CHAPTER 1
GENERAL INTRODUCTION

During the past few decades, the poultry industry has made dramatic progress in the areas of nutrition, genetic engineering, management and disease control thereby increasing the efficiency of growth performance and total yield output. According to Halley (2005), improvement in broiler chicken growth rate has reached an annual increase of 0.1 kg in body weight gain, 3% in breast meat yield and approximately an annual reduction of 2 points in feed conversion ratio (FCR). In parallel to this, as revealed by the Food and Agriculture Organisation (FAO) statistics, the global chicken meat production in 2005 reached 60 million tones, accounting for nearly 27% of total meat production (186 million tonnes) of the world (FAO, 2006).

It is without doubt that the intensive rearing system (NASC, 1999) coupled with the aggressive genetic selection for growth traits has led to a compromised immune system (Emmerson, 1997). Therefore, in-feed antibiotics have been routinely included in broiler diets to maintain health and production efficiency in the last few decades (Ewing and Cole, 1994). However, shortly after the initiation of widespread use of antibiotics in animal industries, they were placed under increased scrutiny because of concern over development of bacterial resistance to the usual microbiocidal effects of the antibiotics. Ever since then, there has been an increasing outcry from the consumers and governments to eliminate that practice from poultry and livestock production.

The notion has been made that non-use of in-feed antibiotics would lead to consequences that outweigh those of resistance, which include increased incidence of diseases at endemic levels (Kaldhusdal and Lovland, 2000) as well as huge economic losses due to slower growth and higher disease challenges (Thomke and Elwinger, 1998). Therefore, the broiler industry has been confronted to come up with alternative solutions to maintain production efficiency without the use of in-feed antibiotics. 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). Since antibiotics have a direct effect on gut microflora, a greater understanding on the composition and function of gut microflora as well as its interaction with other intestinal physiology such as mucin dynamics will facilitate the development of these strategies.

Gut microflora has significant effects on host nutrition, health, and growth performance (Barrow, 1992), by interacting with nutrient utilisation and the gut development of the host. The mucous layer, composed of a complex mixture of large amounts of different types of mucin glycoproteins, is another important component of the non-immune gut barrier that functions to protect the intestinal lining from digestive proteases, bacterial translocations and prevent dehydration of the underlying mucous membranes (Toribara *et al.*, 1993). Both gut microflora and intestinal mucins interact in a very complex way and, depending on the composition of gut microflora and mucin subtypes, they can have either positive or negative effects on the health and growth of birds.

Therefore, an increased momentum in experimenting the use of various gut microflora modulators, mucin and immunomodulators that may be used to alleviate the problems associated with withdrawal of in-feed antibiotics from poultry diets is deemed necessary. One such product is mannooligosaccharide (MOS) of yeast cell wall origin. The positive effects of MOS on growth performance of broiler chickens have been confirmed by meta-analysis and holo-analysis over a 10-year period (Hooge, 2004a; Rosen, 2007). To date, the beneficial effects of MOS on intestinal mucin dynamics, gut microflora, nutrient utilisation and growth performance have been found to be greatly associated with gut integrity (Iji *et al.*, 2001b; Uni and Smirnov, 2006; Solis de los Santos *et al.*, 2007) and modulation of enteric immune system and disease resistance (Spring *et al.*, 2000; Kocher, 2004).

Despite of all these findings, the mechanisms by which MOS promotes growth is not fully known and requires a more systematic examination of the possible interactive effects of MOS with dietary nutrients, such as threonine, on other mechanisms associated with intestinal mucin biosynthesis, gut motility and intestinal nutrient utilisation and uptake. Studies providing direct evidence for intestinal mucus discharge by MOS are scarce. Recent advances in molecular techniques for the evaluation of gut microflora (Torok *et al.*, 2005) and in measuring parameters associated with mucin secretion and turnover (Smirnov *et al.*, 2006) may enable a more detailed examination of these changes.

Therefore, this study was designed to test the general hypothesis that MOS interacts with dietary threonine to exert its growth-promoting effects in broiler chickens through the modulation of intestinal microflora, mucin dynamics and nutrient utilisation.

In this study, commercially available mannooligosaccharide, Bio-MOS®, produced by Alltech Biotechnology, Nicholasville, Kentucky, USA will be used as the source of MOS.

The objectives of this research were:

- To determine the complementary effects of MOS with dietary threonine on mucin dynamics in relation to the development of intestinal mucosa and overall growth performance.

- To evaluate the interactive effects of MOS with dietary threonine on mucus production, standardised ileal digestibility of indispensable amino acids, intestinal nutrient utilisation and uptake, and feed passage rate.

- To characterise and evaluate, in comparison to zinc bacitracin (ZnB), the response of intestinal microflora and mucins to MOS.

CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

2.1.1 Morphology of the chicken small intestine

The small intestine of the chicken is anatomically divided into three regions, the duodenum, jejunum and ileum. The duodenum pursues a horseshoe-shaped course encompassing the pancreas and becomes continuous with the jejunum that constitutes the next part of the small intestine. The ileum is in continuation with the jejunum and the junction between these two segments is clearly demarcated by the presence of the Meckel's diverticulum.

The wall of gastrointestinal tract (GIT) of chickens consists of five distinct layers, namely mucosa, muscularis mucosae, submucosa, muscularis externa and serosa (Plate 2.1). The continuous innermost epithelial layer of the chicken small intestine is heavily lined by specialized epithelial cells called enterocytes that form a semipermeable surface for selective passage of fluids, electrolytes, and dissolved nutrients. Along the length of the tract, the epithelium differentiates into a variety of cells with special functions that include the endocrine and goblet cells for secretion of various fluids, electrolytes, digestive enzymes and mucus. The epithelium together with the underlying supportive lamina propria, a structural network that stabilizes the epithelium and contains nerve fibres as well as an abundance of immune cells, constitutes the mucosa of the gut (Koutsos and Arias, 2006).

The surface area of the mucosal lining is greatly expanded through an extensive microscopic folding to form a carpet of somewhat flattened, finger-like projections called villi (Turk, 1982). The luminal surface of each villus is, in turn, increased by many microvilli to facilitate absorption on the surface of the cells. Between the villi are the crypts of Lieberkuhn that secrete electrolytes and fluids. Near the bottom of the crypts, the cells rapidly divide and differentiate to regenerate and replace damaged cells or those lost to normal attrition. Daughter cells from this area are pushed up along the side of the villus (Turk, 1982).

The muscularis mucosae and submucosa regions are heavily populated by lymphoid follicles such as B and T lymphocytes that constitute part of the gut-associated lymphoid tissue (GALT), the largest secondary immunological organ in the body (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).

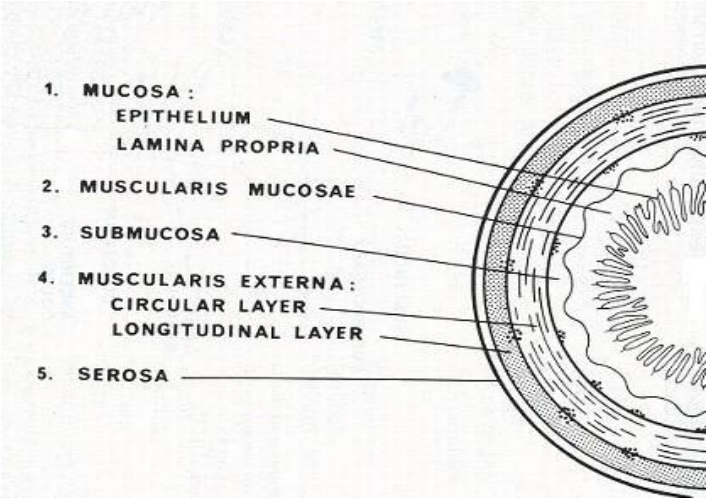


Plate 2.1 The principal morphologic features of the digestive tract in transverse section
Sourced from Jensen (1976)

2.1.2 Structural and functional integrity

The entire life of a bird is constantly being exposed to many variables ranging from dietary (nutrients and non-nutrients) to environmental and disease challenge that, in turn, also have a great impact on growth (Choct *et al.*, 1996; Iji *et al.*, 2001a; Fernandez *et al.*, 2002). Most of these variable factors exhibit their effects through impairment of the gut integrity and consequently alter nutrient uptake and immune function and thus, the growth of chickens. However, the epithelium of the chicken gut is covered by a continuous layer of mucus that functions to protect against the entry of these foreign materials, bacterial translocations and prevent dehydration of the underlying mucous membranes (Toribara *et al.*, 1993).

In addition, the gastrointestinal lumen of an adult broiler chicken harbours nearly 10^{11} to 10^{12} bacteria per gram of intestinal content amongst which there are more than 600 bacterial species (Apajalahti *et al*., 2004; Zoetendal *et al*., 2004). The presence of a fully developed and healthy gut microflora serves to reduce the chance of intestinal colonisation by non-indigenous microflora, especially pathogens (van der Waaij *et al*., 1971; Snel *et al*., 2002). This has been termed colonisation resistance (van der Waaij *et al*., 1971) or competitive exclusion (Lloyd *et al*., 1977) and is the basis for pre- and probiotic strategies. The inability of pathogenic microorganisms to attach and colonise the epithelial surface reduces their ability to cause disease in the host animal (Spring, 1999; Spring *et al.*, 2000).

One of the most convincing demonstrations of the role of the gut microflora in resistance to disease was provided by Collins and Carter (1978) who demonstrated that a germ-free mouse 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. Although colonisation resistance (Corrier *et al*., 1991) was proposed as one of the main mechanisms, other mechanisms include: (1) secretion of antimicrobial compounds such as volatile fatty acids (van der Wielen *et al*., 2002) and bacteriocins (Gusils *et al*., 1999); (2) direct stimulation of immune system (Jeurissen *et al*., 2002); (3) interaction with goblet cells for the production of mucus barrier (Freitas and Cayuela, 2000), and (4) gut peristalsis (Mathew, 2001). The continuous unidirectional flow of materials through the lumen of the upper and middle regions of the small intestine is also a strong influence in preventing microbial communities from developing diseases unless they can attach to the underlying epithelial structures (Savage, 1977).

2.1.3 Intestinal microflora and gut development

The presence of normal gut bacteria has a direct impact on intestinal development and morphology by contributing to a thicker gut wall, heavier intestinal weight, reduced absorptive capacity, and a more rapid mucosal cell replacement rate (Commission on Antimicrobial Feed Additives, 1997). There is a direct evidence to show that the intestinal wall of the germ-free chicks was generally thinner and lighter than that of the conventional controls (Palmer and Rolls, 1983). Microscopic evaluation of germ-free intestine reveals a more regular and slender villus structure, with a thinner lamina propria and fewer mitoses in the crypt (Rolls *et al*., 1978; Ratcliffe, 1985). The cause of these effects is unknown but may result from host responses to bacterial antigens or metabolic by-products, such as ammonia, phenolic and aromatic compounds, and bacterial toxins (Anderson, 2002).

The resident intestinal microorganisms have been demonstrated to increase epithelial cell turnover but it is unknown whether the intestinal microflora has a role in the differentiation of crypt stem cells into specific lineages of epithelial cells (Mahida, 2004). There are available data indicating that intestinal microbes affect goblet cell dynamics as well as the glycocalyx (a network of glycoprotein fibers) layer directly via the local release of bioactive factors or indirectly via activation of host immune cells (reviewed in Deplancke and Gaskins, (2001).

2.2 THE RELATIONSHIPS BETWEEN GUT MICROFLORA AND THE HOST

2.2.1 Establishment of gut microflora

Prior to hatching, the intestinal tract of chicks is usually sterile and the intestinal flora is derived exclusively from the environment with the first sources being the egg shell, incubator, chick box and litter of the poultry house (Elsayed, 2002).

The intestinal tract of the day-old chick contains a large population of faecal *Streptococus, Enterobacteriaceae* and *Clostridia* (Smith, 1965; Barnes *et al.*, 1978) and the small intestine harbours 10^8-10^9 coliforms and faecal streptococci per gram of digesta (Smith, 1965; Barnes *et al.*, 1978). However, this is not a stable population. As the chick grows, the microflora changes and different parts of the intestine become populated by microflora characteristic of a particular region. Twenty-four hours after the chick is fed, a developing population of lactobacilli is found both in the lumen and attached to the epithelium of the crop. By the time the bird is 3 days old, lactobacilli become the predominant flora (Smith, 1965; Fuller, 1973) and the numbers of streptococci and coliform decline to become only a minor component of the flora. Within a week, lactobacilli become the dominant flora in the small intestine, reaching 10^7 per gram, while streptococci and coliforms make up only about 10^4 or less per gram of luminal contents (Barnes *et al.*, 1978).

Salanitro *et al.* (1978) reported that in the caeca, anaerobic counts exceeded aerobic counts by at least a factor of 10^2. The lower part of chicken GIT harbours diverse communities of bacteria. These microflora provide resistance against enteric pathogens, and include bacteroides, eubacteria, Gram-positive cocci plus minor groups such as *Clostridium* and *Lactobacillus* species (Barnes *et al*., 1978).

2.2.2 Intestinal mucosa associated microflora

The microflora of the gut has a proximal to distal gradient in bacterial numbers. In addition, there are also radial distributions of microbes within each segment of the gut (Gaskins, 2001). The four microhabitats for the commensal flora include: (1) the intestinal lumen; (2) the unstirred mucous layer of the mucosa; (3) the deep mucous layer found in the crypts, and (4) the surface of the intestinal epithelial cells (Gaskins, 2001).

What has not received sufficient attention is the large number of unattached microbes existing within the mucous layer which lines the lumen of the intestine, a habitat that has been called the "mucous blanket". King (1905) made the first report on the existence of microflora in the intestinal mucosa of common fowl and found to be made up of about 50 different species of bacteria with its composition remaining relatively constant.

Lactobacilli predominate the commensal flora in the crop (Fuller, 1973). In the duodenum, enterococci were shown to be attached to the gut wall (Fuller *et al*., 1979, 1983). In the jejunum, histological studies have failed to show any bacteria associated with the intestinal wall. However, this may be due to the methods normally used for tissue processing for histological examination that result 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 scanning electron microscopy. However, segmented filamentous bacteria and non-pathogenic gram-positive, anaerobic spore-forming bacteria were usually found at the ileal villous tip of young animals (Heczko *et al.*, 2000). In the caeca, a thick layer of bacteria was seen adjacent to the epithelial surface. This layer was 200 cells deep and contained mainly Gram-positive rods with only occasional Gram-positive cocci and Gram-negative rods present (Fuller and Turvey, 1971).

Colonic mucins do not mix homogenously but rather form a stacked or laminated structure of alternating sialo- and sulphomucins. Bacteria have been consistently observed within the laminated arrays of the outer mucous layer, particularly species that have evolved motile elements which function best in the relatively viscous medium of mucous blanket or other mucus-preferring organisms such as certain fusiform bacteria (Rozee *et al.*, 1982). This indicates the importance of the mucous gel in preventing direct adherence of even commensal gut bacteria to colonic epithelial cells.

Despite all efforts, the knowledge of mucous gel and particularly of the microbial populations that normally or abnormally colonise intestinal mucus is still significantly limited because the fixation of intestinal tissues with conventional, dehydrating aldehyde fixatives results in detachment and loss of the hydrophilic mucous blanket (Deplancke and Gaskins, 2001). Normally, the blanket is also largely removed by fluid shear forces during the preparation of other intestinal microstructures for microscopy. However, with the progress made in the field of molecular technology for microbiological work, a more

complete version of the luminal as well as mucosa-associated gut microbial profiles has been revealed and will be discussed in detail later.

2.2.3 Gut microflora and intestinal adaptations

The presence of bacteria in the GIT affects its motility. In germ-free animals, the rate at which the digesta is moved by peristalsis along the upper GIT is slower than in the conventional counterparts (Falk *et al.*, 1998). When germ-free animals are inoculated with the caecal contents of a conventionally raised animal, the gut motility is restored (Huseby *et al.*, 1994). This may be related to the end-products of microbial fermentation. It is known that besides the contribution of short-chain fatty acids (SCFAs) to energy and colonocyte nutrition, these fermentation by-products also have other important implications on gut health. These include the stimulation of gut motility to protect the ileum against the growth of certain opportunistic pathogens introduced through colonic-ileal reflux (Kamath *et al.*, 1988), reducing diarrhoea by stimulating reabsorption of water and sodium (Roediger and Moore, 1981), and modulating intestinal mucosal growth (Scheppach, 1994).

The fermentation of carbohydrates in the chicken gut produces a large amount of volatile fatty acids (60 to 80 mM) (Yang, 2007), lactic acid, and gases (hydrogen, carbon dioxide and methane) varying in concentration and relative proportion depending on the gastrointestinal section. Lactic acid is the main organic acid in the small intestine while volatile fatty acids (VFAs) including acetic, propionic and butyric acids predominate in the colon and caeca. A typical ratio of 75% acetic: 10% propionate: 15% butyrate has been described for the lower chicken GIT (Yang, 2007).

The effects of SCFAs on gut motility, including systemic humoral and neutral pathways as well as local reflexes and myogenic responses, have been studied extensively (Yajima, 1985; Cherbut *et al.*, 1996; Cherbut *et al.*, 1997). In rats, SCFAs reduce the amplitude and threshold and increase the frequency of peristaltic contractions of the terminal ileum (Dass *et al.*, 2007). This finding is consistent with studies using dogs where SCFAs are found to stimulate the longitudinal, but not circular, ileal and colonic smooth muscle contractions via a direct effect on the smooth muscle (McManus *et al.*, 2002; Fich *et al.*, 2005).

In pigs, the possible effects of reflux of colonic SCFAs on ileal motility were evaluated by Cuche and Malbert (1999) and Cuche *et al*. (2000). It was found that SCFAs exerted similar contractile effects at the ileal level. Furthermore, SCFAs inhibited the gastric motility via

humoral pathway and was dose-dependent with acetic acid being the most potent. In contrast to this, SCFAs were shown not to have any stimulating effects on proximal small bowel motility in humans (Masliah *et al.*, 2005).

Studies on the influence of lactic acid on intestinal motility are limited but the presence of lactobacilli, described as one of the main bacteria species in the chicken GIT particularly in the ileum (Shapiro and Sarles, 1949), is known to be related to the microfloral effect on gut motility (Lesniewska *et al.*, 2006).

The rank-order of SCFAs' activity on gut motility was determined to be highest and equal amongst acetate (C2), proprionate (C3) and butyrate (C4) compared with pentanoate (C5) and formate (C1) (Dass *et al.*, 2007). These ranking effects may be associated with the chain length. Richardson *et al*. (1991) used acetic (C2), butyric (C4), hexenoic (C6) and caprylic (C8) acids to examine food transit time from the stomach to the caecum in rats. They showed that the longer the chain length of SCFAs, the poorer their effects on food transit time.

The production of butyrate, acetate and propionate via bacterial fermentation increases glucose uptake and the expression of glucose transporters in rats and dogs (Reimer *et al.*, 1997; Massimino *et al.*, 1998). Furthermore, the supplementation of total parenteral nutrition formulas with SCFAs increases glucose uptake and the expression of glucose transporters in parenterally fed dogs and rats. Several signals may be involved in the intestinal adaptation, among these, increased messenger RNA (mRNA) levels for proglucagon and several early-response genes, including c-myc and c-fos, are seen in animals receiving the SCFA-supplemented total parenteral nutrition.

Short-chain fatty acids are also shown to increase ileal glucose transporter 2 (GLUT2) mRNA expression and proglucagon mRNA expression compared with the orally fed group. It is known that SCFAs exert their effects by increasing the rate of transcription or by affecting mRNA stability or turnover (Drozdowski *et al.*, 2002). Little is known about the association between SCFAs and glucose uptake in broiler chickens. However, it appears that under normal condition the intestinal uptake capacity changes little after 7 d of age (Noy and Sklan, 1996). Thus, feed intake may be the major factor controlling nutrient uptake in chicks.

2.2.4 The intestinal microflora and metabolic cost

The metabolically active gut microflora together with a high turnover rate of epithelial cells and a continuous renewal of protective mucous coat confer an effective innate defence against luminal threats. These innate defence functions of the gut epithelium, however, are provided at the expense of animal growth efficiency. Gut tissues represent approximately 5% of body weight but account for 15 to 30% of whole body oxygen consumption and protein turnover because of the high rate of epithelial cell turnover and metabolism (Gaskins, 2001). Only 10% of the total protein synthesised by GIT is accumulated as new mass. Most proteins are lost as sloughed epithelial cells or as secreted products such as mucus (Reeds *et al.*, 1993).

In addition to the metabolic costs associated with immunostimulation and the production and secretion of immunoglobulin, even under ideal conditions there are additional metabolic costs associated with the gastrointestinal microflora, such as the direct competition for nutrients ingested by the host (Anderson *et al.*, 2000; Anderson, 2002; Gabriel *et al.*, 2006). The microflora may consume 10 to 20% of the ingested carbohydrates and protein that would otherwise be available to the host (Apajalahti *et al.*, 2004). For example, bacterial use of glucose produces lactic acid, which reduces the energy available to the host epithelium, resulting in a loss of as much as 6% of the net energy in pig diets (Vervaeke *et al.*, 1979; Saunders and Sillery, 1982). Thus, a lower gut mass index (relative intestinal weight) as the result of a slower rate of renewal of epithelial cells may have a beneficial effect on basal energy expenditure and energetic efficiency of nutrient utilisation (Shives *et al.*, 1986).

Dietary energy can also be lost via deconjugation and dehydroxylation of bile salts by microbial bile salt hydrolase (BSH) (Tannock *et al.*, 1989), resulting in impaired lipid absorption by the host animal and production of toxic secondary bile salts (Eyssen, 1973; De Boever *et al.*, 2000). As demonstrated, BSH activity in the ileal contents of conventional mice was decreased 86% by the elimination of lactobacilli from the microflora (Tannock *et al.*, 1989). Indeed, the inhibition of bile acid biotransformation in the gut is proposed as an important mechanism by which antibiotics enhance animal growth (Visek, 1978). In broiler chickens, a particular emphasis is placed on lactobacilli, particularly *L. salivarius* (Engberg *et al.*, 2000), for being largely responsible for dietary energy lost via its bacterial metabolism (De Smet *et al.*, 1995). This becomes important as *L. salivarius* is also found to be abundant in the mucosa of most regions of the upper GIT and represents 63% of the cloned sequences

in the crop, 19% in the duodenum, 22% in the jejunum and 12% in the ileum (Gong *et al.*, 2007).

The BSH is located mainly intracellularly in *C. perfringens* (Gopal-Srivastava and Hylemon, 1988) as reported for the majority of bacteria (Grill *et al.*, 1995), however, extracellular BSH activity has also been demonstrated for *C. perfringens* (Masuda, 1981). Masuda (1981) reported that the extrcellular BSH activity in *C. perfringens* gradually increased during their growth. In addition, there is evidence for multiple BSH activities for *C. perfringens* (Coleman and Hudson, 1995) including 7-dehydroxylation of primary bile salts into secondary bile salts and further conversion of secondary bile salts into 3-keto bile salts (Hirano *et al.*, 1981; Masuda, 1981) which are highly toxic to the hosts (Kandell and Bernstein, 1991; Pazzi *et al.*, 1997; De Boever *et al.*, 2000). Collectively, the effect of these extra BSH activities posed by *C. perfringens* species is likely to be more detrimental to the host than lactobacilli where the majority of the breakdown products of BSH activity are precipitated and excreted in the faeces (Begley *et al.*, 2006).

Lactobacillus species from mammals and chickens show a large variability in BSH activity. For instance, *L. arabinosus* (Midtvedt and Norman, 1967) and *L. salivarius* (Gilliland and Speck, 1977) deconjugate both taurocholic and glycocholic acids, whereas *L. brevis* (Midtvedt and Norman, 1967) deconjugates only glycocholic acid. Gilliland and Speck, (1977) also reported that *L. acidophilus* strongly deconjugated taurocholic acid but had only sparing effect on glycocholic acid. As in the case of *L. casei*, only a moderate deconjugating effect was shown on glycocholic acid and practically no effect on taurocholic acid (Gilliland and Speck, 1977). Strains of *L. delbrueckii* do not deconjugate either of the two bile salts (Midtvedt and Norman, 1967). All in all, there seems to be a strong correlation between growth promotion and bile salt hydrolase activity (Feighner and Dashkevicz, 1987), thus any attempt to selectively differentiate the enrichment of hydrolase-negative strains of gut bacteria may have an overall beneficial growth-promoting effect to the host.

2.3 FUNCTIONS AND SYNTHESIS OF INTESTINAL MUCINS

2.3.1 The chemistry and structure of mucins

The mucous gels of the gastrointestinal tract consist of 95% water by weight, with organic materials such as high molecular weight glycoproteins (mucins), lipids, ions and enzymes of

epithelial secretions, and exfoliated and disrupted cells making up the rest. The viscoelastic, polymer-like properties of mucus come from the glycoproteins (mucins) (Forstner *et al.*, 1995).

Mucins range in molecular weights from 0.5 x 10^6 to over 2.0 x 10^6 Da (Harding *et al.*, 1999). They contain large amounts of carbohydrates that account for nearly 70 to 80% of the mucin glycoprotein weight, compared to proteins and ester sulphates which constitute 12 to 25% and up to about 5% mucin glycoprotein weight, respectively (Harding *et al.*, 1999). According to Allen (1984), undegraded mucins are composed of multiples of monomers linked by disulphide bonds. The 3-D polymeric structure is responsible for the gel formation since glycoprotein subunits reduced by mercaptoethanol or proteolytic enzymes lack the ability to form gels (Plate 2.2).

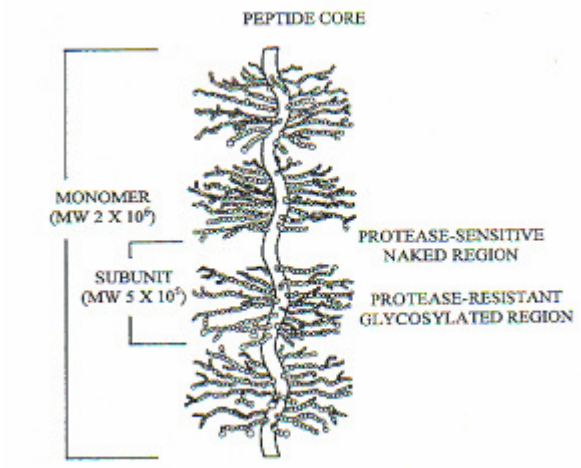


Plate 2.2 The 'bottle-brush' structure of an intestinal mucin protein
Sourced from Lamont (1992)

Two different models have been proposed for the polymeric structure of mucins, which exists in a flexible spheroidal shape in solution. Allen (1981) proposed a four bladed windmill structure in which the protein backbones of all four mucin subunits are linked in a common area known as the non-glycosylated 'naked' region. Harding *et al*. (1983) and Carlstedt and Sheehan (1984) proposed that mucins are linked into linear arrays of long peptides containing several glycosylated and non-glycosylated regions.

Although linear, mucin molecules in solution are loosely and randomly coiled into a spheroidal, highly swollen (by water imbibition) domain (Harding *et al*., 1999). Mucin polymers overlap and are nonconvalently joined to provide the structural basis of the gel.

Table 2.1 The structure-function relationships of intestinal mucins

Structural Feature	Property of Function
Very high molecular weight	Enhances polymerisation
Extended rod-like structure	High viscosity
Glycosylated repeating sequences	Protease resistance
Branched oligosaccharides	Binding of toxins, bacteria
Hydrophobic domains	Lipid binding

Sourced from Lamont (1992)

These nonconvalent interactions are resistant to osmotic pressure and solubilisation but not against gel spreading or mechanical disruption (Bell *et al*., 1985). The structure-function relationships of intestinal mucins are summarised in Table 2.1.

Within the primary structure of mucins, there are two major protein regions. The glycosylated (native) region that accounts for over 95% of the glycoprotein and about 65% of the total protein in mucins, is consisted of the linear polypeptide backbone of protein sequences containing large amount of serine and threonine acting as the sites for the *O*-glycosylation. Another part of this region, comprising large amounts of proline, assists in the coiling of the mucin molecule (Harding *et al.*, 1983). Serine and threonine comprise 57% of rat small intestinal mucins and 41% of human small intestinal mucins while proline may account for up to 15% of the total amino acid residues (Specian and Oliver, 1991).

The *O*-linked carbohydrate chains may contain up to five different saccharides, namely D-galactose, L-fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine and sialic acid. As branched oligosaccharides, they are covalently attached via *O*-glycosidic linkages from *N*-acetylgalactosamine to serine and threonine residues of the protein core. Sialic acid, which is acidic sugar (in gastrointestinal mucins usually either *N*-acetyl or *N*-glycollyl-neuraminic acid), is usually in a terminal position on the carbohydrate chain, whereas ester sulphate residues occur in a more internal position (Harding *et al.*, 1983). It is this special arrangement that accounts for the polyanionic nature of mucins at neutral pH and contributes to giving the molecule a net negative charge which is thought to be of importance in interactions with polycationic materials (Forstner *et al.*, 1995).

Other potential residues for bacterial mucoadhesive interaction are the carbonyl (hydrogen bonding) and methyl (hydrophobic bonding) groups on the *N*-acetyl residues (*N*-acetylglucosamine, *N*-acetylgalactosamine and sialic acid) and another methyl group in fucose (Harding *et al.*, 1983).

The non-glycosylated (naked) region represents about 35% of the total protein and about 4-5% of the total glycoprotein. This region has an amino acid composition similar to that of the average globular protein, rich in glutamate and aspartate and unique in that it is rich in cysteine. This is consistent with its role in the formation of the polymeric structure of mucus via the joining of mucin subunits by disulphide bridges (Allen, 1981; Lamont, 1992). Because this region lacks the protection of closely packed oligosaccharide side chains, it is protease sensitive. The globular peptide also has considerable hydrophobic regions, which may be involved in lipid binding and also in protein-protein interactions (Lamont, 1992).

2.3.2 Classification and histochemical staining effects of mucins

Classification of mucins according to the histochemical staining effects was best described by Kierrnan (1990). Small intestinal mucins can be broadly classified into neutral and acidic mucins. Neutral mucins can be further categorised into hexose- or sialo-neutral mucins depending on the presence of the two types of carbohydrate. During Periodic Acid Schiff's (PAS) staining, neutral mucins containing vicinal glycol groups (hexose or sialo) or their amino or alkylamino derivatives are oxidised by periodic acid to form dialdehydes, which combine with Schiff's reagent to form an insoluble magenta compound. The chromphoric groups of basic fuchsin in Schiff's reagent are broken by sulphuration to form a colourless solution. In the presence of free aldehyde groups an insoluble coloured compound similar to the original dye is formed.

In the presence of PAS with mild acid hydrolysis, the α-2-glycosidic linkage by which sialic acids are attached to the subjacent sugars of the oligosaccharide chains of glycoproteins is easily broken by acid-catalysed hydrolysis (sections are treated with aqueous acid (pH 2.5 or lower) for 1 or 2 hour at 70-80°C). In this process, all sialic acid residues are removed, including those that resist digestion by neuraminidase, but most other glycosidic linkages remain intact, thus making it possible to differentiate pure sialo mucins from hexose-sialo mucins.

As opposed to neutral mucins, acidic mucins owe their histochemical property to the presence of carboxyl or sulphate groups that bind to Alcian Blue (AB) basic dye at pH 2.5. Alcian blue is a copper phthalcyanin dye and contains positively charged groups capable of linking with certain polyanions. These polyanions consist of sulphate and carboxyl radicals of the acidic mucins. In contrast, the phosphate radicals of the nucleic acids do not react.

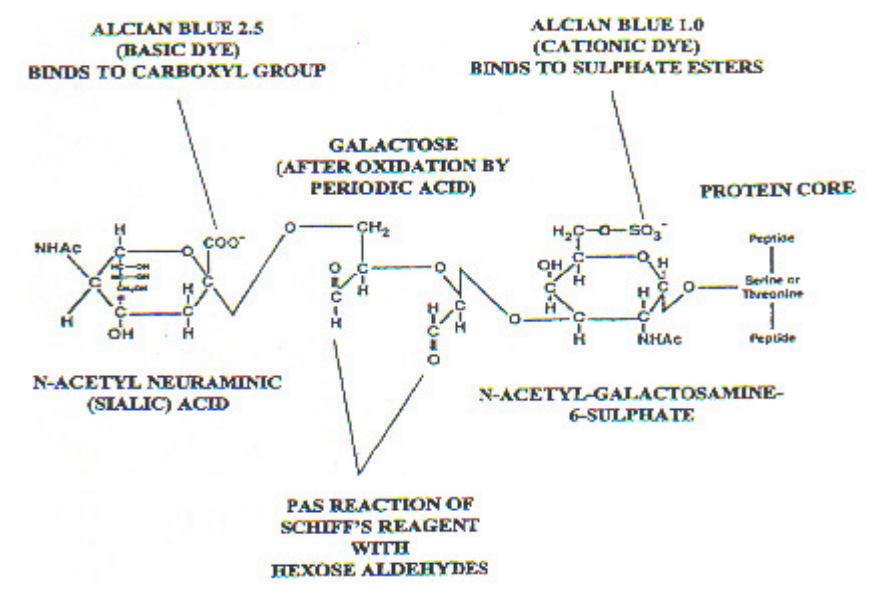


Plate 2.3 Oligosaccharide attachment to mucin protein core and sites of histochemical stain binding

Sourced from Spicer and Schulte (1992)

Consequently, only the acidic mucins are stained. By lowering the pH to 1.0, the sulphate ester groups will be selectively dyed thus, revealing the sulphated-acidic mucins. High Iron Diamine (HID) also selectively stains sulphated-acidic mucins. Importantly, a brown colour stain, which compared to the blue purple stain of Alcian Blue pH 1.0, is a better staining combination that contrasts well with the blue stain of Alcian Blue pH 2.5. A summary of the relationship between classification and histochemical staining effects of mucins is shown in Plate 2.3.

Staining mucins with Alcian Blue pH 2.5 in the presence of methylation and saponification treatment identifies whether those acidic mucins stained at both pH 1.0 and 2.5 owe their acidity only to sulphate groups or to both sulphate and carboxyl groups. Before staining, pre-treatment with hot, acidified methanol removes all the sulphated-ester and most of the sialic acids with esterified carboxyl groups. Nothing will be stained with Alcian Blue. When the methylated sections are subjected to saponification, the methyl groups are hydrolysed with the carboxyl groups restored. These will regain their staining ability by Alcian Blue at pH 2.5. The sulphated-esters, however, are irreversibly removed and will no longer be detectable.

