CHAPTER 1
GENERAL INTRODUCTION

The broiler industry is growing rapidly around the world. The success of this rapid growth is attributed to the dramatic improvements in genetics, intensive production, and nutrition. The tight coordination of these improvements also makes broiler production more efficient compared to other livestock industries, such as the pig industry (Martinez, 1999).

The intensive raising system causes various stresses to birds (NASC, 1999) and genetic selection for growth traits leads to a weakened immune system (Emmerson, 1997), therefore antibiotic growth promotants (AGP) have been routinely included in broiler diets (Ewing and Cole, 1994) to maintain health and production efficiency in the last few decades.

However, because of the development of resistance by pathogenic bacteria, which can impact public health, AGP are being taken out of broiler diets around the world. beginning in 1986 (Dibner and Richards, 2005). The withdrawal of AGP has brought up many problems, such as the increased incidence of clinical or subclinical necrotic enteritis (Kaldhusdal and Lovland, 2000) and decreased feed efficiency (Thomke and Elwinger, 1998a), which directly impair animal productivity. A big issue that the broiler industry needs to confront is how to maintain the production efficiency without the use of AGP. It is generally believed that multiple strategies are required to achieve the results that are comparable to the use of antibiotics (Dibner and Richards, 2005), for example, it may be necessary to adopt improved hygiene standards and animal husbandry practices, combined with feed additives which can promote health and growth.

Gut microflora has significant effects on host nutrition, health, and growth performance (Barrow, 1992), by interacting with nutrient utilization and the development of gut system of the host. This interaction is very complex and, depending on the composition of gut microflora, it can have either positive or negative effects on the health and growth of birds. There is an increased focus on animal microbiology to study the profile and function of gut microflora as well as its interrelationship with gut immune system. There is also an increased effort in animal nutrition to modulate the gut microflora through diet and thus improve the health and growth of the host. Of particular interest is the search for feed additives which may replace AGP in terms of growth-promoting effects. The focus of alternative strategies has been to prevent proliferation of pathogenic bacteria, thus maintaining health, immune

status and performance (Ravindran, 2006). Various gut microflora modulators, immunomodulators, or the combination of these two have been evaluated in broilers.

Mannanoligosaccharide (MOS), of yeast cell wall origin, was first applied in broiler diets in the year 1983 (Hooge, 2004a). The positive effects of MOS on the growth performance of broiler chickens have been confirmed by meta-analysis and holo-analysis over a 10-year period (Hooge, 2004a; Rosen, 2007). The research about the mechanisms elucidating the growth-promoting effects of MOS is increasing in recent years; however, the available data lack of consistency and correlation, and require a systematic examination of the role of MOS in the growth and gut physiology of birds.

Two major suggested mechanisms of action of MOS are 1) pathogen exclusion, and 2) immune modulation, both of which directly or indirectly affect the composition of gut microflora. Therefore, the work reported in this thesis was designed to test the general hypothesis that MOS exerts growth-promoting effects on broiler chickens through the modulation of gut microflora. Two major objectives of the present study were:

1) the evaluation of MOS effects on energy and nutrient utilization of birds, and 2) the evaluation of MOS effects on the development of gut microflora and gut mucosal morphology of birds.

CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

The broiler industry is growing rapidly around the world. In the US, for example, broiler production increased dramatically from approximately 5 billion in 1962 to nearly 48 billion in 2002 (USDANASS, 2003). It is agreed that the achievement of this growth is almost impossible without the advancement of modern management techniques and genetic engineering. Modern strains of broilers today, though raised at high rearing density rate of 10,000 - 20,000 chicks per house (USDANASS, 2003), can still achieve their full genetic potential and reach a liveweight of over 2.4 kg at 42 days of age. According to the FAO, 75% of the world's poultry are maintained in intensive operations using confinement systems (Shane, 2006). Intensification of the poultry industry has brought increased risks of both clinical and subclinical enteric disease as stressed animals are vulnerable to potentially harmful micro-organisms such as *Escherichia coli*, *Salmonella* spp., *Clostridium perfringens* and *Campylobacter sputorium* which, though, can be predominantly controlled with antibiotics (NASC, 1999). Therefore, it is without doubt that the use of in-feed AGP (Leeson, 1991) has become part and parcel of today's intensive poultry industries.

2.2 THE APPLICATION OF ANTIBIOTICS IN POULTRY PRODUCTION

The term "antibiotic" was first used to define naturally occurring chemical substances which are produced by various microorganisms and which suppress the growth of bacteria. However, modern common usage extends the term to include synthetic agents such as sulfonamides, nitrofurans and quinolones, which were formerly known as antibacterial or antimicrobial agents (JETACAR, 1999).

Antibiotics are used in animal husbandry in four ways 1) for therapy, 2) for disease prevention (prophylaxis), 3) as growth promotants, and 4) for coccidiosis control (JETACAR, 1999). The level used for therapy and prophylaxis is higher than the level (sub-therapeutic level) used for growth improvements (JETACAR, 1999). In the US, for example, the sub-therapeutic levels of antibiotics employed for growth promotion purposes range from 2 to 200 g/ton of feed (2.2-220 ppm). For prophylaxis against infection in so called "stressed" animals that are undergoing shipment, weaning or abrupt environmental change,

the concentration used increases to 100 to 400 g/ton (110-440 ppm) and are applied for 3-5 days and 2-3 weeks for chickens and livestock, respectively. For therapeutic effect against active infection, these drugs are usually given in a higher dose of 200-1000 g/ton (220-1100 ppm) of feed (DuPont and Steele, 1987).

2.2.1 Past, present and future use

In the early development of antibiotics, accurate figures on their total use, and particularly on the rationale for their use, are impossible to obtain. One of the great economic developments in the field of antibiotics is the use of these drugs in feeds for the promotion of animal growth (Jukes, 1955). Moore *et al.* (1946) were apparently the first to show that inclusion of antibiotics in the feed of chickens caused increased weight gain. A number of investigators subsequently documented the growth stimulation effect of antibiotics in pigs and chicks (Groschke and Evans, 1950; Stokstad and Jukes, 1950; Whitehill *et al.*, 1950, cited by Jones and Ricke, 2003).

Some antibiotics given to animals at growth-promoting levels may help prevent diseases increased by intensive poultry production. Common animal production practices such as overcrowding and high stocking rates, driven largely by economics, add significantly to animal stress and raise concerns about overall animal health and welfare (Wallinga, 2002). Putting antibiotics in feed or water can help reduce the possibility of infection in these stressed animals (JETACAR, 1999) making it possible for the sustainability of these practices. Antibiotics added to feed can, as a result, almost always be justified as "disease prevention," at least when administered for limited periods of time (Wallinga, 2002).

Later, attention was drawn to the separation of antibiotics into "feed" and "therapeutic" categories. The former legally sold without prescription, and the latter were to be available only on prescription (Vanbelle, 2000). In 1980s, approximately 45% of the 2.1-2.5 million kilograms of antimicrobial agents used in the United States annually were used in animal feed supplementation. Eighty percent of poultry was estimated to have been fed an antimicrobial agent at some time during their life; the percentage was the highest compared to other animal industries (swine, feedlot cattle and dairy calves) (DuPont and Steele, 1987).

Common antibiotics used in poultry feed include avoparcin, bacitracin, chlortetracycline, erythromycin, lincomycin, neomycin, oxytetracycline, penicillin, streptomycin, tylosin and virginiamycin (JETACAR, 1999). Most of these antibiotics are used in poultry as growth

promotants, especially in broiler chickens. However, there are some specific growth promotants such as avoparcin, virginiamycin and zinc bacitracin, which are also used to control necrotic enteritis (Kaldhusdal and Lovland, 2000). Around the world, almost all chicken feed contained both an antibacterial feed additive and a coccidiostat before 1986 (Greko, 2000), the year Sweden first initiated to ban in-feed AGP due to the issue of bacterial resistance to antibiotics. Since then, the use of antibiotics as growth promoters varies dramatically around the world (Hughes and Heritage, 2003).

Barnes (1958) and Elliott and Barnes (1959) were among the first report on resistance to tetracycline when growth-promoting levels of antibiotic were fed to chickens. Thirty years later, an association between use of the glycopeptide avoparcin and prevalence of resistance was clearly demonstrated (Greko, 2000). Based on these facts and on the grounds that resistance development as a consequence of antibiotic use in animals might endanger human health (Vanbelle, 2000), the European Union (EU) banned the use of avoparcin in 1997. In 1999, the EU banned the use of four additional antibiotics (bacitracin, spiramycin, tylosin and virginiamycin) used for prevention of disease and enhancement of productivity (Bafundo and Cervantes, 2006). The EU withdrew approval for the remaining AGP, including avilamycin, flavomycin, salinomycin and monensin, in EU member nations on January 1, 2006, however, the therapeutic use of antibiotics is still permitted (Ferket, 2006). In Australia, registration of the antibiotic, avoparcin, ended in 2000 and virginiamycin in 2005 (Frost & Sullivan, 2006). Zinc bacitracin or avilamycin are still permitted to be used to improve weight gain, feed conversion ratio (FCR) as well as to control necrotic enteritis by *C. perfringens* in the broiler industry. In the U. S., a high number (32) of antimicrobial compounds are approved for use in broiler feeds without veterinary prescription, among them listed as growth promotants that are also used in human medicine are bacitracin, chlortetracycline, erythromycin, lincomycin, novobiocin, oxytetracycline, and penicillin (Jones and Ricke, 2003).

The effects of an antibiotic ban on bacteria resistance differ markedly around the world. A comparison of the prevalence of resistance in faecal indicator bacteria (*E. coli* and enterococci) was conducted with pigs in the Netherlands and Sweden (van den Bogaard *et al.*, 2000). The authors concluded that the Swedish ban on antibacterial feed additives was effective in reducing the degree of resistance and their data indicated that the prohibition did not lead to an increase in the use of therapeuticals to such extent that the selection pressure is higher than in countries using antibiotics for growth promotion (Greko, 2000). In contrast,

tetracycline use in Denmark increased from 12,100 kg in 1998 to 27,000 kg in 2001. Currently, Denmark has mounting tetracycline resistance in human pathogens, such as *Salmonella typhimurium* and *Campylobactor jejuni* (Ferket, 2004). In the U.S., where relatively little regulatory activity has been done regarding AGP use, the National Antimicrobial Resistance Monitoring System (NARMS) shows resistance patterns in animal pathogens have been relatively low and stable since monitoring began in 1996 (CDC, 2002). According to the World Health Organization (WHO) global principles for the containment of antimicrobial resistance in animals intended for food, a risk assessment should be carried out before the withdrawal from food animal production of AGP that are in classes also used to treat human disease; a national level of risk assessment studies and establishment of surveillance programs to monitor AGP use and antimicrobial resistance in bacteria from food animals should be implemented (Dibner and Richards, 2005).

The notion has been made that non-use of AGP would lead to consequences that outweigh those of resistance. The ban on antibiotic feed additives in Scandinavia has had a negative effect on animal health and wellbeing. Necrotic enteritis in poultry briefly increased to what was termed "epidemic" rates throughout the EU before settling to new significantly higher endemic levels (Kaldhusdal and Lovland, 2000). The Animal Health Institute of America has estimated that, without the use of growth promoting antibiotics, the USA would require an additional 452 million chickens to reach the levels of production attained by the current practices (Hughes and Heritage, 2003). According to Garland, cited by Thomke and Elwinger (1998b), the financial advantage, in terms of feed costs by using antibiotic promotants in the UK broiler chicken industry may be in the region of 0.035 Euro per bird. On the basis of an annual production of 660 million birds, one arrives at an extra yield of 23100 tons of bird liveweight, and a saving of about 91000 tons feed, which equals a gross financial saving of 25 million Euro in the UK. Obviously, the broiler industry will experience big, if not huge, economic losses without the use of AGP.

2.2.2 Mechanisms of action

Antibiotics exert no benefits on the performance of germ-free (GF) animals, an aspect which clearly points to their effect being centred on antimicrobial activities rather than being caused by direct interaction with the physiology of the animal (Bedford, 2000). Antibiotics suppress the growth of bacteria in two ways: 1) by arresting growth and preventing bacteria from dividing to produce new progeny (bacteriostatic), and 2) by killing the bacteria (bacteriocidal) (JETACAR, 1999). Although the means whereby the various growth

promoters exert their antimicrobial effect differs for the various products (Table 2.1), virtually all achieve their effect on Gram-positive species, the majority of intestinal bacteria, in contrast to Gram-negative organisms (Armstrong, 1985; Bedford, 2000). Antibiotics generally inhibit the colonization of gut bacteria but its effects on the populations of certain species of bacteria are variable among experiments (Jukes, 1955).

Table 2.1 Mechanism of action of different groups of antibiotics (adapted from JETACAR, 1999)

Mode of action	Antibiotic group[1]
Inhibit cell wall synthesis	B-lactams (penicillins, cephalosporins, carbapenems, monobactams), bacitracin, glycopeptides
Inhibit protein synthesis	Aminoglycodies, aminocycitols, amphenicols, macrolides, lincosamides, streptogramins, tetracyclines
Interfere with cell membrane function	Polypeptides
Interfere with DNA/RNA synthesis	Quinolones, rifamycins
Inhibit metabolism	Sulfonamides, sulfones, trimethoprim, nitrofurans, nitroimidazoles
Unknown	Polyethers

[1] this grouping does not predict cross-resistance between groups or within groups.

However, the mechanisms by which AGP act in animals are not totally understood. At least four mechanisms have been proposed as explanation of antibiotic-mediated growth enhancement: 1) inhibition of subclinical infections, 2) reduction of growth-depressing microbial metabolites, 3) reduction of microbial use of nutrients, and 4) enhancement of the uptake and use of nutrients through a thinner intestinal wall associated with antibiotic-fed animals (Gaskins *et al.*, 2002). Based on these four mechanisms, AGP can induce a variety of microbiological, physiological, nutritional and metabolic effects (Table 2.2). The systematic as well as non-systematic effects of AGP were extensively reviewed by Thomke and Elwinger (1998a; 1998b). With the recent technological development in microbial analysis, the interrelationship between AGP and gut physiology of birds is revealed at cellular and molecular levels (Smirnov *et al.*, 2005; Miles *et al.*, 2006).

Table 2.2 Summary of microbiological, physiological, nutritional and metabolic effects of growth promoting antibiotics (adapted from Rosen, 1995)

Microbiological		Physiological	
Beneficial bacteria	+[a]	Gut food transit time	-
Adverse bacteria	-	Gut wall diameter	-
Transferable resistance	± 0	Gut wall length	-
Competition for nutrients by gut flora	-	Gut wall weight	-
Gut floral nutrient synthesis	+	Gut absorptive capacity	+
Clostridium perfringens	-	Feed intake	± 0
Pathogenic *E. coli*	-	Faecal moisture	-
Pathogenic streptococci	-	Mucosal turnover	-
Beneficial lactobacilli	+	Stress	-
Beneficial *E. coli*	+		
Debilitation of pathogens	+		
Nutritional		**Metabolic**	
Energy retention	+	Ammonia production	-
Gut energy loss	-	Toxic ammine production	-
Nitrogen retention	+	Alpha-toxin production	-
Limiting amino acid supply	+	Mitochondrial fatty acid oxidation	-
Vitamin absorption	+	Bacterial cell wall synthesis	-
Trace element absorption	+	Bacterial DNA synthesis	-
Fatty acid absorption	+	Bacterial protein synthesis	-
Glucose absorption	+	Faecal fat excretion	-
Calcium absorption	+	Liver protein synthesis	+
Plasma nutrients	+	Gut alkaline phosphatase	+
		Gut urease	-

[a] +, an increase; -, a reduction; 0, no change.

The overall effects, estimated 1-11% improvement in weight gain (McEwen and Fedorka-Cray, 2002), of AGP on the growth performance of animals may not be realized amid modern production practices. Response to AGP of a specific type in recent time seems to be of the same magnitude as compared to 20 years ago, although for some preparations the recommended doses have increased (Thomke and Elwinger, 1998a). Rosen (1995) estimated the ratio in response between a very good and a poor environment at 1:2. Furthermore, more reports showed that the effect of a growth promotant on live performance of broiler may be more pronounced at younger ages than later in life (Eyssen and De Somer, 1963; Bartov,

1992; Belay and Teerer, 1996). All these factors indirectly affect birds' growth responses to AGP by influencing the profile and activity of gut microflora.

2.3 INTERRELATIONSHIPS BETWEEN GUT MICROFLORA AND ANIMAL GROWTH

The relationships between the intestinal microflora and the host are complex. Germ-free and gnotobiotic animals were used several decades ago to elucidate the interrelationship between the microflora and the growth. The following sections will review this complex interrelationship from two intestinal levels of gut lumen and gut wall.

2.3.1 Influence in the gut lumen

The animal alimentary tract has evolved as an adaptation enabling the animal to secure food and limit consumption by other animals. This allows the retention and digestion of ingested food, followed by absorption and metabolism of digestion products, while feeding and other activities continue. As living organisms, bacteria need nutrients for their own growth. Since bacteria grow rapidly under favourable conditions in the gut, they could become serious competitors for the animal's food. On the other hand, substance(s) synthesized by bacteria and/or the fermentation products may contribute nutrients to the host.

2.3.1.1 Competition for nutrients

Bacteria compete with the host for amino acids. Bacterial catabolic or synthetic capabilities can degrade amino acids or incorporate them into microbial protein (Coates, 1980). Twenty five percent of amino acid content in chicken excreta was estimated to be of microbial origin (Parsons *et al.*, 1982). However, it is difficult to evaluate the true effects of bacteria on the utilization of amino acid in the feed because the gut microflora of chicks probably serves an important role in the degradation of endogenous proteins (Salter and Fulford, 1974). When fed protein deficient diets, GF birds excreted considerably greater amounts of endogenous nitrogen (+20%) than conventional (CV) birds (Larbier and Leclercq, 1994). Protein supplements with poor digestibility will undergo more microbial fermentation than highly digestible material (Williams, 1995).

