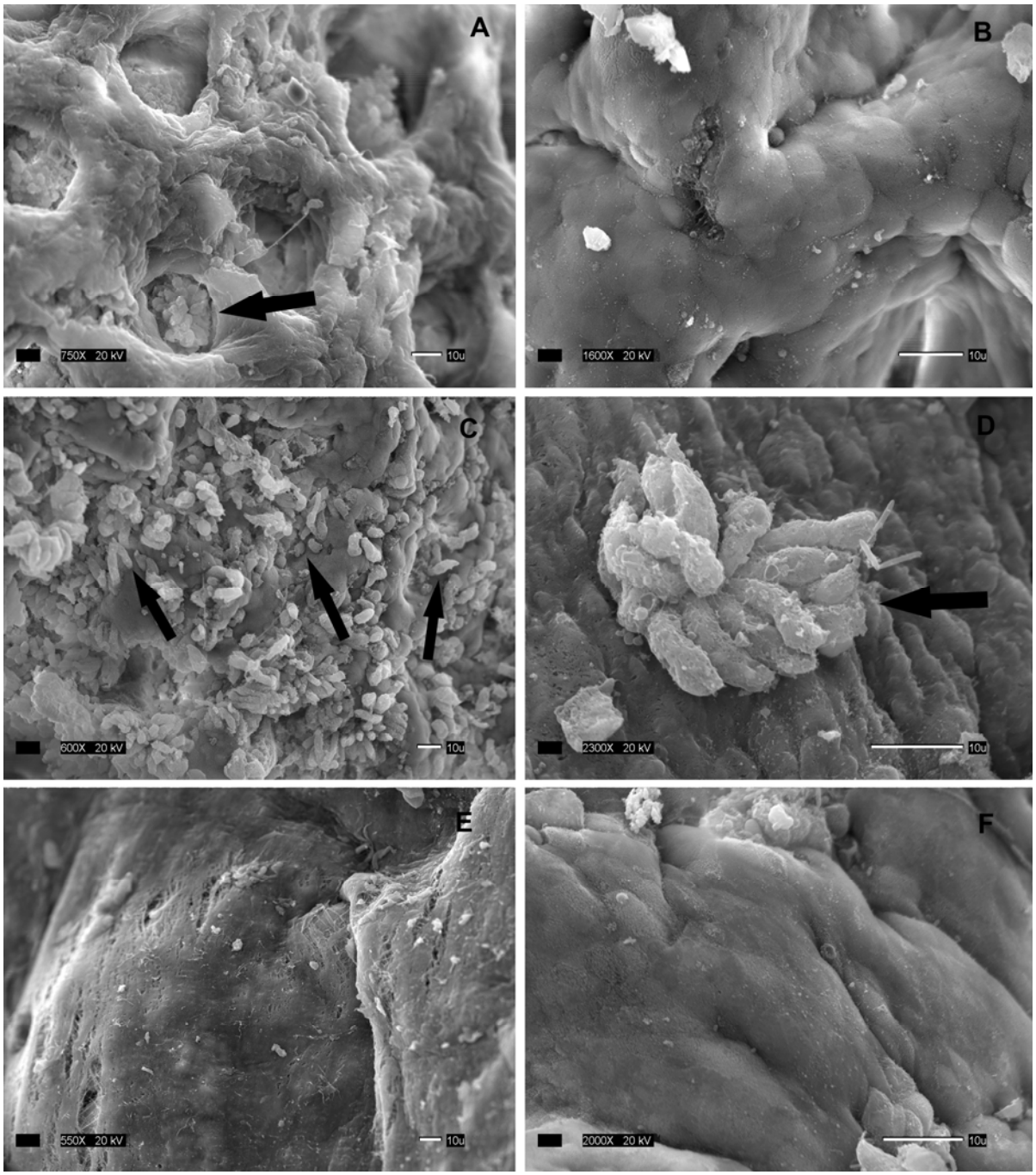

Figure 7. SEM micrographs of the cecal surfaces of 21 d old chicken broilers. A: CON, arrows indicate bacterial colonization sites; B: CON, higher magnification, goblet cells not associated with any microorganisms; C: DFM, very dense bacterial colonization, arrows indicate several attachment sites for microbes; D: DFM, arrow indicates bacteria colony attached to the mucous producing cell; E: SAL, altered cecal surface, very few transverse furrows, no bacteria colonies visible; F: SAL, higher magnification, several goblet cells, not associated with any bacteria colonies.