2.3.3 Distribution of goblet cells and mucins

The neonatal chick only has acidic mucins (Uni *et al.*, 2003). From hatch to 7 days of age, the proximal, middle and distal segments of the small intestine contain similar proportions of goblet cells producing both acidic and neutral mucins (Uni *et al.*, 1995, 1999, 2003)). The goblet cell density increases along the duodenal to ileal axis.

In the production cycle of broiler chickens, a similar distribution pattern of neutral and acidic mucins was observed from 1 to 21 days of life (Mendez and Garcia, 2001). Neutral mucins diminish gradually from the third week onwards. Acidic mucins remain active in intestinal villi, but lose their activity in the crypts, except in the ileum.

In the small intestine, sulphomucins are more active in the villi than in the crypts for the first two weeks of life and their activities diminish after week 2. Surprisingly, in the caeca, sulphomucins are equally active in both the villi and crypts throughout the bird's productive lifespan (Mendez and Garcia, 2001).

Suprasert *et al.* (1986) studied the distribution of mucin glycoconjugates in the chicken colonic epithelium and demonstrated that most of the colonic goblet cells contained acidic and neutral glycoconjugates with sulphate and vicinal diol groupings, α-D-mannose and α-D-glucose residues and sialic acid-galactose dimmers. These goblet cells undergo changes in histochemical reactivity during upward migration along the crypts with increase in the amounts of α-D-mannose and α-D-glucose residues and terminal sialic acid-galactose dimers (Suprasert *et al.*, 1986).

The mammalian gastric mucosa expresses mainly neutral mucins (Sheahan and Jervis, 1976). Acidic mucins are expressed throughout the intestinal epithelium, although the large intestine is the dominant site for them (Sheahan and Jervis, 1976).

This distribution pattern of mucins along the intestine may perhaps explain why pathogens preferentially colonise different regions of the intestine. Thus, it is possible to manipulate gut microflora composition via mucin secretion through diet or feeding system (Mendez and Garcia, 2001; Montagne *et al.*, 2004).

The mucous layer varies in thickness depending on the gut section. For instance, in mammals, the gastric mucous layer can reach up to 450 μm (Forstner *et al.*, 1995), whereas the small intestine is covered with a thinner or discontinuous mucous layer (Peyer's patches

are apparently not covered). It gradually increases in thickness in the large intestine with the thickness finally reaching up to 285 μm in the rectum (Forstner *et al.*, 1995).

In broiler chickens, using a scoring system, Mendez and Garcia (2001) demonstrated that mucous secretion decreases along the gastrointestinal tract from the duodenum to the caecum with age. However, in a recent study by Smirnov *et al.* (2004), it was observed that the thickness of the mucous adherent layer ranged from 68 to 76 μg Alcian Blue/cm^2 of the intestinal tissue (the unit of thickness is defined as the amount of Alcian blue dye being absorbed into the mucus per cm^2 of the intestinal tissue). The thickness of the adherent mucous layer did not differ between the small intestinal regions (Smirnov *et al.*, 2004).

2.3.4 Control of mucin secretion

In humans, mucins are secreted from the apical surface of specialized columnar epithelial cells (goblet cells) by two distinct processes, baseline secretion and compound exocytosis. Baseline secretion involves the constitutive release of newly synthesised mucin granule mass (Forstner *et al.*, 1995). On exposure to a mucin secretagogue, goblet cells undergo compound exocytosis, an accelerated secretory event resulting in the acute release of centrally stored mucin granules (Forstner *et al.*, 1995).

A wide array of bioactive factors, including hormones, bacterial products, neuropeptides and inflammatory mediators (cytokines and lipids) (Temann *et al.*, 1997) can induce compound exocytosis (Forstner *et al.*, 1995). The regulated expression of mucin (MUC) genes via transcriptional levels is the main mucins secrectory control pathway that contributes to the dynamic nature of the mucous layer (Temann *et al.*, 1997).

2.3.5 Functions of mucins and intestinal mucus

Mucus constitutes a protective barrier against harmful intraluminal microorganisms, physical and chemical attacks. The mucous layer prevents bacterial translocation by competitive binding through carbohydrate moieties with mucus-colonising pathogens (such as *Campylobacter jejuni* and *Salmonella enteritidis*). The release of mucus into the gastrointestinal tract draws the luminal contents away from the epithelium, thus preventing the pathogens from invading the epithelial cells (Gill, 2003).

Mucus also supports a group of mucus- and crypt-dwelling microorganisms that competitively exclude mucus colonising pathogens, such as *C. jejuni* and *S. enteritidis,* from

colonising the chicken gut (Hariharan *et al.*, 2004). These include mucus-adapted, curved bacteria resembling campylobacters called K-bacteria (Aho *et al.*, 1992) and members of *Enterobacteriaceae* that are capable of using mucins as sole substrate for growth and producing anti-*Campylobacter jejuni* metabolites (Schoeni and Doyle, 1992).

Furthermore, intestinal mucus as a lubricant aids the passage of digesta along the digestive tract, thus influencing gastrointestinal peristalsis and the frequency of defaecation. Intestinal motility plays an important role in limiting the growth of microorganisms in the gut. Most of environmental pathogens entering the gastrointestinal tract are destroyed at the port of entry by gastric acidity. The presence of a functional intestinal mucous layer coupled with sound intestinal movements would remove those organisms that escape the gastric acid barrier, and thence prevent stagnation and bacterial overgrowth. Disturbances in gut motility are known to reduce the anaerobic (normal) gut microflora (Gorbach *et al.*, 1970). In addition, decreased motility is associated with bacterial overgrowth in the small intestine (Ventrappen *et al.*, 1981).

Another mechanism by which the mucous layer excludes antigens or microbes from the gut is through the formation of antigen-antibody complexes in the mucous coat, coupled with peristalstic movement (Gill, 2003). This function of an intact mucous layer depends on both its quantitative (thickness) and qualitative (sugar composition of carbohydrate chains) aspects. The physiologic relevance of distinct mucin subtypes is not well understood. It is suggested that acidic mucins protect against bacterial translocation because highly sulphated-mucins appear less degradable by bacterial glycosidases and host proteases (Robertson and Wright, 1997; Fontaine *et al.*, 1998). Therefore, the composition of mucins is a key factor in determining the maturity of mucins, resistance of mucosa to bacterial infection and has an influence on the gut bacterial colonisation (Deplancke and Gaskins, 2001).

As mentioned earlier, the quantitative (thickness) aspect of mucus is an important factor in determining its functions. Mucous gels are always in a dynamic balance between mucin synthesis and secretion from goblet cells of the underlying epithelium and erosion on the luminal side releasing mucin in the gut lumen (Piel *et al.*, 2005). This appears to constitute a diffusion barrier between luminal nutrients and digestive and transport sites on the brush border membrane of the enterocytes. However, it has now been well researched that mucous gels provide a microclimate, which differs from the luminal bulk phase in their

physiochemical properties. This microclimate is pH constant and acts as a medium in which digestion and absorption processes occur close to the brush border membrane and helps to transport nutrients and even protonated SCFAs between the luminal contents and epithelial lining (Forstner *et al.*, 1995). The unstirred mucin-water layer is also envisaged to protect digestive enzymes immobilised on the enterocyte surface from degradation by pancreatic proteases in the lumen (Alphers and Tedesco, 1975; Moran, 1985). In addition, mucin glycoproteins can act as a selective barrier to the absorption of certain nutrients, like water and electrolytes (Forstner, 1978; Proust *et al.*, 1984). A diagrammatic illustration of the influence of intestinal mucus on starch digestion is shown in Plate 2.4.

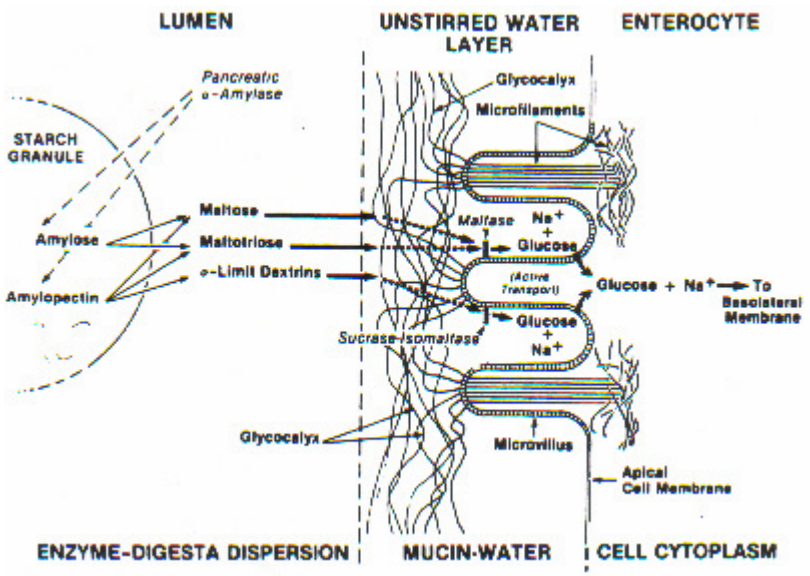


Plate 2.4 Diagrammatic illustration of the influential roles of intestinal mucus on the digestion of starch in fowl

Sourced from Moran (1985)

2.3.6 Intestinal mucin genes and their expression

Nine epithelial MUC genes have been identified in humans. Based on the deduced amino acid sequences, these nine MUC genes can be broadly categorised into 3 families: gel-forming (MUC2, MUC5AC, MUC5B and MUC6), soluble (MUC7) and membrane bound (MUC1, MUC3, MUC4 and MUC12) (Moniax *et al.*, 2001).

Among these, MUC2 and MUC3 are the predominant ileocolonic mucins. The MUC2 mucin polypeptide contains a repetitive peptide of 23 amino acids and is rich in threonine and proline residues. It is heavily glycosylated, and is flanked by cysteine-rich domains (Gum, 1995). The MUC2 gene is expressed in goblet cells of the small and large intestines (Chang

et al., 1994). In contrast, MUC3 has a 17-amino acid tandem repeat rich in threonine and serine residues with a cysteine-rich carboxyl terminal domain that shows homology to epidermal growth factor. MUC3 is not highly expressed in the colon (Ho *et al.*, 1993) but shows expression in both goblet cells and enterocytes of the small intestine (Chang *et al.*, 1994).

In chickens, mucin gene work is scanty. A fragment of a chicken intestinal mucin gene (67% homologous to human MUC5AC, 54% to rat MUC2 and 49% to human MUC2 genes) was first sequenced by Smirnov *et al.* (2004). The slight differences between chicken intestinal mucin and mammalian mucin genes might be due to evolutionary divergence as suggested by Smirnov *et al.*, (2004). However, despite the differences in homology, the chicken mucin protein included in the location of the predicted domain was found to contain a sequence corresponding to the sequence of the von Willebrand factor type D domain (VWD) (Smirnov *et al.*, 2004). This domain is required for the multimerisation process (Titani and Walsh, 1988), thus the chicken mucin protein apparently maintains the polymerising ability (Smirnov *et al.*, 2004).

It was later further identified by Lang *et al.* (2006) the existence of three transmembrane (MUC4, MUC13 and MUC16) and four gel-forming mucins (MUC6, MUC2, MUC5AC and MUC5B) in chickens. Lang *et al.* (2006) also confirmed that in chickens, the gel-forming mucins are encoded within a locus similar to the corresponding human mucins. However, the chicken has an additional gene inserted between MUC2 and MUC5AC that encodes the ∝-subunit of ovomucin, a protein similar to MUC2, but it is lacking a proline, threonine and serine (PTS) domain.

The ability to form gels is dependent on the capacity of monomers to form polymeric structures. Gel-forming mucins have three VWD domains in their N-terminal ends that are involved in polymerisation through intermolecular disulphide-bonds (Lang *et al.*, 2006). They also have a cysteine knot (CK) domain at their C-terminal ends (Perejz-Villar and Hill, 1999). The gel-forming mucins and the VWD factor dimerise with help of their C-terminal VWD domains in the endoplamic reticulum and oligomerise through their N-terminal VWD domains in the acidic compartments of the Golgi complex (Perejz-Villar and Hill, 1999).

2.3.7 Tools for analysis of intestinal mucin gene expression

Relative reverse transcription-polymerase chain reaction (RT-PCR) is a popular method for analysing gene expression. The assumption behind relative RT-PCR is simple: start with equal amounts of RNA from multiple samples, use identical RT-PCR conditions on each, and amplify the same target from each sample. When the products are analysed, the amount of products from each reaction should be proportional to the abundance of the RNA transcript in the samples. The data obtained by relative RT-PCR are commonly expressed as "percent (or x-fold) difference" or in arbitrary units by normalising the density of the PCR products to the density of the internal control (housekeeping) gene. This invariant endogenous control gene is used to correct for sample to sample variations in RT-PCR efficiency and errors in sample quantitation.

This method of analysis is best described as semi-quantitative and is highly dependent on the amplification efficiency and determination of amplification stage. In many cases the amount of products is not related to the amount of input DNA, making this type of PCR a qualitative tool for detecting the presence and absence of a particular DNA.

Unlike other end-point methods, real-time RT-PCR, as the name suggests, detects the reverse transcription PCR products on a real-time basis and thus, has been proven to be a more preferred quantitative method for validating results obtained from array analysis and other techniques that valuate gene expression changes and other global scale.

In real-time RT-PCR, the cycle that corresponds to logarithmic increase in fluorescence (and therefore cDNA) is measured. This is known as the threshold cycle (C_T) at which the fluorescence crosses the threshold. The array results are usually validated using $\Delta\Delta C_T$ or Pfaffl method that requires endogenous control, which is a gene target used to normalise quantitative PCR results. The endogenous control should consistently express (little C_T variability across the samples) in all samples and the expression level should not be altered by any experimental condition, such as a drug treatment. In order to use Pfaffl method, a validation experiment is necessary to ensure that the amplification efficiencies of endogenous control and the target sequences are the same (Pfaffl, 2001). This invariant endogenous control gene is also used to correct for sample to sample variations in RT-PCR efficiency and errors in sample quantisation or pipette calibration errors.

2.4 MICROFLORA AND INTESTINAL MUCUS

2.4.1 Gut microflora and mucin dyanamics

There is now evidence to prove that in monogastric animals, mucin production is also influenced by gut microflora and gut-associated immune status. It is likely that many enteric bacteria produce mucolytic or glycosidic enzymes that alter the chemical nature of the mucins as both commensal bacteria and pathogens can degrade mucins (Deplancke and Gaskins, 2001; Gaskins, 2001). The bacterial strains differ in their competence in degrading mucins. For instance, *Clostridium* strain of RS42 and *Bacteroides* strains of RS2 and RS13 remove more fucose and less sulphated-carbohydrate domains (Stanley *et al.*, 1986).

Some species of gut microflora induce specific patterns of mucin gene expression that alter the local environment (Bry *et al.*, 1996). For example, adherence of lactobacilli to receptors of epithelial cells can induce expression of genes coding for variants of intestinal mucins that inhibit the adherence of enterophathogenic *E. coli* to the epithelium (Mack *et al.*, 1999).

The gut microflora can also influence mucous biology indirectly via the production of SCFAs and release of bacterial metabolites (Sakata *et al.*, 1999). The most convincing evidence for this comes from histochemical studies in which germ-free animals were compared with conventional controls or were inoculated with mixed microbial populations (Kandori *et al.*, 1996). Goblet cells of the germ-free rodents are fewer in number and smaller in size than those of conventional raised counterparts. As a result, the mucous layer may be up to twice as thick in conventionally raised than in germ-free rodents, indicating greater mucus production (Kandori *et al.*, 1996)

Supplementation of probiotics or antibiotics has been shown to influence the processes of mucin biosynthesis and/or degradation and it is believed that the alteration in mucin is brought about by the changes in the intestinal bacterial populations (Smirnov *et al.*, 2005).

2.4.2 The interaction between mucins and bacterial infections

During the initial phase of infection and colonisation by bacteria, mucins first lose their pool due to mucin-degrading activity of some bacterial strains that produce extracellular glycosidases (Hoskins and Boulding, 1981; Enss *et al.*, 1992). Further development is followed by a stimulation of mucin secretion. During this phase, the initially increased secretion of neutral mucins is attributed to a pronounced release of immature mucin

glycoproteins, while the shift to more acidic mucins is considered to result from stimulated secretion as well as from a selective bacterial degradation of neutral mucin components (Enss *et al.*, 1992).

Studies by Ensgraber *et al.* (1992) and Fernandez *et al.* (2000b) indicated that the carbohydrate moieties of intestinal mucins, especially those associated with *N*-acetyglucosamine residues (acidic mucins), prevent the attachment by *C. jejuni*, *S. typhimurium* and *Yersinia enterocolitica* (Ensgraber *et al.*, 1992; Fernandez *et al.*, 2000b). This is further confirmed by Deplancke and Gaskins (2001) who demonstrated that the major role of acidic mucins in mucosa defence mechanism is, taken as a whole, to help the intestinal mucosa to counteract microorganisms and resist bacterial enzymes.

The actual underlying mechanism involved in the induction and modulation of these mucin dynamics is still unknown. However, the limited data available do provide evidence that a direct crosstalk between intestinal microbes and goblet cells is indeed likely (Freitas *et al.*, 2003). For instance, *Lactobacillus* strains prevent enteropathogenic *E. coli* attachment to HT29 epithelial cells through a post-transcriptional upregulation of MUC2 and MUC3 gene expression (Mark *et al.*, 1999). This could be directly linked to the upregulation of MUC2 gene induced by MOS and probiotics as demonstrated by Uni and Smirnov (2006) and Smirnov *et al*. (2005). Zhou *et al*. (2001) also reported that *Lactobacillus* strains generally lack the ability to degrade mucins as opposed to *Clostridium*, *E. coli* and *Bacteroides* strains that are capable of significant hydrolysis of mucin carbohydrate through the release of mucolytic enzymes that act specifically on sialomucins (Stanley *et al.*, 1986; Dai *et al.*, 2000; Deplancke *et al.*, 2002; Freitas *et al.*, 2005).

2.4.3 Application of new molecular techniques in the study of intestinal microflora

The current knowledge of microbial diversity and ecology is based largely on the anaerobic culture techniques that, as stated by Gaskins (2001), are limited by three major factors: (a) culturing can only be performed on those organisms for which nutritional and growth requirements are known; (b) there is a lack of a phylogenetically based classification scheme, and (c) there is an unavoidable bias introduced by culture-based enumeration and characterisation techniques because of different survival rates *in vitro*. As such, it is estimated that only 10 to 60% of the bacteria in the ceaca of chickens could be cultivated (Barnes *et al.*, 1972; 1978; Zhu and Joerger, 2003). A more comprehensive overview of the

intestinal microflora, may it be in the lumen or associated with the brush border membrane, is of practical importance as it will aid in further understanding of the interactions between gut bacteria and goblet cells and development of effective competitive exclusion products such as pro- and prebiotics.

Modern molecular techniques, such as the comparative sequence analysis of small subunit ribosomal RNA (16S rRNA) molecules, can be used to provide molecular characterisation and provide a classification system that predicts natural evolutionary relationships (Simpson *et al.*, 1999; Tannock, 1999; Pluske *et al.*, 2002). To date, the 16S rRNA gene with nearly 1540 base pairs (bp), is one of the commonest phylogenetic marker used in bacterial molecular analysis. Among these 1540 bp, there are several portions that are highly conserved that can be used as the primer target to expand the rest of the bacterial 16S rRNA sequence. With the help of a PCR-based approach, nearly 16277 small-sub-unit RNA sequences have been listed in the Ribosomal Database Project, RDP. The latest version of RDP is RDP 10.2 (Accessed in 2008).

The PCR approach has also helped to develop a series of modern bacterial analytical techniques that works on the 16S rRNA gene sequence. These include the common approaches of denaturing gradient gel electrophoresis (DGGE) (Deplancke *et al.*, 2002), fluorescent *in situ* hybridisation (FISH) (Zhu and Joerger, 2003), temporal thermal gradient gel electrophoresis (TGGE/TTGE) (Nubel *et al.*, 1996), Single Strand Conformation Polymorphism (SSCP) (Zoetendal *et al.*, 2004), and terminal-restriction fragment length polymorphism (T-RFLP) (Gong *et al.*, 2002a; Lan *et al.*, 2004).

These methods have various advantages and disadvantages and many complement each other. The choice of a particular molecular-based approach depends on the focus of study. Clone libraries can be sequenced to identify the composition of the microflora, often to the species level. Microbial community structure can be analysed via fingerprinting techniques, while dot blot hybridisation or FISH can measure abundance of particular taxonomy.

The major bias involved with these molecular techniques comes from variance in cellular rDNA copy number (Wang *et al.*, 1997), DNA extraction methods (von Wintzingerode *et al.*, 1997), primer selection (Suzuki and Giovannoni, 1996), PCR amplification (Speksnijder *et al.*, 2001), cloning strategy and efficiency (Chandler *et al.*, 1997) and mixed environmental samples or mixed microflora (Farrelly *et al.*, 1995; Zhu and Joerger, 2003). A

summary of the advantages and disadvantages of such molecular approaches is shown in Table 2.2.

Table 2.2 A summary of current techniques used to study complex microbial ecosystems

Methods	Uses	Limitations
Cultivation	Isolation	Not representative; slow & laborious
16S rRNA gene analysis	Phylogenetic Identification	Labourious; subject to PCR sequence biases
DGGE/TGGE/TTGE	Monitoring of community/ population shifts; rapid comparative analysis	Subject to PCR biases; semi-quantitative; identification requires clone library
T-RFLP	Monitoring of community/ population shifts; rapid comparative analysis; very sensitive; potential for high throughput	Subject to PCR biases; semi-quantitative; identification requires clone library
SSCP	Monitoring of community/ population shifts; rapid comparative analysis	Subject to PCR biases; semi-quantitative; identification requires clone library
FISH	Detection; enumeration; comparative analysis possible with automation	Requires sequence information; labourious at species level
Dot-blot Hybridisation	Detection; estimates relative abundance	Requires sequence information; labourious at species level
Quantitative PCR	Detection; estimates relative abundance	Labourious
Diversity microarrays	Detection; estimates relative abundance	In early stages of development; expensive
Non-16S rRNA profiling	Monitoring of community shifts; rapid comparative analysis	Identification requires additional 16S rRNA-based approaches

Sourced from Zoetendal *et al.* (2004)

However, T-RFLP, and particularly T-RFLP using a capillary electrophoresis sequencer, seems to have the advantages of higher throughput of processing 96 samples per run time and reproducibility in monitoring bacterial community. This technique also amplifies the 16S subunit of bacterial ribosomal DNA present in samples and all bacterial sequences

amplified are labeled with a fluorescent dye. The commonly used fluorescent dyes are TET, HFX and 6-FAM (Liu *et al.*, 1997; Moeseneder *et al.*, 1999). The amplified and labeled bacterial sequences are then cut with specific restriction enzymes. The resulting fragments are separated according to size and detected by fluorescence emission from the incorporated dye by a DNA sequencing machine (GeneScan). The results are converted to graphical profiles where peaks represent taxonomically related groups and/or species of bacteria depending on the peak position. The peak height or area represents the proportion of different bacterial groups found in the population and allows an easy comparison between samples to identify changes in bacterial community composition. A simple illustration of how T-RFLP works is shown in Figure 2.1.

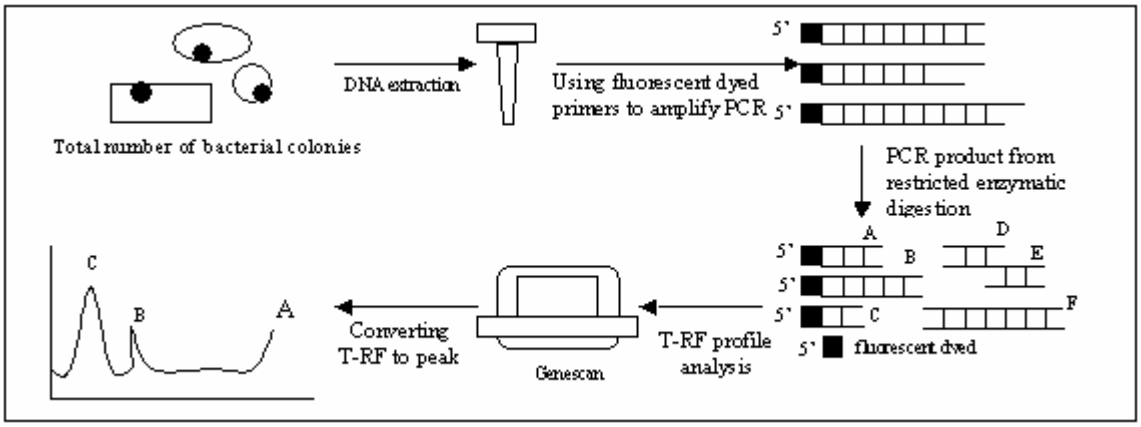


Figure 2.1: Flow diagram of T-RFLP

Sourced from Jia *et al*. (2004)

Like any other PCR tool, T-RFLP is also influenced by both the template (DNA extraction) used and the choice of PCR conditions. Therefore, choice of the DNA extraction method as well as the PCR primers and standardisation conditions can greatly influence the complexity of the resulting profile (Torok *et al.*, 2005).

A study of microbial diversity in the mucosal layer of the pig colon with molecular analysis compared with culture-based methods highlights the problems mentioned earlier. Pryde *et al.* (1999) demonstrated that *Streptococcus* species and *Lactobacillus* species comprised the majority of isolates recovered (54%) from the colon wall when conventional method was applied. However, these groups accounted for only 33% of the sequence variation for the same sample from random cloning. Similarly, Jensen (2001) also demonstrated the shortcomings of traditional culturing techniques, since numerous bacterial species present in the intestine belong to unknown species. As suggested by Jensen (2001), despite the majority

of the larger bacterial population being represented using culturing techniques, it is likely that small populations performing key ecological functions may escape detection.

In broiler chickens, Dumonceaux *et al.* (2006a), using real-time PCR technology, demonstrated an improved profiling of the composition of the chicken intestinal microflora compared with conventional culture technique. At 47 days of age, the small intestinal libraries were dominated by sequences from the *Lactobacillales* (90% of sequences) while the caecal libraries was more diverse and included members of the *Clostridiales* (68%), *Lactobacillales* (25%) and *Bacteroidetes* (6%). Zhu and Joerger (2003) using a FISH-PCR technique were able to demonstrate the diversity and dynamics of the caecal microflora with age in broiler chickens. At d 2, approximately 56%, 34% and 3% of the bacteria detectable (97%) with a universal probe reacted with the probes Enter1432 (enterics), Lacto722 (*Lactobacillus/Streptococcus/Enterococcus*) and Bif164 (*Bifidobacteria*), respectively. With the progression of age, at d 42, approximately 3, 6, 8, 9, 22 and 32% of the bacteria detectable with the universal probe reacted with the probes of Enter1432, Bif164, Bacto1080 (*Bacteroides* groups), Lacto722, Erec482 (*Clostridium coccoides-Eubacterium rectale*), and Clept1240 (*Clostridium leptum* subgroup), respectively (Zhu and Joerger, 2003).

Using the T-RFLP technique, Gong *et al.* (2002a) compared microflora in the lumen and in the mucosa of the ileum of broiler chickens with that in the caeca. It was found that bacteria residing in the ileal mucosa were mainly Gram-positive with low G+C (Guanine + Cytosine) content and differed from those present in the lumen. There were 15 operational taxonomic units (OTUs) among 51 cloned sequences with more than 70% of the cloned sequences related to lactobacilli and *Enterococcus cecorum*. The results also revealed differences among bacterial populations present in the mucosa and lumen of the ileum, indicating that bacterial populations in the ileum (mucosa and lumen) are less diverse than in the caeca. Lactobacilli, *E. cecorum* and butyrate-producing bacteria were the three major groups detected in the ileum and caeca (Gong *et al.*, 2002a).

Using a PCR technique, Amit-Romach *et al.* (2004) demonstrated that in young chicks, the major species present in the small intestines and caeca were lactobacilli (25%) with *Bifidobacterium* population becoming more dominant in the caeca at later age (a total of 40%). Clostridia were detected in some segments of the small intestines in young chicks. In older chickens, *Salmonella, Campylobacter and E. coli* strains (33%) were found in the caeca (Amit-Romach *et al.*, 2004).

2.5 THE INFLUENCE OF THREONINE ON MUCIN DYNAMICS

2.5.1 Dietary factors affecting mucin dynamics

Studies providing direct evidence for the interaction between intestinal mucus discharge and nutrients are scarce. Change in diet, use of different ingredients and inclusion of fibre evoke intestinal mucus secretion. The study by Sharma *et al*. (1997) on the influence of maize- and wheat-based diets with and without enzyme supplementation serves as the pioneer work to evaluate effects of diet on composition and carbohydrate sequences of mucin-like glycoproteins in the chicken intestinal tract. Supplementation of an enzyme in a wheat-based diet elevated crypt-surface glycosylation of the sialic acid residues and also increased the amount of neutral and sulphated-mucins in the goblet cells of the small and large intestines (Sharma *et al*., 1997; Fernandez *et al*., 2000b). These results provide evidence that wheat diets supplemented with xylanase lead to greater changes in the mucin composition and carbohydrate expression of goblet cell glycoconjugates, which in turn, coincided with reduced intestinal viscosity and decreased numbers of *C. jejuni* (Fernandez *et al.*, 2000b).

Non-specific nutrients, such as dietary fibre, may also have an effect on mucins. In rats, adding 5% citrus fibre to a fibre-free diet increased the concentrations of luminal mucin in the stomach and small intestine by 3.5 and 2 fold, respectively (Satchithanandam *et al.*, 1996). In broiler chickens, dietary methylated citrus pectin was also observed to increase the number of goblet cells per villus (Langhout *et al.*, 1999) though there was no change in the percentages of sialo- versus sulphomucin positive goblet cells.

Piel *et al*. (2005) showed that carboxymethylcellulose, a highly viscous polysaccharide, affects mucin biology in weaned piglets where the number of goblet cells per villus increased by 30%. This increase was essentially accounted for by increased numbers of acidic and acidic-sulphated mucin-containing cells with trends toward decreased numbers of neutral and acidic mucin-containing cells in ileal crypts. It is possible that carboxymethylcellulose modulates goblet cell dynamics through direct physicochemical interactions with mucins because there are rheological similarities between carboxymethylcellulose and mucins (Rossi *et al.*, 1996). On the other hand, an indirect effect through changes in the intestinal microflora cannot be excluded.

Perhaps one of the single most important dietary factors that affects the intestinal mucins is threonine (Thr). For instance, 60% of the dietary threonine is utilised by the pig gut and serine and threonine alone account for 40 to 50% of the protein core of mucin glycoproteins (Stoll *et al.*, 1998). Thus, threonine is believed to play an important role in the proper development of the chicken gut.

2.5.2 Chemistry of threonine

Poultry are not capable of synthesising threonine *de novo* which makes it a nutritionally essential amino acid (Kidd and Kerr, 1996). Threonine was one of the last essential amino acids to be discovered and characterised. It is the second most limiting amino acid in wheat-based diets and third in corn-soy diets (Kidd and Kerr, 1997; Kidd *et al.*, 1997). Threonine has a molecular weight of 119.12 and contains two chiral centres. Thus, there are two possible diastereomers of L-threonine. However, the name L-threonine is used for one single enantiomer, (2*S*,3*R*)-2-amino-3-hydroxybutanoic acid. The second diastereomer (2*S*,3*S*), which is rarely present in nature, is called L-*allo*-threonine. Because threonine, unlike most amino acids is not transaminated, animals do not utilise its D-isomer (Kidd and Kerr, 1996), therefore D-threonine is not discussed here. The threonine side chain can undergo *O*-linked glycosylation, making it an important property in the function of mucin glycoprotiens.

2.5.3 Intestinal uptake and metabolism of threonine

The small intestine of the chicken also plays a critical role in amino acid metabolism. In fact, gastrointestinal tissues have one of the highest fractional protein synthesis rates of any tissue in the body (Waterlow, 1967; van der Meulen and Jansman, 1997). Gastrointestinal mucosal protein synthesis is one of the primary reasons for the gut's high fractional synthesis rate. Stoll *et al.* (1998) suggest that in piglets, 60% of the dietary threonine is utilised by the gut, compared to lysine (35%), leucine (32%) and phenylalanine (35%). In the chicken, about 75.5% of the total threonine intake is recovered as threonine in the whole body protein accreted (predicted to be equal to residual threonine) and is constant at all levels of threonine intake between 5 to 95% of the requirement for maximal growth. Out of the 24.5% not recovered, 5.5% can be assigned to the maintenance need (obligatory endogenous gut loss) and the remaining 19% can be assigned to oxidation, primarily via the threonine dehydrogenase pathway (Edwards *et al.*, 1997; Baker, 2004). Thus, threonine utilisation shows no evidence of diminishing returns as the level in the diet is increased. The

contribution of threonine to protein accretion is also fairly constant at 44.1 mg of threonine for every 1 g increase in protein accretion (Edwards *et al.*, 1997).

The cells of the mucosa are capable of utilising both dietary and arterial amino acids for protein synthesis. In the case of threonine, the major metabolic fate of dietary threonine is likely to be incorporation into mucosal and secretory proteins and less likely to be oxidation (Schaart *et al.*, 2005). This incorporation can be into mucins and goblet cells that are not yet secreted or into constitutive proteins in the intestine (Schaart *et al.*, 2005). It has been shown that the gut preferentially uses dietary threonine for mucosal protein synthesis as threonine oxidation represents only 2 to 9% of the total threonine utilised (Stoll *et al.*, 1998; van der Schoor *et al.*, 2002; Schaart *et al.*, 2005).

However, on protein-deficient diets, threonine oxidation, which is the main source of nutritional inefficiency, becomes more significant and accounts for nearly 50% of the metabolic fate of dietary threonine (Schaart *et al.*, 2005). These results suggest that the gut might be an important site, not only for the utilisation of dietary threonine, but also in dietary threonine catabolism that results largely in glucogenic products of pyruvate and propionate (Kidd and Kerr, 1996).

There are two main pathways for L-threonine metabolism. Threonine dehydrase in mitochondria and threonine dehydrogenase in cytosol are the two major enzymes, which play a dominant role in the catabolism of threonine during different metabolic states (Bird and Nunn, 1983). Threonine dehydrogenase accounts for most of the threonine oxidation in mammals in a fed state and threonine dehydrase only becomes important during fasting (Kidd and Kerr, 1996). Carbon skeletons from the catabolism of L-threonine generate pyruvate for energy or glucose production and glycine for metabolic needs (Baker *et al.*, 1968).

2.5.4 Dietary threonine requirement in broiler chickens

As threonine is an essential amino acid required for many key functions within the body, it should be given due consideration in most practical diets. These functions include its essential role in synthesising body protein and muscles, optimising gut health and integrity through its incorporation into mucous glycoproteins and digestive enzymes (Souffrant, 1991). It enhances the immune system through its incorporation into certain immunoglobulins, especially Y-globulins synthesis (Bhargava *et al.*, 1971; Jamroz, 2005).