It is well known that gut microflora reduces the amount of dietary lipid available to the host, both directly by the action of bacterial lipases on dietary and endogenous lipids, and indirectly by biohydrogenation of fatty acids, deconjugation of bile acids and modifications to cholesterol metabolism (Ratcliffe, 1985). Lepkovsky *et al.* (1964) observed lipase of

microbial origin in CV birds. *Streptococcus faecium* adhering to duodenal epithelial cells was reported to be part of the reason for the growth depression in chicks as they can deconjugate bile salts (Fuller *et al.*, 1983; Cole and Fuller, 1984). These authors also found *Clostridium perfringens*, *Streptococci* and some of the *Bifidobacteria* and *Lactobacilli* were able to deconjugate taurine and glycine conjugates whereas the bacteroides deconjugated only the taurine conjugate and the coliforms were completely inactive. A greater degradation of bile salts by microflora and thus a reduction in lipid utilization were noticed when the diet had a high viscosity (Smits *et al.*, 1997; Maisonnier *et al.*, 2003). It was pointed out that microflora did not act as a mediator but acted essentially as a prerequisite by lowering the basal level of intestinal bile salts (Maisonnier *et al.*, 2003). Knarreborg *et al.* (2002a) reported that *C. perfringens* and *Enterococcus faecium* were the dominant bile acid-deconjugating bacteria in the broiler intestinal tract.

The utilization of minerals by the host is reduced by the presence of gut microflora. Retention and intestinal absorption of calcium is greater in the GF state; bacteria may also fix zinc into their bodies at the expense of the host (Coates, 1980). Depending on the type of diet, the gut microflora competes for iron or copper with the host (Coates, 1980).

The availability of nutrients to the host might be decreased by bacteria as they are involved in the rapid inactivation of enzymes in the gut. In GF animals, endogenous digestive enzyme activity persists into the large intestine whereas in CV animals such activity is non-existent or at a very low level beyond the terminal ileum (Ratcliffe, 1991).

2.3.1.2 Contribution to nutrient supply

Microbial fermentation of carbohydrates can provide energy to the chicken in the form of volatile fatty acids (VFAs), but the net efficiency of energy utilisation by this pathway is considerably lower than that of glucose in the small intestine (Carre *et al.*, 1995; Langhout *et al.*, 2000). The net efficiency of utilisation of dietary energy via hind gut fermentation is estimated to be 50% that of glucose absorbed by the intestine in chickens (Jorgensen *et al.*, 1996). The degradation of the non-starch polysaccharides (NSP) is far lower in chickens than found in other animal species such as pigs and rats. The amount of energy available from fermentation of NSP appears to reach a maximum of 42 kJ per day independent of fibre source and level (Jørgensen *et al.*, 1996). When birds are given a high-fibre diet, the release of VFA from fermentation may contribute to the energy which would have been limited in the diet (Muramatsu *et al.*, 1994).

There is ample evidence for the synthesis by gut bacteria of vitamins of the B complex and vitamin K, but the products of synthesis are of little value to the host unless recycled by coprophagy (Coates, 1980). It is unknown how much bacteria would help digestion and utilization of nutrients by the host although an increase in the proteolytic activity and mucosal alkaline phosphatase (AP) activity was noticed in CV birds compared to GF birds (Palmer and Rolls, 1983; Philips and Fuller, 1983).

In conclusion, the contribution of gut microflora to nutrient supply is far outweighed by its competition for nutrients to birds. Germ-free chicks fed diets adequate in energy have lower metabolizable energy intakes, greater rates of protein and energy retention, lower maintenance energy requirements, and ultimately greater rates of growth (5-30%) when compared to conventionally reared chicks (Furuse and Yokota, 1984a, 1984b; Furuse *et al.*, 1991).

2.3.2 Influence at the gut mucosa

Gut microflora have an intimate contact with the host at the GIT surface. Adherent microbes may be present on the surfaces of the more heavily colonized portions of the tract. These can form complex microhabitats, and the role of such a lining in the gut structure and function could be substantial.

2.3.2.1 Development of gut structure and morphology

General description of gut structure and morphology

The GIT consists of five distinct layers (Figure 2.1). These are, from the lumen outwards, the mucosa, the muscularis mucosae, the submucosa, the muscularis externa and the adventitia. The avian intestine lacks the macroscopic level of folding seen in large mammals, but the surface is folded into many structures called villi (Turk, 1982). On the surface of villi, there are epithelial cells (primary absorptive cells), goblet cells, endocrine cells, M cells and intraepithelial leukocytes. In the interior of the villi, beneath the epithelial cells, is the lamina propria, a structural network that stabilizes the epithelium and contains nerve fibres as well as an abundance of immune cells, including plasma cells (immunoglobulin, Ig, A secreting), T lymphocytes (generally $CD4^+$), macrophages, eosinophils, mast cells and dendritic cells (Koutsos and Arias, 2006). Between the villi are the crypts of Lieberkuhn. Within the crypts are areas near the bottom in which the cells are rapidly dividing. Daughter cells from this area are pushed up along the side of the villus. These cells differentiate as they move up the villus becoming either principal or goblet cells (Turk, 1982). The muscularis mucosae and

submucosa regions are heavily infested by lympoid follicles such as B and T lymphocytes (Friedman *et al.*, 2003). The muscularis layer underlies each of these regions and provides structural support and motility along the GIT (Koutsos and Arias, 2006).

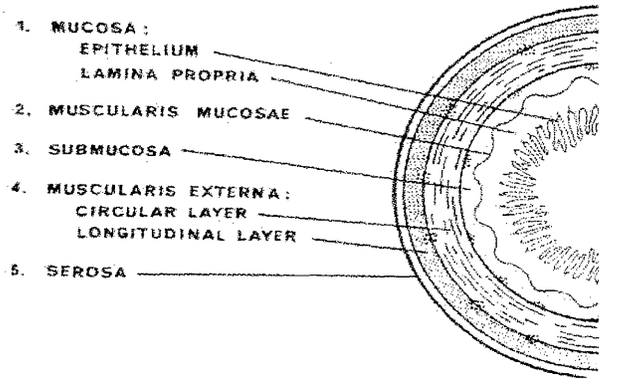

Figure 2.1 The principal morphologic features of the digestive tract (Adapted from Jensen, 1976).

Bacterial 'Catalyst' functions in gut development and structure.

Bacteria have a direct impact on intestinal development and morphology. The intestine of GF chicks was generally lighter than that of CV controls (Palmer and Rolls, 1983). Rolls *et al.* (1978) reported that villus height, crypt depth and number of mitoses in the crypt were generally, although not always significantly, greater for CV chicks than for GF chicks at all sites investigated in the intestine and the rates of epithelial cell migration were higher for CV than for GF chicks. The changes in tract thickness, which were characterized in GF versus CV chicks, included: 1) decreased lamina propria, especially in the portion of lamina which extends into the villi, 2) decreased mucosal surface area, and 3) decreased water and lymphoid tissue, lymphocytes and plasma cells of mucosa and submucosa (reviewed in Ratcliffe, 1985). A "trialogue" among the intestinal microbiota, the self-renewing intestinal epithelium, and the diffuse gut-associated lymphoid tissue (GALT) is probably critical in forming and maintaining this dynamic ecosystem (Umesaki *et al.*, 1997).

The mucous layer is the interface between a host and its microflora in the intestine. It contains an underlying cell coat (or glycocalyx), and glycoconjugates on the apical surface of the enterocyte (Schauer, 1997). Experimental evidence has shown that the mucous layer can be enhanced by the presence of selected commensal bacteria (Kelly and King, 2001a). For example, fucoconjugates were largely absent from weaned GF mice; inoculation with

the commensal bacterium *Bacteroides thetaiotaomicron* restored the same fucosylation pattern as in CV mice (Hooper *et al.*, 2001).

The resident intestinal microorganisms have been shown to increase epithelial cell turnover but it is unknown whether the intestinal microflora have a role in the differentiation of crypt stem cells into specific lineages of epithelial cells (Mahida, 2004). Available data indicate that intestinal microbes affect goblet cell dynamics as well as the glycocalyx layer directly via the local release of bioactive factors or indirectly via activation of host immune cells. Goblet cells of GF rodents are fewer in number and smaller in size than those of conventionally raised mice. As a result, the mucus layer may be up to twice as thick in CV as in GF rodents, indicating greater mucus production. Mucin composition also differs significantly between GF and CV animals (reviewed in Deplancke and Gaskins, 2001). The processes of mucin biosynthesis and/or degradation were changed when either probiotic or AGP was supplied in a diet and it was believed that the alteration in mucin was brought about by the changes in the intestinal bacterial populations (Smirnov *et al.*, 2005).

The development of GALT requires an interaction with the intestinal microflora (Hunter and Bevins, 1999). Germ-free mice have a reduced number of Peyer's patches even though they are fully immunocompetent, but lymphoid follicles and M cells increase in number after transfer of GF mice to a conventional environment (Lu and Walker, 2001). Segmented filamentous bacteria (SFB) resulted in expression of major histocompatability complex class (MHC) II molecules, expansion of intraepithelial lymphocytes (IEL), and increase in IgA-producing cells (Umesaki *et al.*, 1997).

The complex interaction between bacteria and epithelium is becoming a fancinating research around the world and the effort is likely to offer novel and useful means to modulate gut function, particularly mucosal immunity, for protection from, or treatment of a wide variety of human and animal intestinal disorders.

2.3.2.2 Structural and functional integrity

The absorptive ability of the epithelial cells colonized by indigenous bacteria might be improved. Ileal epithelium-associated bacteria may absorb nutrients such as endogenous nitrogen and uric acid; provide these nutrients to the epithelial cells, to stimulate their absorption ability and renewal rate (Yamauchi *et al.*, 1990).

However, the most important function of the normal microflora is its barrier function to control or eliminate an invading pathogen. One of the most convincing demonstrations of the role of the gut microflora in resistance to disease was provided by Carter and Collins (1978). They showed that a GF mice was killed by 10 cells of *Salmonella enteritidis* but it required 10^9 cells to kill a conventional animal with a complete gut microflora. The mechanism by which this occurs is still not fully understood. Amongst the suggestions which have been considered are the competition for limiting carbon sources, the competition for binding sites along the epithelium, the presence of antibacterial compounds, or the production of VFA. On the other hand, bacterial antigens play a very significant role in the proliferation and development of GALT (Gaskins, 1998). Exposure to bacterial antigen is now recognized to be of immense importance, both in early life, in order to prime the immune system in the correct way, and throughout life, to maintain a functional immune system (Kelly and King, 2001a). Immaturity of Paneth cell-mediated innate host defence against luminal microorganisms has been implicated in necrotizing enterocolitis (Mahida, 2004).

When pathogens attach to the mucosa, gut integrity and function will be severely affected. Incubation of isolated intestinal segments with intact *S. typhimurium* bacteria resulted in the loss of mucosal epithelial integrity after as little as 30 min incubation (Droleskey *et al.*, 1994). *In vitro* penetration of the intestinal wall by *E. coli* showed up to 135 *E. coli* organisms counted in a single absorptive cell on severely affected villi in pigs (Staley *et al.*, 1969). In cells adjacent to the areas of *E. coli* attachment, the microvilli were also destroyed, and various degrees of swelling and budding were observed (DeVinney *et al.*, 1999). The importance of microvillous degeneration and exfoliation to the nutritional status of the neonatal animal is undoubtedly considerable. Loss of surface membrane would impair brush-border enzyme production.

Furthermore, when pathogens translocate epithelial cells after attaching, they will cause inflammation, diarrhoea and even death in birds. Three primary mechanisms appear to promote bacterial translocation: 1) intestinal bacterial overgrowth caused by disruption of the gut ecological equilibrium, 2) increased mucosal permeability as a result of a defective barrier function, and 3) deficiencies in the host's immune defences (Shanahan, 2002). Fuller and Jayne-Williams (1970) reported that translocation of bacteria from the intestinal lumen to the liver was restricted to the first 4 days of life; thereafter the incidence of bacteria in the liver fell rapidly in chickens. The innate and acquired immune systems both contribute to the

immune containment of bacterial translocation, but the innate rapid responses may be more important in early life of birds (Friedman *et al.*, 2003).

Indigenous bacteria vary in the rate and efficiency with which they translocate from the GIT. Gram-negative, facultative anaerobes such as the *Enterobacteriaceae*, *E. coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* translocate at a greater rate than other indigenous bacteria, whereas obligate anaerobes and Gram-positive bacteria appear to be less efficient translocators (Shanahan, 2002).

2.3.3 Impact of microflora on immune system

The microbes and their activities have a major impact on the development and function of the intestinal immune system and vice versa. This research area has started to gain momentum as a breakthrough in understanding the relationship between the gut microflora and the immune system will be key to the mechanism of its effects on animals (Gaskins *et al.*, 2002). Basically, the nature of bacteria, be it of pathogenic or non-pathogenic source, will lead to immune responses, irritation or tolerance (Bar-Shira and Friedman, 2005a), that in term will post very different impacts on the immune system and growth.

When a pathogen comes into contact with the host, a struggle between the pathogen and the local innate host defence systems ensues (Lu and Walker 2001). A more urgent and escalated response to bacterial threats is the process of "inflammation." Inflammation is a programmed, tightly choreographed host response to stimuli that may potentially result in tissue damage. The process of inflammation begins when endogenous or exogenous signals of potential danger induce local release of soluble inflammatory mediators and chemotactic agents that serve to increase vascular permeability and attract inflammatory cells, initially neutrophils, and later monocytes and lymphocytes (Neish, 2002). When the pathogen has been eradicated, regulatory mechanisms intervene to stop the inflammatory response and resituate the intestinal barrier integrity (Schiffrin and Blum, 2002). The generalised response to an infection is for the body to redistribute nutritional and energy resources away from anabolic and maintenance processes at the extremities to the vitally important metabolic processes driving immunity and disease resistance (Lochmiller and Deerenberg, 2000). However, when an infection happens, the reduced growth rate is mainly because of a decline (70%) in food intake (Klasing *et al.*, 1987).

Immune response against commensal microflora does not lead to tissue damage (Schiffrin and Blum, 2002). Resident enteric bacteria may be viewed as a component of innate immunity (Neish, 2002). The results of several studies suggest that the resistance to colonization by enteric pathogens prompted by gut commensals results in preventing large inoculums of pathogens that could cause clinical symptoms and pathologic changes (Rolfe, 1996). Furthermore, it is believed that commensal gut bacteria can modulate the immune system, especially mucosal immune system. Immune mechanisms operative at the mucosal surface include cytokines, MHC molecules, intestinal T lymphocytes, the lamina propria and antigen presenting cells, and secretory Ig A (Gaskins, 1998).

Cytokines are a group of natural proteins which are produced by the immune system immediately upon an infectious challenge or vaccination (Hilton *et al.*, 2002). There is a unique regulatory scheme in which host intestinal epithelial cells respond to cues from adherent bacterial populations by altering cytokine production. Several pathogenic bacterial species, such as *Salmonella* spp., stimulated epithelial cell Interleukin-8 (IL-8) responses; whereas noninvasive bacterial species and bacterial lipopolysaccharide (LPS) did not induce an IL-8 response (Gaskins, 1998). A dramatic increase in cytokine expression (IL-2 and IFNγ, indicative of activation and effector functions, respectively) ensues after 4 days of life in broiler chickens (Bar-shira and Friedman, 2005b).

Major histocompatibility complex encodes highly polymorphic class I and class II molecules responsible for rapid allograft rejection, vigorous proliferation in the mixed lymphocyte reaction, appropriate cellular cooperation in production of antibodies, and related T cell-dependent functions (Kaufman, 1996). Matsumoto *et al.* (1992) reported a link between expression of class II MHC molecules on epithelial cells and intestinal colonization by commensal bacteria.

Intestinal T lymphocyte populations include the IEL, lamina propria T cells, and those residing in Peyer's patches (Guy-Grand *et al.*, 1993). Intestinal T cells which reside in the epithelium are generally considered as key components of a front-line of defence at sites of first contact with enteric pathogens (Gaskins, 1998). Intestinal T cell subsets are distinguished by their pattern of ontogenic appearance, their site of maturation, their surface expression of cell differentiation molecules (e.g. CD4 vs. CD8), and by T cell receptor (TCR) subtype ($\alpha\beta$ or $\gamma\delta$). In broiler chicken, the majority of T cells located in the lamina propria are CD4+, whereas CD8+ T cells and natural killer cells predominate within the epithelial

cells (Koutsos and Arias, 2006). The effects of commensal bacteria on the development of intestinal T cells appear to vary according to T cell phenotype. Peyer's patches contain T cell areas with CD4 or CD8 cells, which use the αβ TCR, and these populations are diminished in the GF state along with a generally reduced lymphocyte cellularity (Macpherson *et al.*, 2001). Of the IEL, the number of γδ TCR cells are relatively unaffected by GF conditions, however, αβ TCR-positive cells are reduced in number, and the normal developmental increase in this population does not occur (Gaskins, 2005).

Residing beneath the epithelial cell monolayer in the lamina propria, are diffuse populations of T lymphocytes, B lymphocytes, plasma cells, macrophages, mast cells, eosinophils, and smaller numbers of dendritic cells, and neutrophils, as well as biologically-active fibroblasts (Hinterleitner and Powell, 1991). The function of macrophages may be indicative of an active innate immune system in the gut following hatch (Bar-shira and Friedman, 2005b).