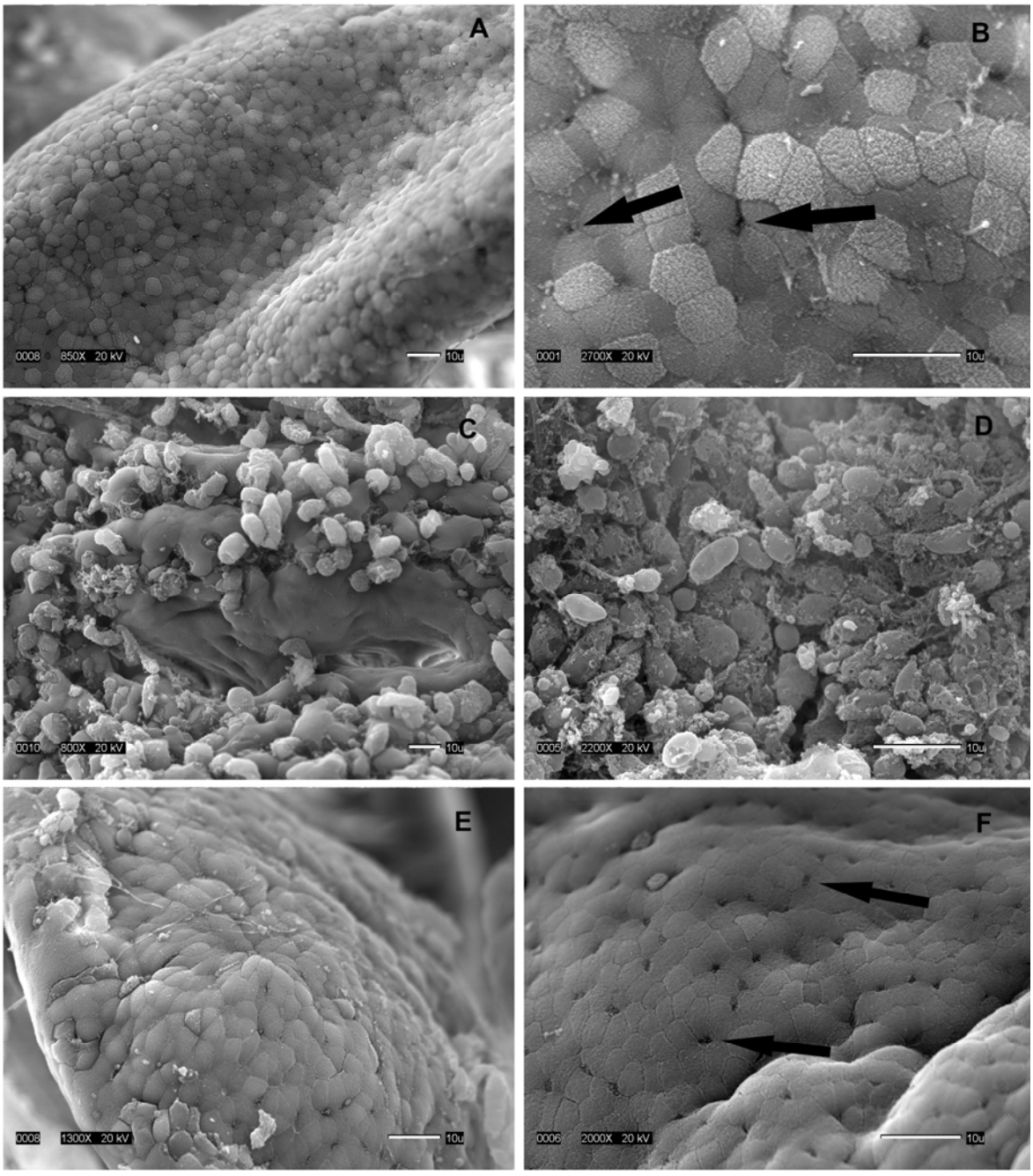

Figure 8. SEM micrographs of the colonic surface of 21 d old chicken broilers. Surface of the colon (goblet cells – arrows). A, B: CON at lower magnification, tissue appears smooth with no bacterial colonization; C: DFM, dense bacterial colonization; D: DFM, multiple microorganisms present in the mucous layer; at higher magnification the preserved mucous layer appears to cover the structural detail of the intestinal surface; E: SAL, no colonization visible; F: SAL, goblet cells present are not colonized by bacteria.