Therefore, threonine requirements can be compartmentalised into those required for maintenance and the rest for protein accretion. In chickens, the portion of threonine required for maintenance is dependent on age and is estimated to be at 5.5 and 7.4% of the total digestible threonine need during 1 to 3 and 3 to 6 weeks of age, respectively (Edwards *et al.*, 1997). Threonine requirement for maintenance comprises those destroyed in the body, excreted in the urine, synthesised for essential body metabolites and lost from the skin and gastrointestinal tract (Moughan, 1989; Fuller, 1991; Chung and Baker, 1992). In growing birds the need for dietary threonine for protein accretion comprises the largest portion of the threonine requirement. However, the contribution of the maintenance component to threonine requirement becomes increasingly important when the birds mature or increase in age and body weight (Kidd and Kerr, 1996; Samadi and Liebert, 2008).

Threonine requirement of broiler chickens for growth and carcass traits has been studied extensively (Kidd and Kerr, 1997; Rosa *et al.*, 2001; Corzo *et al.*, 2003) and critically reviewed (Kidd and Kerr, 1996; Kidd, 2000; Barkley and Wallis, 2001). However, this requirement still remains to be a point of debate. Aspects of experimental procedures such as mathematical concept of calculating requirement (that is break point versus quadratic analysis), chicken age, genotypes, sex and experimental conditions, all contribute to inconsistencies between studies and inaccuracies in estimation (Kidd and Kerr, 1997). Furthermore, the appropriate way to express threonine whether as a percentage of diet, metabolisable energy, crude protein or total lysine is also subject to controversy. Throughout this thesis, threonine requirement will be compared based on % of total lysine.

The ideal protein concept has been well accepted in diet formulation schemes for chickens (Baker, 1997). Based on this concept, published threonine requirement data for body weight gain of broiler chicks and grower range from 63 (Rosa *et al.*, 2001) to 70% (Atencio *et al.*, 2004), and 63 (Atencio *et al.*, 2004) to 65% (Corzo *et al.*, 2003) of total lysine in the diet, respectively. The National Research Council (1994) recommendation for threonine requirement in broiler diets is 0.8% of the diet for 0 to 3 weeks of age and 0.74% of the diet for 4 to 6 weeks of age. Estimates for threonine requirements by Pack *et al.* (2003) for maximal body weight gain, optimal feed utilisation and maximal breast meat yield were at 0.66, 0.68 and 0.70%, respectively. Thus, it appears that it requires more threonine to maximise feed utilisation than body weight gain, although to maximise breast meat yield, an even higher level of threonine is needed.

Notwithstanding that responses to threonine in body weight gains, FCR and carcass traits have been studied extensively in broiler chickens, experiments need to be conducted to establish specific threonine requirements for intestinal maintenance, especially to optimise gut health via mucus production.

2.5.6 Dietary effects of threonine on mucosal growth and mucins

The effect of dietary threonine in broiler chickens has focused mainly on the growth performance rather than impact on intestinal integrity. Thus, discussion on intestinal utilisation and metabolism of threonine in chickens for mucin biosynthesis can only be postulated from previous studies carried out on pigs and rats. In pigs, among the indispensable amino acids, threonine is found to be one of the most abundant amino acids in gastrointestinal mucus secretion (Toribara *et al.*, 1993). In a more recent experiment, Schaart *et al.* (2005) observed that dietary, rather than systemic threonine, was preferentially utilised for protein synthesis in the small intestinal mucosa in piglets that consumed a diet with a normal level of protein.

In humans, essential amino acid losses in the gut are estimated to account for 14-33% of the daily maintenance requirement. In the case of threonine, these losses account for 61% and mucin output accounts for the majority of the endogenous threonine loss (Fuller *et al.*, 1994). This high retention of threonine in the gut reflects the high demand of threonine by the gut for the synthesis of mucin glycoproteins. It has been well proven that the mucous layer and mucin biosynthesis are qualitatively and quantitatively altered in many situations of intestinal stress and nutritional state including intestinal infections, starvation and malnutrition in broiler chickens (Smirnov *et al*., 2004, 2005, 2006). Dietary antibiotics and probiotics have also been shown to influence the mucin gene expression and biosynthesis (Smirnov *et al*., 2005). It is hypothesised that under such conditions, there is an increase in the requirement for threonine that is not met by the normal diet and that intestinal mucin biosynthesis is particularly sensitive to dietary threonine availability.

Faure *et al.* (2005), and Nichols and Bertolo (2008) clearly proved that any dietary restriction of threonine would specifically reduce the rat's intestinal mucin synthesis, particularly at the translational level. Wang *et al.* (2007) also indicated that a deficiency or excess of threonine in pig diets would result in a significant reduction of jejunal as well as skeletal muscle protein synthesis.

2.6 MANNOOLIGOSACCHARIDES

In the light of a possible universal ban on use of in-feed antibiotics, nutritionists are constantly searching for potential alternatives to alleviate problems associated with this withdrawal. One of such alternative products that have been frequently tested for use in broiler chickens is mannooligasachharides (MOS). Mannooligosaccharides are derived from the cell wall of yeasts, *Saccharomyces cerevisiae*. The most popular product, BioMOS®, was introduced as a feed additive for broilers in 1993 (Hooge, 2004b). Since then, a series of experiments has evaluated the effect of MOS on body growth, intestinal development and immune function with positive results (Fernandez *et al.*, 2000a; Spring *et al.*, 2000; Iji *et al.*, 2001b; Kabir *et al.*, 2004).

Mannooligosaccharides are reported to exert their effects through: (1) inhibiting the colonisation of enteric pathogens by blocking bacterial adhesion to gut lining via absorption of pathogenic bacteria containing type 1 fimbriae with mannose-sensitive lectins (Spring *et al.*, 2000); (2) improving of the intestinal functions or gut health (Iji *et al.*, 2001b; Loddi *et al.*, 2002; Uni, 2008); (3) modifying microflora fermentation to favour nutrient availability to the host; (4) modulating the immune functions by stimulating the gut-associated lymphoid tissue and systemic immunity by acting as a non-pathogenic microbial antigen (Savage *et al.*, 1996; Spring and Privulescu, 1998; Ferket, 2002), and (5) reducing enterocyte turnover rates and enhancing the brush border mucin barrier (Ferket, 2002; Uni and Smirnov, 2006; Uni, 2008).

2.6.1 Inhibition of pathogen colonisation by MOS

Mannooligosaccharides act as a high affinity ligand, offering a competitive binding site for a certain class of bacteria (Ofek *et al.*, 1977). Gram-negative pathogens with the mannose-specific Type-1 fimbriae can attach to the gut surface through a mannose specific receptor. Dietary MOS is said to act as a decoy to bind pathogens (Newman, 1994). Mannose was shown to inhibit the attachment of *S. typhimurium* to intestinal cells of day-old chickens *in vitro* as well as *in vivo* (Oyofo *et al*., 1989a, 1989b) and is effective in reducing *Salmonella* infection of birds (Spring *et al.*, 2000). The ability of MOS to interfere with the attachment of pathogenic bacteria in the gut raises the possibility that it could also inhibit the binding between bacteria during plasmid transfer via conjugation and thus, prevent antibiotic resistant gene transfer (Duval-Iflah, 2001; Ferket *et al.*, 2005). Using lactose, Lou (1995)

demonstrated that dietary MOS decreased the proportion of specific groups of Gram-negative antibiotic-resistant faecal bacteria in swine.

2.6.2 MOS and mucin dynamics

Antibiotics and probiotics exert influence on the regulation of mucin gene expression, mucin composition and mucin secretion. Smirnov *et al.* (2005) showed that both probiotics and antibiotics alter processes of mucin biosyntehsis and/or degradation mediated via changes in bacterial populations in chickens. Neither the probiotic nor the antibiotic affected the thickness of the mucous adherent layer. This suggests that the increase in mucin gene expression does not necessarily result in an increase in thickness of the intestinal mucus depending on the activity of the mucolytic bacteria and possibly dietary threonine availability.

The co-culture of probiotics (*Lactobacillus* GG and *L. plantarum* 299v) with colonic epithelial cells *in vitro* enhanced the expression of mucin genes (MUC2 and MUC3). Furthermore, *Lactobacillus* GG and *L. planetarium* inhibited the adherence of enteropathogenic and enterohaemorrhagic *E. coli* to epithelial cells but not to non-epithelial cells, suggesting a protective role for mucins (Mack *et al.*, 1999; Gill, 2003). Whether an upregulation of mucin gene expression is mediated directly by bacterially derived bio-active molecules or indirectly via the stimulation of immune cells is not known (Brockhausen *et al.*, 1998). The ability of pro-inflammatory chemokines, such as interleukin-I (IL-I), to stimulate mucin release and upregulate mucin gene expression has been reported in mammalian colonic cell lines (Jarry *et al.*, 1996; Plaisancie *et al.*, 1998).

Stimulation of secretory immunoglobulin A by the probiotics and prebiotics has also been suggested to have effect on mucin dynamics (McKay and Perdue, 1993; Kudoh *et al.*, 1999). It is hypothesised that prebiotics, such as MOS that have been frequently shown to have effect on the establishment of a sound intestinal microflora and facilitate in the secretion of immunoglobulin A into the gut mucosa (Kudoh *et al.*, 1999), might also have an impact on mucin gene expression. Indeed, a number of studies have shown that MOS increase intestinal mucous layer, goblet cell cup size and density, and MUC2 expression in chickens (Solis de los Santos *et al*., 2007; Ferket, 2002; Uni and Smirnov, 2006). However, interrelationships between the modulating effects of MOS with dietary threonine on mucous microflora and mucin morphometric characteristics are yet to be elucidated.

2.6.3 The effect of MOS on gut tissue integrity and nutrient utilisation

Mannooligosaccharides influence gut microflora, nutrient utilisation and growth performance through their association with brush border morphology. Spring (1996) reported an increase in small intestinal villus height while Iji *et al.* (2001b) observed an increase in the jejunal villus height:crypt depth ratio with MOS supplementation in broilers. Furthermore, dietary MOS also increased the protein to DNA ratio in the jejunal mucosa as well as activities of brush-border enzymes maltase, leucine aminopepidase and alkaline phosphatase (Iji *et al.*, 2001b). This finding was further confirmed by Ferket (2002) who observed a similar impact of MOS on villus morphology in poults where there was a decrease in crypt depth rather than an increase in villus height. Effects of MOS on standardised ileal nutrient digestibility and *in vivo* ileal nutrient uptake have not been studied extensively. Since MOS has significant effects on gut SCFA (Santos and Teixeira, 2004; Yang *et al.*, 2007a) and mucin dynamics (Uni and Smirnov, 2006), it may also influence other intestinal characteristics, such as standardised ileal digestibility and nutrient uptake of nutrients. In fact, recent studies have shown that addition of MOS at 4 g/kg of the broiler diet improved the ileal amino acid digestibility as well as the apparent metabolisable energy (ME) (Biggs and Parsons, 2007; Biggs *et al.*, 2007). In addition, recent findings have shown that broiler diet supplemented with MOS significantly elevated the gene expression and activity of several major brush border enzymes and transporters, including peptide transporter (PepT1), y-Glutamyl transferase (yGT) and sodium/glucose cotransporter (SGLT-1) (Uni, 2008).

2.6.4 Effects of MOS on gut motility

In addition to looking at mucin gene expression and morphometric characteristics of mucins when investigating mucin dynamics, gut motility should also be considered as it plays an important role in the expulsion of microorganisms that adhere to mucus.

Studies in mammals have shown that the restoration of normal intestinal flora via the use of probiotics affects the frequency of defaecation and gut transit time (Takiguchi *et al.*, 1998; Mance *et al.*, 1999). In humans, dietary supplementation with *B. animalis* significantly reduced the total and segmental colonic transit time (Takiguchi *et al.*, 1998; Mance *et al.*, 1999).

In avain species, the effect of pro- and prebiotics on intestinal motility and feed passage rate is yet to be established. A study carried out by Sieo *et al.* (2005) showed that feeding transformed *Lactobacillus* strains reduced the food passage time by 2.2 hours in chickens. Ferket *et al.* (2002) studied the effect of MOS and antibiotics in turkey on the total weight and length and also the mucosa and muscularis weights of the intestine in relation to gut motility. The antibiotic treatments had significant effects on the relative intestinal weights and lengths through either decreasing both the circular and longitudinal muscularis layers or increasing the lamina propria of mucosa. The decrease in intestinal muscularis weight was probably associated with a reduced need for gut motility to control microbial activity, whereas the increase in the mucosa's lamina propria could be related to the stimulatory effect on the lymphatic tissue. In this study, MOS had no significant effect on the intestinal muscularis weight.

In another study, Ferket *et al.* (2002) showed that MOS reduced the thickness of the intestinal external muscularis with an increase in the number of goblet cells per villus. In line with this, Biggs and Parsons (2007) postulated that the increase in true amino acid digestibility in caecectomised roosters fed with MOS is likely to be associated with the improved gut microbial ecology induced by MOS and thereby reducing digesta passage rate and improving amino acid digestibility.

2.7 CONCLUSIONS

Due to public concerns regarding the development of antibiotic-resistant bacteria in humans, restriction in use of in-feed antibiotics is likely to be the trend for future broiler production. As the results of this, increase in cost of production as well as the incidental rate of enteric diseases are likely to be expected. Use of subtherapeutic antibiotics is known for its direct effect on the establishment of optimal gut microflora that are closely associated with improvement in growth and control of diseases.

Although the gut microflora can also be altered through dietary and husbandry manipulations, alternatives to antibiotics are likely to be more effective in maintaining the delicate balance of chicken gut microflora. Thus, there has been increased interest in the applications of biological products such as probiotics, prebiotics, organic acids and even plant extracts as alternatives to in-feed antibiotics. However, the growth-promoting effects of these alternatives are highly variable and dependent on dietary factors and experimental

conditions. Moreover, the exact mechanisms through which they deliver a potential growth-promoting effect are still largely unknown and more comprehensive research in this area is warranted.

In spite of the fact that the growth-promoting potential of MOS in broiler chickens has been well established through series of meta- and holo-analyses, there is still a lack of understanding on the presumptive relationships between the various responses observed and the proposed modes of action. In order to address this shortcoming, a series of experiments outlined in the following chapters were designed and conducted to explore further the interactive effects of MOS with dietary threonine on intestinal mucin dynamics and growth, gut microflora and motility, standardised ileal digestibility of amino acids, uptake of nutrients and performance of broiler chickens.

CHAPTER 3
INFLUENTIAL ROLES OF DIETARY THREONINE AND MANNOOLIGOSACCHARIDES ON GROWTH PERFORMANCE IN RELATION TO INTESTINAL MUCIN DYNAMICS IN BROILER CHICKENS

3.1 INTRODUCTION

Intestinal mucous layer is composed of a complex mixture of different types of mucin glycoproteins. It is an important component of the non-immune gut barrier that functions to protect the intestinal lining from digestive proteases, bacterial translocations, and dehydration of the underlying mucous membranes (Toribara *et al.*, 1993).

Nutritional state appears to be the key factor in regulating the mucous layer and gut barrier integrity. As such, threonine, which is highly retained in the gut, is thought to be in high demand by the gut for the synthesis of mucin glycoproteins (Toribara *et al.*, 1993; Fuller *et al.*, 1994; Stoll *et al.*, 1998). Dietary threonine requirements of broiler chickens for growth and carcass traits have been studied extensively (Kidd *et al.*, 1999; Rosa *et al.*, 2001; Corzo *et al.*, 2003) and critically reviewed (Kidd and Kerr, 1996; Kidd, 2000; Barkley and Wallis, 2001). However, information on dietary threonine requirement for intestinal maintenance and mucin dynamics is scarce.

A number of studies have shown that MOS modulate intestinal mucin dynamics by increasing intestinal mucous thickness, goblet cell “cup” size and density, and MUC2 mRNA expression in chickens (Solis de los Santos, 2007; Ferket, 2002; Uni and Smirnov, 2006). However, the influence of threonine on mucin dynamics discharged by MOS is still unknown.

It is hypothesised that the modulating effect of MOS on intestinal mucin dynamics is particularly sensitive to dietary threonine availability. Therefore, the present study was designed to examine the interaction between MOS and threonine on growth performance and intestinal mucin dynamics.

3.2 MATERIALS AND METHODS

3.2.1 Experimental design and diets

Nine treatments were allocated to each of the starter and grower basal diets, comprising mainly of sorghum, peanut-meal and soybean meal (Table 3.1). A 2-phase feeding programme with the starter diet being fed from d 1 to 21 and grower diet from d 22 to 35 was adopted. All diets were formulated to meet or exceed the minimum National Research Council (NRC) (1994) nutrient recommendations for broiler chickens, except for threonine which was formulated at 80, 90, 100, 110, 120 and 130% of the NRC recommended level (Table 3.2). L-Threonine (Ajinomoto Eurolysine, Paris, France) was supplemented to the basal diets at the respective rate of 0, 0.08, 0.16, 0.24, 0.32 and 0.40 % at the expense of a filler (Zeolite). Two levels of MOS supplementation, with and without MOS, were created by adding Bio-MOS® (Alltech Biotechnology, Nicholasville, Kentucky, USA) at 0 and 0.20 % of the starter diets, and 0 and 0.10 % of the grower diets at the expense of a filler (Zeolite), respectively. In this way, all starter and grower diets were isocaloric at 3050 and 3180 kcal ME/kg with crude protein maintained close to 20.5 and 18.5%, respectively. A summary of the calculated and analysed composition of the experimental diets is shown in Table 3.2.

For the study on MOS and threonine interaction, treatments consisted of two levels of MOS (with and without) and three levels of threonine (deficient-80% NRC, adequate-100% NRC and excess-120% NRC). As for the indicative study on threonine requirements, additional diets with two levels of threonine at 90 and 110% (without MOS) and one level of threonine at 130% (with MOS) of NRC recommendation were included.

All diets were mixed and cold-pelleted at the University of New England. To reaffirm the expected major nutrient levels, all feed samples were analysed for crude protein by the method of Sweeney (1989) using a LECO® FP-2000 automatic nitrogen analyser (Leco Corp., St Joseph, MI, USA). Samples of all the diets were also sent to the Australian Proteome Analysis Facility Ltd. (APAF, Sydney, NSW, Australia) for threonine analysis using a precolumn derivatisation method (AccQTaq, Waters, Milford, MA, USA).

3.2.2 Bird management

A total of 324 day-old male Cobb broiler chicks (Baiada Poultry Pty. Ltd., Kootingal, NSW, Australia), weighing 43.13 ± 0.77 g, was randomly assigned to 54 cages (600 x 420 x 230

cm) in four-tier battery brooders housed in an environmentally controlled room. The birds were transferred to slide-in cages (800 x 740 x 230 cm) in an environmentally controlled room at the end of week 3. Each of the 9 treatments was randomly assigned to 6 cages with 6 birds per cage. An initial room temperature of 34°C was maintained for the first 5 days and gradually decreased to 21°C by the end of the third week. Birds were provided *ad libitum* access to feed and water. The lighting programme was 24 h light for the first day followed by 18 h light and 6 h darkness for the rest of the trial period.

Table 3.1 Composition of experimental basal diets

Ingredient	Starter	Grower
	----------%	----------
Sorghum grain	62.60	66.30
Soybean meal dehulled, 47.5% CP	17.10	16.80
Peanut meal, solvent 45% CP	12.00	7.00
Vegetable oil	3.12	4.82
Filler[1]	0.70	0.60
L-Threonine	0.00	0.03
L-Lysine HCl	0.64	0.60
DL-Methionine	0.30	0.27
Calcium carbonate	1.11	1.11
Dicalcium phosphate	1.76	1.80
Choline chloride	0.05	0.05
Sodium bicarbonate	0.32	0.32
Salt	0.10	0.10
Vitamin and mineral premix[2]	0.20	0.20
Total	100.00	100.00
Calculated nutrient composition	----------%	----------
CP	20.60	18.60
Lysine	1.30	1.18
Methionine	0.55	0.50
Methionine and cystine	0.89	0.80
Threonine	0.64	0.59
Tryptophan	0.23	0.20
Calcium	0.90	0.90
Available phosphorus	0.46	0.45
Sodium	0.15	0.15
Chloride	0.23	0.23
ME poultry, kcal/kg	3050	3180

[1]Filler represents inert space (Zeolite) in the diet to which L-threonine and BioMOS® were added at its expense.
[2]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.

Table 3.2 Calculated and analysed nutrient composition of experimental diets

	Treatment								
MOS	-					+			
Thr	80 (Def[1])	90	100 (Ade[2])	110	120 (Exc[3])	80 (Def)	100 (Ade)	120 (Exc)	130
	% NRC recommendation								
Calculated nutrient composition (% as-is basis)									
Starter									
CP	20.60	20.68	20.76	20.84	20.92	20.60	20.76	20.92	21.00
Thr	0.64	0.72	0.80	0.88	0.96	0.64	0.80	0.96	1.04
Grower									
CP	18.60	18.68	18.76	18.84	18.90	18.64	18.76	18.92	19.00
Thr	0.59	0.67	0.74	0.82	0.89	0.59	0.74	0.89	0.97
Analysed nutrient composition (% as-is basis)									
Starter									
CP	20.80	20.30	20.50	21.20	21.30	20.30	21.10	20.90	20.80
Thr	0.64	0.69	0.80	0.89	0.98	0.68	0.82	0.94	1.08
Grower									
CP	18.40	19.00	19.20	19.10	19.10	18.40	18.90	19.20	19.30
Thr	0.61	0.69	0.76	0.83	0.96	0.61	0.72	0.94	0.99

[1] Deficient.
[2] Adequate.
[3] Excess.

3.2.3 Animal ethics

All the procedures were approved by the Animal Ethics Committee of the University of New England with the approval number AEC 06/143.

3.2.4 Growth performance measurement

Body weight and feed intake (FI) were recorded on a cage basis at weekly intervals. Mortality was recorded daily and feed conversion ratio (FCR) was corrected for mortality.

3.2.5 Sample collection

On d 14 and 28, one bird per cage (6 birds per treatment) was weighed and euthanised by cervical dislocation. The gastrointestinal tract (GIT) was excised with the small intestine dissected into three regions of 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).

The length and weight of the respective intestinal regions were measured. Approximately 2 cm of the mid-point jejunum were removed for gut morphological measurements. The gut samples were flushed with ice-cold buffered phosphate saline (PBS) at pH 7.4 and immediately placed in 10% formalin solution.

For the analysis of adherent mucous thickness, 1 cm^2 of the middle portion of the respective gut regions were collected, rinsed with 10 mL of ice-cold 0.25 mol/L sucrose solution and later, incubated in 2 mL buffered Alcian Blue (AB) solution. The buffered AB solution was made up of 10 g/L Alcian Blue dye (Sigma-Aldrich Chemicals, St Louis, MO, USA) in buffer containing 160 mmol/L sucrose and 50 mmol/L sodium acetate at pH 5.8.

On d 14, approximately 1 g of the middle portion of the jejunum was also excised, flushed with the ice-cold normal saline and collected in RNAlater® (Ambion Inc., Austin, TX, USA). The samples were incubated overnight at 4°C to allow thorough penetration of the tissue and then stored at -20°C for future mucin gene expression assays.

The remaining intestinal regions were again measured for length with pre- and post flushed weights recorded. They were then cut open longitudinally along the mesentery line for the dissection of mucosa and the underlying external muscularis layer using scraping method (Law, 2000). The respective regional mucosa and external muscularis weights were measured and corrected proportionally based on the respective original length and weight of the intestinal regions.

3.2.6 Measurement of adherent mucous layer thickness

The thickness of the adherent mucous layer was estimated using a modified method of Corne *et al*. (1974) as outlined by Ota and Katsuyama (1992), Nam *et al.* (2005) and Smirnov *et al.* (2005). Briefly, after 2 h of soaking in the buffered AB solution, excess uncomplexed dye was removed by two successive washes of 10 mL of 0.25 M sucrose solution for 15 and 45 min, respectively. The absorbed dye was then extracted by incubating in 3 mL of 1% dioctyl sodium sulfosuccinate (DSS) solution (Sigma-Aldrich) overnight at room temperature and cleared by centrifuging at 700 *g* for 20 min. The optical density (absorbance) of the resultant blue solution was then measured at 620 nm using a Hitachi 150-20 Spectrophotometer

(Hitachi Science Systems Ltd., Ibaraki, Japan). A standard curve was developed by plotting the concentrations of a serial dilution of AB standard solutions against the absorbance readings. The mucous thickness layer, expressed in μg of Alcian Blue per $1cm^2$ of intestinal tissue, was then determined by extrapolating the absorbance value on the standard curve.

3.2.7 Enteric morphometric analysis

Tissue slices, 1-2 mm thick, were trimmed from the formalin-fixed jejunal samples, enclosed in a plastic tissue cassette, and later 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, and impregnation with wax. The tissue was then embedded in paraffin wax using Histo Embedding Centre (Leica EG 1160, Leica Microsystems, Bensheim, D-64625, Germany) prior to sectioning on a rotary microtome (Leiz 1516, Leica Microsystems). Separate 5 μm sections were placed on glass slides for staining with: (1) 1% Alcian blue (AB, pH 2.5, 30 min) for the examination of total sulphated- and carboxylated-acidic mucins (blue); (2) 1% Alcian blue (AB, pH 1.0, 30 min) for the selective identification of acidic sulphomucins (blue); (3) periodic acid-Schiff's reagent (PAS, 20 min each) for the identification of hexose and sialo-rich neutral mucins (bright red), and (4) a combination of AB (pH 2.5, 30 min) and PAS (20 min each) for the counting of total number of goblet cells (Kierrnan, 1990).

All staining reagents were purchased from Sigma-Aldrich Chemicals. To ensure comparability between the different groups of birds, the sections from all experimental groups were stained in a single batch and mounted in a DPX medium (Fluka Chemical, Buchs, Australia). They were then analysed using a digital video camera (Sony, Tokyo, Japan) aided binocular microscope (Leica DLMB, Leica microsystems) at 4x, 20x and 40x objective magnifications. Histomorphometric analyses were performed with the help of a calibrated image analysis software (Video Pro 32, Leading Edge Pty. Ltd., Blackwood, SA, Australia). The parameters measured for villus morphometry on 20 well-oriented villus-crypt units stained with PAS and AB, pH 2.5 were external muscularis thickness, villus height, crypt depth, and villus to crypt ratio.

Mucin glycoprotein profiles were measured on 15 well-oriented villus-crypt units stained with the respective reagents and expressed as number of goblet cells per mm^2 of the villus area. In addition, individual goblet cells in 20 segments of the stained field were also subjected to measurements of: (1) goblet cell "cup" size by elliptical transformation of the

maximum and minimum diameters; (2) staining intensity for the determination of stain concentration calculated from the transmittance rate expressed in logarithmic function, and (3) integrated optical density (IOD) to indicate the total amount of stains determined by integrating the staining intensity and stained area together and standardised to one mm^2 of the measured villus surface area.

3.2.8 MUC2 mRNA expression assay

Total mRNA isolation and cDNA synthesis

Tissues were immediately homogenised in TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) with an Ultra-Turrax T10 homogenizer (IKA®-Werke GmbH & Co, Staufen, Germany) and total RNA extraction was performed according to the manufacturers' instructions (Molecular Research Center). Briefly, the homogenate was incubated for 5 min at 25°C and centrifuged (Biofunge 13, Heraeus Sepatech, Germany) at 12,000 *g* for 10 min at 4°C. The supernatant was then transferred to a fresh tube and supplemented with 0.1 mL of bromochlopropane (BCP) (Molecular Research Center), shaken for 15 s and incubated at 25°C for 10 min. It was then centrifuged at 12,000 *g* for 10 min at 4 °C with the supernatants transferred to a fresh tube. Next, 0.5 mL 100% isopropanol (Sigma-Aldrich) were added to the tube, which was shaken well and incubated at 25°C for 10 minutes and later centrifuged at 12,000 *g* for 10 min at 4°C. The supernatant was then carefully discarded and the pellet was washed with 1 mL of 75% ethanol followed by centrifugation at 7,500 *g* for 10 min at 4°C. The ethanol was then carefully removed and the remaining mRNA pellet was briefly air-dried for 3-5 min before dissolving in the non-diethyl pyrocarbonate (DEPC) treated Nuclease Free Water (Ambion) by passing the solution a few times through a pipette tip and incubated for 10 min at 60°C.

The concentration of the resuspended RNA solution was determined by measuring its absorbance (A) at 260 nm and 280 nm using a ND-1000 NanoDrop Spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA). The total RNA had an A_{260}/A_{280} ratio of 1.8–2.2. The RNA integrity was assessed by running the aliquot on 2% agarose gel electrophoresis stained with 5 μL of Gelstar® nuclei acid gel stain (BioWhittaker Molecular Applications, Rockland, ME, USA), visualized on UV illuminator (Chemiluminescence, Vilber Lourmat, Cedex, France) and digitised on an Infinity CN-3000 Gel Documentation System (Vilber Lourmat).

The two-step pre-optimised Custom TaqMan Gene Expression Assays (Applied Biosystems, Foster, CA, USA) was used for the MUC2 gene expression assays. A total of 20 µL reaction volume containing 2 µg of total RNA was reverse transcribed in an Eppendorf thermal cycler (MasterCycler®, Eppendorf AG, Hamburg, Germany), using High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems) as described by the manufacturer, under the following thermal profile parameters: 25°C for 10 min, 37°C for 120 min, 85°C for 5 s and 4°C for ∞. After completion, all the cDNA samples were stored at -20°C.

Primer design and PCR amplification

The second step of the assays involved the use of TaqMan® Universal PCR Master Mix (Applied Biosystems) and Custom TaqMan® Gene Expression Assays (Applied Biosyatems) containing TaqMan® MGB FAM™ dye-labeled probes and forward and reverse primers of chicken MUC2 gene (accession code: XM_421045) or housekeeping genes of β-actin (accession code: L_08165). All the primers of MUC2 and β-actin genes were designed with reference to the published sequences (Smirnov *et al*., 2004, 2005, 2006) using BLAST® search method available at National Center for Biotechnology Information (NCBI, www.ncbi.nlm.gov/blast) (Table 3.3).

A final 20 µL reaction volume for each of the gene expression assays and samples making up the reaction components of 1 µL of Gene Expression Assays (20x working stock solution), 9 µL of 60 ng cDNA template solution and 10 µL of TaqMan® Universal PCR Master Mix (2x) were prepared as described by the manufacturer (Applied Biosystems). In order to validate all the PCR amplifications, the amplification efficiency of PCR reactions for both MUC2 and β-actin were determined by amplifying a duplicated 4 ten-fold serial dilutions (10^1 to 10^4) of pooled cDNA samples under the thermal cycling profiles as listed in Table 3.3 on Rotor-Gene™ 6000 real-time PCR machine (Corbett Research, Sydney, NSW, Australia). To reaffirm the validation of the two amplification efficiencies, the slope (M value) of the relative efficiency plotted as $\log_{10}$ dilution inputs against ΔC_T (β-actin-MUC2) was determined and evaluated. A value of <0.1 for the slope (M value) of the relative efficiency justifies the validation of all the PCR amplifications (Applied-Biosystems, 2005). Once the validation of the amplification efficiencies had been established, amplification of MUC2 and β-actin genes based on the same reaction components and volume using the amplification protocol as stated above were conducted in duplicate and in separate tubes for all the cDNA samples. For the purpose of optimal analysis and troubleshooting, a pair of

non- template control (NTC) reactions was also included for both MUC2 and β-actin assays in each plate run. The real-time fluorescence measurements were taken and a threshold cycle (C_T) value for each sample was calculated by determining the point at which the fluorescence rises appreciably above the background fluorescence.

Analysis of relative quantification of Real Time PCR

The Pfaffl method (Pfaffl, 2001) was employed to compare the relative quantitative analyses of the real time PCR by taking into account the actual difference in efficiencies between the target and the housekeeping genes. Fold change in MUC2 and β-actin mRNA abundances was calculated as $(E_{MUC2})^{\Delta C}{}_T$ and $(E_{\beta\text{-actin}})^{\Delta C}{}_T$, respectively where E represents the amplification efficiency and taken as $10^{(-1/slope)}$ and ΔC_T as the difference in C_T values between the treatment and the control for both MUC2 and β-actin. The treatment group without MOS at adequate threonine level was used as the control. Finally by taking the ratio between the fold changes in MUC2 and β-actin, the internally corrected fold change of MUC2 can then be determined. The general Pfaffl formula used for the calculation is as shown below:

$$\text{ratio} = \frac{\left(E_{\text{MUC2}}\right)^{\Delta C_T \text{ MUC2 (control}-\text{treated)}}}{\left(E_{\beta\text{-actin}}\right)^{\Delta C_T\ \beta-\text{actin (control}-\text{treated)}}}$$

3.2.9 Economic response analysis

The economic model of Kidd *et al*. (1999), with slight modification, was employed to predict and contrast the financial gains as a result of formulating diets near or at the threonine requirement with or without MOS. Quadratic regression equations implemented into the economic model were body weight gain, feed intake and feed conversion ratio from d 1 to 35 as determined herein. As treatment effects on mortality were not significant, livability was standardised to 95%. Since carcass yield was not affected by dietary threonine (Kerr *et al.*, 1999), a dressing percentage of 72% was assumed. Feed costs for broiler starter and grower with baseline threonine level at 80% NRC (1994) recommended level were adjusted to AUD 0.51 and 0.50/kg, respectively. Feed costs were also adjusted accordingly by AUD 0.0004 and 0.012/kg for each 0.01% increase in total dietary threonine and 0.1% inclusion of MOS, respectively.

Table 3.3 Primer sequences and PCR conditions for MUC2 and β-actin genes expression assays

Gene	Primer sequence	TaqMan ®MGB probe sequence	Reporter dye	PCR cycle profile[1]	PCR Product (bp)[2]
MUC2	5'-GGG ATA TTC ACC TGG AGA AAC CAT T 3'-TTG CAT GTC CAT CTG CCT GAA	CAC AGG TGC ATT CTTC	FAM	95°C/15s[3] 60°C/60s	75
β-actin	5'-CCC CGA GGC CCT CTT C 3'-GAT GGA GTT GAA GGT AGT TTC ATG AA	ACT CCA TAC CCA AGA AAG A	FAM	95°C/15s 60°C/60s	75

[1]All PCR runs were performed with a Corbett Rotor-Gene™ 6000 real-time machine and used an initial Amp Erase UNG activation of 50°C for 120s and AmpliTaq Gold Enzyme activation at 95°C for 600s. PCR conditions for each primer pair consisted of 45 cycles followed by melting curve analysis by the Rotor-Gene™ 6000.