Secretory immunoglobulin A (sIg A) is the main immunoglobulin of the humoral immune response, which together with the innate mucosal defenses provides protection against microbial antigens at the intestinal mucosal surface. The production of intestinal sIgA requires the presence of commensal microflora (Macpherson *et al.*, 2001). In GF mice, IgA-plasmocyte number is decreased tenfold as compared with controls (Moreau and Gaboriau-Routhiau, 2001). It is not yet clear, however, how lamina propria B cells are activated to become IgA-secreting plasma cells or how the intestinal microflora influence this process (Adolfsson *et al.*, 2004). It has been shown that the sequential establishment of the digestive flora from birth to weaning is responsible for the progressive increase in IgA plasmocyte numbers in the lamina propria of the small intestine in the growing normal mouse. Gram-negative bacteria such as *E. coli* and *Bacteroides* play an important role in this immunologically non-specific effect (Elson, 1985; Pabst, 1987).

In summary, commensal microbiota is a major stimulus for the postnatal development of immune cell compartments in the intestine. Cell yield and lymphocyte subset patterns from the intestine of GF piglets of 45 days of age were comparable to those of five-day-old CV animals (Rothkotter *et al.*, 1994). However, maintaining a competent immune system and mounting an immune response is a nutritionally demanding process that necessitates trade-off decisions among competing nutrient demands for growth (Lochmiller and Deerenberg, 2000). An antagonistic relationship exists between the physiological processes of immunity and growth (Humphrey and Klasing, 2004). Exposure to high levels of microbial challenges

results in immunological stress, slower rates of growth and decreased accretions of many tissues, especially skeletal muscle (Benson *et al.*, 1993).

Bacterial adherence to mucosa – first line of defence in the body

The mucosa layer could represent a physical barrier and a competitive inhibitor to the epithelial cell. The mucosa accounts for as much as 75% of the total intestinal mass in rat (Steiner *et al.*, 1968). If bacteria are able to bind strongly to components of the intestinal mucus layer, their clearance by the motile and abrasive forces of digestion may be delayed and colonization of the intestinal tract may be favoured (Kelly *et al.*, 1994). For many enteropathogens this is the first step to cause tissue damage or disease to the host.

Mucosal surfaces are characterized by an extensive carbohydrate coat and the bacterial lectin-like adhesions use cellular glycoproteins, glycolipids or the associated mucin glycoprotein as receptors (Adlerberth *et al.*, 2000). Many indigenous and pathogenic bacteria specifically adhere to complex carbohydrates of small intestinal membrane and mucin glycoconjugates (Kelly *et al.*, 1994), such as the mannose-specific adhesin for type 1 fimbriated *E. coli*. Strains of *Lactobacillus plantarum* isolated from the human GIT express a mannose-binding adhesin (Adlerberth *et al.*, 1996). The adhesin pathway for different strains or types of pathogens is different. For example, compared to pathogenic *E. coli*, adhesin of *C. perfringens* is facilitated by its major virulent factor, toxin A (Adlerberth *et al.*, 2000).

The structural units of mucin glycoproteins, the major component of glycoconjugates in mucus, are subunits consisting of a central protein core with glycosylated and non glycosylated regions. The glycosylated region that is resistant to proteolytic attack consists of many carbohydrate side chains (up to 19 sugars per chain in gastric mucus) attached close together by O-glycosidic linkages to serine and threonine residues in the protein core (Allen *et al.*, 1993). The relatively simple glycosylation changes may be sufficient to create, or mask, binding receptors of bacteria (Kelly and King, 2001b). The nonglycosylated region participates in interchain disulfide bridges that join the mucus subunits to form the large-molecular-mass polymeric mucins, forming the viscoelastic properties of the mucous barrier (Allen *et al.*, 1993).

Bacteria possess glucidolytic enzymes to cleave off oligosaccharidic chains of mucin and/or produce a large amount of various proteolytic enzymes to de-mask receptors, which facilitate them to colonize the gut or cause host damage (Adlerberth *et al.*, 2000). On the other hand,

dietary factors may modulate the glycosyl structures of the mucosal surfaces of the gut, especially during early development of the neonate (Kelly and Coutts, 2000), to facilitate or prevent bacterial adherence. For example, phytohaemagglutinin feeding results in incomplete glycosylation of glycoconjugates by accelerating enterocyte turnover; consequently polymannosylated glycan structures enriched in the mucosa and dramatic increases in the numbers of coliforms adherent to the mucosal surface (Kelly *et al.*, 1994).

The exact nutritional role of mucosa in the host is unknown. However, mucin, the major component of mucosa, contributes to basal losses of endogenous protein and specific losses of endogenous protein, which may affect nutrient absorption, and amino acid and energy metabolism (Montagne *et al.*, 2004). Mucosa nutrition (dietary modulation of glycosylation process) and the mechanisms of commensal bacterial adherence is becoming an important subject to investigate as to how to maximize the protective effect of mucus and minimize the metabolic costs associated with mucin production.

It is clear that an important symbiotic relationship exists between the gut microflora and the host. A balanced and stable gut microflora is a prerequisite for birds to possess a functional GALT and gut barrier function. On the other hand, an active GIT microflora component may have an increased energy requirement for maintenance and a reduced efficiency of nutrient utilization. It is generally agreed that gut microflora is a nutritional "burden" in fast-growing broiler chickens (Lan *et al.*, 2005).

Compared to commensal bacteria, pathogens can largely induce growth depression. Chicks grown in a pathogen-free environment grow 15% faster than those grown under conventional conditions where they are exposed to bacteria and viruses (Klasing, 1997). Because there is a delicate balance between beneficial bacteria and pathogens, an optimal microbial balance will favour the optimal growth of animals.

2.4 MICROBIAL COMMUNITY OF BROILER CHICKENS

The bacterial component, particularly in the GIT, to the weight of an animal is 1 to 2% of the animal's weight (Cook, 2004). The population consists of predominant bacteria ($>10^9$ CFU/g), subdominant bacteria (between 10^6 and 10^9 CFU/g) and bacteria in transit ($<10^6$ CFU/g). The first two are the endogenous or resident gut microflora. Depending on the number of bacteria ingested, the transit bacteria will be present in greater or lesser numbers (Bourlioux *et al.*, 2003). Habitats can be divided into three, namely, luminal, epithelial and

cryptal (Ewing and Cole, 1994). For many years, the cultivation method is used to examine the gut microflora. It is generally accepted that only a minority of the GIT microbes have been isolated in pure culture. Recently ribosomal DNA-based molecular techniques have been applied to detect the microbial ecosystems as a whole.

2.4.1 The diversity and development of gut microflora

Bacterial populations as high as 10^{11}/g contents, representing over 600 different species, are found in the hindgut of mammalian species (Zoetendal *et al.*, 2004a). Six hundred and forty different species and 140 different bacterial genera were found in the chicken GIT when tight criteria were used (Apajalahti *et al.*, 2004). This diversity is important for the host to fight against opportunistic pathogens or other pathogens. A recent investigation of the ileal microbiota in neonatal piglets nourished either enterally or parenterally revealed an inverse relationship between bacterial diversity and susceptibility to colonization by the opportunistic pathogen, *C. perfringens* (Deplancke *et al.*, 2002).

Bacteria located in gut lumen

Culture-dependent studies showed that community structure varies among GIT regions in most animals, including chickens. Appreciable variation in the composition among birds and even, at various times, within the same individual was also observed (Salanitro *et al.*, 1974).

The numbers of bacteria were found to be highest in the caeca and progressively lower in the contents of the colon, ileum and duodenum, and lactobacilli appeared to be the most numerous group of bacteria in all regions of the intestinal tract of the chicken, with the exception of the colon (Shapiro and Sarles, 1949). Normal bacteria became established in the duodenum, ileum, caeca, and colon after the chicks had been given food and water for 16 hours (Shapiro and Sarles, 1949). During the first 2 to 4 days, Streptococci and Enterobacteria colonize the small intestine and cecum (Salanitro *et al.*, 1978). When birds were two weeks old, the composition of the microflora stabilizes and lactobacilli predominate in the small intestine with smaller numbers of *Streptococus* and *Enterobacteria* spp.; whereas the caecum is colonized mainly by anaerobes (bacteroides and bifidobacteria) and fewer numbers of facultative bacteria (Barnes *et al.*, 1972; Salanitro *et al.*, 1978). In the small intestine the typical adult microflora becomes established within the first 2 weeks after hatching but the caecal microflora takes much longer to develop. The bacterial fermentation

products (lactate, propionate, acetate and butyrate) in the intestine also become stable within two to three weeks (Snel *et al.*, 2002).

Culture-independent studies mainly focus on caecal and ileal microbiota and the results generally support what had been observed in culture-dependent studies. Enterococci and *E. coli* are the two groups of bacteria to first colonize the gut of birds after hatching; lactobacilli become the predominant bacteria in the small intestine and the species of lactobacilli change depending on the age (Lu *et al.*, 2003). *Clostridiaceae* (low-G+C, gram positive) seems to be the dominant bacteria in the caeca (Lu *et al.*, 2003; Jozefiak *et al.*, 2005). Broiler chickens undergo 3-4 significant succession periods where the dominant species of bacteria are replaced by new communities (Lu *et al.*, 2003; Lee *et al.*, 2005). The community structure becomes more complex when birds get older (van der Wielen *et al.*, 2002; Collier *et al.*, 2003) and the diversity and complexity are much higher than had been reported previously by culture-based studies (Gong *et al.*, 2006). Compartment-specific factors play an important role in the bacterial development of each intestinal region within one chicken (van der Wielen *et al.* 2002).

Bacteria associated with gut mucosa

King (1905) first reported that the microflora of the intestinal mucosa of common fowl was being made up of about 50 different species of bacteria in birds and the composition was constant to a certain extent. Similar to the distribution of the luminal bacteria, a gradual decrease in number from the cecum and colon to the ileum, jejunum, and duodenum was noticed in the gut mucosa of chickens (Fuller and Turvey, 1971; Yamauchi *et al.*, 1990).

Scanning electron microscopy (SEM) did not reveal any bacteria in one-day-old chicks and many bacteria were observed around the apical area of villi in 10-d-old broilers (Yamauchi *et al.*, 1990). In the crop the bacteria formed an almost complete layer, 2-3 cells thick, on the surface of the stratified squamous epithelium (Fuller and Turvey, 1971). These were Gram-positive, rod-shaped bacteria, mainly composed of lactobacilli. These organisms were found adhering to the crop within 24 hr after the chicks began to feed (Fuller and Brooker, 1974). In the duodenum, Enterococci were shown to be attached to the gut wall and some strains were also the reason for the growth depression of chicks fed a diet without antibiotics (Fuller *et al.*, 1979; Fuller *et al.*, 1983). In the jejunum, histological studies did not show any bacteria associated with the intestinal wall but the fault could lie in the methods normally used for tissue processing for histological examination that resulted in the elimination of

bacteria associated with the intestinal wall (Fuller and Turvey, 1971). In the ileum only a few bacteria were seen attached to the epithelial surface by SEM. However, segmented filamentous bacteria, non-pathogenic, gram-positive, anaerobic, spore-forming, were usually found at the ileal villus tip of young animals (Heczko *et al.*, 2000). The SFB were present in high numbers for about 10 days after hatching in chicks (Yamauchi *et al.*, 1990). In the caecum a thick layer of bacteria was seen adjacent to the epithelial surface. This layer was *c.* 200 cells deep and contained mainly Gram-positive rods with only occasional Gram-positive cocci and Gram-negative rods being seen (Fuller and Turvey, 1971).

Recently, mucosa-associated microbiota from different regions of the GIT of adult broilers was studied by analysis of 16S rRNA gene sequences (Gong *et al.*, 2006). A high diversity of bacteria exists in the caecal mucosa, followed by crop, duodenum, jejunum and ileum. Lactobacilli were predominant in the upper GIT and had the highest diversity in the crop. Both *L. aviarius* and *L. salivarius* were the predominant species among lactobacilli. Candidates of the division *Arthromitus* were also abundant in the jejunum and ileum. Compared to luminal bacteria, mucosal bacteria colonize gradually (Vahjen *et al.*, 1998; Smirnov *et al.*, 2005). However, information regarding the composition and succession of mucosa-associated bacteria in the small intestine of all kinds of animals is sparse.

The processes involved in the establishment of microbial populations are complex, involving microbial succession as well as microbial and host interactions and eventually resulting in dense, stable populations inhabiting specific regions of the gut (Mackie *et al.*, 1999).

With regards to the mucosa-associated bacteria, bacterial adhesion occurs first and foremost at the mucin sites that act as soluble or insoluble layers. Under normal conditions, adhesion to epithelial cells does not occur. This has been confirmed by a whole series of experiments that show the normal flora always remain on the surface of the mucus, at the entrances of the villi, but never inside the crypts (Bourlioux *et al.*, 2003). Growth of mucosa-associated bacteria is more or less independent of digesta flow rates; thus, surface or substrate factors determine the amount of bacteria in the mucosal layer of the intestine (Danicke *et al.*, 1999). The predominant mucosa-associated community is host-specific and significantly different from luminal and faecal bacteria communities (Zoetendal *et al.*, 2004a). There exists a positive correlation between the mucosal and luminal microflora in rats and piglets (Mikelsaar *et al.*, 1987). The case, however, might not be true in chicks. A large number of coliforms and/or enterococci was observed in the chicken mucosal sample compared to the

luminal sample in both culture-dependent and culture-independent studies (Untawale and Mcginnis, 1979; Zhu and Joergert, 2003).

Prior to hatching, the intestinal tract of chicks is usually sterile and the intestinal microflora is derived exclusively from the environment. With the advent of modern incubation, the chick is exposed to the first bacteria in the incubator, chick box, and litter of the poultry house. During succession, a dynamic balance exists between the potential beneficial bacteria and the potential harmful bacteria. In fact, some pathogens are autochthonous to the gut ecosystem and can live in harmony with their hosts, becoming pathogenic only when the ecosystem is disturbed in some way (Mackie *et al.*, 1999). The development of a probe system for identifying pathogenic factors seems of great importance to keep the number of pathogenic bacteria to a minimum. Considering the close interaction with epithelial cells, the community and succession of mucosa-associated (potentially) pathogenic bacteria deserves more research in fast-growing meat chickens.

2.4.2 Dietary modulation of gut microflora

Microbial succession in the GIT is influenced by numerous external and internal host-related factors (Mackie *et al.*, 1999). Extrinsic factors include the microbial load of the immediate environment, food and feeding regimen. In addition, dietary and temperature stress can influence the succession of microbes. The most studied external factor that influences the establishment of intestinal microbiota is dietary ingredients, including additives.

Dietary cereals rich in NSP can promote the proliferation of bacteria in the gut lumen and mucosa (Wagner and Thomas, 1978; Hubener *et al.*, 2002). The community structure may also change as molecular technique has shown that corn might favour low G+C clostridia, enterococci and/or lactobacilli but wheat favours higher proportion of G+C bifidobacteria (Apajalahti *et al.*, 2004). Depending on the micronutrients in cereal grains, *C. perfringens* seems to be able to more quickly proliferate than other kinds of bacteria, such as enterococci (Takeda *et al.*, 1995). Birds fed diets based on wheat, rye, oats or barley suffer more severe necrotic enteritis than birds fed maize-based diets (Riddell and Kong, 1992). This may be due in part to increased clostridial proliferation associated with the wheat or barley diets, or to decreased proliferation associated with the corn diet (Annett *et al.*, 2002).

The provision of plant-protein-based feed reduces the risk of bacterial overgrowth in the lower GIT of birds compared to those given animal protein or a combination of animal and

plant protein (Udayamputhoor *et al.*, 2003). Drew *et al.* (2004) found a positive association between crude protein derived from fish-meal and rate of colonization of the ileum and caeca by *C. perfringens*, but no such association existed for soya-derived protein. Glycine and methionine levels in fish-meal are higher than in soya concentrate, and these amino acids are known to stimulate *C. perfringens* growth *in vitro* (Williams, 2005). Lipid source can also affect the population of *C. perfringens* but not that of lactobacilli and enterobacteria (Knarreborg *et al.*, 2002b).

Feed form, feeding method (meal feeding vs. feed restriction) and cereal storage were shown to alter the composition of gut microflora (Kaldhusdal, 2006). Various dietary additives are added to chicken diet to directly or indirectly affect the gut microflora of birds, which is further reviewed in the subsequent sections.

It is obvious that various factors involved in or related to diet can affect the composition of gut microflora. Determining how the diet affects the gut microflora depends upon precise measurement of the species diversity of the flora. The method used to determine gut microflora is changing from cultivation to molecular analysis. The application of culture-independent, mainly 16S rRNA-based approaches, has given us novel insights into the ecology of the GIT. It has been reported that denaturing and temperature gradient gel electrophoresis (DGGE/TGGE) are sensitive enough to represent bacteria that constitute up to 1% of the total bacterial community (Zoetendal *et al.* 2004a). However, it is known that PCR and cloning steps are not without biases. Moreover, whether the compositional stability also indicates a functional stability remains to be investigated (Zoetendal *et al.*, 2004b).

2.5 POULTRY PRODUCTION WITHOUT AGP

Poultry companies are voluntarily eliminating or reducing antibiotics in feeds due to pressure from fast food chains with animal welfare guidelines, the media representing the modern health-conscious consumer, and precedent-setting events in other countries (Hooge, 2004b). In the concept of the production of healthy farm animals without the use of antibiotics, diet, management and alternatives to AGP can be relevant in many different ways.