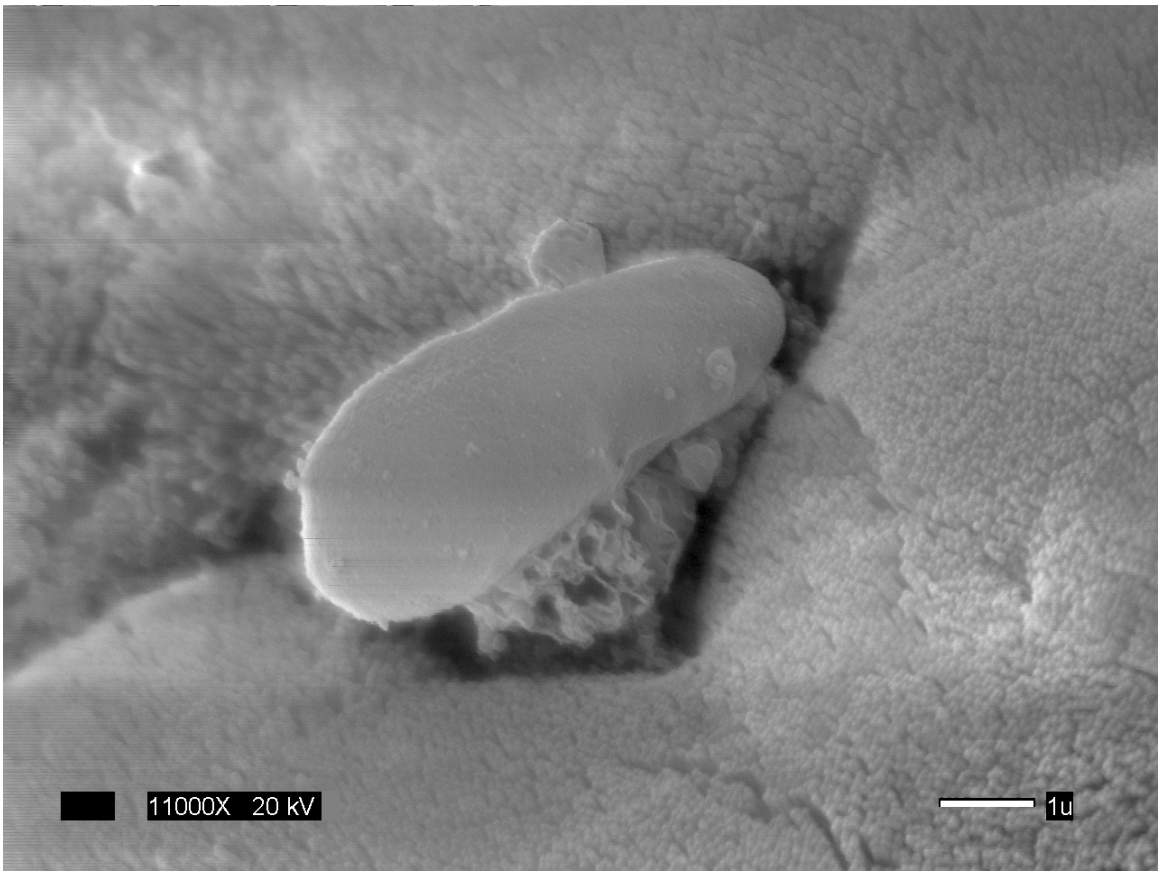

Figure 9. SEM micrograph of the ileal surface of the 21 d old broiler chicken. At high magnification, a rod shaped microorganism can be seen attached to a goblet cell.

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CHAPTER 5

CONCLUSIONS

CONCLUSIONS

The beneficial effects on growth and feed conversion reported in many studies using DFM are likely due to a complex and highly integrated cascade of alterations in the physiological mechanisms of the bird. The first study demonstrates the potential effects of SAL and DFM on intestinal (Table 4, page 64) and whole-body metabolism (Table 5, page 65), as well as expression of cytokines in the innate intestinal immune system (Figure 2, page 67). Although some alterations were noted in intestinal mucosal cytokine production, no comprehensive paradigm emerged from the data that fully explained the decrease in whole-body and ileal O_2 consumption in the DFM vs. the SAL and CON birds. Additionally, salinomycin seems to result in increased whole-body and intestinal energy expenditures. It is possible that this effect may be the because of an induced salinomycin toxicity.

The second study revealed changes in fermentation and function of the lower gastrointestinal tract of broilers treated with DFM or SAL. The results of intestinal fermentation (Table 2-5, pages 91-94), ileal nutrient transport (Table 6, page 95), as well as ouabain specific tissue oxygen consumption estimates (Table 8, page 97) suggest the likelihood of a small, but additive series of beneficial changes in metabolism with DFM.

The third study focused on analysis of histological and ultrastructural changes in intestinal architecture as well as the spatial relationship between DFM microorganisms and the epithelial cells lining the gastrointestinal tract. The data presented here strongly suggest that DFM alters intestinal configuration and ultra-structure (Figure 1, page 122), as well as mucus production and distribution of SFB (Figure 4, page 125). Furthermore,

salinomycin seems to alter both the intestinal colonization by bacteria and the ultrastructure of the surface of intestinal epithelium in a manner detrimental to intestinal function (Figure 7, page 128).

Many parameters indicated non-significant trends between DFM and CON dietary treatments that suggested beneficial effects of DFM. For example DFM treatment numerically decreased whole body and tissue oxygen consumption, passive nutrient absorption, and lowered expression of the pro-inflammatory cytokines in ileum compared to CON. It is likely that trends were biologically significant; however, the complex nature of the measurements precluded estimates using enough experimental units large enough to demonstrate statistical significance.

It is likely that the beneficial effects of direct fed microbials are the result of the summation of a complex, multi-variate series of alterations in gut microbial and whole body metabolism. When considered together these changes constitute a complex and dynamic mechanism of DFM action. Further studies are needed that will require a multidisciplinary approach, combining molecular biology, immunology, physiology, gastroenterology, microbiology and nutrition to fully elucidate the mechanisms of action of DFM consortia. .