[2]bp=base pair.

[3]s=second.

Using the Australian market estimates at the time of reporting, the production costs for day-old-chick, vaccination and medication, and contract growing fees were estimated to be at AUD 1.64 per bird. Other associated production costs including transportation, processing and distribution, and administrative works were estimated to be at AUD 0.85/kg of live bird. A wholesale price of AUD 3.85/kg of dressed carcass was taken as the average value in order to account for the volatile broiler market. Values of net profit margin, calculated as gross profit minus cost of production on per bird basis, were determined based on a dose response manner as total dietary threonine increased in the presence or absence of MOS addition.

3.2.10 Statistical analysis

The six treatments of three levels of threonine (deficient, adequate and excess) and two levels of MOS inclusion (with or without) were analysed using ANOVA of General Linear Models procedure of SAS (SAS/STAT® Version 9.1, SAS Institute Inc., Cary, NC, USA). This factorial arrangement was fitted into a model of $Y_{ijk} = \mu + M_i + T_j + MT_{ij} + e_{ijk}$ where μ is the common mean; M_i is the effect of the ith MOS inclusion; T_j is the effect of the jth threonine level; MT_{ij} is the effect of the interaction of the ith MOS with the jth threonine; and e_{ijk} is the random error. If significant differences were detected, means were separated with repeated *t*-test using Least squares means option of SAS (SAS Institute Inc.).

For the study on threonine requirements, viewed on both dietary % and grams of threonine intake per bird per phase, quadratic responses were measured using General Linear Model procedure of SAS (SAS Institute Inc.). Threonine intakes were calculated from the designated threonine levels (%) based on the actual feed intakes. When quadratic responses ($P \leq 0.05$) were detected, optimisations of dietary threonine requirement were calculated by extrapolating 95% of the upper or lower asymptote. In order to determine dietary threonine requirements or intakes from d 22 to 35, covariate-adjusted regression by means of ANCOVA of General Linear Models procedure (SAS Institute Inc.) was used with body weights at d 21 served as the covariates. The level of significance was considered at $P \leq 0.05$ unless otherwise specified.

3.3 RESULTS

3.3.1 Dietary composition and treatment design

The calculated crude protein and threonine contents of the treatment diets and their respective analysed values are presented in Table 3.2. The analysed values were generally in close agreement (within 5% variation in differences) with the calculated values. The only exception was the grower diet at 120% NRC recommended threonine level without MOS supplementation where the analysed threonine level was 7.8% (absolute difference of 0.05%) higher than the calculated value.

3.3.2 Growth performance

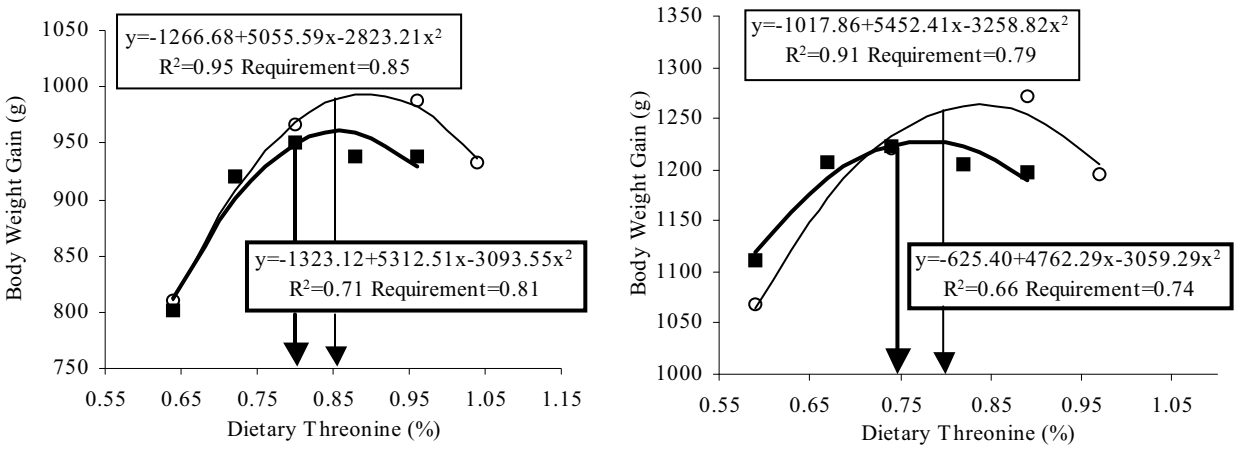
The effects of dietary threonine and MOS on body weight gain (BWG), FI and FCR are presented in Table 3.4. Birds fed the low-threonine diets responded negatively ($P<0.05$) in all performance criteria, except mortality. The main effect of MOS on growth performance significantly improved ($P<0.01$) BWG and FCR in the starter phase but not in the later stage of growth. The positive interactions between MOS and threonine supplements on BWG were apparent in all phases of growth ($P<0.05$) with the highest BWG noticed in birds given the excess-threonine plus MOS diet. Dietary threonine also interacted with MOS to improve ($P<0.05$) the FCR at the grower phase. No significant response to MOS and its interaction with threonine on FI were observed. Mortality was also not affected by treatments and averaged 1.7% across all treatments from 1 to 5 weeks of age.

The proposed regression equations are not intended to provide a comprehensive description of dietary threonine requirements or intakes in broilers. However, despite the limitations of this study, the quadratic effect of dietary threonine requirement and intake was obvious on BWG (Figures 3.1 and 3.2) and FCR (Figures 3.3 and 3.4) in both groups, with and without MOS. By extrapolating from the 95% of upper asymptote of the quadratic equations, the normal dietary threonine requirements without MOS for BWG were determined to be at 0.81 and 0.74% of total threonine (62 and 63% of the total lysine) or 10.50 and 17.33 g of threonine intake per bird per phase for d 1 to 21 and d 22 to 35, respectively. As expected, the normal dietary threonine requirements without MOS for FCR, in comparison to BWG, were higher at 0.83 and 0.77% of total threonine (64 and 65% of the total lysine) or 10.80 and 17.50 g of threonine intake per bird per phase for d 1 to 21 and d 22 to 35, respectively.

Table 3.4 Effects of dietary threonine level supplemented with or without MOS on body weight gain (BWG), feed conversion ratio (FCR), feed intake (FI) and mortality rate of birds fed on the experimental diets [1]

Treatment		BWG (g/bird)			FCR			FI (g/bird)			Mortality (%)		
Thr	MOS	1-21d	21-35d	1-35d	1-21d	21-35d	1-35d	1-21d	21-35d	1-35d	1-21d	21-35d	1-35d
Deficient		807	1085	1902	1.52^{a}	1.84	1.70^{a}	1226^{b}	1989^{b}	3216^{b}	1.17	0.24	1.40
Adequate		958	1235	2193	1.37^{b}	1.72	1.56^{b}	1309^{a}	2124^{a}	3433^{a}	0.05	0.73	1.74
Excess		963	1225	2188	1.33^{b}	1.74	1.56^{b}	1284^{a}	2133^{a}	3416^{a}	0.93	0.48	1.86
	-	891	1173	2070	1.43^{a}	1.78	1.63^{a}	1277	2089	3367	0.70	0.48	1.32
	+	922	1190	2118	1.38^{b}	1.75	1.59^{b}	1268	2074	3343	1.40	0.64	2.01
Deficient	-	802^{d}	1110^{d}	1912^{d}	1.57	1.82^{a}	1.71	1254	2022	3276	0.93	0.00	0.93
Deficient	+	812^{d}	1060^{cd}	1892^{d}	1.48	1.85^{a}	1.69	1198	1956	3155	1.40	0.48	1.86
Adequate	-	950^{c}	1237^{a}	2187^{b}	1.37	1.73^{b}	1.57	1302	2142	3445	0.70	0.97	1.63
Adequate	+	966^{ab}	1232^{ab}	2198^{ab}	1.36	1.71^{b}	1.56	1316	2105	3421	1.40	0.48	1.86
Excess	-	939^{b}	1172^{bc}	2111^{c}	1.36	1.79^{a}	1.60	1276	2104	3380	0.50	0.48	1.40
Excess	+	988^{a}	1277^{a}	2265^{a}	1.31	1.70^{b}	1.52	1291	2161	3452	1.40	0.97	2.32
SEM		11.0	24.1	29.3	0.021	0.033	0.019	19.1	54.8	61.1	0.565	0.501	0.913
Source of variation		----------Probability----------											
Thr		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0050	0.0200	0.0011	NS	NS	NS
MOS		0.0017	NS[2]	NS	0.0063	NS	0.0029	NS	NS	NS	NS	NS	NS
Thr x MOS		0.0340	0.0080	0.0101	NS	0.0300	NS	NS	NS	NS	NS	NS	NS

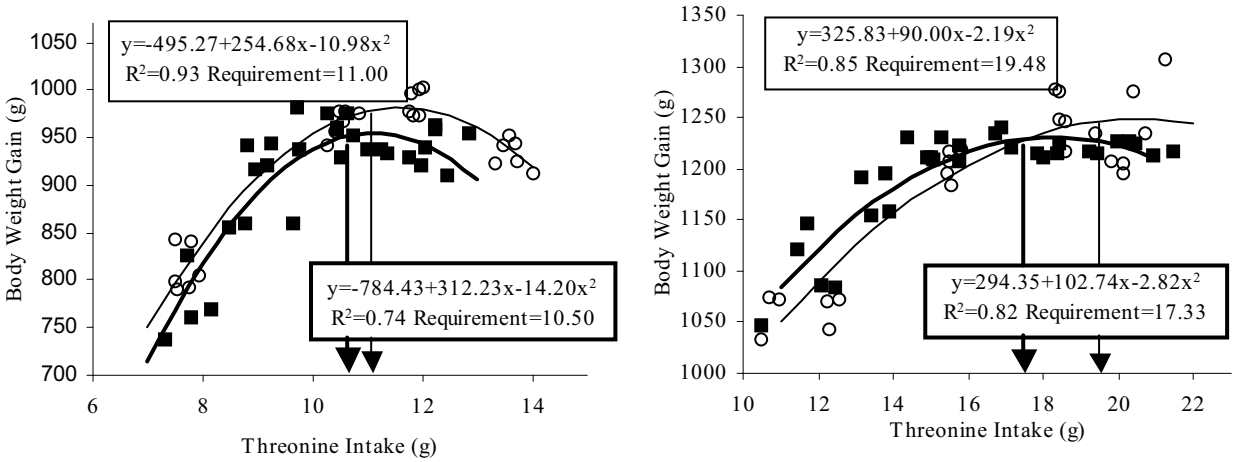
[1]Each value represents the mean of 6 replicates for each treatment group.
[2]NS=Not significant.
[a-d]Means within a continuous column with different superscripts are significantly different (P<0.05).



(a) (b)

Figure 3.1 Estimated threonine requirement (%) for the optimisation of BWG of broiler chickens from (a) 1 to 21 and (b) 21 to 35 days of age

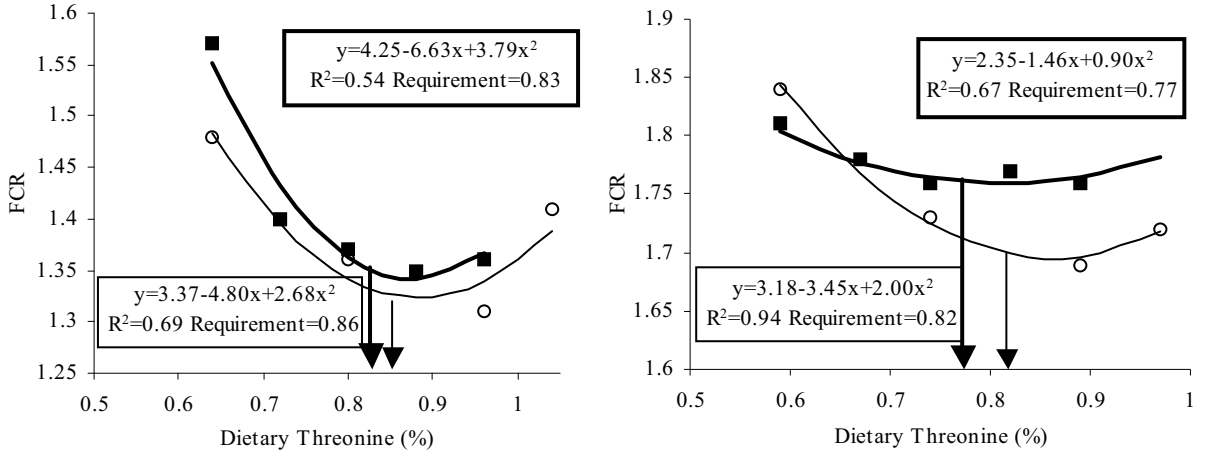
Values were determined by taking 95% upper asymptote of quadratic regression of body weight gain plotted as a function of dietary threonine level with (—) and without (▬) MOS supplementation. Observed mean values (n=6) at different dietary threonine levels with (○) and without (■) MOS supplementation are also shown.



(a) (b)

Figure 3.2 Estimated threonine requirement (g per phase) for the optimisation of BWG of broiler chickens from (a) 1 to 21 and (b) 21 to 35 days of age

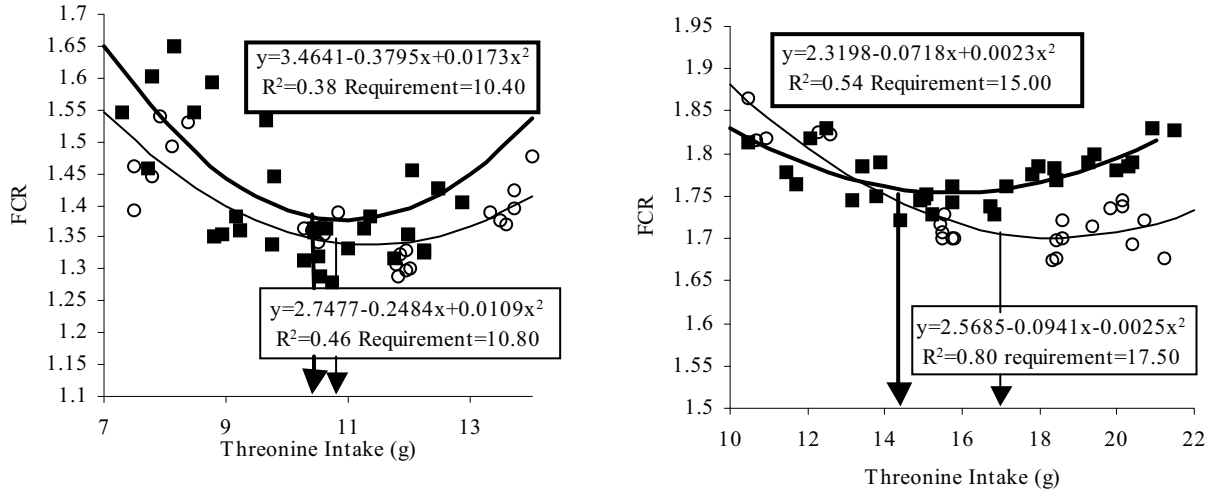
Values were determined by taking 95% upper asymptote of quadratic regression of body weight gain plotted as a function of threonine intake with (—) and without (▬) MOS supplementation. Observed mean values (n=6) at different dietary threonine levels with (❍) and without (■) MOS supplementation are also shown.



(a) (b)

Figure 3.3 Estimated threonine requirement (%) for the optimisation of FCR of broiler chickens from (a) 1 to 21and (b) 21 to 35 days of age

Values were determined by taking 95% lower asymptote of quadratic regression of FCR plotted as a function of dietary threonine level with (—) and without (▬) MOS supplementation. Observed mean values (n=6) at different dietary threonine levels with (○) and without (■) MOS supplementation are also shown.



(a) (b)

Figure 3.4 Estimated threonine requirement (g per phase) for the optimisation of FCR of broiler chickens from (a) 1 to 21 and (b) 21 to 35 days of age

Values were determined by taking 95% lower asymptote of quadratic regression of FCR plotted as a function of threonine intake with (—) and without (▬) MOS supplementation. Observed mean values (n=6) at different dietary threonine levels with (○) and without (■) MOS supplementation are also shown.

As indicated in the regression curves, at normal dietary threonine level, MOS only improved the growth performance of younger birds. However, a higher order of magnitude in growth response towards supplementation of threonine was also observed for MOS-treated birds particularly in the later stage of growth. For instance, in the grower phase, feeding of MOS with an extra 0.05% of total threonine or 2.2 g of threonine intake per bird per phase on top of the normal dietary allowances resulted in an additional BWG of 21 to 33 g per bird and an improvement of 6 points in FCR.

3.3.3 Mucin synthesis

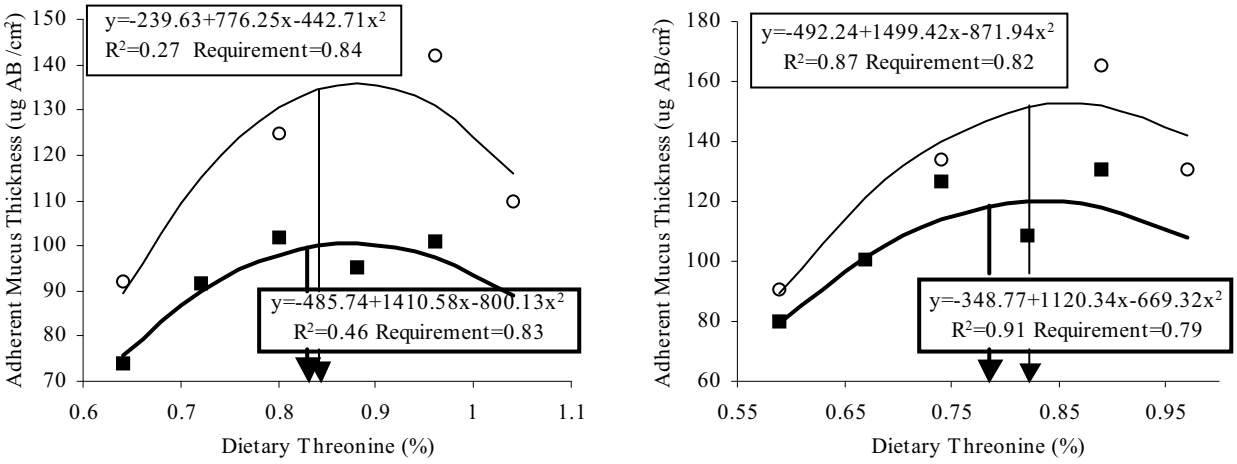
Birds fed the low-threonine diets had consistently thinner ($P< 0.05$) adherent mucous layer in all the three regions of the small intestine (Table 3.5). Supplementation of threonine beyond the adequate level did not result in any further increase in adherent mucous thickness. At d 14, the positive effects of MOS on intestinal adherent mucous thickness averaged at an increase of 30% were observed in all the three regions of the small intestine. The only near significant (P=0.0506) interaction between dietary threonine and MOS on adherent mucous thickness was detected in the jejunum at d 14, whereby an increase in mucous thickness, due to MOS, was observed at supplementary feeding levels of threonine.

In assessing the dietary threonine requirements for the optimisation of mucous synthesis, jejunal adherent mucous thickness measured at d 14 and 28 were taken as the representative values for d 1 to 21 and 22 to 35, respectively. The jejunal adherent mucous thickness with or without MOS supplementation varied in a similar manner as BWG with more obvious change in threonine requirement occurred in the grower phase (Figures 3.5 and 3.6). In younger birds, MOS increased the jejunal mucous thickness by almost 35% (35 μg AB/cm^2) with only a marginal change in threonine requirement or intake. However, in this respect, the older broilers fed the MOS-supplemented diets generally required more threonine amounting to an extra 0.03% of total threonine or an additional threonine intake of 4.40 g per bird per phase.

Table 3.5 Effects of dietary threonine level with or without MOS supplementation on small intestinal adherent mucous thickness of broilers at 14 and 28 days of age [1]

Treatment		Intestinal adherent mucous thickness (μg AB/cm^2)					
Thr	MOS	Duodenum		Jejunum		Ileum	
		14d	28d	14d	28d	14d	28d
Deficient		69.2^{b}	81.7^{b}	82.6^{b}	84.3^{b}	43.8^{b}	48.7
Adequate		84.8^{a}	127.4^{a}	112.6^{a}	124.1^{a}	63.5^{a}	52.9
Excess		83.5^{a}	120.5^{a}	121.7^{a}	148.1^{a}	52.3^{ab}	58.7
	-	68.5^{b}	102.9	92.3^{b}	110.7^{b}	46.5^{b}	51.7
	+	89.8^{a}	116.8	118.9^{a}	126.9^{a}	59.9^{a}	55.2
Deficient	-	57.0	80.2	73.7	80.0	38.0	42.0
Deficient	+	81.3	83.2	90.5	88.5	49.7	55.3
Adequate	-	77.8	117.7	102.9	121.1	55.9	52.8
Adequate	+	91.8	137.2	123.3	127.2	71.2	53.2
Excess	-	70.7	111.0	102.3	131.0	45.7	60.3
Excess	+	96.3	130.0	142.0	165.2	59.0	57.0
SEM		5.82	14.36	5.85	9.78	6.29	7.68
Source of variation		----------Probability----------					
Thr		0.0140	0.0400	0.0001	0.0001	0.0070	NS
MOS		0.0001	NS	0.0001	0.0400	0.0100	NS
Thr x MOS		NS[2]	NS	0.0506	NS	NS	NS

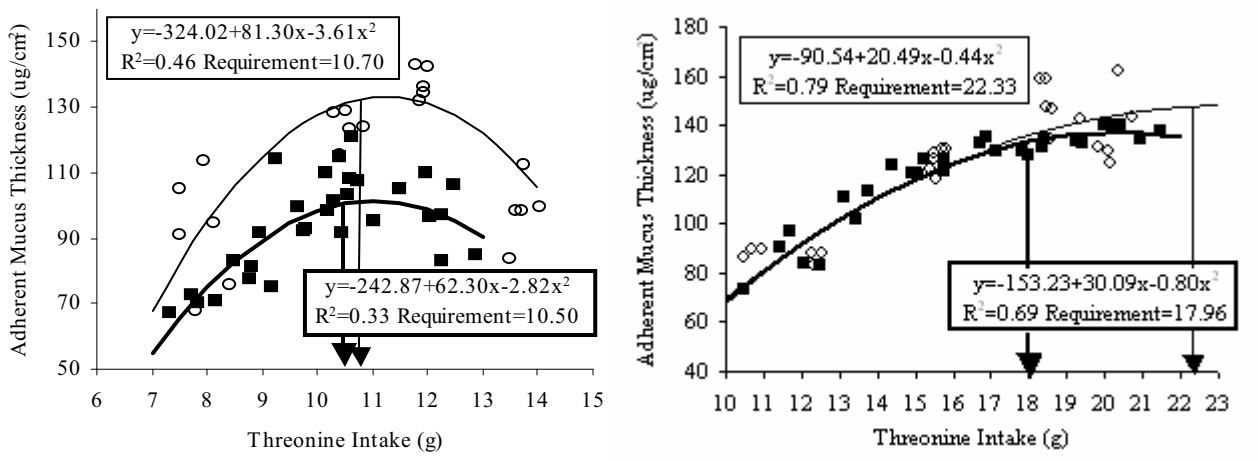
[1]Each value represents the mean of 6 replicates for each treatment group.
[2]NS=Not significant.
[a-d]Means within a continuous column with different superscripts are significantly different at $P<0.05$. Probability values up to $P<0.10$ are also shown if the data suggest a trend.



(a) (b)

Figure 3.5 Estimated threonine requirement (%) for the optimisation of jejunal adherent mucous thickness of broiler chickens from (a) 1 to 21 and (b) 21 to 35 days of age

Values were determined by taking 95% upper asymptote of quadratic regression of jejunal adherent mucous thickness (assayed at 14 and 28 days of age) plotted as a function of dietary threonine level with (—) and without (▬) MOS supplementation. Observed mean values (n=6) at different dietary threonine levels with (○) and without (■) MOS supplementation are also shown.



(a) (b)

Figure 3.6 Estimated threonine requirement (g per phase) for the optimisation of jejunal adherent mucous thickness of broiler chickens from (a) 1 to 21 and (b) 21 to 35 days of age

Values were determined by taking 95% upper asymptote of quadratic regression of jejunal adherent mucous thickness (assayed at 14 and 28 days of age) plotted as a function of threonine intake with (—) and without (▬) MOS supplementation. Observed mean values (n=6) at different dietary threonine levels with (○) and without (■) MOS supplementation are also shown.

3.3.4 mRNA extraction and mucin gene expression

All mRNA isolated from the samples had A_{260}/A_{280} ratio between 1.8 and 2.2 with no signs of degradation when subjected to electrophoresis on 2% agarose gel (Plate 3.1).

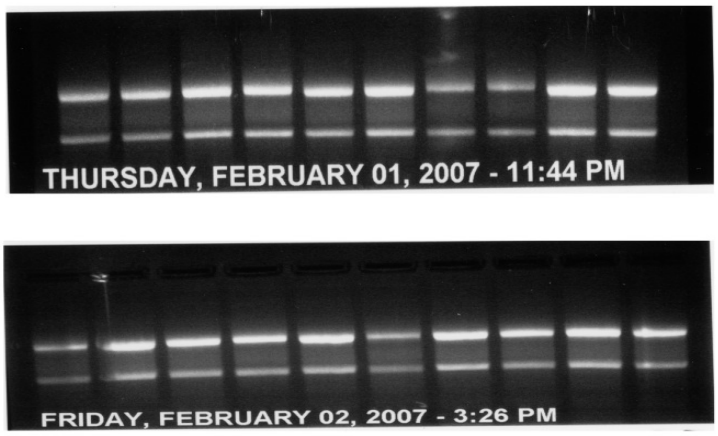


Plate 3.1 Patterns of mRNA electrophoresis on 2% agarose gel

Table 3.6 Slopes and corresponding efficiencies of jenunal-specific PCR amplifications of MUC2 and β-actin as determined by dilution series[1]

	Genes		
	β-actin	MUC2	Relative efficiency[2]
Slope[3]	-3.57	-3.52	0.045
Efficiency[4]	1.91	1.92	
R^2	0.996	0.999	0.980

[1] Each value represents the mean of 2 replicates for each of the 4 serial dilutions
[2] Relative efficiency of MUC2 -β actin (ΔC_T) was determined from equation of ΔC_T=0.573+0.045 x $\log_{10}$ (conc).
[3] Determined from linear equations for MUC2 of C_T=17.40-3.516 x $\log_{10}$ (conc) and β-actin of C_T=17.10-3.566 x $\log_{10}$ (conc)
[4] Corresponding real time PCR efficiencies (*E*) were calculated according to the equation: $E=10^{(-1/slope)}$

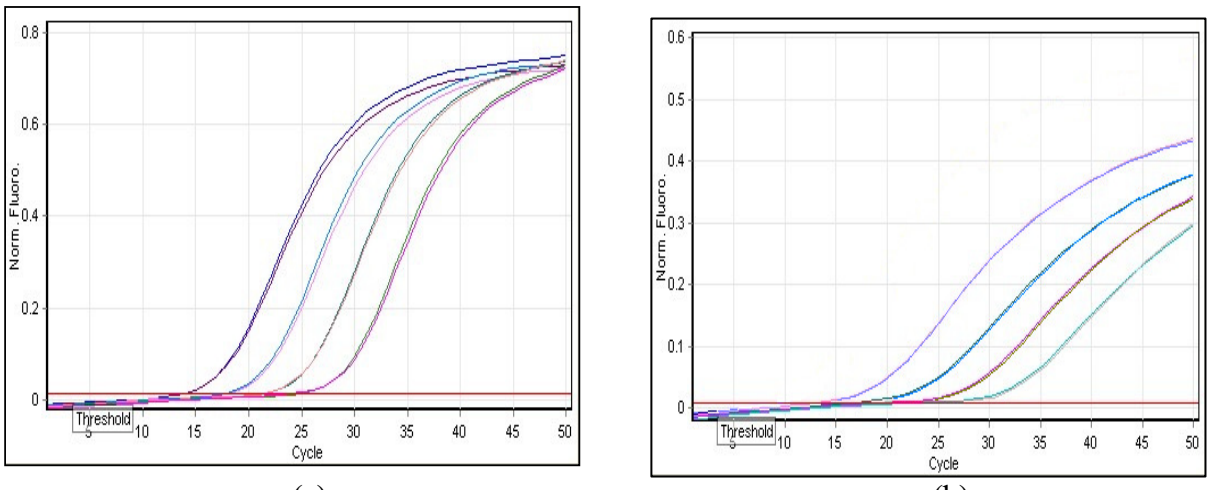


Plate 3.2 Log view of fluorescence history versus thermal cycles curves for PCR amplifications of serial dilutions of (a) MUC2 and (b) β-actin

The amplification efficiencies for both target (MUC2) and housekeeping (β-actin) genes were observed to be at 0.92 and 0.91% with R^2 values of 0.999 and 0.996, respectively (Table 3.6 and Plate 3.2). Since, the absolute value of the slope (M value) of a log input dilution versus $\Delta C_{T\ \beta\text{-actin-MUC2}}$ curve determined at 0.045 was < 0.1, validation of all PCR amplification efficiencies could further be confirmed (Applied-Biosystems, 2005).

The mean, approximate ANOVA F-test of variance (F-ratio), coefficient of variance (CV), slope of regression curve and stability index (product of CV and slope) of the C_T values of β-actin gene were 16.29, 1.59, 3.67%, 0.076 and 0.28, respectively (data not shown). These values, especially the low stability index, statistically confirm the constancy of the expression level of β-actin gene among the sample tissues tested under the current experimental conditions and thus, validate the use of β-actin gene as the choice of housekeeping gene for data normalisation (Brunner *et al.*, 2004).

Using the Pfaffl method, the PCR amplification results showed that threonine had no significant effects on MUC2 mRNA expression. Relative to the untreated groups, expressions of jejunal MUC2 mRNA were consistently enhanced (P<0.01) by more than 100% in all the MOS supplemented groups and were independent of dietary threonine levels (Figure 3.7). No significant interactions between MOS and threonine on jejunal MUC2 mRNA expression were noted.

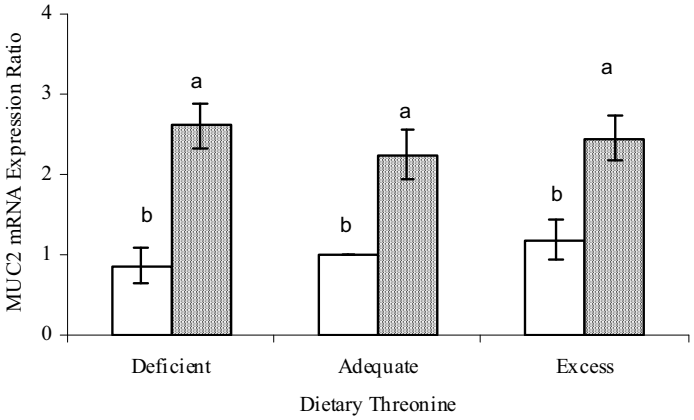


Figure 3.7 Effects of dietary threonine level supplemented with (shaded) or without (open) MOS on jejunal MUC2 mRNA expression of broiler chickens at 14 days of age

Changes in MUC2 mRNA expression normalised to β-actin mRNA and expressed relative to the control (adequate threonine without MOS) were measured using Pfaffl method. Values are means ± SEM, n=4. Only effect of MOS on MUC2 mRNA expression was detected significant at P<0.001. Means on each bar with no common letter differ significantly (P<0.05).

3.3.5 Intestinal growth parameters

Intestinal growth parameters of 14 and 28-day-old broilers as affected by dietary threonine supplemented with or without MOS are shown in Tables 3.7 and 3.8. The absolute values of length and component weight (data not shown) were not significantly modified by any treatment. However, these values became statistically significant when expressed relative to body weight. The length as well as the external muscularis and mucosa weights of all the three small intestinal regions was higher (P<0.05) in threonine-deficient birds than those in threonine-supplemented groups (Tables 3.7 and 3.8). Conversely, MOS reduced (P<0.05) the duodenal length as well as the relative mucosa weight of the ileum at d 28, and the relative muscularis weight of all the three regions of the small intestine at d 14. There were no interactive effects between MOS and threonine on intestinal growth parameters except at d 14 where relative ileal length and, jejunal and ileal muscularis weights were significantly (P<0.05) reduced in the MOS-threonine-excess group (Tables 3.7 and 3.8).

Table 3.7 Effects of dietary threonine level and supplementation of MOS on relative length of small intestinal regions of broilers at 14 and 28 days of age[1]

Treatment		Relative length (cm/100g body weight)					
Thr	MOS	Duodenum		Jejunum		Ileum	
		14d	28d	14d	28d	14d	28d
Deficient		5.41[a]	2.10[a]	12.51[a]	4.99[a]	12.02	4.95
Adequate		4.81[b]	1.91[b]	10.75[b]	4.28[b]	10.52	4.45
Excess		4.92[b]	1.87[b]	10.76[b]	4.38[b]	11.17	4.46
	-	5.25[a]	2.02	11.61	4.51	11.48	4.46
	+	4.84[b]	1.90	11.07	4.60	11.00	4.78
Deficient	-	5.53	2.13	12.54	5.02	11.78[a]	4.69
Deficient	+	5.30	2.06	12.49	4.44	12.26[a]	5.20
Adequate	-	5.02	1.91	11.22	4.97	10.62[b]	4.31
Adequate	+	4.60	1.91	10.29	4.40	10.42[b]	4.58
Excess	-	5.20	2.01	11.09	4.13	12.04[a]	4.38
Excess	+	4.64	1.83	10.43	4.37	10.31[b]	4.55
SEM		0.178	0.091	0.409	0.207	0.458	0.039
Source of variation		----------Probability----------					
Thr		0.0032	0.0440	0.0001	0.0025	0.0055	NS
MOS		0.0072	NS	NS	NS	NS	NS
Thr x MOS		NS[2]	NS	NS	NS	0.0500	NS

[1]Each value represents the mean of 6 replicates for each treatment group.
[2]NS=Not significant.
[a-d]Means within a continuous column with different superscripts are significantly different (P<0.05).