2.5.1 Balanced diet

The nutrition of animals primarily focuses on animals being supplied with all essential nutrients and energy in adequate amounts and appropriate proportion. It is well known that nutrient deficiencies or an unbalanced diet can not only retard the normal growth of animals but also induce metabolic problems, leading to inefficient production.

Genotypes used in the broiler industry have changed significantly in the past 50 years, and continue to change at a slower rate today. Different selection criteria are used by the major breeding companies, leading to widely different genotypes being available to the broiler industry (Gous, 1998). The genetic variance of most commonly used poultry throughout the world is relatively small, being primarily restricted to a relatively few major international poultry companies such as Ross and Cobb (Acamovic, 2002). Relatively little research has been done that take into consideration changes imposed by broiler genetic selection, even though these may have altered dietary requirements (Emmans, 1995).

It is appropriate to formulate diets based on the information provided by the breeders in addition to NRC and/or ARC recommendations, which are considered to be outdated and of little practical use other than to provide a base to work from (Acamovic, 2002). Publications on nutrient requirements offer useful information to further refine a balanced diet. However, fixed requirements usually cannot accurately represent nutrient needs of birds because nutritional requirements of broilers are influenced to a considerable extent by dietary (interaction between/among nutrients), environmental and age effects. A simulation model was proposed by Gous (1998) and, by integrating information about the bird, the feed, and the environment into an accurate theory, accurate predictions of feed intake, growth rate and thus nutrient allowances can be made for any given bird, in any given state, and in any given environment. A number of model systems are available, such as EFG, Novus, etc. (Acamovic, 2002). It is pointed out that the evaluation of bird responses to dietary nutrient concentrations should aim at cost-benefit as such to estimate economic optimum levels, rather than as biological maximums (Fawcett, 1986; Pesti and Miller, 1997). However, the accuracy of prediction of models is likely to be affected by the presence of antinutrients and feed additives to diet (Acamovic, 2002).

Although the common anti-nutritional substances are well known to the poultry industry and have been generally accommodated or their effects ameliorated, much of the nutritional behavior of these antinutrients remains to be understood at a fundamental level and their

effects remain to be explored (Smithard, 2002). Furthermore, when novel feed ingredients are incorporated into diets either to reduce the cost of feed or to make full use of the local ingredients, the presence of unknown antinutrients probably depresses the normal or optimal growth of birds even when the diets are formulated to meet nutrient requirements and given certain safety margins. The accurate evaluation of nutritional availability of single feed ingredients and the compatibility between or among ingredients have a key role in designing a balanced diet to maximize the genetic growth potential of broiler chickens.

Appropriate feed processing and feeding programs can help achieve the economic efficiency of a balanced diet. It is reported that ileal starch digestibility was improved when a wheat-base diet was fed in a mash form, as compared to pellets (Svihus and Hetland, 2001). Early feed restriction of broiler chickens and whole wheat feeding is usually applied to improve efficiency of feed utilization (Bjerrum *et al.*, 2004; Teimouri *et al.*, 2005).

2.5.2 Role of management and husbandry practices

Alternatives to AGP in food animal production also include management practices that reduce the likelihood and effect of infectious diseases and increase the production efficiency (McEwen and Fedorka-Cray, 2002).

Biosecurity

Biosecurity refers to all of the measures that should be taken to prevent viruses, bacteria, fungi, protozoa, parasites, insects, rodents, and wild birds from entering and affecting the wellbeing of poultry (Bojesen *et al.*, 2003). Biosecurity measures can be applied at many points: source of birds, barriers and access to the flock, disinfection station, worker disinfection practices, visitor precautions, disposal of dead birds, disposal of waste, clean-up and disinfection between flocks and contact with other animals. After conducting a survey of biosecurity practices, significantly better broiler flock performance was observed in enhanced biosecurity farms compared to other farms (Tablante *et al.*, 2002). Targeted biosecurity measures seem to be effective in preventing *C. jejuni* colonization among the flock (Newell and Fearnley, 2003).

Vaccination

The modern poultry industry relies heavily on vaccines to protect against a wide range of infectious agents and vaccination is a highly effective tool to protect poultry against viral

infections (Davison, 2003). Many commercial products are available, including vaccines for prevention of Newcastle disease, infectious bursal disease, and infectious bronchitis. Mycoplasma infection, coryza (*Haemophilus paragallinarum*) and fowl cholera (*Pasteurella multocida*) are now mainly controlled by vaccination rather than use of antibiotics (JETACAR, 1999). Increasing reliance on vaccination and development of improved vaccine delivery may provide opportunities to minimize feed-borne medications (Revington, 2002).

Litter management

Good litter management means lower pathogen exposure. Moisture is essential for oocyst sporulation; higher moisture content is commonly believed to facilitate greater sporulation rates while dried or acidified litter can reduce pathogen load (Garrido *et al.*, 2004).

Testing feed ingredients for spore contamination and checking droppings for the "fluid zone" area may be useful for monitoring management programmes (Williams, 2005). Nipple drinker systems have kept water clean, reduced water spillage and litter moisture; water and air quality improvements are also beneficial (Revington, 2002). Other husbandry practices include genetic selection to enhance disease resistance (McEwen and Fedorka-Cray, 2002).

2.5.3 Dietary alternatives to AGP

A balanced diet depends not only on the selection of appropriate dietary ingredients but also on the selection of appropriate dietary additives. The additives, discussed in the following sections, may not be "alternative" to AGP in relation to improved performance, but can be effective in altering the gastrointestinal microbial population. Negative effects of gut bacteria on animal growth become most remarkable when diet quality is less than optimum. The magnitude of growth suppression in birds under nutritional stress can be dramatic as evidenced by a 78% greater rate of growth in GF chicks compared to conventional-reared chicks fed a low-protein diet (Furuse and Yokota, 1984a). By enhancing growth of beneficial microbes or by reduction and removal of potential pathogens, these alternatives possibly can enhance the health and performance of birds.

Microbial enzymes

Enzymes are naturally occurring and are produced by all living organisms for catalyzing chemical reactions. Enzymes were discovered in the latter part of the 19th century and have been used in industry and food processes since the early 1900s. The majority of enzyme products have been derived from fermentation products of alkaliphilic microorganisms (Clarkson *et al.*, 2001). In animals, enzymes are used to break down feedstuff from polymers to simple structures for easy absorption by animals. In poultry, carbohydrases are used to break down complex carbohydrates, such as NSP, into simple sugars for absorption and phytase is used to improve the digestibility of phytate. Ferket (1993) defined enzymes as special proteins that catalyse or accelerate the rate of specific chemical reactions in which the enzyme activity may be dependent on the substrate in a random manner or it may be through very specific sites on substrates such as fat, protein, or carbohydrates.

The effects of enzymes on gut microflora were classified by Bedford (2000) into two phases: an ileal phase and a caecal phase. In the ileum, enzymes simply reduce the number of bacteria by increasing the rate of digestion and limiting the amounts of substrates available to the microflora. In the caecal phase, enzymes produce soluble, poorly absorbed sugars which feed beneficial bacteria. The VFAs produced by such bacteria may be of benefit not only in controlling populations of *Salmonella*, and perhaps, *Campylobacter* species, but also in providing an energy source for the bird.

However, the effects of enzymes on the gut microflora may be far more than those two phases. The composition of gut microflora in the proximal small intestine as well as those associated with the gut wall was shown to be changed by the addition of xylanase (Vahjen *et al.*, 1998; Danicke *et al.*, 1999; Hubener *et al.*, 2002). The authors correlated those effects of xylanase on the gut microflora with its effects on the viscosity of diet, which is well known as one of the major modes of action of enzymes. Inclusion of cereal rich in NSP increases the viscosity of the digesta, reduces the apparent nutrient digestibility, alters bacterial profiles and gut physiology. By adding enzymes into the diet, the viscosity of the content is reduced and nutrient uptake and animal performance are improved (Bedford, 2001). Elimination of cell wall encapsulation is another major mode of action of enzymes (Bedford, 2001). It relies on the fact that the feed manufacturing process of grinding and pelleting does not break open all the cell walls of the endosperm. The addition of enzymes can remove such "encapsulated" material in the gut and hence improve nutrient utilization by the birds.

Therefore, the responses of birds to enzyme mainly depend on cereal quality and quantity in diet. However, quantity and quality of fat, microbial status, bird age and antimicrobial agents can also affect the effects of enzymes (Bedford, 2001). Furthermore, because of cereal quality (e.g. the complexity of carbohydrate) and the thermolabile characteristic of enzymes, improvements are not always observed (Acamovic, 2001).

Supplementation of enzymes generally can lead to 2-5% improvement in feed/gain ratio and 2-3% improvement in growth rate (Broz and Beardsworth, 2002). Reduced incidence of sticky excreta and improved litter conditions are also the benefits of using enzymes, which makes the problem of NSP and associated increased ingesta viscosity more manageable (Morrow, 2001). The development of enzymes is towards a specially-designed stage to use NSP as energy sources and to deactivate anti-nutrients in feed (Choct, 2006).

Prebiotics

Oligosaccharides that predominantly escape digestion in the upper GIT are important sources of energy for bacteria in the caeca-colon. These bacteria express enzymes such as β-fructosidase, β-galactosidase, xylanase and other hydrolases (Delzenne, 2002), which process affects the microbial ecology of the GIT and influences gut metabolism and function.

Gibson and Roberfroid (1995) defined a prebiotic as a nondigestible food ingredient which beneficially affects the host by selectively stimulating the growth of and/or activating the metabolism of one or a limited number of health-promoting bacteria in the intestinal tract, thus improving the host's microbial balance. The growth of endogenous microbial population groups such as bifidobacteria and lactobacilli is specifically stimulated and bifidobacteria and lactobacilli are perceived as being beneficial to animal health. Prebiotics have the advantage, compared with probiotics, that bacteria are stimulated which are normally present in the GIT of that individual animal and therefore already adapted to that environment (Snel *et al.*, 2002). The dominant prebiotics are fructooligosaccharide products (FOS, oligofructose, inulin) (Patterson and Burkholder, 2003); glucooligosaccharides, stachyose, and maltooligosaccharides have also been investigated in broiler chickens (Zhang *et al.*, 2003; Gao and Shan, 2004; Jiang *et al.*, 2006).

Compared to the application of prebiotics in human and pet food, the use of prebiotics in broiler chicken diet is still new. Reports on the effects of prebiotics on the activity of the microflora of broilers are limited. Prebiotics were shown to support the growth of beneficial

bacteria such as lactobacilli (Xu *et al.*, 2003) but failed to stimulate the growth of bifidobacteria (Vidanarachchi *et al.*, 2006). Reduced susceptibility to *Salmonella* colonization was noticed in birds on prebiotic treatment compared to controls (Bailey *et al.*, 1991; Fukata *et al.*, 1999). It was shown that prebiotics can bring about "bifidogenic" effects and a shift in microbial metabolism from "proteolytic" to the more favorable "saccharolytic" in mice (Gibson and Roberfroid, 1995). A combination of various substances with different rates of fermentation will be effective in mimicking some of the antibiotic effects in pigs (Williams *et al.*, 2001). However, no similar research has been reported in broiler chickens. Prebiotics were shown to improve growth performance of birds (Ammerman *et al.*, 1988; Xu *et al.*, 2003; Ao, 2004). In addition, some positive changes in digestive enzymes, gut morphology, and immune system were also noticed in birds given prebiotic-supplemented feed (Gülsen *et al.*, 2002; Xu *et al.*, 2003; Zhang *et al.*, 2003), which is the indirect effects of prebiotics on the gut system.

There are many considerations in supplementing prebiotics in animal feed. These include the type of diet (i. e. the content of non-digestible oligosaccharide); the type and inclusion level to supplement; the animal characteristics (species, age, stage of production); and the hygienic conditions of the farm (Verdonk *et al.*, 2005). The primary one is the type and inclusion level to supplement as high dosage of prebiotics can cause negative effects on the gut system and retard the growth rate of birds. It is reported that rapid fermentation of prebiotics, leading to high concentrations of organic acids, impaired the barrier function, which reduced the ability of rats to resist salmonella infection (Ten Bruggencate *et al.*, 2003).

Probiotics

In animal nutrition, probiotics are defined as viable microorganisms used as feed additives, which lead to beneficial effects for the host by improving its microbial balance (Fuller, 1989) or the properties of the indigenous microflora (Havenaar and Huis In't Veld, 1992). A variety of microbial species have been used as probiotics, including species of *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Eschericia coli*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, a variety of yeast species, and undefined mixed cultures. *Lactobacillus* and *Bifidobacterium* species have been used most extensively in humans, whereas species of *Bacillus*, *Enterococcus*, and *Saccharomyces* yeast have been the most common organisms used in livestock (Simon *et al.*, 2001). There has been an increase in research on feeding *Lactobacillus* to broiler chickens (Jin *et al.*, 2000; Kalavathy *et al.*, 2003).

Possible modes of action of probiotics were extensively reviewed (Jin *et al.*, 1997; Simon *et al.*, 2001; Ghadban, 2002; Edens, 2003). Two basic mechanisms by which probiotics act include maintaining a beneficial microbial population by "competitive exclusion" and immune modulation. Competitive exclusion includes competition for substrates, production of antimicrobial metabolites that inhibit pathogens, and competition for attachment sites. Immune modulatory effects of probiotics are dependent on the strain or species of bacteria included in the probiotics (Edens, 2003). For example, feeding *L. paracasei* to broilers increases phagocytic and bacterial activity whereas *Bifidobacterium lactis* increases Ig A levels.

Probiotics have been shown to be very efficient at controlling intestinal *Salmonella* colonization in poultry (Ghadban, 2002). Reduced caecal coliform populations were noticed in the chickens given the feed supplemented with a probiotic but the populations of other kinds of bacteria were not affected (Watkins and Kratzer, 1984; Jin *et al.*, 1998a; Jin *et al.*, 1998b). Probiotics can improve the growth performance of birds (Jin *et al.*, 1998a; Jin *et al.*, 1998b; Kalavathy *et al.*, 2003) but the effects are dependent on specific probiotics, the application level of probiotics as well as the age of birds. Moreover, there are many factors from nutrition, environment, and management that could compromise the effectiveness of probiotics (Edens, 2003), which can probably explain the variable results in the growth performance and gut physiology observed in birds given prebiotc-supplemented feed.

Bacterial receptor analogues (BRA)

Carbohydrate chains on the animal cell are primary receptors for many microorganisms. The corresponding lectin (adhesin) of a microorganism ensures binding to these chains. Many pathogens use carbohydrate-binding proteins to attach to cells and initiate disease. The first line of defence against these infectious diseases consists of decoy oligosaccharides in the mucous layer that lines all exposed epithelial cells (Zopf and Roth, 1996). The basic theory of BRA applied in animal nutrition is, by blocking the oligosaccharide lectin of pathogens, the infection is inhibited, the integrity of epithelial line is protected and the health and growth performance of animal are improved.

Mannanoligosaccharide, derived from the outer cell wall of *Saccharomcyes cerevisiae* var. *boulardii*, is one kind of BRAs. MOS products are more complex than the name suggests; they are components of the outer layer of yeast cell walls and their components include proteins, glucans and phosphate radicals as well as mannose (Klis *et al.*, 2002). The basic

composition of the wall consists of mannan (30%), glucan (30%) and protein (12.5%). While the ratio of one component to another remains relatively constant from strain to strain, the degree of mannan phosphorylation and the interaction among the mannan, glucan and protein components varies (Lyons, 1994). Mannanoligosaccharide contains protein which has relatively high proportions of serine, threonine, aspartic and glutamic acids, and a paucity of methionine (Song and Li, 2001).

Hooge (2004a) reviewed pen trials conducted with a commercially available dietary MOS (Bio-MOS, Alltech Inc.) from 1993 to 2003 and the meta-analysis showed that MOS improved the growth performance of birds compared to the negative control. Compared to a wide range of AGPs (including avilamycin, bacitracin, bambermycin or virginiamycin at growth promoting concentrations), a significant decrease in mortality was observed for MOS treatment. Three major modes of action by which broiler performance is improved by MOS are proposed: 1) control of pathogenic bacteria or potential pathogenic bacteria which possesses type-1 fimbriae (mannose-sensitive lectin), 2) immune modulation, and 3) modulation of intestinal morphology and expression of mucin and brush border enzymes.

Mannanoligosaccharide does not only prevent the pathogenic bacteria or potential pathogenic bacteria possessing type-1 fimbriae from attaching to gut wall but also displace them from the gut wall. This reduces subclinical or lethal infection. Pathogens, possessing type-1 fimbriae, are much more virulent than those non-fimbriated bacteria. Duguid and co-workers (1976) demonstrated that fimbriation in *S. typhimurium* significantly increases both the number of infections (26% increase) and deaths (40% increase) in mice inoculated orally compared to non-fimbriated organisms from the same parent strain.

Type 1 fimbriae are found in a wide range of isolates of *E. coli*, pathogenic as well as commensal, and in other members of the *Enterobacteriaceae* family (Adlerberth *et al.*, 2000). Of *E. coli* isolated from poultry, 68% possess type 1 fimbriae; 53% of Salmonella species tested possess type 1 fimbriae (Finucane *et al.*, 1999b). Receptors for type 1 fimbriae, i.e., mannose-containing glycoproteins, occur in abundance after mucosal injury, because the newly synthesized immature intestinal epithelial cells carry more glycoproteins with terminal mannose (Baba *et al.*, 1993; Pusztai *et al.*, 1993).