Table 3.8 Effects of dietary threonine level and supplementation of MOS on relative weight of small intestinal mucosa and muscularis externa of broilers at 14 and 28 days of age[1]

Treatment		Relative mucosal weight (g/100 g BW)						Relative muscularis weight (g/100 g BW)					
Thr	MOS	Duodenum		Jejunum		Ileum		Duodenum		Jejunum		Ileum	
		14d	28d	14d	28d	14d	28d	14d	28d	14d	28d	14d	28d
Deficient		0.95	0.58^{a}	1.08^{a}	0.96^{a}	0.58^{a}	0.48^{a}	0.76^{a}	0.33	1.33	0.77^{a}	1.23	0.80^{a}
Adequate		0.93	0.48^{b}	0.97^{ab}	0.82^{b}	0.43^{b}	0.40^{b}	0.65^{ab}	0.33	1.20	0.67^{b}	1.08	0.71^{b}
Excess		0.87	0.47^{b}	0.92^{b}	0.79^{b}	0.44^{b}	0.39^{b}	0.60^{b}	0.31	1.19	0.59^{b}	1.08	0.70^{b}
	-	0.89	0.49	1.03	0.88	0.47	0.45^{a}	0.73^{a}	0.31	1.27	0.67	1.19^{a}	0.72
	+	0.94	0.53	0.95	0.82	0.50	0.40^{b}	0.61^{b}	0.33	1.21	0.68	1.06^{b}	0.74
Deficient	-	0.89	0.53	1.13	0.99	0.54	0.52	0.78	0.33	1.26^{abc}	0.76	1.28^{a}	0.73
Deficient	+	0.10	0.62	1.04	0.92	0.62	0.44	0.74	0.32	1.40^{a}	0.79	1.17^{ab}	0.85
Adequate	-	0.90	0.51	0.93	0.83	0.41	0.41	0.74	0.32	1.25^{bc}	0.65	1.16^{ab}	0.69
Adequate	+	0.96	0.46	0.95	0.80	0.45	0.39	0.55	0.34	1.16^{cd}	0.69	1.12^{b}	0.73
Excess	-	0.88	0.43	0.97	0.83	0.47	0.42	0.66	0.29	1.30^{ab}	0.62	1.13^{b}	0.75
Excess	+	0.85	0.50	0.86	0.75	0.40	0.37	0.53	0.33	1.07^{d}	0.56	0.92^{c}	0.64
SEM		0.049	0.041	0.009	0.053	0.043	0.029	0.067	0.034	0.055	0.048	0.052	0.039
Source of variation		----------Probability----------											
Thr		NS[2]	0.0280	0.0385	0.0010	0.0018	0.0110	0.0500	NS	0.0090	0.0013	0.0069	0.0290
MOS		NS	NS	NS	NS	NS	0.0440	0.0278	NS	0.0124	NS	0.0033	NS
Thr x MOS		NS	NS	NS	NS	NS	NS	NS[2]	NS	0.0021	NS	0.0130	NS

[1]Each value represents the mean of 6 replicates for each treatment group.
[2]NS=Not significant.
[a-d]Means within a continuous column with different superscripts are significantly different (P<0.05).

3.3.6 Enteric morphometric characteristics

At 14 days of age, birds fed the threonine-deficient diets had a lower (P<0.05) number of acidic goblet cells (stained with AB at pH 2.5) in the jejunum, compared to those fed with the two higher levels of threonine (Table 3.9). On the other hand, dietary MOS increased (P<0.05) the acidic and sulphomucin goblet cells densities at d 14 and 28, respectively (Table 3.9). No significant differences in neutral (stained with PAS) and total (stained with PAS-AB, pH 2.5) goblet cells densities attributable to MOS and threonine supplementation were detected. There were also no significant interactions between threonine and MOS on all the goblet cells densities.

Supplementation of MOS consistently increased (P<0.01) the goblet cells' "cup" size (76.60 vs 23.99 μm^2 at d 14 and 80.61 vs 26.15 μm^2 at d 28) and was independent of threonine (Table 3.9). Responses of all the three types of mucin staining intensities and IOD were greatly suppressed by deficiency in dietary threonine, especially at d 14 (Table 3.10). Conversely, the magnitudes of responses on the staining intensity of sulphomucins to MOS were more pronounced at d 28 (P<0.01) (Table 3.10 and Plate 3.3). At d 28, the IOD of sulphomucin goblet cells was also significantly increased (P<0.01) in the MOS supplemented group, compared to the other non-supplemented groups (Table 3.10).

Table 3.11 shows the gut morphology data. Birds fed the low-threonine diets had thicker (P<0.05) jejunal muscularis externa at d 28 and shorter (P<0.01) jejunal villi at d 14 and 28. On the contrary, MOS-fed groups had thinner (P<0.01) jejunal muscularis externa at d 14 and longer villi at both d 14 and 28. In addition, MOS interacted with threonine at supplementary levels to increase the villus height at d 14 (P<0.05). No significant differences in crypt depth attributable to the treatments were observed.

Table 3.9 Effects of dietary threonine level and supplementation of MOS on jejunal goblet cell density and cell "cup" size of broilers at 14 and 28 days of age[1]

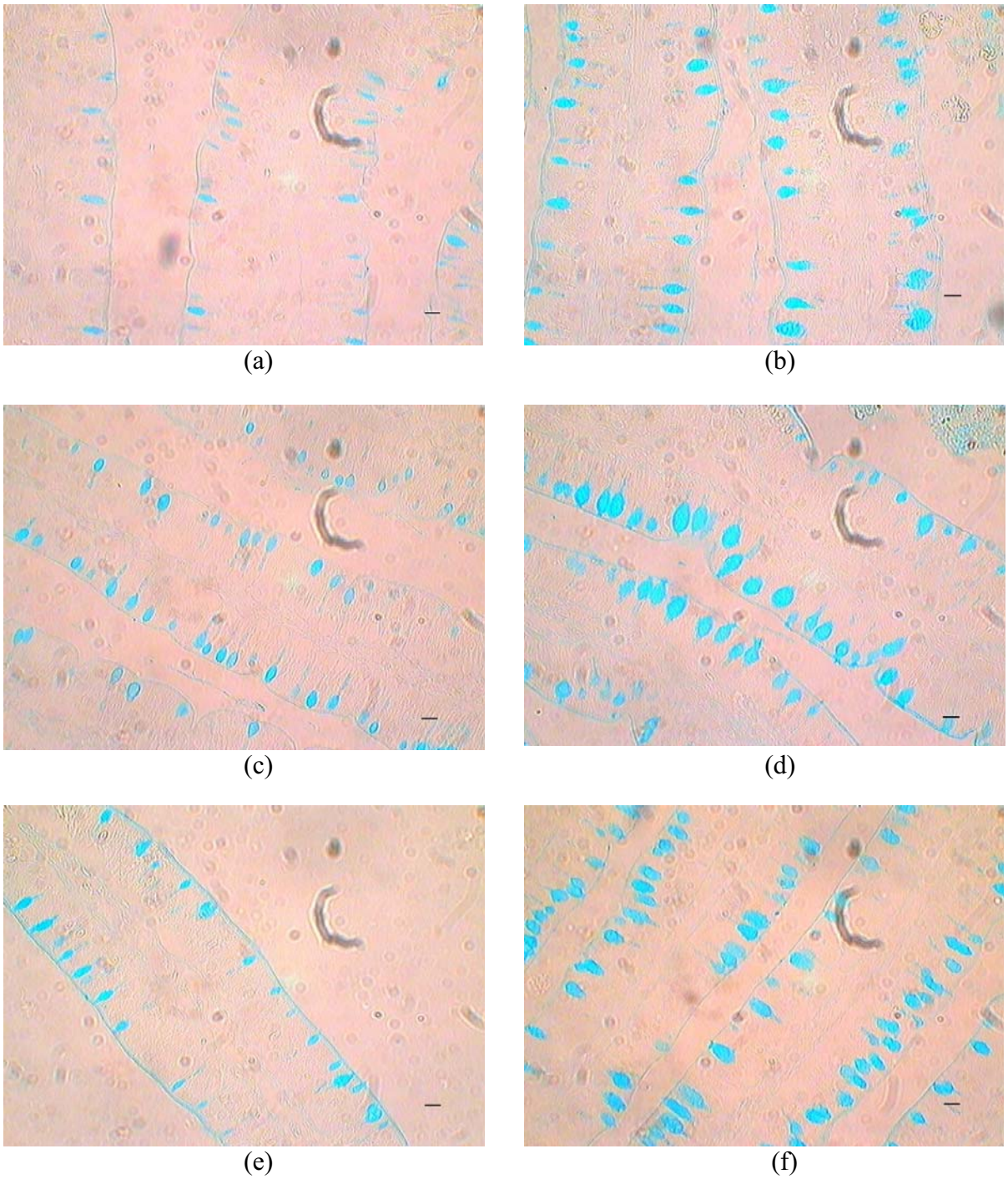
Treatment		Goblet cell density (number/mm^2)								Goblet cell "cup" size (μm^2)	
Thr	MOS	AB, pH 1.0 (Sulphated-acidic)		AB, pH 2.5 (Acidic)		PAS (Neutral)		PAS AB, pH2.5 (Total)			
		14d	28d	14d	28d	14d	28d	14d	28d	14d	28d
Deficient		1633	1376	1678[b]	1469	1323	2198	1588	1684	43.78	54.83
Adequate		1863	1576	2195[a]	1748	1609	2158	1880	2141	51.56	58.23
Excess		1803	1523	1918[a]	1772	1877	2120	1924	1948	55.61	47.08
	-	1799	1410[b]	1772[b]	1608	1700	2277	1760	1920	23.99[b]	26.15[b]
	+	1734	1573[a]	1999[a]	1718	1505	2040	1934	1928	76.60[a]	80.61[a]
Deficient	-	1688	1356	1774	1428	1276	1903	1695	1885	23.63	28.07
Deficient	+	1579	1396	1982	1511	1370	1892	1781	1792	63.81	81.59
Adequate	-	1946	1490	2112	1592	1728	2209	1896	2114	23.49	29.20
Adequate	+	1780	1662	2278	1903	1489	2106	1864	2167	76.64	87.25
Excess	-	1762	1384	1730	1804	2097	2118	1990	1811	24.86	21.28
Excess	+	1844	1663	2305	1740	1656	2122	1957	2085	86.36	72.98
SEM		116.2	110.1	210.5	115.2	189.1	152.9	201.8	187.2	7.049	5.919
Source of variation		----------Probability----------									
Thr		NS[2]	NS	0.0390	NS	NS	NS	NS	NS	NS	NS
MOS		NS	0.0410	0.0027	NS	NS	NS	NS	NS	0.0001	0.0001
Thr x MOS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[1]Each value represents the mean of 4 replicates for each treatment group.
[2]NS=Not significant.
[a,b]Means within a continuous column with different superscripts are significantly different (P<0.05).

Table 3.10 Effects of dietary threonine level and supplementation of MOS on jejunal goblet cell staining intensity and integrated opaque density of broilers at 14 and 28 days of age[1]

Treatment		Goblet cell staining intensity (Log_{10} % transmittance allowed)						Goblet cell integrated opaque density x 10^3 (IOD/mm^2)					
Thr	MOS	AB, pH 1.0 (Sulphated-acidic)		AB, pH 2.5 (Acidic)		PAS (Neutral)		AB, pH 1.0 (Sulphated-acidic)		AB, pH 2.5 (Acidic)		PAS (Neutral)	
		14d	28d	14d	28d	14d	28d	14d	28d	14d	28d	14d	28d
Deficient		0.16	0.15	0.13^{b}	0.22	0.25^{b}	0.30	14.35^{b}	13.80^{b}	14.30^{b}	21.32	30.00^{b}	43.40
Adequate		0.22	0.23	0.22^{a}	0.26	0.29^{a}	0.31	27.15^{a}	24.52^{a}	29.90^{a}	34.00	56.84^{a}	44.31
Excess		0.20	0.20	0.14^{b}	0.24	0.28^{a}	0.30	19.69^{a}	20.64^{a}	18.30^{ab}	32.52	36.59^{b}	42.13
	-	0.20	0.16	0.18	0.26	0.27	0.29	21.90	15.13^{b}	25.00	28.65	45.01	43.00
	+	0.19	0.22	0.42	0.22	0.28	0.31	18.89	24.20^{a}	16.63	30.00	37.65	43.00
Deficient	-	0.16	0.14^{b}	0.14	0.30	0.25	0.30	17.12	11.63	18.22	28.84	34.82	48.38
Deficient	+	0.16	0.16^{b}	0.12	0.13	0.25	0.30	11.54	15.97	19.38	13.81	25.91	38.65
Adequate	-	0.22	0.21^{a}	0.27	0.22	0.29	0.29	27.72	21.05	37.96	24.79	62.88	43.29
Adequate	+	0.21	0.25^{a}	0.17	0.29	0.29	0.33	26.60	27.99	31.44	43.22	50.80	45.32
Excess	-	0.20	0.14^{b}	0.14	0.24	0.28	0.28	20.83	12.65	18.61	32.06	38.80	38.78
Excess	+	0.19	0.26^{a}	0.14	0.25	0.29	0.31	28.56	28.62	18.95	37.00	34.18	45.47
SEM		0.022	0.019	0.018	0.043	0.011	0.013	0.519	0.009	0.572	0.012	0.633	0.008
Source of variation		----------Probability----------											
Thr		NS[2]	0.0008	0.0121	NS	0.0266	NS	0.0446	0.0051	0.0500	NS	0.0028	NS
MOS		NS	0.0011	NS	NS	NS	NS	NS	0.0009	NS	NS	NS	NS
Thr x MOS		NS	0.0157	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[1]Each value represents the mean of 4 replicates for each treatment group.
[2]NS=Not significant.
[a-c]Means within a continuous column with different superscripts are significantly different ($P<0.05$).



(a) (b)

(c) (d)

(e) (f)

Plate 3.3 Representative micrographs showing the effects of dietary threonine and MOS supplementation on sulphomucin (stained with AB at pH 1.0) goblet cell density, "cup" size and staining intensity in the jejunum of broiler chickens at 28 days of age

Treatment diets: (a) deficient threonine without MOS, (b) deficient threonine with MOS, (c) adequate threonine without MOS, (d) adequate threonine with MOS, (e) excess threonine without MOS and (f) excess threonine with MOS. Magnification x400; bar=10 μm.

Table 3.11 Effects of dietary threonine level and supplementation of MOS on jejunal morphometric parameters of broilers at 14 and 28 days of age[1]

Treatment		Jejunal morphometric parameters							
Thr	MOS	Muscularis thickness (μm)		Crypt depth (μm)		Villus height (μm)		Villus/Crypt ratio	
		14d	28d	14d	28d	14d	28d	14d	28d
Deficient		201	245[a]	204	185	837	980[b]	4.12	5.93
Adequate		204	228[b]	228	220	991	1276[a]	4.28	5.89
Excess		197	207[c]	216	205	917	1304[a]	4.30	6.36
	-	233[a]	212	203	211	825	1052[b]	4.09	5.03[b]
	+	168[b]	235	229	197	1006	1321[a]	4.38	7.09[a]
Deficient	-	236	211	201	201	799[c]	869	4.02	4.33
Deficient	+	165	280	208	169	875[bc]	1091	4.22	7.53
Adequate	-	239	239	214	227	924[b]	1149	4.39	5.11
Adequate	+	169	218	241	213	1058[a]	1403	4.18	6.67
Excess	-	224	186	196	204	750[c]	1138	3.86	5.65
Excess	+	170	207	236	207	1084[a]	1470	4.74	7.09
SEM		17.2	19.8	15.8	22.2	42.1	82.7	0.432	1.041
Source of variation		----------Probability----------							
Thr		NS[2]	0.0500	NS	NS	0.0050	0.0009	NS	NS
MOS		0.0001	NS	NS	NS	0.0001	0.0004	NS	0.0150
Thr x MOS		NS	NS	NS	NS	0.0139	NS	NS	NS

[1]Each value represents the mean of 4 replicates for each treatment group.
[2]NS=Not significant.
[a-c]Means within a continuous column with different superscripts are significantly different ($P<0.05$).

3.3.7 Economic response analysis

The economic model employed in this study takes into consideration the monetary inputs typical in the Australian broiler industry. As revealed by the regression equations, the economical optima with or without MOS supplementation are near the respective threonine optima for BWG and FCR (Figure 3.8). In this respect, feeding of MOS from d 1 to 35 with dietary threonine level at 104% of NRC recommendation, compared to the normal 100% NRC recommended feeding level, resulted in an increase of 8.5% [(1.164-1.073)/1.073 x 100] in economic response contributing to an overall improvement in net profit margin by AUD 0.09/bird (1.164–1.073).

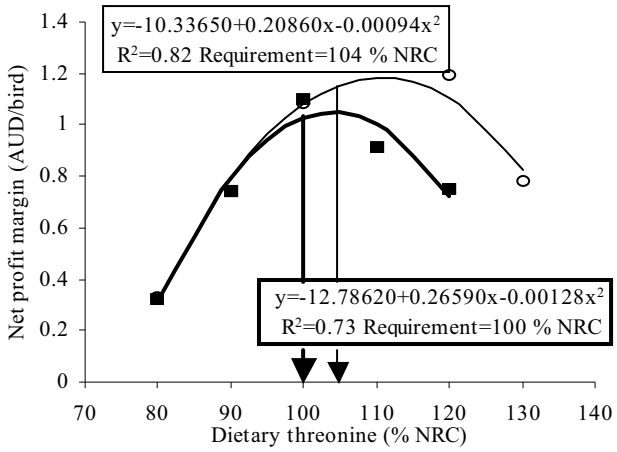


Figure 3.8 Estimated threonine requirement for the optimisation of net profit return of broiler chickens from 1 to 35 days of age

Values were determined by taking 95% upper asymptote of quadratic regression of net profit margin/bird plotted as a function of dietary threonine level expressed as % of NRC recommendation with (---) and without (—) MOS supplementation. Observed mean values (n=6) at different dietary threonine levels with (○) and without (■) MOS supplementation are also shown.

3.4 DISCUSSION

3.4.1 Dietary composition and treatment design

There was a high degree of consistency in the calculated and analysed CP and threonine values for all diets except the one without MOS at 120% NRC recommended threonine level. It is likely that the 7.8% difference in analysed threonine value for this diet was due to sampling error as the other MOS-threonine-excess (120% NRC) grower diet which was prepared from the same titration diet was analysed to have the threonine level close to the calculated value.

3.4.2 Growth performance

Detrimental effects of threonine deficiency on the growth performance were expected as threonine is extensively involved in the synthesis of immunoglobulins (Bhargava *et al.*, 1971; Jamroz, 2005), muscle (Wang *et al.*, 2007), mucin glycoproteins and digestive enzymes (Stoll *et al.*, 1999; van der Schoor *et al.*, 2002; Hamard *et al.*, 2007). Thus, a lack of threonine would result in growth suppression (Kidd *et al.*, 1999; Corzo *et al.*, 2003; Kidd *et al.*, 2003b). It is also generally agreed that threonine is highly retained in the gut and is in high demand by the gut for the synthesis of mucosal protein and mucin glycoproteins in which threonine represents about 40% of the protein core (Carlstedt *et al.*, 1993; Nichols and Bertolo, 2008).

In the study on dietary threonine requirement, the strong quadratic responses noted for BWG and FCR also indicate the presence of diminishing returns associated with the excessive levels of threonine in the diet. This is in concordance with a previous report that diets supplemented with 4 % of L-threonine resulted in 33 and 28% reduction in BWG and FI, respectively (Edmonds and Baker, 1993). An excess of luminal threonine may induce an amino acid imbalance where intestinal uptake of other amino acids is reduced, due to their sharing of the same transport systems (Wu, 1998).

It was reported that growth response to MOS is age-dependent with younger birds responding more than the older ones (Jamroz *et al.*, 2003; Tucker *et al.*, 2003; Yang *et al*., 2007a). A similar trend on this main effect of MOS was also detected in the current experiment. This could be explained by the fact that the gut microflora of younger birds are more transient in nature and less mature than those older birds. Thus, it is postulated that feeding of MOS may help in accelerating the maturation of the gut microflora in younger birds, leading to better growth performance (Yang *et al.*, 2007a). However, a novel and important finding from the current study is the presence of a significant interaction between MOS and threonine at all stages of life, suggesting that the growth-promoting effect of MOS is also threonine-sensitive.

The normal dietary threonine requirement for BWG and FCR, estimated at 62 and 63% of the total lysine for broiler starter, was close to those reported by Rosa *et al.* (2001), Corzo *et al.* (2003), and Çiftci and Ceylan (2004). For the grower phase, the estimated threonine requirement of 63 and 65% of total lysine for the respective BWG and FCR was also in good agreement with those reported by Kidd and Kerr (1997), Corzo *et al.* (2003) and Muhl and Liebert (2007). Although the respective normal dietary threonine requirements of 0.81 and 0.74% total threonine for the body weight gain of broiler starter and grower were near the NRC (1994) recommended levels (0.80 and 0.74%), they were much lower than when expressed as a fraction of the total lysine contents. This is largely because the NRC recommendations for total lysine (1.10% for starter and 1.00% for grower) are generally considered to be too low for today's lean genotype (Dozier *et al.*, 2007).

A greater response in growth towards MOS with only a marginal change in threonine need generally suggests the adequacy of normal dietary threonine level for MOS to optimise the growth of younger birds. In order to make valid estimations on threonine requirements for older birds, it would have been preferable to use more dose-response points and chicks

grown on the same starter diet. Nonetheless, despite these limitations, use of covariant adjusted regression revealed a strong quadratic growth response of broiler growers towards threonine both with and without MOS supplementation. A significant increase in the growth performance of MOS-treated adult birds in response to a higher inclusion of dietary threonine further confirms the presence of a positive interaction between threonine and MOS at this stage of growth. It is possible that in older birds the lack of a homeostatic mechanism in regulating their body threonine (Yamashita and Ashida, 1971) coupled with the increase in threonine need for mucosal maintenance and growth (Kidd and Kerr, 1996; Samadi and Liebert, 2008) may have rendered the change in metabolic requirement for threonine even more sensitive to the modulating effects of MOS on mucin dynamics.

3.4.3 Mucin synthesis and mucin gene expression

The negative effects of threonine deficiency on intestinal adherent mucous thickness observed in this study further support the findings of Fuller *et al.* (1994), Stoll *et al.* (1998), and Wang *et al.*(2007). It is likely that under such conditions, no compensatory mechanism was operating at the whole-body level to mobilise threonine and thereby supply the gut with enough threonine (Faure *et al.*, 2005). Excessive feeding of threonine resulted in a diminishing return in mucin synthesis. This is probably due to the presence of an upward limitation in the adaptive regulation of intestinal amino acid catabolism in response to high amino acid intakes. As a result, the surplus of luminal amino acids might eventually challenge the capacity for amino acid catabolism in the portal-drained viscera and liver to an extent that would result in overt toxicity (Yu *et al.*, 1990; Hoerr *et al.*, 1993; Faure *et al.*, 2005).

In contrast to the quadratic response seen on jejunal adherent mucous thickness, fluctuations in dietary threonine levels did not result in any significant change in MUC2 mRNA expression, indicating that theonine may exert its influence on mucin synthesis only at the translational level and not at the transcriptional level. Faure *et al*. (2005) and Todd (2008) also reported similar findings where intestinal MUC2 mRNA expressions were not affected by dietary threonine restriction or supplementation.

Conversely, a constant up-regulation of intestinal MUC2 mRNA expressions independent of threonine observed in birds fed the MOS-supplemented diets strongly suggests that the modulating effects of MOS on mucin dynamics are more likely to occur at the transcriptional level. During dietary threonine restriction, the up-regulation of MUC2 mRNA

expression induced by MOS did not correspond to an expected increase in thickness of intestinal adherent mucous layer. It is likely that under such a condition, mucin production was limited at the translational level because globally, MUC2 mRNA levels were similar among groups. Therefore, it can be deduced that the enhancement of MUC2 mRNA expression by MOS would not translate into an overall improvement in mucin synthesis unless luminal threonine is readily available to act as the substrate for the completion of this anabolic process.

The modulating effects of MOS on intestinal mucin dynamics observed in this study further supports the finding of Uni and Smirnov (2006) who reported a significant increase in jejunal adherent mucous thickness coupled with a constant up-regulation of MUC2 mRNA expression by MOS. It is still unknown whether MOS exerts this positive effect through a direct effect on the epithelial cells or indirectly through the modification of intestinal microflora.

It is interesting to note that, regardless of MOS supplementation, similar trends in threonine demand for BWG and FCR were observed for the optimisation of jejunal adherent mucous thickness. Faure *et al.* (2005) also observed a quadratic response in fractional synthesis rate of intestinal mucins in rats fed diets with graded levels of threonine. This correlation points to the importance of optimising intestinal mucous layer for the maximisation of growth. The same explanation for observations made on extra threonine demanded by MOS for growth can also be applied to the case of mucous production.

A significant increase in adherent mucous thickness is important as the main function of the mucus is to protect the mucosa from digestive proteases, bacterial translocations and prevent dehydration of the underlying mucous membranes (Toribara *et al.*, 1993). Mucus also supports a group of mucus and crypt dwelling microorganisms that competitively exclude mucus-colonising pathogens such as *Campylobacter jejuni* and *Salmonella enteritidis* from colonising the chicken gut (Hariharan *et al.*, 2004). Furthermore, intestinal mucus as a lubricant also aids the passage of digesta along the digestive tract, thus preventing stagnation and bacterial overgrowth. The establishment of sound gut integrity through restoration of a balanced resident microflora would generally mean a lower tissue turnover rate, less competition for vital nutrients between the bird and the microbes (Ferket, 1991) and less mucosal inflammatory immune response (Klasing, 1988; Cook, 2000). These, in turn, help to

save on nutrients that can be made readily available for more productive purposes such as muscle accretion which would otherwise be wasted (Bedford, 2000).

The adherent mucous layer appears to constitute a diffusion barrier between luminal nutrients and digestive and transport sites on the brush border membrane of the enterocytes. However, it has also been well understood that mucous gels provide a microclimate, which differs from the luminal bulk phase in their physiochemical properties. This microclimate is pH constant and acts as a medium in which digestion and absorption processes occur close to the brush border membrane and helps to transport nutrients between the luminal contents and epithelial lining (Forstner *et al.*, 1995). In addition, mucin glycoproteins can act as a selective barrier to the absorption of certain nutrients, like water and electrolytes (Forstner, 1978; Proust *et al.*, 1984).

3.4.4 Enteric morphometric characteristics

Dietary threonine was found to have little effects on the density of all types of goblet cells but significantly affected their staining intensities. The first part of these observations concurs with those of Hamard *et al*. (2007). Again, this finding reiterates the importance of threonine in serving as a substrate for the synthesis of mucin glycoproteins.

Excessive threonine ingestions did not result in any further increase in the goblet cell density and mucin staining intensity depicting the existence of an upward limitation in the regulation of mucin dynamics. The ability of MOS to differentially enhance the expression of acidic and sulphomucin goblet cell densities is another important contribution of MOS in conferring a better protection to the gut against bacterial translocation. It is known that acidic mucins, in particular the more mature sulphate subtypes, are highly viscous in nature and thus, are less degradable by bacterial glycosidases and host proteases (Robertson and Wright, 1997; Fontaine *et al.*, 1998). The increase in goblet cell "cup" size by MOS might also have compounded the effect of an already increased sulphomucin goblet cells density in expanding the mucin storage capacity as indicated by a higher value of sulphamucin IOD.

The negative effect of threonine deficiency on intestinal villus height agrees with the observation made by Hamard *et al.* (2007). Iji *et al.* (2001b), and Yang *et al.* (2007a) also reported similar findings on the effect of MOS in improving the small intestinal villus height. The increased height of villi points to the increased absorptive area and improved gut function, which might have contributed to the overall improvement in BWG and FCR

frequently seen in birds supplemented with MOS. It is likely that threonine might have a direct effect on the intestinal morphology mediated through its obligatory involvement in the fractional as well as absolute synthesis of intestinal mucosa and mucins (Faure *et al.*, 2005; Wang *et al.*, 2007; Nichols and Bertolo, 2008), whereas MOS might exert its main influence on villus morphology via its modulating effects on intestinal mucin dynamics and microbial-mucosal interactions (Uni and Smirnov, 2006).

The decrease in thickness of jejunal external muscularis induced by MOS further confirms the observation of Ferket *et al*. (2002) and it is likely to be associated with an overall improvement in gut viability where there is a reduced need for gut motility to control gut microbial activities.

3.4.5 Intestinal growth parameters

In the present investigation, it was observed that the trend in modification of relative component weight of the small intestine occurred more as a function of the body weight rather than the actual weight of the gut tissue itself. This is in contrast to observations in young pigs where dietary threonine levels did not affect the relative weight of the gut tissues (Hamard *et al.*, 2007; Wang *et al.*, 2007). This discrepancy is possibly due to the absence of a significant improvement in the gross body weight as pigs are generally more tolerant to excess feeding of threonine (Edmonds and Baker, 1993; Baker, 2004; Wang *et al.*, 2007).

During the restriction of dietary threonine, an increase in mucosal degradation rate (Visek, 1978; Law *et al.*, 2007) and a reduction in mucosal fractional synthesis rate (Faure *et al.*, 2005; Wang *et al.*, 2007; Nichols and Bertolo, 2008) may have caused more wastage of net energy and amino acids (Stoll *et al.*, 1997; Bruins *et al.*, 2000; Wang *et al.*, 2007), resulting in a poorer body weight gain and thus, significantly higher relative gut tissue weights.

The reduction in the relative external muscularis weight of birds fed with MOS further re-affirms the thinning effect of MOS on intestinal muscularis layer, especially at earlier stage of bird life (Ferket *et al.*, 2002). It also implies that MOS-supplemented birds receiving an adequate level of dietary threonine had a lower gut mass to maintain. Correspondingly, the net energy spared from gut maintenance can be made readily available for growth (Stutz and Lawton, 1984; Iji *et al.*, 2001a; Miles *et al.*, 2006).

3.4.6 Economic Responses

Although the economic model employed in the present study takes into account the monetary inputs typical in the Australian broiler industry, the growth responses, as affected by threonine and MOS, incorporated in the current model were controlled experimental data which may not reflect the actual variations seen in the field. However, it is imperative to note that the economical optima estimated in the current study are near the threonine optima for BWG and FCR in both MOS-treated and untreated groups.

Although the economic inputs reflect typical prices within the Australian context, farm prices and feed costs may vary greatly depending on market fluctuations in the commodity prices and competitive pressures on farm prices. Thus, the costs of increasing the threonine and MOS minima may differ from that projected in the current model of AUD 0.0004 and 0.012/kg of diet for each 0.01 and 0.1% increase in total dietary threonine and MOS, respectively. Nonetheless, in line with the results of Kidd *et al*. (1999), the economic model used in this study demonstrates the importance of feeding adequate dietary threonine to ensure economic profitability. Although there is only a marginal gain of AUD 0.09/bird in net profit margin, a significant improvement of 8.5% in economic response may justify the benefit of feeding MOS with dietary threonine level adjusted to 104% NRC recommendation from the normal 100% NRC recommendation.

3.5 CONCLUSIONS

The findings from this study strongly indicate the importance of optimising intestinal mucin dynamics for the maximisation of growth. Dietary threonine exerts its effects on growth performance by influencing intestinal growth through its direct involvement at the translational stage of intestinal mucin synthesis. The growth-promoting effects of MOS were both age-dependent and threonine-sensitive, especially at the later stage of growth. Thus, feeding of MOS with threonine supplemented to meet the extra amount demanded by MOS resulted in additional BWG and better FCR and was economically justified based on the improved net profit return. It is likely that the interaction between MOS and threonine on growth is linked to its modulating effects on mucin dynamics where a constant up-regulation of MUC2 mRNA expressions in the absence of adequate threonine supply would not translate into an overall improvement in mucin production. MOS was also found to increase the goblet cell “cup” size and selectively enhance the differentiation of acidic and sulphated-

acidic mucins goblet cells. Furthermore, MOS was also observed to have additional effects in reducing the jejunal external muscularis weight and thickness of the younger birds.

Therefore, future studies are warranted to explore further the interactions between MOS and threonine on other physiological parameters such as feed passage rate, standardised ileal digestibility of amino acids and intestinal nutrient uptake. In addition, detail studies designed to determine whether MOS influenced intestinal mucin dynamics directly or indirectly through the modulation of intestinal microflora is also deemed necessary.

CHAPTER 4
CHARACTERISATION AND RESPONSE OF INTESTINAL MICROFLORA AND MUCINS IN BROILER CHICKENS ON DIETS SUPPLEMENTED WITH MANNOOLIGOSACCHARIDES AND ANTIBIOTIC

4.1 INTRODUCTION

As shown in Chapter 3, MOS-induced growth response is age-dependent, with younger birds responding more than older ones. This may be related to a progressing maturation of gut microflora in younger birds. The profile of the gut microflora continues to change as birds grow but at a very gradual pace. For example, it takes about 2 weeks for lactobacilli to become the predominant bacteria in the small intestine (Barnes *et al.*, 1972). Therefore, it is proposed that MOS exerts its influence on growth by accelerating the maturation of both the gut microflora as well as the GIT (Solis de los Santos *et al*., 2007; Yang *et al.*, 2007a).

Dietary MOS is also known to modulate the intestinal mucin synthesis by up-regulating the MUC2 mRNA expression, enhancing the goblet cell density as well as selectively differentiating the expression of acidic and sulphomucin goblet cell types (Chapter 3). The actual underlying mechanism involved in the modulation of intestinal mucin dynamics by MOS is unknown. The physiologic relevance of distinct mucin subtypes is likely linked to the intestinal microflora. It is suggested that acidic mucins confer a better protection against bacterial translocations through the epithelium because particularly, sulphated subtypes appear to be less degradable by bacterial glycosidases and host proteases (Robertson and Wright, 1997; Fontaine *et al.*, 1998). There is also evidence for the presence of a direct crosstalk between intestinal microbes and goblet cells (Freitas *et al.*, 2003). For example, it has been reported that *Lactobacillus* strains can prevent the attachment of enteropathogenic *E. coli* to the HT29 epithelial cells through a post-transcriptional up-regulation of MUC2 and MUC3 gene expressions (Mack *et al.*, 1999).

It is hypothesised that MOS helps to establish a functional ecosystem between the host and the intestinal microflora through intestinal mucins (Uni and Smirnov, 2006). Thus, the current study was designed to use the conventional culture-based technique for the assessment of MOS-induced changing pattern of the gut microflora, in comparison to ZnB, in younger

birds. A molecular technique, the terminal-restricted fragment length polymorphism (T-RFLP), was also employed to aid in providing precise bacterial community fingerprints for the characterisation of the mature patterns of gut microflora attributable to the treatments. In order to further substantiate this newly defined mode of action of MOS, enteric morphometric parameters were also characterised and discussed in line with any detectable change in the constituents of the gut microflora.