The immune modulatory effects of MOS are based on the following two aspects: 1) its mannan and glucan components have antigenicity characteristics, and 2) MOS prevents colonization of specific pathogens but allow them to be presented to immune cells as

attenuated antigens (Ferket, 2004). The immunostimulatory effects of MOS were demonstrated by Spring and Privulescu (1998) in GF vs. CV piglets (Table 2.3). A unique character of MOS in immune modulation is that MOS enhances the protective antibody response to enhance disease resistance while at the same time suppress the acute phase (fever) response (Ferket *et al.*, 2002). Mannaoligosaccharides have also been shown to enhance macrophage response in different animal species (Spring and Pirvulesu, 1998).

Table 2.3 Effects of MOS (Bio-MOS) on immune responses of conventional and germ-free piglets (Adapted from Privulescu, 1999)

Item	Without MOS	With MOS	Change
Cellular immune responses			
Phagocytotic index	41.4	78.2	89% increase
Lymphoblast transformation	1.60	3.09	93% increase
Proportion T lymphocytes	37.6	24.7	34% decrease
Cytokines dose in lymphocyte cultures (relative doses)			
IL-2	1	2	100% increase
IFN-γ	1	3	200% increase
Humoral immune responses:			
Serum IgG (mg/100mL)	200	916	358% increase
Serum IgM (mg/100mL)	Trace	106	>500% increase
Serum IgA (OD by ELISA)	163	364	123% increase
Lysozyme (μg/mL)	1.38	3.22	133% increase

Although positive effects of MOS on growth performance and gut physiology were observed (Table 2.4), the information available is still very limited and some results are also quite variable. There is only scanty information on the effect of MOS on nutrient digestion, availability and retention of birds. Studies by Kumprecht and Zobac (1997a) showed that faecal digestibility of fibre was increased by MOS but the digestibility of fat, nitrogen-free extract and crude protein was not affected. While Samarasinghe *et al.* (2003) observed that energy and protein utilization was improved by MOS, Shafey *et al.* (2001b) reported that MOS supplementation had no effect on nitrogen utilization of birds. A number of studies demonstrated that MOS is effective in reducing *Salmonella* infection of birds (Fernandez *et al.*, 2000; Spring *et al.*, 2000; Fernandez *et al.*, 2002; Kumar *et al.*, 2002). Only a few studies investigated the effect of MOS on intestinal and faecal microbial populations of broilers (Stanley *et al.*, 1996; Spring *et al.*, 2000; Song and Li, 2001; Jamroz *et al.*, 2003; Peuranen

et al., 2006) and the results are variable. Iji *et al.* (2001) found that MOS supplementation could affect gut morphology and function, however, MOS did not appear to improve the growth performance of birds. The effects of MOS on the immune organ or response were observed by Kumar *et al.* (2002), Ao (2004), Kocher *et al.* (2004) but not Shafey *et al.* (2001a). The variances observed on the effects of MOS in broiler chickens seem to be related to differences in diets, rearing conditions as well as the age of birds. More detail studies are needed to systematically examine the role of MOS in the gut system of birds in order to explain its growth-promoting effects and thus making its dietary application more successfully.

Table 2.4 The effects of MOS in broiler chickens

Effects	References
Enhance growth performance	Stanley *et al.*, 1996; Ao, 2004; Samarasinghe *et al.*, 2003; Pelicano *et al.*, 2004; Kocher *et al.*, 2004
Improve nutrient utilization	Kumprecht and Zobac, 1997a; Samarasinghe *et al.*, 2003; Kannan *et al.*, 2005
Alter gut microflora	Spring *et al.*, 2000; Song and Li, 2001; Fernandez *et al.*, 2002; Jarmoz *et al.*, 2003; Samarasinghe *et al.*, 2003
Alter gut morphology	Iji *et al.*, 2001; Loddi *et al.*, 2002; Pelicano *et al.*, 2005
Stimulate immune system	Shafey *et al.*, 2001; Kumar *et al.*, 2002; Ao, 2004; Kocher *et al.*, 2004
Exclude pathogenic bacteria	Fernandez *et al.*, 2000; Spring *et al.*, 2000; Fernandez *et al.*, 2002

Symbiotics

A symbiotic is, in its simplest definition, a combination of probiotics and prebiotics (Collins and Gibson, 1999; Schrezenmeir and de Vrese, 2001). This combination could improve the survival of the probiotic organism, because its specific substrate is available for fermentation. This could result in advantages to the host offered by the live microorganism and the prebiotic. Bengmark (2001) defines symbiotics as products of fermentation. Since in mixtures of pre- and Probiotics, the prebiotics will be fermented when the appropriate choice

of products is used, this definition may also be plausible. Examples of symbiotics are FOS and bifidobacteria, and lactitol and lactobacilli (Collins and Gibson, 1999). Bailey *et al.* (1991) used a combination of FOS and competitive exclusion flora to reduce *Salmonella* colonization in chickens. The combination was more effective in reducing *Salmonella* colonization than FOS or competitive probiotic alone. However, no reports have been published regarding the growth-promoting effects of symbiotics in broiler chickens. A symbiotic (the combination of FOS and MOS) has been shown to improve the colonic health and immune status of dogs (Swanson *et al.*, 2002).

Phytobiotics

Plant products have been used for centuries by humans as food and to treat ailments. Natural medicinal products orgininating from herbs and spices have also been used as feed additivies for farm animals in Asian countries for the same length of time. To differentiate from the plant products used for veterinary purposes (prophylaxis and therapy of diagnosed health problems), phytobiotics were redefined by Windisch and Kroismayr (2006) as plant-derived products added to the feed in order to improve performance of agricultural livestock. Around the world, phytobiotics have been investigated as natural sources of biologically important chemicals since efforts are being made to ban all types of AGP in many countries. Compared with synthetic antibiotics or inorganic chemicals, these plant-derived products have proven to be natural, less toxic, residue free, and are thought to be ideal feed additives in food animal production (Wang *et al.*, 1998).

With respect to biological origin, formulation, chemical description and purity, phytobiotics comprise a very wide range of substances and four subgroups may be classified: 1) herbs (product from flowering, non-woody and nonpersistent plants), 2) botanicals (entire or processed parts of a plant, e.g. root, leaves, bark), 3) essential oils (hydro distilled extracts of volatile plant compounds), and 4) oleoresins (extracts based on non-aqueous solvents) (Windisch and Kroismayr, 2006). The active compounds of phytobiotics are secondary plant constituents.

Antimicrobial activity and immune enhancement probably are the two major mechanisms by which phytobiotics exert positive effects on the growth performance and health of animals. Compounds (phytochemicals) in phytobiotics are well known to have antimicrobial ability (Cowan, 1999). Polysaccharide components are considered to be the most important immunoactive components (Xie and Niu, 1996; Xue and Meng, 1996). In diseased chickens

(either infected with avian *Mycoplasma gallisepticum* or *Eimeria tenella*), Guo and his colleagues (Guo *et al.*, 2004a, 2004b, 2004c) demonstrated that plants and their extracts could improve the growth performance, reduce the populations of potential harmful bacteria, and enhance both cellular and humoral immune responses of chickens. Some herbal extracts have also been shown to possess a coccidiostatic activity (Allen *et al.*, 1997; Youn and Noh, 2001; Christakia *et al.*, 2004).

A common feature of phytobiotics is that they are a very complex mixture of bioactive components. For example, hawthorn fruit, a common growth-enhancing and digestion modifier, has been shown to contain more than 70 kinds of organic chemicals along with some unidentified factors and active bio-ingredients (Wang *et al.*, 1998). Therefore they may exert multiple functions in the animal body. Increased feed intake and digestive secretions are also observed in animals offered phytobiotic-supplemented feed (Windisch and Kroismayr, 2006). The growth enhancement is probably the result of the synergistic effects among complex active molecules existing in phytobiotics (Gauthier, 2002). However, the exact growth-promoting mechanisms of phytobiotics in broiler chickens are poorly understood.

Among phytobiotics, essential oils have been applied into chicken feed in Europe and USA (Hooge, 2004b). Essential oils function mainly as antimicrobials and antioxidants; its antimicrobial ability may modulate the gut ecosystem to affect fat digestibility (Lee *et al.*, 2004). A commercial preparation of essential oil components reduced faecal *C. perfringens* counts of broilers in a field study (Mitsch *et al.*, 2002) but essential oils do not always enhance growth in all studies.

Four factors may affect the effectiveness of phytobiotic additives: 1) plant parts and their physical properties, 2) location, 3) harvest time, and 4) compatibility with the other ingredient (s) in the feed (Wang *et al.*, 1998), which may also explain why 50% difference in BWG and 63% difference in FCR could happen when different kinds of phytobiotics are used in chicken diet (Xing, 2004).

Although phytobiotics are a group of natural additives, research into their mechanisms of action, compatibility with diet, toxicity and safety assessment (based on the fact that some phytobiotic might have harmful substance(s)) needs to be done before they can be applied more extensively in chicken feed.

2.6 CONCLUSIONS

It is certain that the cost of animal production will increase with a universal ban on in-feed antibiotics which, through their direct effects on the establishment of optimal gut microflora, are closely associated with improvement in growth and production. Although dietary as well as environmental factors also influence the delicate balance of gut microflora, alternatives to AGP may play a bigger role in keeping the balance of gut microflora and health status of broiler chickens. All the observed effects of alternative AGP products are highly variable depending on the specific conditions of the experiment, such as diet characteristics and hygienic condition of the rearing environment. More research into the exact mechanisms and mode of actions of these products is urgently needed so as to reduce the outcome variability and to maximize the efficacy of their application.

Although the number of studies involved in evaluating the potential of MOS in broiler production has increased quite rapidly recently, there is still a lack of linkage among the various responses to MOS. In order to address this shortcoming, the experiments outlined in the following chapters were designed and conducted with the intention to elucidate the relationships among MOS, gut microflora, gut morphology, nutrient digestibility, and performance of broiler chickens.

CHAPTER 3
EFFECTS OF DIFFERENT DIETARY LEVELS OF MANNANOLIGOSACCHARIDE ON GROWTH PERFORMANCE AND GUT DEVELOPMENT OF BROILER CHICKENS

3.1 INTRODUCTION

The withdrawal of AGP has led to problems related to animal health and welfare as well as serious economic losses (Dibner and Richards, 2005). The problems might be alleviated by in-feed additives other than AGP. Mannanoligosaccharide was reported to be a promising alternative in terms of production performance and mortality (Hooge, 2004a; Rosen, 2007), but the supplemental levels of MOS varied by trials and by feeding phases in different studies, ranging from 0.5 g MOS/kg diet to 5g MOS /kg diet. The dose-response studies of MOS showed that the optimal dosage for growth performance is approximately 2 g MOS /kg diet (Kumprecht *et al.*, 1997b; Tucker *et al.*, 2003). However these authors did not examine the effects of MOS on the gut development, which are closely related to the body growth of fast-growing broiler chickens (Sklan, 2001).

Studies on the effects of MOS on the gut development of broiler chickens are limited. Iji *et al.* (2001) examined the effects of MOS (0, 1, 3 and 5g MOS/kg diet) on the intestinal structure and function of birds during a 21-day feeding period. Improvements in the intestinal structure and function were noticed in birds fed diets supplemented with medium or high levels of MOS but the effects of MOS on the growth performance were minimal. It should be pointed out that the birds did not receive the MOS-supplemented feed until they were 7 days old. In broiler chickens, the gut system is under rapid development during the first week and matures around the second week (Sklan, 2001). In-feed additives can exert their effects on the development of the gastrointesstinal system at an early age and thus affect subsequent performance of birds (Monsan and Paul, 1995).

The present study was designed to 1) identify the optimal level of MOS that will maximize the growth performance of birds, and 2) examine changes in nutrient digestibiligy, general morphology of GIT, intestinal mucosal morphology and the concentrations of caecal VFAs, which may explain the growth response induced by the addition of MOS.

3.2 MATERIALS AND METHODS

3.2.1 Experimental design and diets

The basal diets, consisting mainly of corn, wheat and soybean meal, were used in a 2-phase feeding programme with the starter feed provided for 1-3 weeks of age and the finisher diet for 4-6 weeks of age (Table 3.1).

Table 3.1 Composition (g/kg) and nutritive value of basal diets

	Feeding period, wk	
Component	1-3	4-6
Basal concentrate		
Wheat	50.0	50.0
Corn	585.0	645.0
Soybean meal	250.0	195.0
Meat meal	80.0	85.0
Limestone	7.3	3.4
Lysine-HCl	3.0	3.0
DL-Methionine	3.0	2.5
Salt	0.7	0.3
Sodium bicarbonate	4.5	3.5
Choline Chloride	3.0	1.0
Vegatable oil	11.0	9.0
L-Threonine	0.5	0.3
Vitamin and mineral premix[1]	2.0	2.0
Calculated chemical composition		
ME, MJ/Kg	12.56	12.75
Crude protein	220.00	200.00
Crude fibre	28.00	26.60
Crude fat	45.00	46.00
Lys	13.00	11.80
Met+Cys	9.70	8.90
Ca	10.30	9.50
P available	5.10	4.80
Na	1.90	1.80
Cl	2.20	2.40

[1]Supplied per kg of diet (mg): vitamin A (as *all-trans* retinol), 12,000 IU; cholecalciferol, 3,500 IU; vitamin E (as d-α-tocopherol), 44.7 IU; vitamin K3, 2 mg; thiamine, 2 mg; riboflavin, 6 mg; pyridoxine hydrochloride, 5 mg; vitamin B12, 0.2 mg; biotin, 0.1 mg; niacin, 50 mg; D-calcium pantothenate, 12 mg; folic acid, 2 mg; Mn, 80 mg; Fe, 60 mg; Cu, 8 mg; I, 1mg; Co, 0.3 mg; and Mo, 1 mg.

The six experimental diets tested are shown in Table 3.2. A commercial xylanase product (Allzyme PT, Alltech Pty Ltd, Australia) was included in the basal diet at the recommended level (0.5g/kg feed). Titanium dioxide, 5g/kg, was incorporated in the basal diet as a marker for the calculation of digestibility coefficients.

Table 3.2 Dietary treatments

Treatment	Description
1	Negative control (NC)
2	Positive control with zinc bacitracin (ZnB) added at 50 ppm from 0 to 21 d and at 30 ppm from 21 to 42 d
3	Low MOS with MOS added at 0.5 g/kg from 0 to 42 d
4	Medium MOS with MOS added at 1.0 g/kg from 0 to 42 d
5	High MOS with MOS added at 2.0 g/kg from 0 to 42 d
6	Step-down MOS with MOS added at 2.0 g/kg from 0 to 7 d, 1.0 g/kg from 8 to 21 d and 0.5 g/kg from 22 to 42 d

3.2.2 Bird management

Three hundred and eighty four (384) one-day-old male Cobb broiler chickens (42.6g±3.55) were obtained from a local hatchery (Baiada hatchery, Kootingal, NSW) and randomly assigned to 48 cages in four-tier battery brooders housed in an environmentally controlled room. Each of the 6 dietary treatments was randomly assigned to 8 cages (600×420×230 cm) with 8 birds per cage. The birds were transferred to slide-in cages (800×740×460 cm) in an environmentally controlled room at the end of week 3. Room temperature was at 34±1 °C on the first day and gradually decreased to 24 °C by the end of the third week. The lighting program was 18 h light and 6 h darkness throughout the trial.

3.2.3 Animal ethics

The experiment complied with the guidelines of the University of New England with respect to animal experimentation and care of chickens under study (Approval No.: AEC05/048).

3.2.4 Growth performance and AME measurement

Body weight and feed intake (FI) were recorded on a cage basis at weekly intervals. Feed and water were offered *ad libitum* during the 42-d trial period. Mortality was recorded daily and feed per gain values were corrected for mortality.

All excreta were collected for the determination of apparent metabolizable energy (AME). Feed intake and excreta output were measured quantitatively per cage over 4 consecutive days (d 28 to d 32). Excreta were pooled within each cage and dried at 80 °C in a forced-air oven. Dried samples were ground to pass through a 0.5 mm screen and stored in airtight plastic containers for gross energy (GE) analyses. The AME values were calculated using

the following formula. Appropriate corrections were made for differences in dry matter content.

$$AME = \frac{FI \times GE_{diet} - Excreta\ output \times GE_{excreta}}{FI}$$

3.2.5 Sampling procedure

At 7, 21 and 42 days of age, one bird per cage (8 birds per treatment) was killed by cervical dislocation, and the GIT was excised. The proventriculus and gizzard were emptied and weighed. The small intestine was divided into three segments: duodenum (from gizzard outlet to the end of the pancreatic loop), jejunum (from the pancreatic loop to Meckel's diverticulum), and ileum (from Meckel's diverticulum to the ileo-caecal junction). Approximately 2 cm of the proximal jejunum and proximal ileum were flushed flushed with ice-cold phosphate-buffered saline (PBS) at pH 7.4 and fixed in 10% formalin for morphological measurements.

Each intestinal segment was emptied by gentle pressure and the length and weight were recorded, as was the body weight of the birds from which they were excised. The weight of pancreas, liver, spleen, and bursa were also measured. The contents of the jejunum and ileum were collected, freeze-dried, and milled (0.5mm screen); the contents of the caeca were stored at -20 °C until VFA analysis was performed.

3.2.6 Analytical methods

Gross energy, protein and starch

Gross energy of the feed and digesta was determined using a bomb calorimeter (Calorimeter C7000, Protech group, Australia) standardised with benzoic acid. The nitrogen contents were determined using a Leco FP-2000 analyzer and the protein contents were calculated using a multiplication factor of 6.25.

Starch was determined as glucose using a glucose oxidase and peroxidase method, with GPD-Perid kit supplied by Boehringer-Mannheim Australia (Castle Hill, NSW, Australia). Finely ground samples (0.5 mm) were weighed accurately into screw-capped reaction tubes (30 mL) and wet with 0.2 mL aqueous ethanol (80% v/v). A further 3 mL of thermostable α-amylase in MOPS buffer (sodium salt, Sigma M9381; 50 mM, pH 7.0) were added and the samples were incubated in a boiling water bath for 6 min. After cooling, 4 mL acetate

sodium buffer (200 mL, pH 4.5) were added, followed by 0.1 mL of amyloglucosidase (E. C. 3.2.1.3., Megazyme) and incubated at 50 °C for 30 min. Glucose was determined colorimetrically after incubating an aliquot (0.1 mL) with 3 mL of GOPOD reagent (Megazyme) at 50 °C for 20 min and reading the absorbance at 510 nm against a reagent blank (glucose oxidase assay).

Digestibility

Titanium dioxide of the feed and digesta was determined by the method of Short *et al.* (1996). Approximately 0.1 g of dried digesta was ashed and dissolved in 7.4 M sulphuric acid. Hydrogen peroxide (30% vol) was subsequently added, resulting in the typical orange colour, the intensity of which is dependent upon the titanium concentration. Aliquots of the solutions obtained and of similarly prepared standard solutions were analysed using a UV spectrophotometer (Model 50-120, Hitachi, Japan) by measuring the absorbance at 410 nm. Small intestinal digestibility of starch and protein was then estimated using the formula:

$$Digestibility = (1 - \frac{digesta\ nutrient\ (g/kg)/digesta\ AIA\ (g/kg)}{diet\ nutrient\ (g/kg)/diet\ AIA\ (g/kg)}) \times 100$$

Histology

Tissue slices, 1-2 mm thick, were prepared from each section of the gut sample, enclosed in a plastic tissue cassette, and processed over a 19-hour period in an automatic tissue processor (TOSCO, Thomas Optical & Scientific Co., Melbourne & Sydney, Australia). Processing involved serial dehydration with ethanol, clearance with histolene, then impregnation with wax. The tissue was embedded in paraffin wax prior to sectioning on a microtome. Separate 7 μm sections were placed on a glass slide for staining with haematoxylin and counter-staining with eosin, then mounted in a DPX medium (Fluka Cheme, Buchs, Australia). The images were captured at 4 x and 5 x magnification using a Leica DLMB Microscope in bright field mode and a Spot RT digital camera. The images were stored and the Spot software was used to calibrate and then measure the villus height and crypt depth. About 15 villi and 15 crypts were measured in each sample (chicken).

VFA analysis

Concentrations of acetate, propionate, and butyrate were determined by gas chromatography (GC, Model CP 3800, Varian Analytical Instruments, Palo Alto, CA, USA). Approximately 2 to 3 g of thawed digesta were suspended in 3 mL of 0.1 M sulphuric acid in a screw-

capped tube and centrifuged (15 min at 12,000 ×*g*) at 4 °C. Caproic acid, 0.1 mL, was added to 1 mL of the supernatant, which were transferred into Thundberg tube. The sample was frozen in liquid nitrogen, vacuum-sealed, and bathed overnight in liquid nitrogen. Subsequently, the sublimated sample was thawed for analysis by GC. The GC was equipped with a flame ionization detector and a polyethylene glycol packed column (1.5 m long, 5.6 mm ID). The column was operated at 100 to 150°C with high purity helium, at 20 mL/min, as the carrier gas.

3.2.7 Statistical analyses

The software package SPSS, (Version 12.0, SPSS Inc.), was used to perform the statistical analysis of data obtained in this study. Data were subjected to analysis of variance (ANOVA) and the differences between mean values were identified by the least significant difference (LSD). Differences among treatments were deemed to be significant only if the P-value was less than 0.05. All results were expressed as means.

3.3 RESULTS

3.3.1 Growth performance

In the first three weeks, diet did not affect the FI of birds (Table 3.3). However, compared to the negative control, the high MOS diet improved ($P<0.05$) body weight gain (BWG), whereas the other MOS treatments had no significant effects on BWG. There were no significant differences in BWG between any of the MOS treatments and the positive control and no significant effect of the treatments on FCR was observed.

In the last three weeks, there were no significant differences in the growth performance of birds among MOS treatments and the negative control (Table 3.3). Birds fed the positive control diet had higher ($P<0.05$) BWG and lower ($P<0.05$) FCR than those in the negative control and/or MOS groups.

By d 42, birds given the ZnB diet had the highest body weight gain and the lowest FCR among all the treatments (Table 3.3). No significant differences were noticed in AME values and mortality rates as a result of diet.

Table 3.3 Feed intake (FI), body weight gain (BWG), feed conversion ratio (FCR), energy utilization and mortality of birds fed the experimental diets[1]

	NC	Low MOS	Medium MOS	High MOS	Stepdown MOS	ZnB	SEM	P values
0-3 wk								
FI (g/bird)	1160	1208	1167	1203	1141	1169	33.8	0.34
BWG (g/bird)	826^{b}	872^{ab}	835^{ab}	877^{a}	836^{ab}	847^{ab}	19.2	0.05
FCR (g/g)	1.41	1.39	1.40	1.37	1.37	1.38	0.022	0.35
4-6 wk								
FI (g/bird)	3466	3438	3439	3406	3368	3482	80.5	0.76
BWG (g/bird)	1936^{ab}	1918^{b}	1922^{b}	1910^{b}	1894^{b}	1995^{a}	31.1	0.04
FCR (g/g)	1.79^{a}	1.79^{a}	1.79^{a}	1.78^{a}	1.78^{a}	1.75^{b}	0.015	0.04
0-6 wk								
FI (g/bird)	4626	4646	4606	4609	4509	4651	94.5	0.70
BWG (g/bird)	2761^{b}	2790^{ab}	2757^{b}	2788^{ab}	2729^{b}	2841^{a}	34.7	0.05
FCR (g/g)	1.68^{a}	1.67^{a}	1.67^{a}	1.65^{ab}	1.65^{ab}	1.64^{b}	0.013	0.05
Mortality (%)	1.6	6.3	3.1	6.3	6.3	6.3	-	-
AME (MJ/kg DM)	14.2	14.7	14.1	14.5	14.4	14.6	0.44	0.88

[1]Values are means of 8 replicates.
[a,b]Means within the same row with no common superscript differ significantly (P<0.05).

3.3.2 Gross morphology of gastrointestinal tract

Only the relative weight of the liver and the relative length of the small intestine were affected by MOS and the effects were dependent on the age of birds as well as the dosage level of MOS. Thus, at 7 days of age, the MOS treatments significantly reduced (P<0.05) the relative weight of the liver (Table 3.4). A similar result was noticed for ZnB treatment. No significant differences in the relative weight of the visceral organs were observed among the treatments at 21 or 42 days of age. Dietary additives also did not affect the relative weight of the small intestine at different ages (Table 3.5). However, the relative length of the duodenum was significantly reduced (P<0.05) by step-down MOS treatment compared to ZnB treatment at 21 days of age. At 42 days of age, birds fed the medium MOS diet tended (P<0.08) to have shorter duodenum than both controls. A shorter (P<0.05) ileum was also noticed in birds fed the medium MOS diet compared to the negative control. The addition of ZnB reduced (P<0.05) the relative length of jejunum and ileum compared to the high MOS or/and low MOS treatments (Table 3.5).

Table 3.4 Effects of dietary treatments on the relative weight (%BW) of visceral organ of broiler chickens on days 7, 21 and 42[1]

	NC	Low MOS	Medium MOS	High MOS	Stepdown MOS	ZnB	SEM	P values
Day 7								
Proventriculus	1.09	1.04	0.97	0.97	1.09	1.01	0.057	0.11
Gizzard	4.52	4.00	4.23	4.02	4.11	4.17	0.191	0.11
Pancreas	0.53	0.45	0.52	0.48	0.53	0.52	0.047	0.49
Liver	5.19[a]	4.32[b]	4.51[b]	4.43[b]	4.24[b]	4.67[b]	0.254	0.01
Spleen	0.10	0.10	0.10	0.10	0.09	0.09	0.016	0.62
Bursa	0.15	0.20	0.19	0.16	0.19	0.16	0.022	0.21
Day 21								
Proventriculus	0.45	0.46	0.46	0.45	0.43	0.43	0.027	0.88
Gizzard	2.03	2.07	2.16	2.12	2.10	2.05	0.113	0.89
Pancreas	0.25	0.25	0.27	0.26	0.26	0.24	0.016	0.61
Liver	3.01	2.94	3.09	3.06	2.88	2.82	0.220	0.81
Spleen	0.09	0.10	0.09	0.10	0.08	0.09	0.000	0.57
Bursa	0.23	0.23	0.26	0.26	0.24	0.22	0.027	0.59
Day 42								
Proventriculus	0.34	0.35	0.34	0.37	0.32	0.31	0.027	0.48
Gizzard	1.74	1.66	1.76	1.77	1.68	1.73	0.111	0.91
Pancreas	0.19	0.17	0.17	0.17	0.19	0.19	0.016	0.38
Liver	2.45	2.36	2.43	2.56	2.31	2.32	0.160	0.64
Spleen	0.11	0.11	0.12	0.12	0.12	0.12	0.016	0.96
Bursa	0.16	0.16	0.16	0.20	0.16	0.16	0.027	0.46

[1]Values are means of 8 replicates.
[a,b] Means within the same row with no common superscript differ significantly ($P<0.05$).

Table 3.5 Effects of dietary treatments on the relative weight (%BW) and relative length (cm/%BW) of the small intestine of broiler chickens on days 7, 21 and 42[1]

	NC	Low MOS	Medium MOS	High MOS	Stepdown MOS	ZnB	SEM	P values
Relative Weight (%BW)								
Day 7								
Duodenum	2.09	2.05	2.06	2.03	2.14	2.08	0.096	0.87
Jejunum	2.76	2.54	2.84	2.80	2.74	2.64	0.140	0.30
Ileum	1.70	1.75	1.63	1.64	1.81	1.66	0.162	0.59
Day 21								
Duodenum	0.88	0.90	0.91	0.95	0.88	0.79	0.050	0.08
Jejunum	1.50	1.44	1.44	1.40	1.45	1.33	0.096	0.60
Ileum	0.93	0.95	0.96	0.94	0.92	0.82	0.055	0.11
Day 42								
Duodenum	0.54	0.51	0.48	0.49	0.46	0.53	0.032	0.15
Jejunum	0.91	0.89	0.87	0.96	0.89	0.90	0.057	0.75
Ileum	0.66	0.66	0.67	0.68	0.69	0.65	0.039	0.89
Relative length (cm/%BW)								
Day 7								
Duodenum	11.30	11.26	11.66	10.72	11.60	11.67	0.720	0.76
Jejunum	26.23	25.43	26.82	25.90	27.76	25.83	1.718	0.79
Ileum	25.15	23.10	25.37	24.91	26.75	24.00	1.930	0.53
Day 21								
Duodenum	2.79^{abc}	2.98^{ab}	2.86^{abc}	2.76^{bc}	2.66^{c}	3.04^{a}	0.129	0.05
Jejunum	6.57	6.70	6.73	6.23	6.48	6.64	0.384	0.80
Ileum	5.71	6.18	6.27	5.77	5.95	6.00	0.356	0.57
Day 42								
Duodenum	1.16	1.17	1.06	1.08	1.08	1.17	0.050	0.08
Jejunum	2.60^{abc}	2.65^{ab}	2.62^{abc}	2.72^{a}	2.51^{c}	2.58^{bc}	0.063	0.05
Ileum	2.62^{abc}	2.67^{ab}	2.35^{d}	2.70^{a}	2.50^{bcd}	2.46^{cd}	0.097	0.007

[1]Values are means of 8 replicates.
[a,b] Means within the same row with no common superscript differ significantly ($P<0.05$).

3.3.3 Intestinal digestibility of nutrient, morphological measurement and caecal VFA profile

The effects of treatments on the digestibility of nutrients are shown in Table 3.6. There were no significant differences in apparent jejunal and ileal digestibility coefficient of protein and starch among the diets at different ages.

Table 3.6 Effects of dietary treatments on the apparent digestibility of protein and starch in the jejunum and ileum of broiler chickens on days 7, 21 and 42[1]

	NC	Low MOS	Medium MOS	High MOS	Stepdown MOS	ZnB	SEM	P values
Jejunum								
Day 7[2]								
Protein	66	75	76	69	69	68	-	-
Starch	70	73	73	72	71	69	-	-
Day 21								
Protein	66	70	72	69	69	75	4.9	0.57
Starch	80	81	83	84	84	81	4.0	0.86
Day 42								
Protein	76	79	74	75	67	77	5.9	0.44
Starch	88	90	90	89	83	88	3.9	0.43
Ileum								
Day 7[2]								
Protein	85	84	85	79	80	84	-	-
Starch	95	96	96	95	96	97	-	-
Day 21								
Protein	75	79	81	83	82	79	3.4	0.19
Starch	99	98	99	99	99	98	0.3	0.67
Day 42								
Protein	80	82	85	86	84	82	2.9	0.40
Starch	98	98	99	96	97	98	1.6	0.57

[1]Values are means of 8 replicates.
[2]The samples from the same treatment were pooled for the nutrient determination because of the little amount of digesta collected from one week old birds. Hence, one replicate per treatment was used.

Table 3.7 shows the mucosal morphology data. In the jejunum, birds given the medium and high MOS diets had longer ($P<0.05$) villi than birds in the two control groups at the end of week 1. The same trend was observed in 21-day-old birds compared to the negative control but not the positive control. At 42 days of age, birds given the medium MOS diet had significantly shallower ($P<0.05$) crypt depth. At the same age, lower ($P<0.05$) villi in the ileum were noticed in birds given the negative control diet compared to MOS treatments and the positive control. There was a tendency ($P=0.08$) for high MOS treatment to reduce the crypt depth in the jejunum at 7 days of age

Table 3.7 Effects of dietary treatments on the gut morphometry of the small intestinal mucosa of broiler chickens on days 7, 21 and 42[1]

	NC	Low MOS	Medium MOS	High MOS	Stepdown MOS	ZnB	SEM	P values
Jejunum								
Day 7								
Villus height (μm)	874^{b}	904^{ab}	984^{a}	980^{a}	959^{ab}	901^{ab}	42.3	0.05
Crypt depth (μm)	124	125	113	105	109	126	8.5	0.08
Day 21								
Villus height (μm)	1457^{b}	1502^{ab}	1560^{a}	1553^{a}	1548^{a}	1505^{ab}	59.4	0.03
Crypt depth (μm)	135	132	126	126	130	139	21.9	0.89
Day 42								
Villus height (μm)	1650	1591	1659	1659	1672	1695	121.2	0.79
Crypt depth (μm)	117^{ab}	122^{a}	103^{c}	114^{ab}	109^{bc}	109^{bc}	8.7	0.02
Ileum								
Day 7								
Villus height (μm)	565	549	531	542	540	538	42.7	0.81
Crypt depth (μm)	115	96	96	97	100	101	16.2	0.34
Day 21								
Villus height (μm)	748	778	723	780	788	789	72.4	0.56
Crypt depth (μm)	93	95	98	98	106	102	11.9	0.50
Day 42								
Villus height (μm)	855^{b}	922^{ab}	998^{a}	958^{a}	979^{a}	949^{a}	71.5	0.01
Crypt depth (μm)	84	93	97	91	93	99	10	0.15

[1]Values are means of 8 replicates.
[a,b] Means within the same row with no common superscript differ significantly ($P<0.05$).

The concentrations of individual VFAs in the caeca were affected by diet in the younger (21 days old) but not the older (42 days old) birds (Table 3.8). The high MOS and/or step-down MOS treatments significantly increased ($P<0.05$) the concentration of propionate as well as the concentration of butyrate compared to both controls at 21 days of age. However, the total VFA concentrations were not affected by MOS. The medium MOS treatment significantly increased ($P<0.05$) the molar ratio of propionate in 42-day-old birds compared to the negative control. In contrast, ZnB supplementation significantly reduced ($P<0.05$) the molar ratio of butyrate.

Table 3.8 Concentrations of caecal volatile fatty acids in broilers fed the experimental diets at different ages[1]

	NC	Low MOS	Medium MOS	High MOS	Stepdown MOS	ZnB	SEM	P values
Concentration (μmol/g digesta)								
Day 21								
Acetate	52.6	61.1	60.8	73.0	60.5	49.8	10.5	0.33
Propionate	5.8^{b}	6.8^{ab}	5.5^{b}	7.5^{a}	7.4^{a}	5.5^{b}	1.27	0.02
Butyrate	10.4^{b}	11.5^{b}	10.5^{b}	16.4^{a}	12.2^{b}	9.7^{b}	3.54	0.03
Total VFA	76.6	82.5	80.9	100.5	83.1	70.6	22.8	0.34
Day 42								
Acetate	64.3	51.0	59.2	69.6	67.1	69.9	29.8	0.86
Propionate	3.7	3.4	6.0	6.0	4.9	6.3	2.77	0.31
Butyrate	13.0	9.1	4.4	11.3	11.0	7.7	6.23	0.26
Total VFA	83.3	65.2	74.2	90.1	85.5	88.9	37.8	0.84
Molar ratios of VFA (% of total VFA)								
Day 21								
Acetate	73.0	72.5	73.1	71.5	72.6	70.4	4.33	0.89
Propionate	7.9	7.9	8.2	7.7	8.9	8.9	2.94	0.76
Butyrate	15.0	14.2	13.9	17.3	14.6	15.8	3.35	0.53
Day 42								
Acetate	78.1	77.5	77.8	78.0	77.6	80.1	3.39	0.80
Propionate	4.7^{b}	4.9^{b}	7.6^{a}	5.5^{b}	5.4^{b}	6.3^{ab}	1.66	0.05
Butyrate	14.2^{ab}	15.5^{a}	10.6^{bc}	12.4^{abc}	12.5^{abc}	8.9^{c}	3.67	0.05

[1]Values are means of 8 replicates.
[a,b] Means within the same row with no common superscript differ significantly (P<0.05).