4.2 MATERIALS AND METHODS

4.2.1 Bird management and experimental diets

One hundred and forty four (144) day-old male Cobb broiler chicks (Baiada Poultry Pty. Ltd., Kootingal, NSW, Australia), weighing 44.25 ± 0.29 g, were randomly assigned to three treatment groups consisting of a negative control (NC), a positive control supplemented with zinc bacitracin (Albac 15%, Alphama, Australia) at 50 ppm (ZnB), and another group supplemented with mannooligosaccharides (Bio-MOS®, Alltech Biotechnology, Nicholasville, Kentucky, USA) at 0.20% diet (MOS). Each treatment had 6 replicates of 8 birds per cage measuring 600 x 420 x 230 cm in dimension. All the brooding cages were housed in an environmentally controlled room with initial room temperature of 34°C maintained for the first 5 days and then gradually decreased to 21°C by the end of the experiment at week 3. Birds were provided *ad libitum* access to feed and water. The lighting programme was 24 h light for the first day followed by 18 h light and 6 h darkness for the rest of the trial period. The experimental starter diets, consisting mainly of corn and soybean meal, were used throughout the grow-out period and are shown in Table 4.1.

4.2.2 Animal ethics

All experimental procedures were approved by the Animal Ethics Committee of the University of New England with the approval number AEC 06/143.

4.2.3 Growth performance measurement

Growth performance parameters were assessed as described in Section 3.2.4.

4.2.4 Sample collection

Three birds per cage were randomly selected, weighed and euthanised by cervical dislocation at 7 and 21 days of age. The small intestine was excised and ileum, from Meckel's

Table 4.1 Composition of experimental starter diets

Ingredient	NC	MOS	ZnB
	----------%----------		
Corn	63.50	63.50	63.50
Soybean Meal Dehulled, 47.5% CP	29.39	29.39	29.39
Oil, vegetable	2.26	2.26	2.26
Limestone	1.06	1.06	1.06
Dicalcium phosphate	1.86	1.86	1.86
L-Threonine	0.10	0.10	0.10
L-Lyscine-HCl	0.39	0.39	0.39
L-Methionine	0.26	0.26	0.26
Salt	0.19	0.19	0.19
Sodium bicarbonate	0.25	0.25	0.25
Choline chloride	0.05	0.05	0.05
Filler, Zeolite	0.50	0.30	0.47
MOS	-	0.20	-
Zinc Bacitracin	-	-	0.03
Vitamin and mineral premix[1]	0.20	0.20	0.20
Total	100.00	100.00	100.00
Calculated nutrient composition	----------%----------		
Crude protein	20.00	20.00	20.00
Crude fibre	3.63	3.63	3.63
Crude fat	5.01	5.01	5.01
Lysine	1.30	1.30	1.30
Methionine + cystine	0.89	0.89	0.89
Threonine	0.85	0.85	0.85
Calcium	0.90	0.90	0.90
Available phosphorus	0.45	0.45	0.45
Sodium	0.16	0.16	0.16
Chloride	0.23	0.23	0.23
ME poultry, kcal/kg	3050	3050	3050

[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.

diverticulum to the ileo-caecal junction, was dissected out. The caeca were also separated from the ileo-caecal junction. Approximately 2 cm of the mid-point ileum were collected from the first bird of each replicate, flushed with ice-cold buffered phosphate saline at pH 7.4 and immediately fixed in 10% formalin solution for villus and mucin morphometric analyses.

Caecal contents were inverted directly into a sterile container. Ileal contents were collected into sterile containers by carefully cutting open the remaining parts of the ileum longitudinally without damaging the mucosal layer. The respective ileal and caecal contents from the same replicate were combined, homogenised and divided into 2 equal aliquots. One

aliquot of the sample was immediately frozen in liquid nitrogen and kept at –20°C for subsequent DNA extraction. About 1 g of fresh samples from another aliquot were further diluted in 15 mL MacCartney bottles containing 10 mL of anaerobic broth prepared as described by Yang (2007) for bacterial cultivation and enumeration.

Two strips of the emptied mid ileum, each measuring about 10 cm, were dissected and processed to harvest ileal mucosa-associated bacterial cells. One section of the emptied ileum was briefly washed with 0.9% saline solution and gently scraped off its mucosa by using a sterile microscopic slide. The mucosal samples were collected into a sterile container for direct bacterial cultivation and enumeration. Another section of the emptied ileum was processed to harvest ileal mucosa-associated bacterial cells for DNA extraction. It was prepared essentially by the method of Gong *et al*., (2002b). Briefly, the samples were washed three times in sterile 0.9% saline solution to remove any unattached or loosely attached bacteria from the ileal wall. Bacterial cells were then released from the ileal wall by two washes in sterile 0.9% saline solution containing 0.1% Tween 80 with vigorous hand shaking for 30 s per wash. The homogenates were then centrifuged (27,000 *g*, 20 min) at 4°C to pellet the bacterial cells.

4.2.5 Media and culture techniques

A suspension of the diluted digesta or mucosal scraping was transferred into a plastic bag and homogenised under CO_2 atmosphere for 2 min using a bag mixer (Interscience, St. Norm, France). The homogenised suspension was then 10-fold diluted by mixing 1 mL of the homogenised suspension with 9 mL of anaerobic broth and serially diluted from 10^{-1} to 10^{-5}. From the last three diluted samples, 0.1 mL each, were plated on the appropriate medium for enumeration of microbial populations except mucosal samples where samples from the first three dilutions were plated.

Total anaerobic bacteria were determined using anaerobic roll tubes containing Wilkins-Chalgren anaerobe agar (CM 0619) incubated at 37 °C for 7 d. Lactobacilli, lactic acid bacteria and *C. perfringens* were enumerated on Rogosa agar (CM 0627), De Man Rogosa and Sharp (MRS) agar (CM 0361), and perfringens agar (CM 0587 TSC and SFP) incubated anaerobically at 39°C for 48 h except for *C. perfringens,* which were incubated for 24 h. Coliforms (red colonies) and lactose-negative enterobacteria (colourless colonies) were grown on MacConkey agar (CM 0007) incubated aerobically at 39°C for 24 h. All the media

were obtained from Oxoid (Basingstoke, Hampshire, UK). After the incubation, colonies formed on the respective media were carefully counted, converted into logarithmic equivalents and expressed as number of colony forming units (CFU) per gram of wet intestinal content.

4.2.6 Enteric morphometric analysis

All the ileal samples were analysed for villus and goblet cell morphometric parameters using the same techniques as described in Section 3.2.7. In addition, two combined staining techniques were also included: (1) high iron diamine (HID, 16 h at room temperature) and Alcian blue, pH 2.5 (AB, 5 min) for the differentiation of carboxylated- and/or sialic (blue) and sulphated- (brown) acidic mucins, and (2) PAS with mild acid hydrolysis treatment (PAS-MA, 0.5% sulpharic acid for 1 h at 80°C) for the counting of pure hexose containing neutral mucins (Kierrnan, 1990).

4.2.7 Terminal-restriction fragment length polymorphism (T-RFLP)

DNA extraction

Bacterial genomic DNA was extracted using QIAamp® DNA Stool Mini Kit (QIAGEN GmbH, Hilden, Germany) as directed by the supplier with slight modifications. Briefly, 0.2 g of the digesta or ileal mucosal extract was homogenised in buffer ASL and heated at 95°C for 5 min. In order to improve the lysis of bacterial cells, 140 μL of 10 mg/mL lysozyme (Sigma-Aldrich Chemicals, St Louis, MO, USA) were added to the homogenates and incubated at 37°C for 30 min. InhibitEx tablets were added to remove any potential inhibitors and the lysates obtained were then treated with proteinase K and buffer AL and incubated at 70°C for 10 min to remove the protein and polysaccharides. Subsequently, the DNA were precipitated using ethanol and washed with buffer AW1 and AW2 before dissolving in buffer AE. To stabilise the purified DNA, 4 μL of 40 mg/mL Bovine serum albumin (BSA, Sigma-Aldrich) and 2 μL of 40 mg/mL of Ribonuclease-A (Sigma-Aldrich) were added and the DNA preparations were immediately stored at –20°C.

PCR amplification of 16S rRNA

The primers used for the PCR amplification of 16S rRNA (rDNA) gene sequences were supplied by Proligo Australia Pty. Ltd. (Lismore, NSW, Australia). The WellRED D4-PA dye labeled forward primer, S-D-Bact-0008-a-S-20 (5'-AGA GTT TGA TCM TGG CTC AG-3')

and reverse primer, S-D-Bact-0926-a-A-20 (5'-CCG TCA ATT CCT TTR AGT TT-3') were used for the PCR amplificantion (Leser *et al.*, 2000; Gong *et al.*, 2002a; Gong *et al.*, 2002b).

Amplification reactions, in triplicate, were performed each in a total volume of 50 μL containing 50 ng of dissolved DNA, 1.25 U HotStarTaq™ DNA polymerase (QIAGEN GmbH), 5 μL of 10x HotStarTaq™ buffer (QIAGEN GmbH), dNTP mixture of 10 mM each (QIAGEN GmbH), 10 pmol each of the forward and reverse primers, an additional 1.25 mM of Mg^{2+}, 10 μg of BSA (Sigma-Aldrich) and nuclease free water (Ambion, Inc, Austin, TX, USA) to make up the rest of the volume. The 16S rRNA genes were amplified in an Eppendorf thermal cycler (MasterCycler®, Eppendorf AG, Hamburg, Germany) using the following programme: (1) initial DNA incubation at 95^0C for 15 min; (2) 30 cycles of denaturation at 94°C for 30 s, annealing of DNA at 57°C for 45 s and extension at 72°C for 2 min, and (3) a final extension period at 72C° for 10 min. Amplified DNA were verified by subjecting the aliquots of PCR mixtures (2 μL) to 2% agarose gel electrophoresis.

Restriction digests

The PCR products of the triplicates were pooled and purified using QIAquick™ PCR Purification Kit (QIAGEN GmbH) according to the manufacturer's instructions. In brief, a volume of 250 μL buffer PBI and 750 μL buffer PE were added to 50 μL of the PCR products contained in a QIAquick™ spin column and later double centrifuged at 18,000 *g* for 1 min. Subsequently, the purified DNA was eluted in a final volume of 50 μL Buffer EB (10 mM Tris-Cl, pH 8.5). The DNA concentrations were measured and adjusted to approximately 20 ng/μL. About 200 ng of the purified PCR products were digested with 20 U of either *Hha*I or *Msp*I (New England Biolabs, Beverly, MA, USA) in a total volume of 20 μL incubated at 37°C for 12 h followed by enzyme inactivation at 65°C for 20 min (Moyer *et al.*, 1996). To increase the detection of terminal restriction fragment (T-RF) peaks, the digests were desalted by ethanol precipitation method as recommended in the supplier's handbook (Beckman-Coulter Inc., Fullerton, CA, USA) and re-suspended in 40 μL of sample loading solution (Beckman-Coulter Inc.).

T-RFLP analysis

The T-RFs in the range of 50 to 650 base pairs (bp) were determined by mean of capillary electrophoresis on a CEQ 8000™ DNA automatic sequence analyser (Beckman-Coulter Inc.). Briefly, 3 μL of the re-suspended T-RFs digests were mixed with 36.5 μL of sample loading

solution (Beckman Coulter Inc.) and 0.5 μL of 600 DNA size standard mix (Beckman-Coulter Inc.) before loading onto the CEQ 8000™ DNA automatic sequence analyser for the detection of T-RF peaks using the following programme: denatured at 90°C for 2 min followed by injection time of 15 s at 2 kV and separated at 4.2 kV for 85 min with a capillary temperature of 50°C.

The sizes of T-RFs were estimated using Local Southern method of Beckman-Coulter DNA analysis system version 5.0.345 (Beckman-Coulter Inc.). All the T-RFs data including the fluorescence amplitude and peak area were exported out and further analysed according to Kitts (2001) method for normalising and excluding peaks that represent less than 0.5% of the total DNA present in the sample. Fragments were then resolved to ±2 base pair by manual alignment of the standard peak size. Theoretical bacterial species were predicted based on *in silico* restriction of known bacterial 16S rRNA genes using TAP-tTRLP function of the Ribosomal Database Project II (Cole *et al.*, 2003) and public database (http://mica.ibest.uidaho.edu/).

4.2.8 Statistical analysis

Data on growth performance, enteric morphometric parameters and bacterial enumerations were analysed by one-way analysis of variance using the General Linear Models (GLM) procedures of SAS (SAS/STAT® Version 9.1, SAS Institute Inc., Cary, NC, USA). Differences between means were tested using Fisher's least significant difference test.

T-RFLP profiles were converted into binary form for community relatedness analysis using Cluster and Tree procedures of SAS (SAS Institute Inc.) where Jaccard similarity coefficients were determined and dendograms were constructed using Ward algorithm (Blackwood *et al.*, 2003; Lan *et al.*, 2004).

The relative abundances of specific bacterial groups reflected through T-RF peak area expressed as percentage values of the normalised total peak area were compared. Values were calculated as means only for those replicates showing the peaks and compared across treatment groups using Mann-Whitney *U*-test of Nar1way procedure (SAS Institute Inc.) for statistical significance, since we do not know whether the distribution of these datasets are approximate to a normal distribution or not (Lan *et al.*, 2004). The variances of the relative abundance were also compared between treatments by mean of *F* test (SAS Institute Inc.).

Treatment effects on the population of specific bacterial groups were assessed based on the assumption that the proportions of different bacterial groups were conserved in the PCR step. Cumulative T-RF peak areas of all bacterial species belonging to the same bacterial group were calculated as mean for the respective treatments and expressed as % of the total peak area detected. The GLM procedure of SAS was used for the data analysis (SAS Institute Inc.).

Diversity was considered as the number of peaks in each sample once standardised and the differences in frequencies (prevalence) of detection of these peaks across the treatment groups were examined using Fisher's exact test of Freq procedure (SAS Institute Inc.) (Lan *et al.*, 2004). Undetected peaks were taken as 0 values for Fisher's exact test.

Similarity indices of intra- and intergroup were calculated based on Sorenson's (Dice) coefficient (C_s) using Distance procedure of SAS (SAS Institute Inc.). The C_s values were determined using the equation:

$$C_s = \left[\frac{2n_{AB}}{(n_A + n_B)} \right] \text{x } 100$$

where n_{AB} is the number of T-RF peaks in common for samples A and B, n_A is the total number of peaks in sample A and n_B is the total number of peaks in sample B. Thus, two identical profiles create a C_s value of 100%, whereas completely different profiles result in a C_s value of 0%. Each sample was compared with every other sample; therefore, mean percentage similarities (C_s values) can be compared for each treatment group (intragroup) and in relation to all other groups (intergroup) (Leser *et al.*, 2000; McCracken *et al.*, 2001). The GLM procedure of SAS (SAS Institute Inc.) was used to compare differences due to treatment effects for all indices.

A significant difference was declared for *P* values of ≤0.05 unless otherwise stated.

4.3 RESULTS

4.3.1 Growth Performance

Growth performance and mortality data are summarised in Table 4.2. Dietary inclusion of MOS or ZnB significantly ($P<0.05$) increased the BWG and FCR of the birds during week 2 as well as when evaluated from d 1 to 21 of age. There were no differences in FI and mortality rate attributable to the treatments.

Table 4.2 Feed intake (FI), body weight gain (BWG), feed conversion ratio (FCR) and mortality of broilers fed the experimental diets[1]

Treatment	BWG (g/bird)				FCR				FI (g/bird)				Mortality (%)			
	1-7d	7-14d	15-21d	1-21d	1-7d	7-14d	15-21d	1-21d	1-7d	7-14d	15-21d	1-21d	1-7d	7-14d	15-21d	1-21d
NC	132	300^{b}	499	931^{b}	1.09	1.37^{a}	1.52	1.41^{a}	143	410	756	1308	0.00	0.00	1.60	1.60
MOS	137	324^{a}	518	978^{a}	1.06	1.29^{b}	1.45	1.34^{b}	144	416	748	1309	0.00	0.00	0.00	0.00
ZnB	133	321^{a}	512	966^{a}	1.08	1.25^{b}	1.46	1.33^{b}	143	402	746	1291	0.00	1.60	000	1.60
SEM	3.1	5.2	17.2	19.3	0.023	0.019	0.018	0.021	3.1	9.8	17.1	19.3	-	-	-	-
Probability	NS[2]	0.0100	NS	0.0100	NS	0.0500	NS	0.0100	NS	NS	NS	NS	NS	NS	NS	NS

[1]Each value represents the mean of 6 replicates for each treatment group.
[2]NS=Not significant.
[a,b]Means within the same column with different superscripts are significantly different (P<0.05).

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Table 4.3 Effects of dietary treatments on the gut morphometry of the ileal mucosa of broiler chickens at 21 days of age[1]

Treatment	Villus-Crypt (μm)				Goblet cell size (μm^2)	Goblet cell density (number/mm^2)					
	Muscularis thickness	Villus height	Crypt depth	Villus/ Crypt	"cup" area	Acidic	Sulphated-acidic	Sialo-acidic	Neutral	Hexose-neutral	Total
NC	243	538^{b}	195	2.84	28.43^{b}	1812^{b}	1189^{b}	497^{a}	1764	1899	1756^{b}
MOS	192	663^{a}	175	3.97	61.66^{a}	2092^{a}	1708^{a}	20^{b}	2005	2025	2230^{a}
ZnB	184	644^{a}	183	3.61	41.52^{a}	2243^{a}	1842^{a}	19^{b}	1866	2212	2138^{a}
SEM	19.6	29.9	16.8	0.361	2.473	99.6	75.9	21.44	87.6	145.6	106.6
Probability	NS[2]	0.0300	NS	NS	0.0100	0.0300	0.0100	0.0100	NS	NS	0.0200

[1]Each value represents the mean of 6 replicates for each treatment group.
[2]NS=Not significant.
[a,b]Means within the same column with different superscripts are significantly different (P<0.05).

Table 4.4 Effects of MOS and ZnB on ileal goblet cell staining intensity and integrated opaque density of broiler chickens at 21 days of age [1]

Treatment	Goblet cell staining parameters			
	Density (Log_{10} % of transmittance allowed)		Integrated opaque density x10^3 (IOD/mm^2)	
	Neutral (PAS)	Acidic (AB, pH 2.5)	Neutral (PAS)	Acidic (AB, pH 2.5)
NC	0.27	0.20	28.55	25.13[b]
MOS	0.28	0.21	35.28	32.43[a]
ZnB	0.26	0.22	29.53	36.23[a]
SEM	0.078	0.009	2.031	2.673
Probability	NS[2]	NS	NS	0.0400

[1]Each value represents the mean of 6 replicates for each treatment group.
[2]NS=Not significant.
[a,b]Means within a continuous column with different superscripts are significantly different ($P<0.05$).

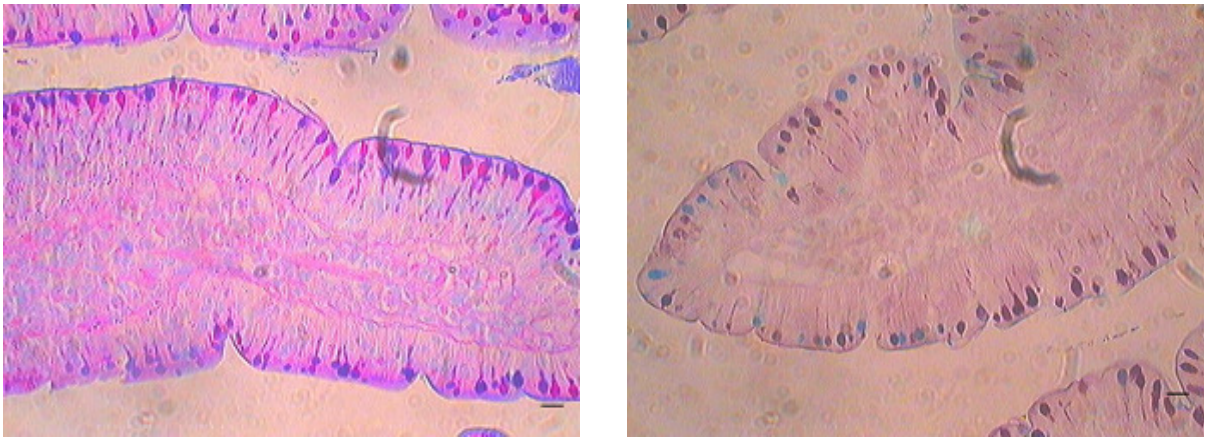
4.3.2 Enteric morphometric analysis

The results of ileal morphometric parameters are shown in Tables 4.3 and 4.4. At d 21, birds fed the MOS or ZnB-supplemented diets had longer ($P<0.05$) villi than those in the NC group. Although statistically non-significant, both MOS and ZnB-treated birds had numerically thinner ileal muscularis layer than their untreated counterparts. Neither MOS nor ZnB had any significant effects on the crypt depth and villus height to crypt ratio. In terms of goblet cell measurements, birds supplemented with MOS or ZnB had more ($P<0.05$) ileal goblet cells with larger "cup" size compared to those in the NC group (Table 4.3 and Plate 4.1). The total number of neutral goblet cells was not affected by the treatments. However, supplementation of MOS and ZnB increased ($P<0.05$) the density of acidic goblet cells. Although no significant changes in the staining intensity of neutral and acidic goblet cells were detected among the treatments, both MOS and ZnB-treated groups displayed higher ($P<0.05$) integrated opaque density (IOD) values for acidic mucins, reflecting an increase in the total content of acidic mucins in the ileum of those birds (Table 4.4).

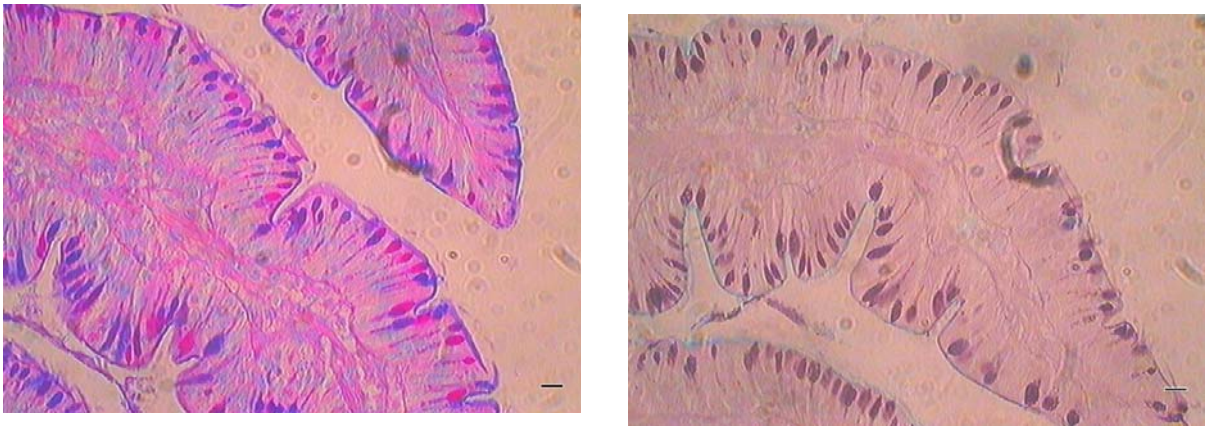
In comparison to the NC group, addition of MOS or ZnB affected goblet cell differentiation where the density of sulphated-acidic goblet cells in the ileum of birds was markedly enhanced at the expense of the sialomucin subtype ($P<0.01$) (Table 4.3 and Plate 4.1).

(a) PAS-AB, pH 2.5 (b) HID-AB, pH 2.5

NC



MOS



ZnB

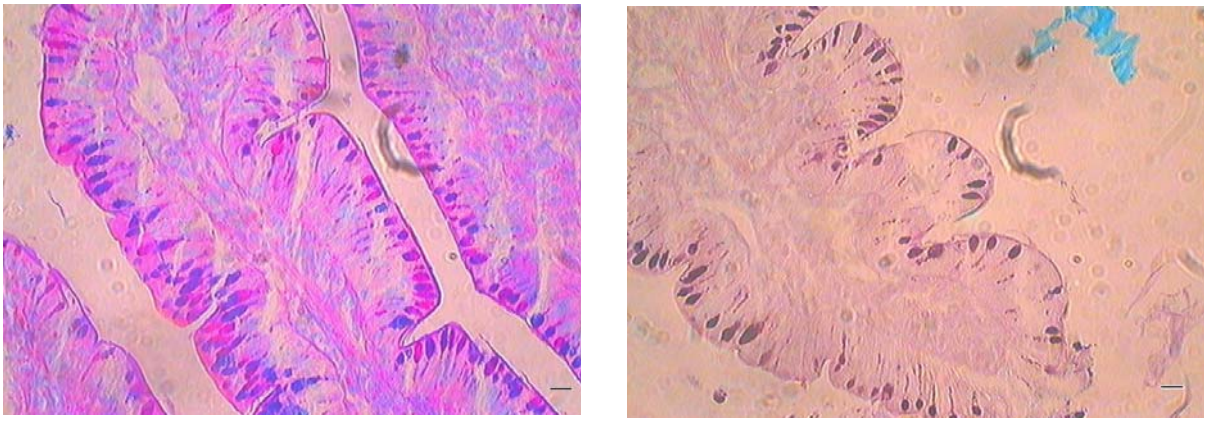


Plate 4.1 Representative micrographs showing the effects of dietary MOS and ZnB supplementation on chicken ileal goblet cells analysed using combination of various histochemical stains

Histochemical stain: (a) PAS-AB, pH 2.5 to differentiate neutral (bright red) and acidic (blue purple) goblet cells. (b) HID-AB, pH 2.5 to differentiate sulphomucin (dark brown) and sialomucin (blue) goblet cells. Magnification 400x; bar=10 μm.

Table 4.5 Effects of dietary treatments on the counts of ileal and caecal luminal, and ileal mucosa-associated bacteria of broiler chickens at 7 and 21 days of age[1]

Treatment	Total anaerobes		Lactobacilli		Lactic-acid bacteria		Lactose-negative enterobacteria		Coliforms		*C. perfringens*	
	Log_{10} CFU/g digesta or mucosa											
	7 d	21 d	7 d	21 d	7 d	21 d	7 d	21 d	7 d	21 d	7 d	21 d
Ileum												
NC	8.40	7.56^{a}	7.83	8.12^{a}	8.50^{b}	7.96^{ab}	6.03	5.75	3.78^{a}	3.65	2.76	4.59^{a}
MOS	8.84	8.03^{a}	7.84	8.52^{a}	9.34^{a}	8.48^{a}	5.05	5.55	3.39^{b}	3.35	3.31	3.34^{b}
ZnB	8.52	7.09^{b}	8.00	6.95^{b}	8.84^{ab}	7.11^{b}	5.32	5.61	2.77^{b}	3.32	3.12	2.89^{b}
SEM	0.148	0.278	0.221	0.322	0.169	0.339	0.261	0.465	0.208	0.391	0.402	0.436
Probability	NS[2]	0.0400	NS	0.1000	0.0200	0.0100	NS	NS	0.0100	NS	NS	0.0400
Ileal Mucosa												
NC	8.20	5.60	6.10	5.96^{b}	7.90	6.10	5.20	4.90	3.96	3.43	2.72	1.95
MOS	8.02	6.03	6.86	6.86^{a}	8.38	6.70	4.51	4.16	3.59	3.00	2.74	2.40
ZnB	7.62	5.70	6.72	5.75^{b}	7.77	5.81	5.17	4.17	3.72	2.73	2.62	2.15
SEM	0.181	0.312	0.207	0.248	0.192	0.189	0.333	0.522	0.181	0.209	0.253	0.172
Probability	NS	NS	NS	0.0100	NS	NS	NS	NS	NS	NS	NS	NS
Caeca												
NC	9.07^{b}	8.42	8.48	8.76^{a}	9.33	8.70	8.90	7.59	8.12^{a}	6.23	7.77^{a}	6.52^{a}
MOS	9.51^{a}	8.83	8.81	8.91^{a}	9.71	9.00	8.97	7.90	7.85^{b}	5.29	6.96^{a}	5.21^{ab}
ZnB	9.62^{a}	8.74	9.06	8.13^{b}	9.91	8.52	8.94	7.85	8.27^{a}	4.55	4.29^{b}	4.85^{b}
SEM	0.122	0.129	0.219	0.171	0.211	0.173	0.228	0.291	0.113	0.589	0.504	0.483
Probability	0.0200	NS	NS	0.0200	NS	NS	NS	NS	0.0300	NS	0.0100	0.0500

[1]Each value represents the mean of 6 replicates for each treatment group.
[2]NS=Not significant.
[a,b]Means within the same column with different superscripts are significantly different at $P<0.05$ unless otherwise specified.

4.3.3 Characterisation of intestinal microflora by plate counts

The bacterial counts in the ileal and caecal contents as well as the ileal mucosal samples collected at d 7 and 21 are shown in Table 4.5. Regardless of age and dietary treatments, lactobacilli predominated the bacterial communities in both the lumen as well as the mucosa of the ileum whilst *C. perfringens*, coliform and lactose-negative enterobacteria were more abundant in the caeca of the birds.

At d7, ZnB and MOS-supplemented birds harboured less coliforms in the ileum than those in the NC group ($P<0.01$). The number of lactic acid bacteria in the ileum of MOS-treated birds was also significantly ($P<0.05$) higher compared to those in the NC group. All the bacterial groups analysed in the ileal mucosa were not significantly altered by the treatments. However, inclusion of MOS or ZnB in the diets markedly increased the total anaerobic counts in the caeca of the birds. In addition, ZnB and MOS suppressed ($P<0.01$) the proliferation of *C. perfringens* and coliforms in the caeca of the chickens, respectively.

The suppressive effects of ZnB on the proliferation of gut bacteria were more pronounced at d 21 where the numbers of total anaerobes, lactobacilli, lactic acid bacteria and *C. perfringens* present in the ileum were greatly reduced. Zinc bacitracin reduced ($P<0.05$) the number of lactobacilli and *C. perfringens* in both the ileum and ceaca. Unlike ZnB, MOS influenced the gut microflora by promoting the growth of lactobacilli in the ileal mucosa and at the same time inhibiting the proliferation of *C. perfringens* in the caeca of the birds at d 21. No significant differences in the number of lactose-negative enterobacteria in all the regions studied which could be attributable to the treatments were detected.

4.3.4 T-RFLP pattern analysis and tentative bacterial identification

Only the luminal contents of the ileum and caeca, and the ileal mucosal samples collected at d 21 were subjected to T-RFLP analysis. Based on *in silico* restriction using Ribosomal Database Project II (RDP-II) (http//rdp.cme.msu.edu) and public database (http://mica.ibest.uidaho.edu/), the T-RF lengths created by either *Hha*I or *Msp*I digestion were theoretically identified and are shown in Appendix 1. Figure 4.1 and Appendix 2 show the representative T-RFLP profiles generated by *Hha*I and *Msp*I digestions, respectively.

The combination of amplification with primers pair of forward S-D-Bact-0008-a-S-20 and reverse S-D-Bact-0926-a-A-20 followed by digestion with *Hha*I or *Msp*I generated a

reasonably large number of unique 5' T-RFs (Liu *et al.*, 1997). However, the automated DNA sequencer used in this study was limited to a threshold detection limit of up to 650 bp only. Thus, it was initially thought that this combination would compliment each other in alleviating the drawback of the machine in peak detections. Despite the good coverage of *Msp*I digestion in containing peaks less than 650 bp for all the major bacterial groups, several unresolved peaks in the range of 93-97, 141-143, 179-182, 477-478, 492-497, 520-522, 578 and 595 bp were detected where one peak corresponded to more than one operational taxonomic unit (OTU) or bacterial group (Appendix 1). This had resulted in the poor resolution of T-RFLP patterns and making it difficult to be differentiated accurately for assessing any attributions by treatments (Appendix 2). As such, distinctively defined and differentiated polymorphisms derived from digestions with *Hha*I were used solely for the analysis of microbial ecosystems with *Msp*I digestion served to re-confirm the tentatively identified peaks.

4.3.5 Structural relatedness of the bacterial communities

Based on the Ward's algorithm, cluster analysis of the *Hha*I digested T-RFLP profiles clearly distinguished and clustered the polymorphisms of the intestinal microbial communities according to treatments (Figure 4.2). Distinct clusters by diets were observed in all the three intestinal regions, depicting that addition of MOS or ZnB differentially altered the gut microbial populations in such a way that they were unique to each other. The effects of MOS and ZnB regimens on the gut microflora can be further assessed by comparisons of Sorenson's pairwise similarity coefficients (C_s) as presented in Figure 4.3. Compared to the NC group, the intragroup C_s values (comparison across samples within a treatment) of MOS and ZnB-treated birds were significantly greater ($P<0.05$) in the ileal and caecal regions, respectively. In the ileum, the intergroup C_s value of the comparison between ZnB and NC groups was significantly lower ($P<0.05$) than when comparing MOS with the NC group. Conversely, the opposite was observed for the ileal mucosal and caecal regions of ZnB-treated birds.