3.4 DISCUSSION

The current study adds to the findings that MOS can improve the growth performance of broiler chickens compared to a negative control (Hooge *et al.*, 2003; Kocher *et al.*, 2004; Hooge, 2004a). Furthermore, the growth-promoting effects are dependent on the MOS supplemental level. Birds fed the low (1g/kg) MOS diet had a greater BWG in the last three weeks but birds fed the step down MOS diet had a better FCR in the first three weeks than those in the negative control group. However, an improvement in both BWG and FCR was observed with birds given the high (2g/kg) MOS diet and the effects were comparable to ZnB treatment in the first three-week feeding period. This observation agrees with the report that the optimal dosage of MOS for the growth performance of broiler chickens is approximately 2 g/kg feed (Kumprecht and Zobac, 1997a; Tucker *et al.*, 2003).

It is reported that birds respond more to MOS supplementation from 1 to 21 d than in the 22-42 d period (Tucker *et al.*, 2003; Jamroz *et al.*, 2003). A similar trend was observed in the current experiment, suggesting that the effects of MOS on the growth performance are related to the age of birds. Early evaluation of the effects of antibiotics on the growth performance of birds showed similar results (Jukes, 1955). It is postulated that this is due to a less mature gut system, e.g. the gut microflora, in younger birds compared to older birds (Jukes, 1955). For broiler chickens, a mature gut system can favourably support the fast body growth. Therefore, MOS may positively affect the maturation of the gut microflora of young birds and thus improve their growth performance. In the current study, a smaller liver weight was noticed in young birds fed MOS diets as well as the ZnB diet compared to the negative control. The liver size is dependent on the gastrointestinal microflora and/or its fermentation products (Jozefiak, 2006) and reduced liver was reported in GF rats and chickens vs. CV individuals (Wostman, 1981; Muramatsu *et al.*, 1983). The authors speculated that the activity of liver was higher in the presence of gut microflora, possibly due to the load of toxic substances such as ammonia and amines produced by intestinal bacteria and transported to the liver to be detoxified. The addition of MOS or ZnB may reduce the concentrations of ammonia or amines by stabilizing the gut microflora of young birds, thus reducing the size of the liver.

The relative weight of the other visceral organs, including the small intestine, was not affected by MOS, which is in agreement with the report by Iji *et al.* (2001) and Juskiewicz *et al.* (2003). However, the relative length of the small intestine of old birds seemed to be reduced in some MOS treatments compared to the negative control and a similar result was found on ZnB treatment. It has been reported that the length of the small intestine was generally shorter in the presence of antibiotics without any effects on digestive/absorptive function (Gordon and Bruckner-kardoss, 1961; Stutz *et al.*, 1983).

No significant differences were found in the small intestinal digestibility of protein and starch between MOS treatment (s) and the negative control. This is in agreement with the findings of Alves *et al.* (2003). On the other hand, an improvement in the villus height of small intestine was observed on the medium and high MOS treatment, which agrees with the reports by Spring (1996) and Iji *et al.* (2001). Similar results were observed with birds fed the ZnB diet. The increased height of villi points to the increased absorptive area (Yasar and Forbes, 1999) and improved gut function, which might contribute to the improved nutrient

digestibility in the small intestine. However, neither MOS nor ZnB seem to improve the digestibility of starch and protein in the small intestine in the current trial.

Dietary MOS and ZnB had different effects on the caecal VFA profile of birds. In general ZnB reduced the individual and total VFA concentrations, but MOS increased propionate concentration in the caeca of birds. In agreement with these findings, Santos *et al.* (2004) reported that propionic acid concentration was higher in MOS group when birds were raised on re-utilized litter. A significant negative correlation was found between caecal propionic acid concentration and caecal *Salmonella* colonization with birds less than 10 days old (Nisbet *et al.*, 1996). However, it is not clear whether there is a direct relationship between MOS treatment and the caecal propionic acid concentration, although 53% of Salmonella species tested possess type 1 fimbriae and they are supposed to be adsorbed by MOS (Finucane *et al.*, 1999). On the other hand, MOS also increased the caecal butyrate concentration of birds. Volatile fatty acids, especially butyrate, can exert a trophic effect not only on the local epithelial cells but also on jejunal structure (Lan *et al.*, 2005). In the current trial, an improved mucosal structure was noticed in the jejunum of birds on some MOS treatment(s).

3.5 CONCLUSIONS

The findings from this study suggest that the growth response of birds was more obvious to the high dosage level of MOS than to the low or medium MOS or step-down program during the first 3 weeks post-hatch. The effects of MOS supplementation appear to be more pronounced in younger than in older birds. The addition of MOS or ZnB showed some positive effects on gut development of birds, however, their effects on the caecal VFA profile differed widely.

CHAPTER 4
INTERRETIONSHIPS AMONG MANNANOLIGOSACCHARIDE, PERFORMANCE AND GUT DEVELOPMENT OF BROILER CHICKENS RAISED ON NEW LITTER

4.1 INTRODUCTION

It was shown in the previous experiment that dietary supplementation with MOS improved the growth performance of broiler chickens, especially in early life. The decrease in growth response of broiler chickens towards MOS with progression of age may be related to a well balanced gut microflora in older birds. The profile of the gut microflora continues to change as birds grow; for instance, it takes about 2 weeks for lactobacilli to become the predominant bacteria in the small intestine (Barnes *et al.*, 1972).

It is well established that majority of bacteria with type 1 fimbriae bind to mannose-based receptors in the intestine (Ofek *et al.*, 1977) and that MOS can act as a receptor analogue to prevent harmful bacteria possessing type-1 fimbriae from colonizing in the gut (Spring *et al.*, 2000), thereby helping birds to maintain a healthy gut. However, only few studies have looked at the effects of MOS on the development of normal gut microflora of birds (Kocher *et al.*, 2005).

The present experiment was designed to test the hypothesis that the addition of an optimal dietary level of MOS (2g/kg, identified in the last experiment), in the absence of prophylactic antibiotics, will improve intestinal microflora and gut function, thus allowing for a more consistent production response.

4.2 MATERIALS AND METHODS

4.2.1 Birds and diets

Three hundred and eighty-four (384) day-old male Cobb broiler chickens were included in the feeding experiment. The dietary treatments consisted of a negative control without MOS or a positive control (ZnB, the same dosage as in Chapter 3), and an addition of 2g MOS /kg diet. Each treatment had 4 replicates of 32 chickens per pen measuring 2.0 by 1.5 m in dimension. At the start of the experiment, the stocking density, excluding the area occupied by feeders (1.2 × 0.7 m), was approximately 15 birds/m^2.

The basal diet was the same as shown in Chapter 3 except that a coccidiostat (Monensin, 1g/kg) was included in place of corn. The chickens were individually wing-banded and maintained as described in Section 3.2.2.

The experiment was approved by the Animal Ethics Committee of the University of New England with approval number of AEC05/048.

4.2.2 Sampling procedure

At the end of weeks 2 and 5, 5 birds from each pen were randomly selected and processed according to the procedure outlined in Section 3.2.4, to obtain the pooled digesta, visceral organs (liver, spleen, and bursa) and intestinal tissue samples for histology. In addition, one section, about 2 cm, was taken from the proximal part of jejunum, rinsed with PBS to remove the digesta and then wrapped in aluminium foil before immersing in liquid nitrogen to keep the samples frozen at -80 °C until analysis of brush-border enzymes. The relative weight of visceral organs of a replicate was obtained by averaging the relevant data of four birds in the replicate (pen); the histological and BBMV enzyme data was obtained by averaging the relevant data of two birds in the replicate.

4.2.3 Measurements and analyses

Feed intake, mortality and AME measurement

Feed intake and mortality were measured as described in Section 3.2.3.

At 20 days of age, 3 birds from each pen were moved into individual AME cages, which gave 12 birds per treatment, for the AME evaluation. Birds were given 4 d to adapt to their new environment. During the subsequent four days, the procedure as described in Section 3.2.3 was followed to determine the AME of the grower diet.

Flock uniformity

The birds were individually weighed at 1, 21 and 42 days of age. Bird uniformity was calculated from the coefficient of variation of their individual body weight according to the following equation as described by Jackson *et al.* (2004).

$$\text{Uniformity} = 100 - \left(\frac{\text{standard deviation}}{\text{mean}} \times 100\% \right)$$

Bacterial Analysis

Fresh digesta samples weighing about 1 g, from the duodenum, ileum and caeca were transferred into 15 mL MacCartney bottles containing 10 mL of anaerobic broth (see Appendix 1 for the composition). The suspension was homogenized for 2 min in CO_2-flushed plastic bags using a bag mixer (Interscience, St. Norm, France). One millimeter of the homogenized suspension was then transferred into 9 mL of anaerobic broth and serially diluted from 10^{-1} to 10^{-5} (for duodenal samples) or 10^{-1} to 10^{-7}(for ileal and caecal samples). From the last three diluted samples, 0.1 mL each, were plated on the appropriate medium for enumeration of microbial populations.

Total anaerobic bacteria were determined using anaerobic roll tubes containing Wilkins-Chalgren anaerobe agar (CM0619) incubated at 37 °C for 7 d. Lactic acid bacteria were enumerated on MRS agar (CM0361) incubated in an anaerobic condition at 37 °C for 48 h. Coliforms and lactose-negative enterobacteria were counted on MacConkey agar (CM 0007) incubated aerobically at 37 °C for 24 h as red and colourless colonies, respectively. Numbers of *C. perfringens* were counted on perfringens agar (CM0543 OPSP) incubated anaerobically at 37 °C for 24 h. All the media were supplied by Oxoid (Sydney, Australia).

pH Analysis

Immediately following slaughter, fresh digesta samples weighing about 0.5 g, from the duodenum, ileum and caeca were transferred into 15 mL containers and 4.5 mL distilled water were added and mixed. The pH value of the suspension was then measured using a combined glass/reference electrode (Ecoscan, Eutech, Singapore).

Fat analysis

The contents of fat in the jejunal and ileal digesta were determined according to AOAC (1994) with minor modifications. Frozen digesta samples were freeze-dried and ground to pass through a 0.5 mm screen. Cellulose thimbles with a wad of cotton inserted into them were placed in 105 °C oven for 24 h with the weights recorded at the end of the period. Approximately 2 g of the sample was weighed into each pre-dried thimble and dried in 105 °C oven for 24 h. The dry weight of the sample together with the thimble was recorded. The thimble was then placed in a Soxhlet fat extraction system, which uses chloroform as the extractant, for 24 h and later dried in the oven at 105 °C for another 24 h. The oven-dry weight was recorded and fat content calculated.

Brush-border enzyme analyses

The method described by Shirazi-Beechey *et al.* (1991), with minor modifications by Iji (1998), was adopted for the analysis of brush-border enzymes.

Preparation of brush-border membrane vesicles (BBMV)

About 1-2 g of fresh frozen samples were cut into small pieces and defrosted in 25 mL of buffer (100 mM mannitol, 2 mM *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid] (HEPES)/Tris, pH 7.1). The mixture was vibromixed for 2 x 30 seconds and filtered through a Buchner funnel of 1 mm pore size. The filtrate was blended in a homogenizer (Utra Turrax T25 basic, IKA, Labortedrik, NC, U.S.A) for 30 seconds at high speed mode (setting 2). One mL of the homogenate was used for the assessment of protein. To the rest magnesium chloride, 2.5 M stock, was added until a final concentration of 10 mM was reached. The mixture was vigorously mixed and allowed to stand for 20 minutes before being centrifuged on a JA20 rotor in a J2-21 centrifuge (Beckman Instruments, Palo Alto, CA, U.S.A) at 3000 $\times g$ for 15 minutes. The supernatant was then removed and centrifuged again at 30 000 $\times g$ for 30 minutes. The pellet was re-suspended in a second buffer (100 mM mannitol, 2 mM HEPES/Tris, 0.1 mM $MgSO_4$, pH 7.4) and made to pass through a 25G needle until the mixture became homogenous. The sample was then centrifuged for 45 minutes at 30 000 $\times g$. After discarding the supernatant, the final pellet was re-suspended in 500 µL of a buffer containing 300 mM mannitol, 20 mM HEPES/Tris, and 0.1 mM $MgSO_4$, pH 7.4. Finally, the recovered membranes were made homogenous by sequentially passing through 25 and 27G needles and membrane vesicles were collected in Eppendorf tubes and stored in liquid nitrogen until ready to use.

Specific activities of enzymes

An enrichment factor was derived to ascertain the purity of BBMV because the specific activity of membrane-bound enzymes should be higher in BBMV than in the crude homogenate as a mark of purity. The enrichment factor was determined by analysing and comparing the specific activities of maltase and AP in the mucosal homogenate with activities in the BBMV. The activities of maltase and AP were enriched by 2.5 and 3 times on d 14, respectively, and 9 and 6 times on d 35, respectively, indicating that membrane vesicles had been purified from the homogenate samples in the present experiment.

Protein The recovery rate of the membranes was assessed by estimating the protein concentrations in both the homogenates as well as the vesicles according to the modified

techniques of Bradford (1976). The assay utilized the red form of Coomassie Brilliant Blue G-250 (CBB), which turns blue on binding to protein. The reaction was started by adding 2 mL of Bradford reagent (a mixture of Coomassie Brilliant Blue, 95% ethanol, 85% phosphoric acid, and distilled water, 2:1:2:15 ratio in mL) to 40 µL of dilute vesicles or homogenates, vibromixing and reading at 595 nm after 5 minutes but within 1 hour. Data generated were analysed with the aid of a computer software, Lowry (Elsevier BIOSOFT, Cambridge, UK).

Maltase and sucrase The disaccharidases, α-glucosidase (maltase, EC. 3.2.1.20) and β-fructofuranosidase (sucrase, EC. 3.2.1.26) were assayed using the method described by Dahlqvist (1964). The incubated mixture was freshly prepared from 100 mM maltose or sucrose, respectively in succinate buffer (4 mM sodium succinate, 90 mM sodium chloride, pH 6.0). Homogenates or vesicles, measuring 25 µL, were incubated in 500 µL of the substrate-buffer for 30 minutes at 39 °C. After 30 min, incubation was terminated by pipetting in 5 folds of 0.2 % Triton X-100 (w/v) in 0.5 M Tris buffer, pH 7.02 at 39 °C to release glucose which was then estimated by GPD-Perid kit supplied by Boehringer-Mannheim Australia (Castle Hill, NSW, Australia). The amount of glucose released was measured spectrophotometrically at 610 nm after 30 minutes of colour development at room temperature.

Alkaline phosphatase Alkaline phosphatase was assayed in line with the modified methods described by Forstner *et al.* (1968) and Holdsworth (1970). The assay system consisted of 50 mM $MgCl_2$, 50 mM Tris (pH 10.1) and the substrate, 10 mM paranitrophenol phosphate (PNP, Sigma 104). The standard used in this experiment was paranitrophenol (Sigma 104-1). The reaction was initiated by incubating 20 µL of dilute vesicles or homogenates with 0.8 mL of Tris buffer, 0.1 mL of $MgCl_2$ and 0.1 mL of PNP at room temperature. After 20 min, the reaction was terminated with 0.1 mL 40 % trichloroacetic acid and further colour development was accomplished by adding 2.0 mL of 0.4 M NaOH to 0.1 mL of the primary reaction mixture, which was then vibromixed and read at 410 nm.

For all protein and enzymatic assays, the samples and the standards were read on a 150-20 spectrophotometer (Hitachi, Japan) and in duplicate.

Gross energy, nutrient digestibility, histology, and VFA analyses

Analytical methods for these variables are described in Section 3.2.5.

4.2.4 Statistical analyses

All the data were statistically analyzed as outlined in Section 3.2.6.

4.3 RESULTS

4.3.1 Growth performance

The effects of treatments on the growth performance, mortality, culled birds and AME are summarized in Table 4.1. In general, regardless of diets, all the birds were in a very healthy condition and had no significant difference in mortality and culling rates.

In the first three weeks of the experiment, no significant differences were observed in FI and FCR among all the treatments although both ZnB and MOS tended (P=0.07) to increase BWG of birds compared to the negative control. Also, no significant effects of the treatments on the growth performance were observed in the last three weeks of the trial. The addition of MOS or ZnB did not affect the AME values of the diet.

Table 4.1 Effects of dietary treatments on feed intake (FI), body weight gain (BWG), feed conversion ratio (FCR), mortality and culling rates, and AME of broiler chickens[1]

	NC	MOS	ZnB	SEM	P values
1-21 day					
FI (g/bird)	1181	1257	1244	45.1	0.25
BWG (g/bird)	855	925	916	28.0	0.07
FCR (g/g)	1.38	1.36	1.36	0.023	0.59
22-42 day					
FI (g/bird)	3643	3799	3643	104.7	0.28
BWG (g/bird)	1991	2069	1993	65.7	0.44
FCR (g/g)	1.83	1.85	1.83	0.031	0.81
0-42 d					
Dead birds	4/128	3/128	3/128	-	-
Culled birds[2]	5/128	1/128	4/128	-	-
AME (MJ/kg DM)[3]	13.54	13.40	13.50	0.099	0.25

[1]Values are means of 4 replicates of 22 birds each for 1-21 d and 19 birds each for 22-42 d.
[2] Culled birds were those that had either leg problems and/or water belly.
[3]Values are means of 12 replicates (cages).