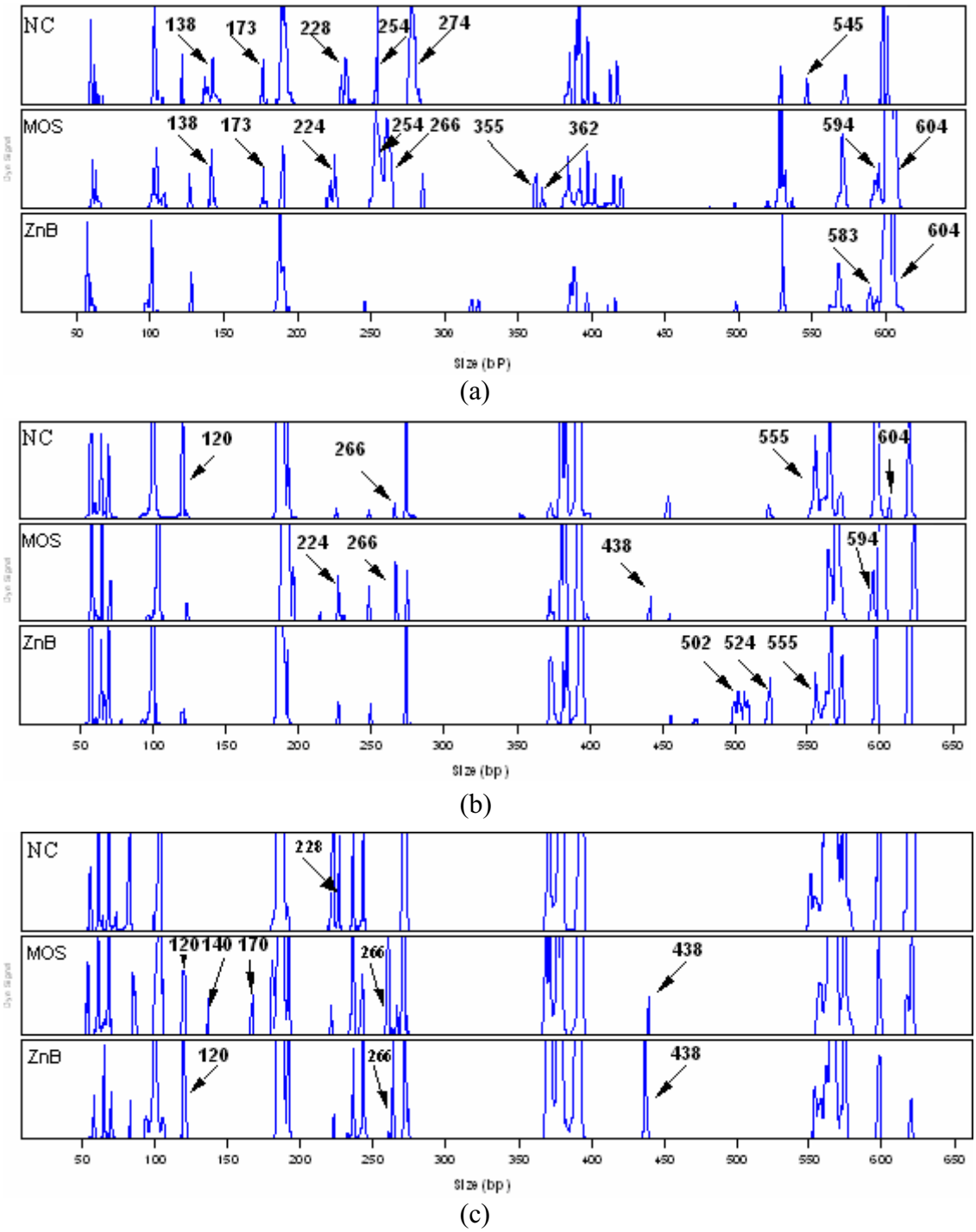
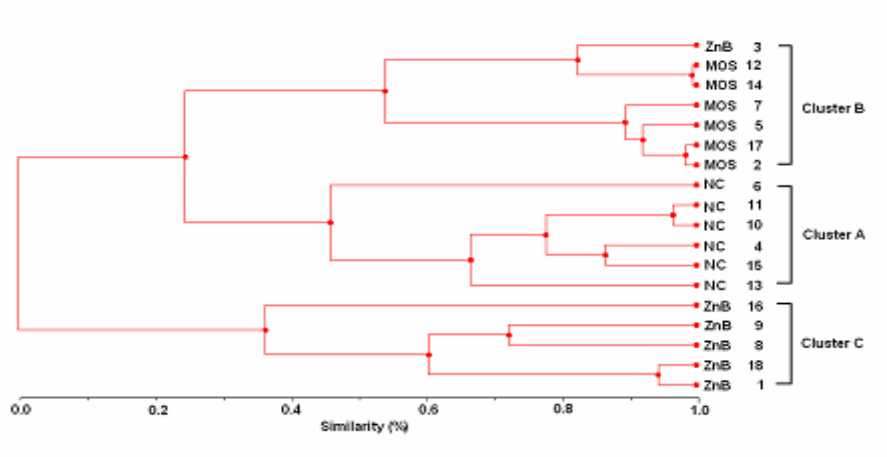
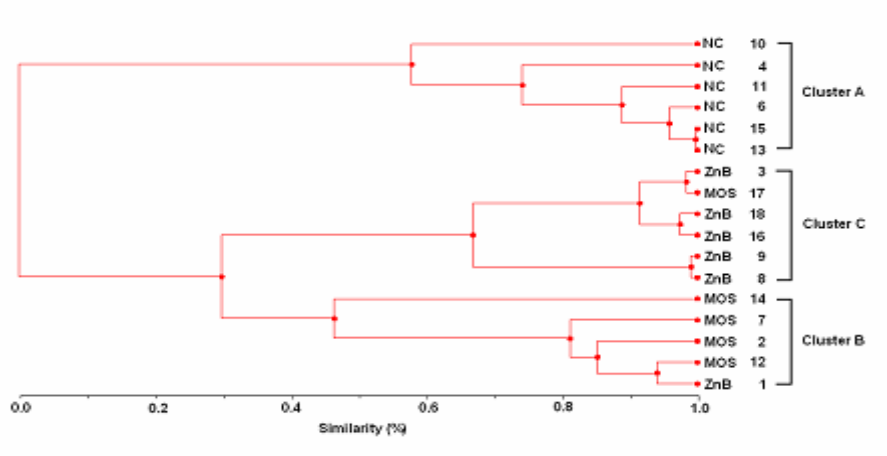


Figure 4.1 Representative T-RFLP patterns of bacterial communities present in (a) ileal digesta, (b) ileal mucosa and (c) caecal contents after digestion with *Hha*I in NC, MOS and ZnB-supplemented broiler chickens at 21 days of age

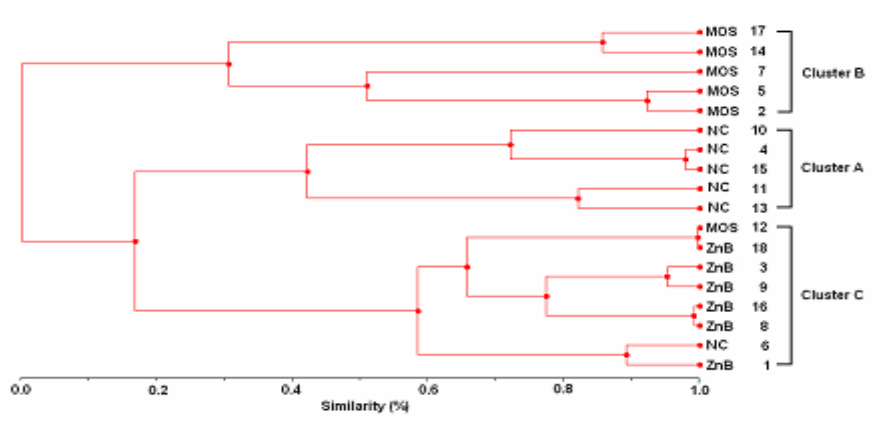
Arrows indicate presence of major peaks with fragment lengths in bp.



(a)



(b)



(c)

Figure 4.2 Dendrograms illustrating the effects of MOS and ZnB on bacterial communities in the (a) ileal digesta, (b) ileal mucosa, and (c) caecal contents of broiler chickens at 21 days of age

The dendrograms, in % similarity value, were constructed by clustering the *Hha*I digested T-RFLP patterns of 6 replicates (except for ileal mucosa-MOS with 5 replicates).

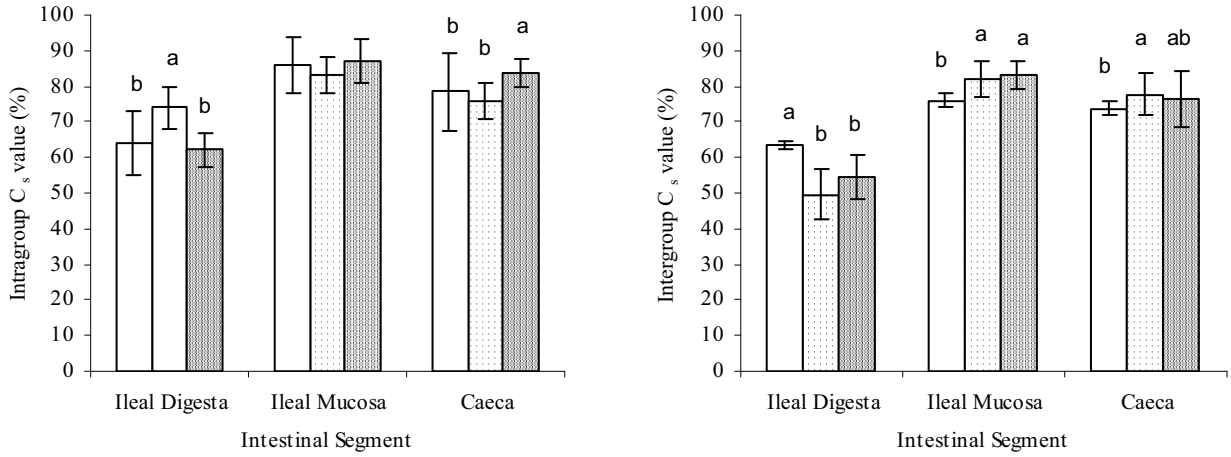


Figure 4.3 Calculated Sorenson's similarity indices (C_s) for the comparisons of average percent similarities of 16S rRNA PCR-T-RFLP peak patterns within each treatment group (intragroup) and between treatment groups (intergroup)

Values represent averages and SEM for 6 replicates from each treatment group at 21 days of age. (a) Intragroup: NC (open), MOS (dotted) and ZnB (darkened). (b) Intergroup: NC vs. MOS (open), NC vs ZnB (dotted) and MOS vs ZnB (darkened). Values that do not have common letter differ at $P \leq 0.05$.

4.3.6 Alterations in the composition and diversity of ileal bacterial populations

As presented in Figure 4.1 and Table 4.6, ileal bacterial communities were predominated by *Lactobacillus* species. Compared to the NC group, inclusion of MOS enriched the diversity of *Lactobacillus* phylotypes in the ileum by increasing the prevalence of additional species of theoretically identified *L. acetotolerans, L. delbrueckii* subsp. *lactis,* and *L. sakei* subsp. *sakei* ($P<0.05$) (Table 4.6).

Mannooligosaccharides also modified the relative abundance of ileal lactobacilli where tentatively identified *L. acidophilus* group constituted 23% of the total T-RF area with another large share of 21% coming from the presumed *L. plantarum* and *L. casei group.* The presumed *L. acidophilus* group comprises several phylogenetically closely-related *Lactobacillus* species such as *L. acidophilus*, *L. amylovorus*, *L. gallinarum* and *L. crispatus,* whereas *L. casei* group consists of *L. casei, L. paracasei* and *L. rhamnosus*. As opposed to MOS, ZnB greatly suppressed the diversity of the *Lactobacillus* species colonising the ileum with the *L. plantarum* and *L. casei* group being the dominant species abundantly present at the rate of 48.9% of the total T-RF area. In relation to MOS and ZnB, the relative abundance of the predicted *L. maltaromicus* was significantly ($P<0.05$) higher in the ileum of NC group. Along with that, the ileum of the untreated birds was also numerically harbouring more *L.*

agilis and *L. aviarius* than their counterparts in the other two treatment groups. No significant differences in the relative abundance of *Clostridium* species attributable to the treatments were detected. However, the theoretically identified *C. butyricum* was detected exclusively in the ileum of the NC group with a relative high abundance of 10.1% of the total T-RF area. Although statistically not significant, the *Clostridium* phylotypes in the ileum of MOS-treated birds were also less diverse in nature with the elimination of *C. butyricum* and *C. ramosum*. Generally, peaks corresponded to *C. perfringens* and *Escherichia* species were not detected in the ileum of all the treatment groups.

Collectively, the proportion of lactobacilli which constituted over 60% of the total ileal microbial population (Figure 4.4) in the ileum of MOS and ZnB-treated groups were markedly higher than those estimated at 43.8% for the NC group ($P<0.05$). In contrast, untreated birds harboured significantly ($P<0.05$) more clostridia (19.3 %) in the ileum than their MOS or ZnB-treated counterparts (10.6 and 12.4%, respectively).

4.3.7 Alterations in the composition and diversity of ileal mucosal bacterial populations

As shown in Figure 4.1 and Table 4.7, lactic acid bacteria, clostridia and bacteroides were the dominant bacterial groups colonising the ileal mucosa of the chickens. In addition, the microbial community structures of the ileal mucosa were also phylogenetically different and less diverse than in the ileum. For example, the theoretically identified *L. vaginalis, L. delbrueckii* subsp. *delbrueckii, L. maltaromicus, Flexibacter flexilis, C. ramosum, C. butyricum., and Chloroflexi bacterium* present in the ileum of the NC birds were not detected in the ileal mucosal region. Likewise, the presumed *L. fructivurans*, *Eubacterium* species, *Gammaproteobacteria* and *Thermoanaerobacteriacease* bacterium were only found in the ileal mucosa and not in the ileum of the NC group.

With reference to the relative abundance, all the major bacterial groups dominating the ileal mucosa were not significantly altered by the treatments except for the *Gammaproteobacteria* group which was significantly more abundant ($P<0.05$) in the MOS-treated group (Table 4.7).

Similar trends were also observed in ileal mucosa where MOS significantly enriched the *Lactobacillus* phylotypes with the presence of two extra *Lactobacillus* species presumed to be *L. delbrueckii* subsp. *Lactis* and *L. acetotolerans* ($P<0.05$) (Table 4.7). Compared to the NC

and MOS groups, the *Lactobacillus* phylotypes of the ileal mucosa of ZnB-treated group was less diverse. Despite being suppressed, two unidentified T-RF peaks of 502 and 524 bp were detected only in the ileal mucosa of ZnB-supplemented group (Figure 4.1 (b)).

In relation to the overall proportion of bacterial groups present in the ileal mucosa, no significant differences attributable to the treatments were observed (Figure 4.4). Irrespective of the treatments, lactobacilli, clostridia and bacteroides constituted almost an equal average share of 24, 23 and 22% of the total T-RF peak area present in the ileal mucosa, respectively.

4.3.8 Alterations in the composition and diversity of caecal bacterial populations

In comparison to the ileum and ileal digesta and mucosa, the composition of the caecal microflora was generally more complex in nature with a higher number of unidentified or uncultured peaks detected (Figure 4.1 and Table 4.8). Again, there were no significant changes in the caecal microbial populations attributable to the treatments except for *C*lostridium subcluster XIVa which was detected to be the major *Clostridium* species in the caeca of the NC group constituting 17.6% of the total T-RF peak area (Table 4.8). Based on computer simulation, *Clostridium* subcluster XIVa comprises mostly *Ruminococcus* species as well as some butyrate-producing bacteria (Gong *et al.*, 2002b; Lan *et al.*, 2004).

Although the lactobacilli that resided in the caecal region were phylogenetically less diverse, inclusion of MOS significantly increased its diversity with additional members of tentatively identified *L. ingluviei* and *L. mucosae* ($P<0.05$) (Table 4.8). On the other hand, *Kitastosphora* species was also detected more frequently in the caecal region of both MOS and ZnB-supplemented groups ($P<0.05$) (Table 4.8).

Regardless of the treatments, lactobacilli represented less than only 10% of the overall bacterial population present in the caeca of the birds. As opposed to this, *Clostridium* and *Bacteroides* species accounted for nearly 45% of the total bacterial population (Figure 4.4).

Table 4.6 Effects of MOS and ZnB on microflora in the ileal contents of broiler chickens at 21 days of age

Bacterial identification		T-RF peak (bp)	Frequency (Percentage of T-RFs, %)[3]		
Bacterial groups	Compatible bacteria[1]	*In silico* restriction (Observed restriction)[2]	NC	MOS	ZnB
Lactic acid bacteria	*L. vaginalis*	61 (60)	4/6(1.8±0.45)[b,e]	6/6(1.9±0.6)[b,e]	4/6(4.8±2.4)[a,d]
	L. delbrueckii subsp. *lactis*	223 (224)	1/6[g](1.3)	6/6[f](2.4±2.0)	ND[g]
	L. delbrueckii subsp. *delbrueckii*	254 (253, 254)	4/6(6.1±4.3)	4/6(6.0±4.2)	ND[4]
	L. agilis, L. aviarius	393, 394 (393)	5/6(10.3±2.9)	5/6(4.6±1.2)	3/6(7.2±2.5)
	L. maltaromicus	586 (587)	1/6(3.5)[a]	3/6(1.8±0.3)[b]	1/6(1.7)[b]
	L. acetotolerans	595 (594, 595)	ND[g]	6/6[f](4.1±3.2)	ND[g]
	L. acidophilus group[1]	596, 597 (596)	5/6[f](13.5±10.2)	6/6[f](23.0±6.6)	2/6[g](17.4±19.0)
	L. plantarum, L. casei group[1]	598, 599 (598, 599)	4/6(24.2±7.5)[b,e]	6/6(21.0±5.4)[b,e]	5/6(48.9±11.7)[a,d]
	L. sakei subsp. *sakei*	604 (602, 604)	ND[g]	5/6[f](6.7±3.6)	2/6[g](3.5±1.0)
	Enterococcus spp.	216, 218 (216, 218)	ND	ND	1/6(5.9)
	Lactococcus garvieae	583 (583)	ND	ND	2/6(3.3±2.2)
Clostridium and relatives	*C.* subcluster XIV_a	186 (185, 186, 187)	6/6(8.5±6.6)	6/6(6.0±4.4)	6/6(9.1±4.0)
	C. clostridioforme, C. symbiosum	190 (188, 189, 190)	6/6(5.4±2.5)	6/6(4.7±2.4)	4/6(5.6±1.8)
	C. ramosum	228, 229 (228)	3/6(3.7±1.0)	ND	ND
	C. cochlearium	363 (362)	ND	2/6(0.6±0.1)	ND
	C. butyricum	544 (545)	2/6(10.1±8.4)	ND	ND

Table 4.6 Effects of MOS and ZnB on microflora in the ileal contents of broiler chickens at 21 days of age (continued)

Bacterial identification		T-RF peak (bp)	Frequency (Percentage of T-RFs, %)[3]		
Bacterial groups	Compatible bacteria[1]	*In silico* restriction (Observed restriction)[2]	NC	MOS	ZnB
Bacteroides and relatives	*Cytophaga & Bacteroides*	100, 101 (99, 100)	6/6(12.9±13.6)	6/6(4.8±2.9)	4/6(4.7±2.4)
	Flexibacter flexilis	248 (248)	5/6[f](7.6±8.2)	3/6[f](3.8±5.2)	1/6[g](7.1)
Fibrobacter	*Fibrobacter succinogenes*	139 (138)	3/6(2.2±0.8)	2/6(1.9±0.4)	ND
Proteobacteria	*Escherichia* spp.	371 (371)	ND	1/6(15)	ND
Other bacteria groups	*Nocardiopsis* spp. / *Pseudonocardia* spp.	355 (355)	ND[g]	3/6[f](1.1±0.3)	ND[g]
	Chloroflexi bacterium	531 (531)	2/6(3.4±2.1)	5/6(1.8±1.1)	2/6(3.5±1.2)

[1]Theoretically identified major chicken gut bacteria with T-RFs corresponded to known 16S rDNA sequences in the database from the Ribosomal Database Project (RDP) 8.1 software and public database (http://mica.ibest.uidaho.edu/). *L. acidophilus* group comprises several phylogenetically closely-related *Lactobacillus* species such as *L. acidophilus*, *L. amylovorus*, *L. gallinarum* and *L. crispatus,* whereas *L. casei* group consisted of *L. casei, L. paracasei* and *L. rhamnosus*. Other peaks of 57, 120, 173, 266, 274, 380, 388, 411, 416, 538 and 567 were either uncultured/unidentified bacterium or not detected with *Msp*I digestion.

[2]*In silico* restriction was performed using the tap-tRFLP tool (RDP II) and real restriction were obtained using *Hha*I enzymatic restriction of the PCR products.

[3]Frequency of detection expressed as numerator (number of ileal samples yielding the T-RFF peak)/denominator (number of samples examined). In brackets relative abundances of T-RF peaks expressed as % of total normalised peak areas. Value of mean±SD is calculated only considering the birds showing the peaks.

[4]ND=not detected.

[a-c]Statistically significant at $P<0.05$ level when compared the variance of the T-RF peak areas across the groups (*F* test).

[d,e]Statistically significant at the $P<0.05$ level when compared with the percentage of T-RF peak areas across the groups (Mann-Whitney *U*-test).

[f,g]Statistically significant at the $P<0.05$ level when compared the frequency of detection across the groups (Fisher's exact test).

Table 4.7 Effects of MOS and ZnB on microflora in the ileal mucosa of broiler chickens at 21 days of age

Bacterial identification		T-RF peak (bp)	Frequency (Percentage of T-RFs, %)[3]		
Bacterial groups	Compatible bacteria[1]	*In silico* restriction (Observed restriction)[2]	NC	MOS	ZnB
Lactic acid bacteria	*L. fructivorans*	68 (69)	2/6(1.9±0.2)	3/5(1.4±0.7)	3/6(1.6±0.6)
	L. delbrueckii subsp. *lactis*	223 (224)	ND[g]	2/5[f](0.8±0.1)	1/6[g](1.2)
	L. ingluviei, L. mucosae	266, 267 (266, 267)	3/6(1.7±0.3)	1/5(0.9)	ND
	L. agilis, L. aviarius	393, 394 (393, 394)	6/6(10.1±6.3)	5/5(9.3±6.0)	6/6(10.3±8.3)
	L. acetotolerans	595 (594, 595)	ND[g]	3/5[f](11.5±3.4)	ND[g]
	L. acidophilus, L. plantarum,	597, 598, 599 (597, 598,	5/6(11.0±5.6)	5/5(8.2±5.6)	6/6(8.9±4.7)
	L. sakei subsp. *sakei*	604 (602, 604)	1/6(2.3)	ND[4]	ND
Clostridium and relatives	*C.* subcluster XIV_a	186 (185, 186, 187)	6/6(15.0±4.8)	5/5(13.0±2.3)	6/6(15.9±3.4)
	C. clostridioforme, C. symbiosum	190 (188, 189, 190)	6/6(8.6±3.0)	5/5(8.2±1.6)	6/6(9.2±3.3)
	Eubacterium spp.	245, 377 (245, 377)	5/6(2.6±1.9)	5/5(1.8±0.5)	6/6(1.5±0.6)
Bacteroides and	*Cytophaga and Bacteroides*	100, 101 (99, 100)	6/6(22.3±6.4)	5/5(19.0±14.0)	6/6(18.6±3.4)
Other bacteria groups	*Gammaproteobacteria*	369, 370 (369)	5/6(0.8±0.6)[b,e]	2/5(4.5±3.3)[a,d]	5/6(2.5±0.9)[ab,e]
	Kitasatospora spp.	438(438)	ND	1/5(2.4)	ND
	Thermoanaerobacteriacease	620(620)	3/6(1.65±0.4)	4/5(3.7±2.1)	4/6(4.9±1.3)

[1]Theoretically identified major chicken gut bacteria with T-RFs that corresponded to known 16S rDNA sequences in the database from the Ribosomal Database Project (RDP) 8.1 software and public database (http://mica.ibest.uidaho.edu/). *L. casei* group comprises several phylogenetically closely-related *Lactobacillus* species such as *L. casei, L. paracasei* and *L. rhamnosus*. Other peaks of 57, 64, 120, 274, 380, 388, 502, 524, 555, 567 and 574 bps were either uncultured/unidentified bacterium or not detected with *Msp*I digestion.

[2]*In silico* restriction was performed using tap-tRFLP tool (RDP II) and real restriction were obtained using *Hha*I enzymatic restriction of the PCR products.

[3]Frequency of detection expressed as numerator (number of ileal samples yielding the T-RFF peak)/denominator (number of samples examined). In brackets relative abundances of T-RF peaks expressed as % of total normalised peak areas. Value of mean±SD is calculated only considering the birds showing the peaks.

[4]ND=not detected.

[a-c]Statistically significant at $P<0.05$ level when compared the variance of the T-RF peak areas across the groups (*F* test).

[d,e]Statistically significant at the $P<0.05$ level when compared with the percentage of T-RF peak areas across the groups (Mann-Whitney *U*-test).

[f,g]Statistically significant at the $P<0.05$ level when compared the frequency of detection across the groups (Fisher's exact test).

Table 4.8 Effects of MOS and ZnB on microflora in the caecal contents of broiler chickens at 21 days of age

Bacterial identification		T-RF peak (bp)	Frequency (Percentage of T-RFs, %)[3]		
Bacterial groups	Compatible bacteria[1]	*In silico* restriction (Observed restriction)[2]	NC	MOS	ZnB
Lactic acid bacteria	*L. fructivorans*	68 (69)	3/6 (1.0±0.3)	4/6(1.1±0.4)	3/6 (1.2±0.3)
	L. delbrueckii subsp. *lactis*	223 (224)	2/6(1.9±0.2)	2/6(1.1±0.5)	1/6(0.8)
	L. ingluviei, L. mucosae	266, 267 (266, 267)	ND[g]	5/6[f](1.4±0.6)	2/6[g](1.1±0.1)
	L. agilis, L. aviarius	393, 394 (391,392)	6/6(8.0±3.8)	6/6(7.6±3.4)	6/6(9.8±3.4)
	L. plantarum, L. casei group	598, 599 (598, 599)	3/6(1.1±0.6)	2/6(2.1±1.9)	1/6(1.6)
	Enterococcus spp.	170, 216 (172, 216)	ND[4]	1/6(2.0)	ND
	Bacillus subtilis	240 (238)	4/6(0.7±0.2)	3/6(1.2±0.3)	3/6(1.0±0.1)
Clostridium and relatives	*C.* subcluster XIV_a	186 (185, 186, 187)	5/6(17.6±3.6)[a]	6/6(13.6±7.5)[ab]	6/6(10.1±2.7)[b]
	C. clostridioforme, C. symbiosum	190 (188, 189, 190)	6/6(8.7±3.1)	6/6(7.6±4.9)	6/6(8.7±3.0)
	C. ramosum	228, 229 (228)	2/6(2.5±1.8)	ND	ND
	Eubacterium spp.	245 (245)	5/6(1.3±0.8)	3/6(1.2±0.7)	6/6(2.0±1.4)
Bacteroides	*Cytophaga & Bacteroides*	100, 101 (99, 100)	6/6(20.7±8.9)	6/6(21.5±6.2)	6/6(14.7±3.4)
Proteobacteria	*Escherichia* spp. other enteric	370, 371, 373 (369, 373)	4/6(2.4±1.5)	6/6(4.6±2.5)	6/6(4.9±2.5)
Other bacteria groups	*Kitasatospora* spp.	438(438)	ND[g]	2/6[f](0.8±0.2)	4/6[f](1.3±0.5)
	Chloroflexi bacterium	551(553)	1/6(0.7)	ND	4/6(0.9±0.3)
	Thermoanaerobactriacease	620(620)	3/6(8.8±5.3)	3/6(5.1±3.8)	3/6(1.6±1.0)

[1]Theoretically identified major chicken gut bacteria with T-RFs corresponded to known 16S rDNA sequences in the database from the Ribosomal Database Project (RDP) 8.1 software and public database (http://mica.ibest.uidaho.edu/). Other peaks of 57, 64, 78, 120,140, 167, 274, 377, 380, 386, 557, 560, 564, 567 and 574 bps were either uncultured/unidentified bacterium or not detected with *Msp*I digestion.

[2]*In silico* restriction was performed using the tap-tRFLP tool (RDP II) and real restriction were obtained using *Hha*I enzymatic restriction of the PCR products.

[3]Frequency of detection expressed as numerator (number of ileal samples yielding the T-RFF peak)/denominator (number of samples examined). In brackets relative abundances of T-RF peaks expressed as % of total normalised peak areas. Value of mean±SD is calculated only considering the birds showing the peaks.

[4]ND=not detected.

[a-c]Statistically significant at $P<0.1$ level when compared the variance of the T-RF peak areas across the groups (*F* test).

[d,e]Statistically significant at the $P<0.05$ level when compared with the percentage of T-RF peak areas across the groups (Mann-Whitney *U*-test).

[f,g]Statistically significant at the $P<0.05$ level when compared the frequency of detection across the groups (Fisher's exact test).

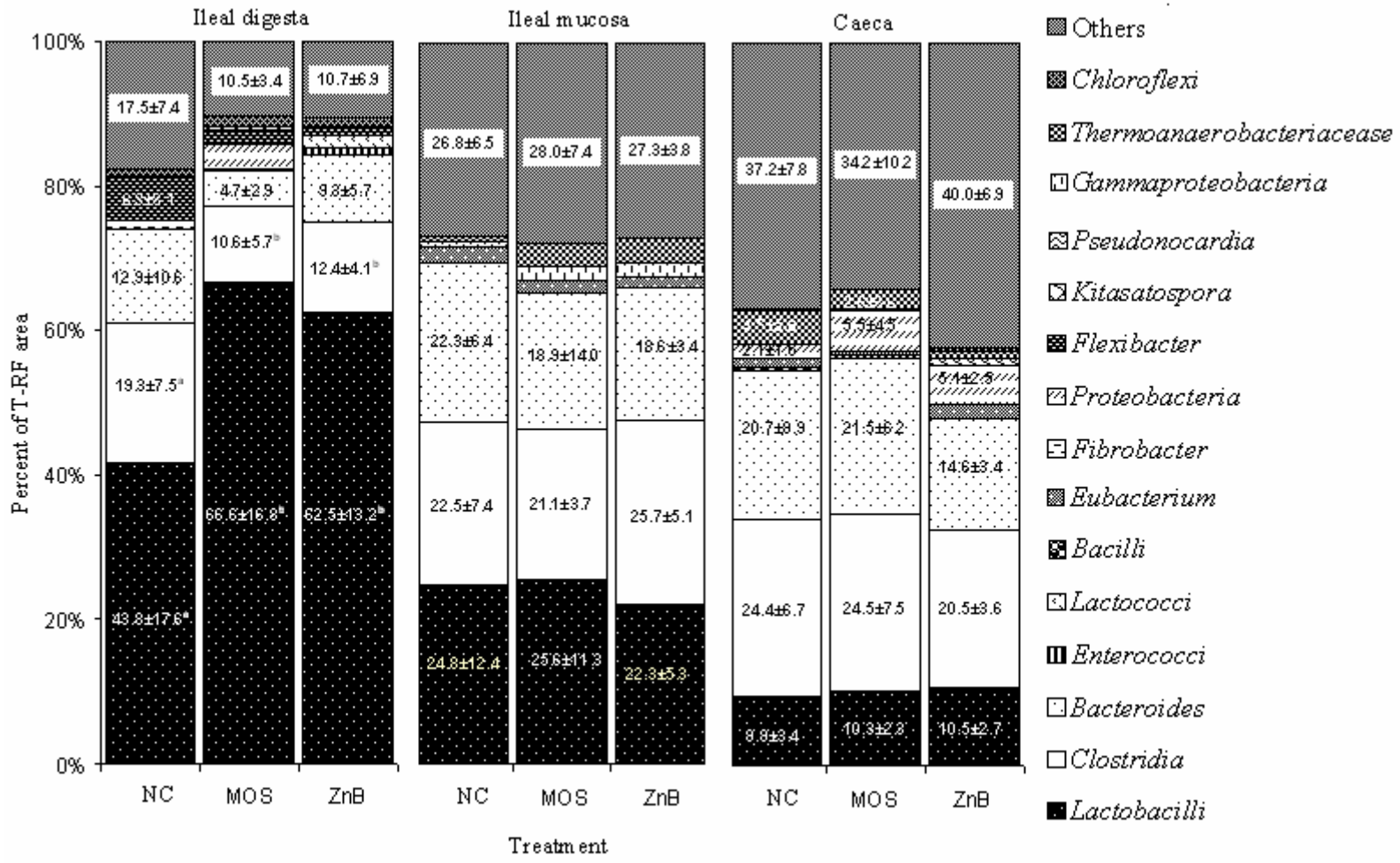


Figure 4.4 Percentage of T-RF peak areas of various groups of bacterial communities present in the Ileal digesta, ileal mucosa and caecal contents after digestion with *Hha*I in NC, MOS and ZnB-supplemented broilers at 21 days of age[1]

[1]Values are mean±SD of 6 replicates.
[a-c]Statistically significant at $P<0.05$ level when compare variance of mean T-RF peak areas across treatment groups (GLM).

4.4 DISCUSSION

4.4.1 Growth performance

The positive response of bird performance to MOS and ZnB observed in this study further confirms their potential growth-promoting effects in the early stage of growth as demonstrated in the previous experiment and by others (Engberg *et al.*, 2000; Jamroz *et al.*, 2003; Yang *et al.*, 2007a). These findings also support the general statement that, under controlled experimental conditions, the growth-promoting effect of MOS is comparable to that of ZnB (Hooge *et al.*, 2003; Sims *et al.*, 2004; Yang *et al.*, 2007a).

4.4.2 Enteric morphometric analysis

The significant increases in ileal villus height and villus to crypt ratio of birds fed the MOS or ZnB diets generally indicate their ability to accelerate the maturation of the chicken gut. Early maturation of the gut is closely associated with an overall improvement in intestinal digestion and nutrient absorption as villi play a crucial role in these processes (Uni *et al.*, 1999; Noy *et al.*, 2001; Sklan, 2001). It is likely that ZnB has a direct effect on the growth of the intestinal villi mediated through the control of gut microbial activities that, in turn, reduces the competition in nutrient utilisation and uptake with the host.

Although the effects of MOS in accelerating gastrointestinal maturation have been well studied (Iji *et al.*, 2001b; Solis de los Santos *et al.*, 2007; Yang *et al.*, 2007a), the actual reasons for this response are still unknown. Certain biochemical alternations due to the oligosaccharide molecule may be responsible for the long-term regulation of the GIT structure (Iji and Tivey, 1999). One possibility is associated with the reduction in the prevalence of intestinal colonisation by potential pathogenic agents through its agglutinating effect on those mannose-sensitive pathogens that possess Type 1 fimbriae, exemplified by *E. coli* and *Salmonella* species (Spring *et al.*, 2000). Furthermore, the accumulation of toxic metabolites produced by these bacteria also damages enterocytes that require diversion of extra energy and nutrients from growth for their replacement (Simon, 2007).

The pronounced increase in ileal goblet cell density and “cup” size in birds supplemented with MOS or ZnB is consistent with the findings of Uni and Smirnov (2006), Solis de los Santos *et al*. (2007), and Thymann *et al.* (2007). These observations may, in part, correlate to the increase in thickness of the intestinal adherent mucous layer. This layer acts as the first

major barrier and displays an important feature in the protective scheme against adherence of enteropathogenic bacteria (Toribara *et al.*, 1993). The decrease in number of goblet cells observed in the NC group may need to be interpreted with caution as literally it can be seem as a direct reduction in goblet cell number or indirect increase in the number of goblet cells experiencing an accelerated acute or complete release of centrally stored mucus (compound exocytosis). The emptied goblet cells were not histochemically stained and thus, could not be counted. The latter explanation seems logical as during high bacterial challenge, a wide array of bioactive factors including inflammatory mediators (cytockines and lipids), hormones and neuropeptides, can act as effective mucin secretagogues causing the goblet cells to undergo compound exocytosis rather than experiencing the normal baseline secretion (simple exocytosis) that involves only a constitutive release of newly synthetised mucin granules (Forstner *et al.*, 1995).