The observed flock uniformity of approximately 90% in all the treatment groups throughout the 6-week experimental period was considered relatively high (Table 4.2). In the first half

of the trial period, MOS slightly improved (P=0.10) the flock uniformity, whereas a decrease (P>0.05) in flock uniformity was noticed in both control groups. No further changes in flock uniformity were observed in the later half of the trial period.

Table 4.2 Flock uniformity (%) of broilers fed the experimental diets at different ages[1]

Age (d)	NC	MOS	ZnB	P values
1	91.7±1.04	90.6±1.65	93.4±1.32	0.06
21	89.2±0.71	91.5±0.65	91.1±1.89	0.10
42	90.7±4.25	91.4±4.45	90.8±2.54	0.96

[1]Values are Means ± SD; 4 replicates of 22 birds each on d 1 and 21, 19 birds each on d 42.

4.3.2 Relative weight of liver, spleen and bursa

A significant reduction (P<0.05) in the relative weight of the liver was noticed in 35-day-old birds on MOS or ZnB treatments compared to the negative control (Table 4.3). The size of the spleen and bursa was not affected by the treatments.

Table 4.3 Effects of dietary treatments on the relative weight (%BW) of liver, spleen, and bursa of broiler chickens[1]

	NC	MOS	ZnB	SEM	P values
Liver					
14 d	3.89	3.91	3.75	0.161	0.44
35 d	2.47[a]	2.31[b]	2.26[b]	0.076	0.004
Spleen					
14 d	0.09	0.09	0.09	0.010	0.40
35 d	0.11	0.10	0.09	0.014	0.49
Bursa					
14 d	0.20	0.20	0.22	0.021	0.37
35 d	0.18	0.18	0.21	0.021	0.19

[1]Values are means of 4 replicates.
[a,b] Means within a row not sharing a common superscript letter are significantly different (P<0.05).

4.3.3 Small intestinal digestibility of fat

On d 14, both MOS and ZnB tended (P=0.09) to increase the digestibility of fat in the jejunum, whereas ZnB also significantly increased (P<0.05) the fat digestibility in the ileum compared to the negative control (Table 4.4). No significant differences in fat digestibility were observed among the treatments in 35-day-old birds.

Table 4.4 Effects of dietary treatments on the intestinal digestibility of fat of broiler chickens[1]

	NC	MOS	ZnB	SEM	P values
Day 14					
Jejunum	65	73	77	5.8	0.09
Ileum	70[b]	75[ab]	82[a]	3.1	0.003
Day 35					
Jejunum	73	75	79	4.9	0.29
Ileum	79	81	84	2.7	0.14

[1]Values are means of 4 replicates.
[a,b] Means within a row not sharing a common superscript letter are significantly different (P<0.05).

4.3.4 Bacterial populations

Diet did not affect the counts of total anaerobic bacteria, lactic acid bacteria, lactose-negative bacteria and *C. perfringens* in the small intestine of birds at 14 and 35 days of age (Table 4.5). However, on d 14, birds in the MOS group had significantly higher (P<0.05) coliform counts in the duodenal and ileal digesta by 1 to 1.5 log CFU compared to both controls.

4.3.5 Intestinal pH and VFA concentrations

Although dietary treatments had no significant effects on the intestinal pH (Figure 4.1), there was a general trend observed in the change in pH from the proximal to the distal region of the GIT. At two weeks of age, digesta pH was 6.41, 6.71, 8.26 and 7.19 in the duodenum, jejunum, ileum and caeca, respectively. Corresponding values at five weeks of age were 6.31, 6.67, 8.17 and 6.69. It was also observed that the pH values in the small intestine were generally lower in older birds (35 days old) than in younger birds (14 days old).

Table 4.5 Effects of dietary treatments on the counts (log CFU/g of digesta) of selected bacteria in the digestive tract of chicken on days 14 and 35[1]

Bacteria and segment	NC	MOS	ZnB	SEM	P values
		Day 14			
Anaerobic bacteria					
Duodenum	7.24	7.10	7.29	0.292	0.68
Ileum	8.12	8.30	8.03	0.189	0.20
Caeca	8.90	9.05	9.04	0.268	0.77
Lactic acid bacteria					
Duodenum	7.30	7.17	7.28	0.396	0.89
Ileum	8.18	8.22	7.84	0.401	0.45
Caeca	8.43	8.75	8.56	0.330	0.51
Coliform bacteria					
Duodenum	5.08[b]	6.34[a]	5.45[b]	0.443	0.02
Ileum	6.92[b]	7.84[a]	7.20[b]	0.280	0.01
Caeca	8.67	8.85	8.78	0.206	0.60
Lactose-negative enterobacteria					
Duodenum	3.10	3.30	3.08	0.311	0.53
Ileum	5.10	5.19	5.08	0.152	0.60
Caeca	5.73	5.42	5.89	0.810	0.74
Clostridium perfringens					
Duodenum	3.10	3.09	3.08	0.101	0.97
Ileum	4.00	4.07	4.04	0.059	0.43
Caeca	5.09	5.09	5.08	0.028	0.76
		Day 35			
Anaerobic bacteria					
Duodenum	7.29	7.49	7.28	0.367	0.76
Ileum	8.49	8.42	8.56	0.169	0.71
Caeca	9.13	9.00	8.88	0.186	0.45
Lactic acid bacteria					
Duodenum	7.79	7.73	7.62	0.326	0.87
Ileum	8.74	8.56	8.48	0.224	0.52
Caeca	9.22	8.96	8.94	0.261	0.52
Coliform bacteria					
Duodenum	5.82	6.03	6.26	0.391	0.55
Ileum	7.76	7.76	8.14	0.347	0.48
Caeca	8.82	8.86	8.87	0.151	0.95
Lactose-negative enterobacteria					
Duodenum	3.98	3.54	4.19	0.690	0.65
Ileum	5.27	5.47	6.03	0.485	0.31
Caeca	7.14	6.67	6.62	0.453	0.48
Clostridium perfringens					
Duodenum	2.86	2.97	2.99	0.071	0.19
Ileum	3.87	3.95	3.94	0.074	0.56
Caeca	4.89	5.02	4.92	0.074	0.24

[1] Values are means of 4 replicates.

[a,b] Means within a row not sharing a common superscript letter are significantly different ($P<0.05$).

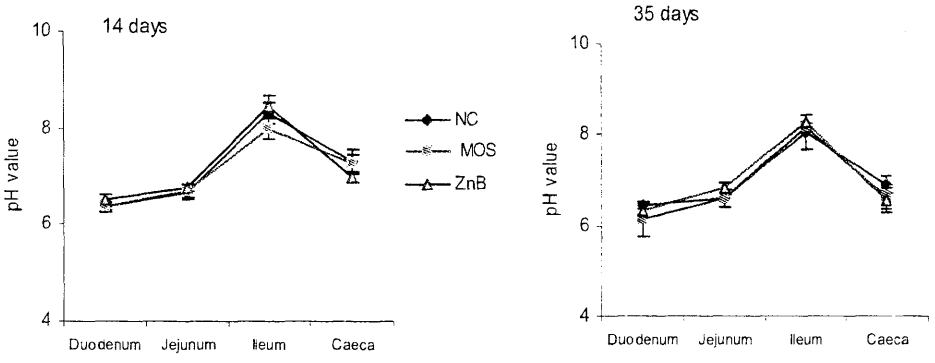

Figure 4.1 pH values of contents of the small intestine of birds fed the experimental diets at different ages (Mean values, n= 4; error bars indicate SD)

Although the concentrations of three major VFAs in the caeca were not affected by the treatments (Table 4.6), birds fed the MOS-supplemented diet had a significant increase ($P<0.05$) in the molar ratio of acetic acid compared to the negative control on d 14. A similar trend was observed in those birds on ZnB treatment at both ages.

4.3.6 Intestinal morphology and brush-border enzyme activities

In general, MOS or ZnB had no significant effects on the gut morphology of birds (Table 4.7). On d 35, ZnB significantly increased ($P<0.05$) the villus height:crypt depth ratio in the jejunum compared to MOS.

Table 4.6 Effects of dietary treatments on caecal VFA concentrations and molar ratios of broiler chickens at different ages[1]

	NC	MOS	ZnB	SEM	P values
Concentrations (μmol/g digesta)					
Day 14					
Acetate	24.9	25.3	27.2	8.92	0.63
Propionate	1.9	1.5	1.2	0.409	0.18
Butyrate	4.5	4.4	4.5	1.097	0.99
Total VFA	42.2	39.1	44.0	4.64	0.48
Day 35					
Acetate	48.5	45.6	58.2	5.478	0.11
Propionate	4.3	3.8	3.6	0.509	0.38
Butyrate	12.6	12.4	12.1	1.860	0.97
Total VFA	84.2	81.5	82.5	5.617	0.89
Molar ratios of VFA (% of total VFA)					
Day 14					
Acetate	57.9[b]	64.6[a]	62.0[a]	1.753	0.01
Propionate	4.4	5.0	2.8	1.140	0.11
Butyrate	10.9	11.2	10.3	2.295	0.92
Day 35					
Acetate	52.8[b]	56.3[b]	70.2[a]	6.262	0.02
Propionate	5.2	4.7	4.4	0.658	0.46
Butyrate	14.9	15.3	14.6	1.975	0.93

[1]Values are means of 4 replicates.
[a,b] Means within a row not sharing a common superscript letter are significantly different ($P<0.05$).

Table 4.7 Effects of dietary treatments on villus height, crypt depth, and villus height:crypt depth ratio (Ratio) of the small intestine in broiler chickens[1]

	NC	MOS	ZnB	SEM	P values
Jejunum					
Day 14					
Villus height (μm)	1185	1230	1224	67.5	0.77
Crypt depth (μm)	102	107	104	4.2	0.51
Ratio	12.7	10.6	11.8	1.33	0.34
Day 35					
Villus height (μm)	1383	1264	1433	73.6	0.11
Crypt depth (μm)	114	112	108	2.6	0.15
Ratio	12.0^{ab}	11.1^{b}	13.2^{a}	0.79	0.03
Ileum					
Day 14					
Villus height (μm)	537	539	548	25.7	0.91
Crypt depth (μm)	94	91	91	3.7	0.71
Ratio	6.0	5.9	6.0	0.30	0.90
Day 35					
Villus height (μm)	753	754	738	55.1	0.95
Crypt depth (μm)	96	89	93	3.6	0.24
Ratio	7.8	8.1	7.9	0.60	0.86

[1]Values are means of 4 replicates.
[a,b] Means within a row not sharing a common superscript letter are significantly different ($P<0.05$).

The protein content and specific activities of BBMV enzymes in the jejunum on d 14 were not affected by diet (Table 4.8). However, on d 35, the specific activity of AP was significantly higher ($P<0.05$) in birds fed the ZnB-supplemented diet than those in the MOS treatment group.

Table 4.8 Effects of dietary treatments on jejunal protein content (mg/g tissue) and specific activities (μmol/mg protein/min) of membrane-bound digestive enzymes[1]

	NC	MOS	ZnB	SEM	P values
Day 14					
Total protein[2]	79.7	86.4	77.1	7.74	0.39
BBMV protein[3]	2.44	2.83	2.99	0.337	0.28
Maltase	4.67	6.79	5.66	0.899	0.11
Sucrase	0.23	0.22	0.26	0.148	0.94
Alkaline phosphatase	5.36	5.25	4.41	0.835	0.33
Day 35					
Total protein[2]	53.1	59.9	67.9	13.58	0.46
BBMV protein[3]	0.55	0.64	0.64	0.098	0.57
Maltase	24.0	22.8	23.0	4.34	0.94
Sucrase	1.32	1.73	1.55	0.643	0.76
Alkaline phosphatase	5.37[b]	5.30[b]	5.97[a]	0.304	0.05

[1]Values are means of 4 replicates.
[2] Protein content in the mucosal homogenate.
[3] Protein content in the brush-border membrane vesicle (BBMV).
[a,b] Means within a row not sharing a common superscript letter are significantly different ($P<0.05$).

4.4 DISCUSSION

4.4.1 Growth performance, AME and flock uniformity

All the birds were in very good health, reaching an average 21-day body weight, which was 4% higher than the value reported in the Cobb broiler nutrition guide (Teeter and Wiernuz, 2003). This superior performance would have made it difficult for a further response to be attained through supplementation. The results of flock uniformity were consistent with those reported by Ao (2004), who also observed an overall improvement in the variable as a result of supplementation with MOS. The addition of MOS had no significant effects on the dietary AME values, which is in agreement with the reports by Hughes (2003) and the results found in Chapter 3. In contrast, Ferket *et al.* (2004) reported significantly higher AME values in 12-week-old turkey poults when MOS were added at 2g/kg to the diet. These inconsistencies may be due to differences in species, diets, age of birds, and rearing conditions.

4.4.2 Microbial composition and activity

The populations of total anaerobic bacteria and lactic acid bacteria in the small intestine were not affected by diet. Similarly, Ceylan *et al.* (2003) found no significant differences in

the populations of the caecal microflora in birds fed a range of feed additives including probiotics, organic acids, MOS and antibiotics. There were no significant differences in the counts of *C. perfringens* between MOS treatment and both controls in this experiment and the values determined were very close to the detection limit of approximately 10^3 bacteria/g digesta due to the inclusion of coccidiostat in the basal diet.

Contrary to the findings of Stanley *et al.* (1996), it was observed in the current study that MOS increased the counts of intestinal coliforms compared to both controls, although this was noticed only in younger birds. On the other hand, Song and Li (2001) and Jamroz *et al.* (2003) reported that MOS significantly decreased the number of *E. coli* in either the small intestine or excreta contents of chickens.

As a receptor analogue, it is suggested that MOS can act as a decoy for those pathogenic or potentially pathogenic bacteria possessing type-1 fimbriae (mannose-sensitive lectins) and thus prevent them from attaching to the gut mucosa to colonize the gut and cause disease. It was reported that approximately 68% of the tested *E. coli* isolated from poultry possess type 1 fimbriae (Finucane *et al.*, 1999). With *E. coli* being the major component of coliform bacteria (Shapiro and Sarles, 1949), MOS was expected to reduce the number of coliforms in the small intestine. However, the opposite trend was observed in the present experiment. An increase in the intestinal coliform population was noticed in some antibiotic studies with a positive growth response taking place simultaneously (Lindgren, 1954).

One possible reason for the increase in the count of coliform bacteria in young birds might be that MOS does not only bind but also displace those type 1 fimbriae bacteria attached to the gut wall. *In vitro* studies examining the attachment of *E. coli* to the epithelial cells strongly suggested that the bacteria could be displaced from the epithelial cells within 30 minutes when exposed to a mannan derivative (Ofek and Beachey, 1978). Similar results were found when using the same commercial MOS product as in the present study (Peuranen *et al.*, 2006).

Recently, Brzoska *et al.* (2005) reported that birds given MOS had more *E. coli* compared to those fed an antibiotic-containing diet and a similar trend was also noticed in birds fed diets containing lactic acid bacteria and organic acids. Hence, another possibility may be that, although MOS increased the number of coliforms in the gut, the profile of coliform bacteria might be adjusted to a population of coliform bacteria potentially beneficial to growth performance. For example, some *E. coli* species in the coliform group can improve growth in

the absence of antibiotics (Jukes, 1955) and *E. coli*-derived phytase was reported to help nutrient utilization of chickens (Ravindran *et al.*, 2006).

Lactate and VFA, two indicators of microbial activity, are the major end products of bacterial fermentation. In the current trial, MOS did not influence the concentrations of individual VFAs but significantly increased the molar ratio of acetate. Ao (2004) reported that MOS increased day 35 caecal propionic acid level when birds were fed a sorghum-based diet. In the previous experiment, MOS increased the individual VFA concentrations and levels in the caeca, suggesting that the gut microflora was altered by the addition of MOS.

4.4.3 Gut morphology and function

As a whole, MOS did not affect gut structure and function in birds. However, it is reasonable to suggest that birds respond differently to in-feed additives in a clean as compared to a dirty experimental condition. The extent of bird response might not be evident when tested under hygienic experimental conditions, as was the case in the present experiment.

Alkaline phosphatase has been used as an indicator of intestinal maturation (Henning, 1987). Iji *et al.* (2001) reported that the specific activity of AP improved as a result of dietary supplementation with MOS, an observation that was not supported by the current experiment. In contrast, a significant increase in the specific activity of AP was noticed in birds on ZnB treatment compared to MOS treatment on d 35. At the same age, birds fed the ZnB-supplemented diet also had higher ($P<0.05$) villus height:crypt depth ratio than those in the MOS group. As the measurements of villus height and crypt depth generally give an indication of the likely maturity and functional capacity of the enterocytes (Hampson, 1986), it can therefore be deduced that the significant differences in the specific activity of AP and gut morphology may suggest that birds in the ZnB group have a relatively higher proportion of mature enterocytes, which would be expected to improve the capacity for digestion and absorption of nutrients from the small intestine, but no significant differences in fat digestibility were observed between ZnB and MOS treatments on d 35.

However, on d 14, a significant increase in the ileal digestibility of fat was noticed in birds fed the ZnB diet. Engberg *et al.* (2000) suggested that fat digestibility might be improved by the addition of ZnB as an inhibition of lactobacilli was noticed in birds fed diets supplemented with ZnB, which means that the degree of bile acid deconjugation is reduced

and in return fat absorption is improved. However, the populations of lactobacilli were not determined in the current trial.

4.5 CONCLUSIONS

Supplementation with MOS altered the gut microflora of birds in the early stage of life. However, there were no other signficant effects of MOS on the growth performance and gut development of birds, due probably to the optimal experimental conditions of the current study.