Mucins are classified into neutral and acidic subtypes; the latter are further differentiated into sulphated (sulphomucins) or non-sulphated (sialomucins) subtypes (Robertson and Wright, 1997). As reflected by a significant increase in the acidic mucin IOD value in the ileum of both MOS and ZnB-treated birds, it is likely that the increase in goblet cell "cup" size may have compounded the effect of the already increased acidic goblet cell density and its staining intensity which eventually resulted in an overall increase in the total tissue content of acidic mucins. The significant increase in acidic mucins predominated by sulphated subtype in the ilea of MOS and ZnB-treated birds may further enhance the inhibition of bacterial invasion. This is because acidic mucins, particularly the more mature sulphomucins, are less degradable by bacterial glycosidases and host proteases thus, confer a better epithelial protection against bacterial translocations (Robertson and Wright, 1997; Fontaine *et al.*, 1998).

The actual mechanism involved in the induction and modulation of these mucin dynamics is unknown. The presumptive close relationships between the degradation and induction of the glycoconjugates initiated by gut microflora (Freitas and Cayuela, 2000) may be closely linked to the modulating effects of MOS and ZnB on mucin dynamics. This is evidenced by the existence of a direct crosstalk between the intestinal microbes and the goblet cells (Freitas *et al.*, 2003). For instance, it has been clearly demonstrated that *Lactobacillus* strains possess the capability to prevent enteropathogenic *E. coli* attachment to HT29 epithelial cells via the post-transcriptional up-regulation of MUC2 and MUC3 gene expressions (Mack *et al.*, 1999). Moreover, *Lactobacillus* strains generally do not degrade mucins, unlike *Clostridium*, *E. coli*

and *Bacteroides* strains which are capable of hydrolysing sialomucins through the release of mucolytic enzymes (Stanley *et al.*, 1986; Deplancke *et al.*, 2002; Freitas *et al.*, 2005). Therefore, it can be implied from these findings that birds in the control group that were more intense in sialomucins might be more susceptible to enteric infections, especially in the presence of a high intestinal load of coliforms and clostridia.

4.4.3 Characterisation of intestinal microflora by plate counts

In relation to the NC group, supplementation of MOS significantly reduced the coliform counts in the ileum and caeca of 7-day-old birds, highlighting its capability in nurturing the juvenile gut microflora to quickly assume the mature stage. This is evidenced by observation that the gut microflora of 21-day-old birds fed the MOS diets were generally more mature with a higher prevalence of lactobacilli in the ileal mucosal region coupled with a low abundance of *C. perfringens* in the lumen of the ileum. A lower incidence of *C. perfringens* in the small intestine of the MOS-treated birds may also reduce the likelihood of birds developing necrotic enteritis (Elwinger *et al.*, 1998).

Generally, *Clostridium* species do not agglutinate to MOS but several studies have indicated a reduction in the numbers of this species in response to MOS treatment (Finucane *et al.*, 1999; Sims *et al.*, 1999; Biggs *et al.*, 2007). Several individual isolates of *Clostridium* species with weak agglutinating properties have been identified, independent of mannose inhibition, suggesting other mechanisms of bacterial adhesion associated with the cell wall extract of *Saccharomyces cerevisiae* (Simon, 2007). Thus, it is likely that the immunostimulatory effects of MOS may also have a part to play in reducing intestinal clostridia (Kocher and Tucker, 2007).

On the contrary, ZnB markedly suppressed the growth of all the major gastrointestinal bacterial groups, especially at 21 days of age. This is in good agreement with the report by Gong *et al.* (2008) where inclusion of ZnB in the diet significantly reduced the abundance of lactobacilli in the caeca of the birds. These observations support the concept that in-feed antibiotics achieve its growth-permitting effects by reversing the growth depression processes through the inhibition or elimination of these metabolically active microflora (Coates, 1980).

As reviewed by Anderson *et al.* (2000), Anderson (2002) and Gabriel *et al.* (2006), the growth of indigenous beneficial bacteria, especially those in the group of lactic acid bacteria, can be metabolically expensive as they depress growth by competing with the bird for

nutrients (Hughes *et al.*, 2001), promoting bile acid biotransformation (Fuller *et al.*, 1984; Tannock *et al.*, 1989) and producing growth-depressing microbial metabolites such as vasoactive amines, ammonia, phenolic and aromatic compounds (Macfarlane and Macfarlane, 1997). Lactobacilli are regarded as being largely responsible for dietary energy lost via their bile salt deconjugating and dehydroxylating activities (De Smet *et al.*, 1995).

This explanation challenges the proposed beneficial effects of lactobacilli seen in MOS-fed birds. Therefore, there may exist a well-balanced cooperative and competitive relationships between the host animal and the metabolically active microflora (Anderson, 2002). The search for this trade-off mechanism remains the impetus of the rest of this study.

4.4.4 Structural relatedness of gut bacterial communities

Due to the complex growth requirements involved in the culture-based technique, typically only around 10 to 50% of the gut bacteria can be cultured under laboratory conditions (Amann *et al.*, 1995; Vaughan *et al.*, 2000). The employment of a higher resolution molecular-based T-RFLP technique in this study aided in revealing the complex structure of the gut microflora and thus, offered a better understanding on the different mechanisms employed by MOS and ZnB in modulating the gut microflora to achieve the same growth-promoting effects.

The groupings of three distinct clusters by diets in all the three intestinal regions indicate the significant differences in the heterogeneity of the microbial community augmented by the treatment effects. A higher ileal intragroup C_s value observed in MOS group reflects a significant higher homogeneity of microflora inhabiting this region of the gut, compared to the other two treatment groups. This finding may be linked directly to the MOS-related improvement in flock uniformity frequently observed under controlled experimental conditions (Ao, 2004; Yang, 2007). A lower ileal mucosal and caecal intergroup C_s values (NC vs. MOS compared with NC vs ZnB) noted in MOS group suggests that the bacterial composition of these intestinal regions were generally more distinctive than in the ZnB group when compared to the control. Similar findings on this effect of MOS on pig oesophageal mucosal microbial ecology have also been reported (Pettigrew and Miguel, 2007).

As opposed to this, the caecal microflora of ZnB-treated birds were more homogeneous (higher intragroup C_s value) compared to the NC and MOS groups. The ileal digesta and ileal mucosal microflora of ZnB-treated birds were also more distinctive (lower intergroup C_s

value) than that of the MOS-treated birds when compared to the control. It is generally believed that dietary antibiotics induce a more homogenous gut microflora (higher intragroup C_s value) (Collier *et al.*, 2003a). However, this effect was only noted in the caeca of ZnB-treated birds and not in the ileum where the suppressive effects of ZnB on gut bacteria are considered more significant. The actual reasons for this observation are unclear. In a similar study, McCracken *et al*., (2001) postulated that the decrease in the number of peaks detected in these birds might have caused the C_s values to become statistically more sensitive to the presence and absence of individual peaks. Collectively, the data support the view of Collier *et al.* (2003a) that microbial homogenisation may ultimately be proven to be a target mechanism for the selection of alternatives such as MOS to antibiotics.

4.4.5 Alterations in the intestinal and caecal bacterial populations

Results from T-RFLP analysis at d 21 demonstrated that MOS increased the proportion of lactobacilli in the total microbial population of the ileum and simultaneously reduced the abundance of clostridia. Dogs supplemented with MOS also tended to have greater faecal *Lactobacillus* populations (Swanson *et al*., 2002). The culture-based analysis indicated a significant reduction in clostridial counts with no change in the numbers of total anaerobes in the MOS-fed group, suggesting that the increase in the abundance of lactobacilli is likely to have occurred at the expense of clostridia. Again, this finding underlines the importance of MOS in accelerating the maturation of gut microflora as the number of lactobacilli increases with age and it takes at least about 2 weeks for lactobacilli to become the predominant bacteria in the small intestine (Barnes *et al.*, 1972).

The suppressive effects of in-feed antibiotics on growth of intestinal lactobacilli and clostridia in broiler chickens are commonly known (Engberg *et al.*, 2000; Knarreborg *et al.*, 2002b; Wise and Siragusa, 2007). However, it is interesting to note that despite the suppression, lactobacilli still represented a relatively high proportion (62.5%) of the total bacterial population colonising the ileum of birds treated with ZnB. This reflects, in part, that even under such a suppressive environment there exists a differential selection for the growth of certain *Lactobacillus* species particularly those in the group of *L. plantarum* and *L. casei* group. Similar findings have also been reported in pigs where tylosin suppresses the growth of gut bacteria in general, but significantly increase the relative proportion of lactobacilli (Collier *et al.*, 2003a). Wise and Siragusa (2007) also reported that use of a combination of bacitracin, nicarbazin and roxarsone in broiler diets increased the relative proportion of ileal

lactobacilli to 45 to 58% at the expense of enterobacteriaceae which only constituted 16% of the total sequences detected.

The species, *L. salivarius* was not detected in the *Hha*I generated T-RFLP profiles due to its relatively large fragment size of 1122 bp which is beyond the detection limit of the DNA sequencer used in the current study. A T-RF peak of 565 bp detected in the *Msp*I digestion indicates the presence of *L. salivarius* in the ileal and caecal regions of all the treatment groups. However, this could not be confirmed due to the poor resolution of the peak which also corresponds to some *Enterococcus* species. *L. salivarius* has been reported to constitute about 5.26% of the total ileal microflora of chickens at 21 days of age (Lu *et al.*, 2003). The estimations of the proportional abundance of lactobacilli reported herein may have been slightly underestimated due to the absence of *L. salivarius.* In spite of this, the predicted 43.8% of the total *Lactobacillus* population in the NC group is in good accordance with those (45%) reported by Gong *et al*. (2002a) where the maximum size detection for T-RF was also limited at 650 bp, indicating the validity of the present results.

Effects of MOS and ZnB on *C. perfringens* and *Escherichiaceae* were not observed as the peaks corresponded to these bacterial species were not detected by T-RFLP in both the ileal digesta and ileal mucosal samples. It is possible that the intestinal load of these bacteria in the range of 10^2 to 10^4, as determined by the plate counts method, may be below the reported DNA detectable level of 10^5 CFU (Hume *et al*., 2003; Dumonceaux *et al*., 2006a, 2006b). Thus, it is evident that the culture-dependent and independent techniques complement each other and allow a detailed analysis of the intestinal microflora in relation to dietary treatments.

4.4.6 Composition and diversity of the intestinal and caecal bacteria

The microbial community structures present in the three intestinal regions examined in the current study differed phylogenetically with the ileal luminal microflora being more diverse than those inhabiting the ileal mucosa. The microbial communities of the caecal region were also highly diverse with more uncultured or unidentified bacterial species present.

***Lactobacillus* species**

An insight into the composition of the *Lactobacillus* population revealed an increase in the diversity of the *Lactobacillus* phylotypes and *vice versa* for the clostridia in the ileum and ileal mucosa of MOS fed birds. Castillo *et al*. (2006) observed similar findings in pigs fed

diets with butyrate and plants extracts as prebiotics. The diversity of beneficial bacteria is one of the most important factors for the establishment of a stable ecosystem in the intestinal tract. This has arisen from the observation that until the bacterial populations are fully established, young birds have fewer bacterial species in the intestinal tract than adult birds, making their gut microflora more susceptible to disturbances (Mead, 1989).

In the present investigation, the prevalence and biodiversity of gut microflora varied, but both MOS and ZnB differentially favoured the growth of certain specific bacterial species. For example, MOS favoured the proliferation of *L. acetotolerans, L. acidophilus* group*, L. delbrueckii* subsp. *lactis* and *L. sakei*, while ZnB preferentially favoured the growth of *L. plantarum* and *L. casei* group. In addition, MOS preferentially selected *L. delbrueckii* subsp. *lactis* in the ileal mucosa. In agreement with this, a stimulating effect of MOS on *L. casei*, *L. acidophilus* and *L. delbrueckii,* and in addition *Bifidobacterium longum* was also reported (Schrezenmeir and De Vrese, 2001 cited in Rekiel *et al.,* 2007). Recently, the results of Pettigrew and Miguel (2007) demonstrated a consistent appearance or disappearance of specific DGGE-bands when MOS was added to the pig diet. However, bacterial species that corresponded to these bands are yet to be determined. In addition, MOS and yeast extract were also shown to have the tendency to differentially select against *L. aviarius* in the ileum of chickens (Owens *et al.*, 2008). Another undisclosed prebiotic was shown to increase the microbial richness and diversity in the colon and caecum of weaning piglets (Mohnl, 2008). Therefore, it is safe to conclude that prebiotic or MOS is capable of changing the intestinal microbial composition.

Using PCR-DGGE, Pedroso *et al.* (2006) and Gong *et al.* (2008) reported that dietary bacitracin had no significant impact on the diversity, but altered the microbial composition of the small intestine of the chickens. In fact, in the Gong *et al.* (2008) study, ZnB was shown to differentially select against *L. vaginalis* in the ileum of 3 day-old chicks. In pigs and young chicks fed with tylosin, an apparent selection of *L. amylovorus* and *L. gasseri* was also noted (Collier *et al*., 2003a, 2003b; George *et al*., 2007). Although Lee *et al.* (2002) reported a similar finding where birds fed virginiamycin contained significantly fewer *Lactobacillus* species (absence of *L. acidophilus*) in the ileum than their untreated counterparts, the ratio of the dominant *Clostridium* species to the dominant *Lactobacillus* species was significantly higher in the virginiamycin-treated group. This contradicts the current finding and is not surprising as Pedroso *et al.* (2006) demonstrated that microbial structure tended to cluster

according to the type of antibiotics depicting that different antibiotics were capable of modifying the birds' intestinal microbial composition in their very own unique way.

It is interesting to note that *Lactobacillus* species originated from the intestinal tract of mammals and chickens demonstrated a large variability in bile salt tolerance as well as bile salt hydrolase (BSH) activity (Moser and Savage, 2001; Begley *et al.*, 2006). Thus, it is likely that the differential selections of these *Lactobacillus* species by MOS and ZnB may have some influence on the overall ileal BSH activities and fat digestion. Since ileal bile salt dehydrolysing activities were not determined in this study, it is therefore not possible to establish the extent to which the abundance as well as the prevalence of these differentially selected *Lactobacillus* species had contributed to the overall growth performance of those birds supplemented with MOS or ZnB.

The actual mechanisms underlying these differential selections induced by MOS and ZnB remain unclear. For ZnB, the mechanism is likely to be associated with the replacement of antibiotic-susceptible strains by resistance strains (Collier *et al.*, 2003a). In fact, resistance of *Lactobacillus* species to different types of antibiotics displayed a strain- and drug-dependent manner. For example, certain strains of *L. plantarum* are more resistance to bacitracin than any other *Lactobacillus* species examined. As postulated, certain *Lactobacillus* species are more sensitive to the presence of bile salts than others which eventually may impair their membrane permeability and thus, increase their susceptibility to antibiotics (Elkins and Mullis, 2004).

***Clostridium* species**

The significance of a high prevalence of clostridia in the ileum and caeca of the NC group, especially those of the *C. perfringens* as revealed by the plate count method may hold an even greater responsibility than lactobacilli in suppressing the growth of the birds as most *Clostridium* species possess additional extracellular BSH-activities (Coleman and Hudson, 1995; Knarreborg *et al.*, 2002a). This is exemplified by *C. perfrimgens* which have high BSH-activity on almost all types of bile salts including taurocholic acid, taurochenodeoxycholic acid, taurodeoxycholic acid, glycocholic acid, glycochenodeoxycholic acid and glycodeoxycholic acid (Ridlon *et al.*, 2006). There are also evidence to support that clostridia, in particular *C. perfringens,* are capable of further breaking down the secondary bile salts into 3-keto bile salts (Hirano *et al.*, 1981; Masuda, 1981; Doerner *et al.*, 1997) which are highly toxic to the hosts by causing DNA damage or

result in impaired intestinal mucosal function (Kandell and Bernstein, 1991; Pazzi *et al.*, 1997; De Boever *et al.*, 2000).

It is noteworthy to point out that the presence of *C. butyricum* only in the NC group may have some significant negative effects on the growth of the birds. Although some strains of *C. butyricum* have been used as probiotics for humans and animals, their close association with neonatal necrotising enterocolitis (Howard *et al.*, 1977) and cecitis in chickens and quails (Popoff *et al.*, 1985; Bousseboua *et al.*, 1989; Corthier *et al.*, 2000) have also been frequently reported. Thus, the presence of *C. butyricum* warrants the future need to be distinct from the other *Clostridium* species. Furthermore, the ecological and physiological role of some of the prominent unidentified peaks present largely in the caeca of the birds may also need to be elucidated in future research.

4.5 CONCLUSIONS

As revealed by the plate culture method, MOS accelerated the maturation of gut microflora by promoting the growth of lactobacilli in the ileal mucosa and *vice versa* on ileal and caecal clostridia. Unlike MOS, ZnB suppressed the growth of intestinal bacteria, especially those of the lactobacilli and clostridia. Use of T-RFLP further revealed that MOS increased the diversity of lactobacilli in the ileal contents and mucosa but the opposite was observed for ZnB. Despite these differences, both MOS and ZnB increased the proportional abundance of ileal lactobacilli at the expense of the clostridia. It also appears that MOS and ZnB possessed a common ability in differentially favouring the growth of certain *Lactobacillus* species and increasing the homogeneity of the gut microflora. The modulating effects of MOS and ZnB on the gut microflora may, in part, responsible for the selective increase in the intensity of sulphated-acidic mucin goblet cells. However, future experiments conducted under germ-free environment are warranted to determine whether MOS or ZnB has any direct effect on intestinal mucin dynamics. Collectively, improvement in intestinal microbial homogeneity and mucin synthesis coupled with the differential selections for certain specific beneficial bacterial species may ultimately be proven to be the target mechanisms for the search of more effective alternatives to antibiotics.

CHAPTER 5
NUTRIENT UTILISATION IN RESPONSE TO MANNOOLIGOSACCHARIDES AND DIETARY THREONINE IN BROILER CHICKENS

5.1 INTRODUCTION

The results obtained in Chapter 3 indicate that graded levels of dietary threonine produced a quadratic response in growth performance as well as in intestinal mucosal development. It was also shown in the last two chapters that MOS, through the modulation of intestinal mucin dynamics and microflora composition, had beneficial effects on the gut development and growth of young birds. However, the effects of these supplementation in relation to mucin dynamics on nutrient utilisation have not been fully investigated.

Excessive mucin secretion increases endogenous nutrient loss and impairs nutrient absorption (Jeurissen *et al.*, 2002). Because MOS and dietary threonine have positive effects on mucin dynamics, there is a concern that the two could have negative impact on the digestibility and intestinal uptake of nutrients. However, more recent data generally support the fact that intestinal mucous layer does not disturb nutrient absorption (Morita *et al.*, 2006) and does not affect the apparent digestibility of the diet (Montagne *et al.*, 2004 cited in Uni, 2008).

Therefore, the present study aims to evaluate the effect of MOS and dietary threonine on the growth performance in relation to the intestinal mucin turnover as well as the apparent and standardised ileal amino acid digestibility. In addition, the impact of these supplementation on the intestinal uptake of threonine and glucose in broiler chickens was also investigated.

5.2 MATERIALS AND METHODS

5.2.1 Bird management and experimental diets

A completely randomised 3 x 2 factorial design was employed in this study where six treatments were allocated to each of the starter basal diets, comprising mainly of sorghum, peanut-meal and soybean meal (Table 5.1). All diets were formulated to meet or exceed the minimum National Research Council (NRC, 1994) nutrient recommendations for broiler chickens, except for threonine. Three levels of dietary threonine were formulated at 70 (Deficient), 100 (Adequate) or 130% (Excess) of the NRC recommended level (Table 5.2)

where L-threonine (Ajinomoto Eurolysine, Paris, France) was supplemented to the basal diets at the respective rate of 0, 0.25 or 0.50 % at the expense of L-alanine and L-glutamic acid (1:1) (Sigma-Aldrich Chemicals, St Louis, MO, USA).

Two levels of MOS supplementation, with and without MOS, were created by adding Bio-MOS® (Alltech Biotechnology, Nicholasville, Kentucky, USA) at 0 and 0.20 % of the experimental diet at the expense of a filler (Zeolite), respectively. In this way, all the diets were isocaloric at 3050 kcal/kg of ME and isonitrogenous at 19.6% CP (Barkley and Wallis, 2001; Atencio *et al.*, 2004; Faure *et al.*, 2005). A set of the same experimental diets marked with 0.5% (w/w) titanium dioxide (TiO_2) (Sigma-Aldrich) was also prepared for the calculation of ileal amino acid flows and mucin turnover.

Another diet containing enzymatically hydrolysed casein (EHC) (New Zealand Pharmacueticals Ltd., Palmerston North, New Zealand) as the sole nitrogen source was used to determine the basal endogenous ileal amino acid losses (Moughan *et al.*, 1990; Butts *et al.*, 1991; Ravindran *et al.*, 2004a) (Table 5.2). Sodium bicarbonate and salt were used as the cationic sources to maintain a dietary electrolyte balance of 250 mEq/kg diet and also to improve feed intake (Ravindran and Morel, 2006). Titanium dioxide (0.50%) was also included in the EHC diet as an inert marker for the calculation of endogenous flow of amino acids. All diets were mixed at the University of New England. Except for the EHC diet which was fed in mash form, all the other diets were cold-pelleted. The major nutrient levels of all the experimental diets were reaffirmed as described in Section 3.2.1. A summary of the experimental diets is shown in Table 5.2.

Two hundred and seventy day-old male Cobb broiler chicks (Baiada Poultry Pty. Ltd., Kootingal, NSW, Australia), weighing 47.23 ± 0.57 g, were randomly assigned to 30 cages (600 x 420 x 230 cm) in four-tier battery brooders housed in an environmentally controlled room. Each of the 6 treatments was randomly assigned to 5 cages with 9 birds per cage. An initial room temperature of 34°C was maintained for the first 5 days and gradually decreased to 21°C by the end of the experiment at 21 d. Birds were provided *ad libitum* access to the feed and water. The lighting programme was 24 h light for the first day followed by 18 h light and 6 h darkness for the rest of the trial period.

5.2.2 Animal ethics

All experimental procedures were approved by the Animal Ethics Committee of the University of New England with the approval numbers AEC 07/156 and AEC 07/157.

Table 5.1 Composition of the experimental diets given to the broiler chickens

Ingredient	Basal starter	EHC
	----------%----------	
Sorghum grain	55.80	-
Maize starch	9.00	53.00
Soybean meal dehulled, 47.5% CP	8.75	-
Peanut meal, solvent 45% CP	17.50	-
Dextrose[3]	-	10.00
Cellulose[3]	-	3.00
Vegetable oil	2.81	6.00
EHC[1]	-	22.00
Filler[2]	0.20	-
Titanium dioxide	0.50	0.50
L-Threonine	0.00	-
L-Lysine HCl	0.92	-
DL-Methionine	0.34	-
Alanine/glutamic acid (1:1)[4]	0.60	-
Calcium carbonate	1.09	1.30
Dicalcium phosphate	1.82	1.70
Choline chloride	0.03	0.10
Sodium bicarbonate	0.40	2.00
Salt	0.05	0.20
Vitamin and mineral premix[5]	0.20	0.20
Total	100.00	100.00
Calculated nutrient composition	----------%----------	
CP	19.60	18.91
Lysine	1.30	1.28
Methionine	0.50	0.55
Methionine + Cysteine	0.85	0.86
Threonine	0.55	0.89
ME poultry, kcal/kg	3050	3109

[1]Enzymatically hydrolysed casein (Casein peptone) obtained from New Zealand Pharmacueticals Ltd., Palmerston North, NZ; molecular mass, <5000 Da. According to the manufacturer, the molecular mass profile was (%): 1-200 Da (43), 200-500 Da (45), 500-2000 Da (13), >2000 Da (0).
[2]Filler represents inert space (Zeolite) in the diet to which BioMOS® was added at its expense.
[3]Sigma-Aldrich Chemicals, St Louis, MO, USA.
[4]L-Threonine was added at the expense of alanine/glutamic acid (1:1).
[5]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.

Table 5.2 Calculated and analysed nutrient values of the experimental diets

	Treatment						
MOS	-				+		
Threonine	Deficient	Adequate	Excess	EHC	Deficient	Adequate	Excess
Calculated nutrient composition (% as-is basis)							
CP	19.60	19.60	19.60	18.91	19.60	19.60	19.60
Lysine	1.30	1.30	1.30	1.28	1.30	1.30	1.30
Methionine	0.50	0.50	0.50	0.55	0.50	0.50	0.50
Threonne	0.55	0.80	1.05	0.89	0.55	0.80	1.05
Analysed nutrient composition (% as-is basis)							
CP	19.20	19.10	19.40	18.56	19.30	19.20	19.50
Lysine	1.30	1.25	1.25	1.22	1.29	1.38	1.26
Methionine	0.44	0.45	0.42	0.57	0.43	0.43	0.44
Threonine	0.55	0.82	1.06	0.85	0.56	0.81	1.09

5.2.3 Growth performance measurement

Body weight gain (BWG), feed conversion ratio (FCR), feed intake (FI) and mortality were determined as described in Section 3.2.4.

5.2.4 Protein and threonine efficiency ratios

Protein and threonine efficiency ratios (PER and TER) were calculated by expressing the body weight gain (g) as a fraction of the actual crude protein and threonine intake (g), respectively.

5.2.5 Apparent and standardised ileal amino acid digestibility

Conduct of trial

At d 15, same set of adjacent replicates were created by transferring three birds from each of the original cages to new cages housed in an environmental controlled room. To this new set of replicates, the purified EHC diet was gradually introduced from d 15 to 18 by diluting the respective experimental diets with the EHC diet at a progressive daily rate of 25, 50 and 75%. The aim of using the diluted EHC diet was to enable the birds to adjust to the changeover EHC diet and ensure a satisfactory feed intake levels when purified diets were fully introduced.

By d 18, the EHC diet was fully introduced and offered to the birds for 3 d *ad libitum*. Water was available at all times. From d 18 to 21, the experimental starter diets fed to those birds

kept in the original cages were also withdrawn and the respective TiO_2 marked experimental starter diets were introduced and fed for 3 d.

Sample collection and processing

On d 21, all the birds fed on the EHC diet were euthanised by cervical dislocation and digesta from the ileum (from Meckel's diverticulum to the ileo-caecal junction) were collected. Samples from birds within a cage were pooled, homogenised and frozen immediately at –21°C. Subsequently, the samples were freeze-dried and the endogenous protein fraction was extracted according to the method described by Cowieson and Ravindran (2007).

Basically, the freeze-dried samples were re-suspended in deionised water and acidified to pH 3.5 with 9M H_2SO_4. The samples were stored overnight at 4°C and then centrifuged at 1,450 *g* for 45 min at 0°C. Each supernatant fraction was decanted off and retained. The precipitate was washed with 10 mL deionised water and centrifuged at 1,450 *g* for 30 min at 0°C. The second supernatant was added to the first and the precipitate was stored at –20°C. The combined supernatant fractions were ultrafiltrated using a Centriprep-10 ultrafiltering device (MW cut-off filter, 10,000 Da; Amicon Inc., Beverly, MA, USA) according to the manufacturer's instructions. The precipitate from the centrifugation step was added to the retentate (>10,000 Da) from the ultrafiltration step. Later, the material was freeze-dried, ground to pass through 0.5 mm sieve and stored in airtight containers at –20°C for chemical analysis of TiO_2 and amino acid (lysine, threonine and methionine) contents as described in Sections 5.2.6 and 3.2.1, respectively.

Also on d 21, three birds per cage fed on the TiO_2 marked experimental diets were also euthanised by cervical dislocation. The gastrointestinal tract was excised and the small intestine dissected into three regions of duodenum, jejunum and ileum with reference points as described in Section 3.2.5. The digesta from the ileum were collected, pooled within a cage, homogenised, and stored at –20°C. Later, the samples were freeze-dried, ground to pass through 0.5 mm sieve and stored in airtight containers at –20°C for chemical analysis of TiO_2 and amino acids (lysine, threonine and methionine) as described in Sections 5.2.6 and 3.2.1, respectively.

A sample from each of the TiO_2 marked experimental and EHC diet was also obtained and processed through the same procedure as described for the digesta sample.

Calculations

The apparent ileal digestibility (AID) of the amino acids expressed as % was calculated from the following equation (Stein *et al.*, 2007):

$$AID = \left[1 - \left(\frac{AA_i}{AA_d} \times \frac{TiO_{2d}}{TiO_{2i}}\right)\right] \times 100$$

where AA_i is the amino acid concentration (g/kg DM) in the ileal digesta, AA_d is the amino acid concentration (g/kg DM) in the diet, $TiO_{2\,d}$ is the TiO_2 concentration (g/kg DM) in the diet and $TiO_{2\,i}$ is the TiO_2 concentration (g/kg DM) in the ileal digesta.

The ileal endogenous amino acid flow (EF) expressed as g/kg DM intake in birds fed the EHC diet gave the estimations of the basal endogenous concentrations in the ileal digesta and was calculated using the following equation (Moughan *et al.*, 1992; Ravindran *et al.*, 2004b; Stein *et al.*, 2007):

$$EF = AA_{EHC\,i} \times \frac{TiO_{2\,EHC\,d}}{TiO_{2\,EHC\,i}}$$

where $AA_{EHC\,i}$ is the amino acid concentration (g/kg DM) in the ileal digesta of the birds fed the EHC diet, $TiO_{2\,EHC\,d}$ is the TiO_2 concentration (g/kg DM) in the EHC diet and $TiO_{2\,EHC\,i}$ is the TiO_2 concentration (g/kg DM) in the ileal digesta of birds fed the EHC diet.

The standardised ileal digestibility (SID) expressed as % was calculated from the following equation (Stein *et al.*, 2007):

$$SID = AID + \left[\left(\frac{EF}{AA_d}\right) \times 100\right]$$

5.2.6 Titanium dioxide analysis

The TiO_2 content of the sample was measured using the Short *et al*. (1996) method. Basically, approximately 0.1 g of the freeze-dried digesta or feed sample were ashed in porcelain crucible for 13 h at 580°C and dissolved in 5 mL 7.4 M sulphuric acid upon cooling. The sample was then gently boiled for approximately 20 min until completely dissolved. After

cooling, the solution was poured quantitatively into a small beaker each containing 15 mL distilled water. The content of the beaker was poured into a 50 mL volumetric flask through a filter paper (Whatman 541). Hydrogen peroxide (30% v), measuring 10 mL in volume, was subsequently added to each flask and the contents diluted to 50 mL with distilled water 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 Hitachi 150-20 UV spectrophotometer (Hitachi Science Systems Ltd., Ibaraki, Japan) by measuring the absorbance at 410 nm. The TiO_2 content, measured in mg/mL, was determined from the standard curve and converted to mg/g of the sample.

5.2.7 Crude mucin turnover and dry matter output

Crude mucins were isolated by a modification of procedures presented by Allen (1981), and Miller and Hoskins (1981) as described by Lien (1995). Approximately 2 g of freeze-dried ileal digesta, obtained as described in Section 5.2.5, were weighed and combined with 25 mL of 0.15M NaCl containing 0.02M sodium azide and maintained at 4°C. Samples were homogenised for 1 min using an Ultra-Turrax T10 homogenizer (IKA®-Werke GmbH & Co, Staufen, Germany) and immediately centrifuged at 12,000 *g* for 30 min at 4°C. The supernatant was decanted into a second 50 mL centrifuging tube and centrifuged once again at 12,000 *g* for 30 min at 4°C to ensure the complete removal of insoluble material. Sixteen mL of the aqueous fraction were pipetted into a pre-weighed 50 mL centrifuge tube, cooled in an ice-bath. Twenty-four mL of the ice-cold absolute ethanol were added to the aqueous supernatant to yield a final concentration of 60% ethanol (v/v). The samples were allowed to precipitate overnight at –20°C.

On the following day, samples were centrifuged at 1,400 *g* for 10 min at 4°C and the precipitates were recovered by decanting the supernatant. The pellet was resolubilised in 16 mL 0.15M NaCl, cooled in an ice-bath and 24 mL of the ice-cold ethanol was added to achieve a final concentration of 60% ethanol (v/v). The samples were again allowed to precipitate overnight at –20°C and the crude mucins were again recovered by centrifuging at 1,400 *g* for 10 min at 4°C. Successive rinsing was continued until a clear supernatant was obtained. The final precipitate was resolubilised in 2 mL of distilled water, lyophilised and weighed. The crude mucins recovered expressed as crude mucin concentration (g/100g DM digesta) and crude mucin output (g/kg DM intake) were computed based on the following equations:

$$\text{Crude mucin concentration} = \frac{\text{Crude mucin}_{\text{digesta}}}{\text{DM}_{\text{digesta}}} \text{ x } 100$$

and

$$\text{Crude mucin output} = \frac{\left(\text{Crude mucin}_{\text{digesta}} \text{ x } \text{TiO}_{2\,\text{diet}}\right)}{\text{TiO}_{2\,\text{digesta}}} \text{ x } 1000$$

where Crude $\text{mucin}_{\text{diegsta}}$ is the amount of crude mucin (g) extracted out from 1 g of DM digesta ($\text{DM}_{\text{digesta}}$) and $\text{TiO}_{2\ \text{diet}}$ or $\text{TiO}_{2\ \text{digesta}}$ is the TiO_2 concentration expressed as mg/g DM of the diet or digesta as determined for the standardised ileal digestibility calculation.

The DM output expressed as g/100g wet digesta was calculated based on the following equation:

$$\text{Dry matter (DM) output} = \frac{\left(\text{DM}_{\text{wet digesta}} \text{ x } \text{TiO}_{2\,\text{diet}}\right)}{\text{TiO}_{2\,\text{digesta}}} \text{ x } 100$$

where $\text{DM}_{\text{wet diegsta}}$ is the amount of DM (g) of 1 g of wet digesta and $\text{TiO}_{2\ \text{diet}}$ or $\text{TiO}_{2\ \text{digesta}}$ is the TiO_2 concentration expressed as mg/g DM of the diet or digesta as determined for the standardised ileal digestibility calculation.

5.2.8 Enteric morphometric and gross morphology parameters

From the first bird (fed on experimental diets) of each replicate euthanised, the length and weight of the respective intestinal regions were measured as described in Section 3.2.5.

Approximately 2 cm of the proximal ileum were also removed from the second bird (fed on experimental diets) for gut histo-morphological measurements. The gut samples were flushed with ice-cold buffered phosphate saline at pH 7.4 and immediately placed in 10% formalin solution before being further processed as outlined in Section 3.2.7.

5.2.9 Ileal nutrient uptake

Conduct of trial

At the end of the experiment at d 21, the remaining birds kept in their original cages were fasted overnight for 12 h but allowed access to drinking water. One bird per cage and three replicates per treatment were randomly selected for the intestinal nutrient uptake assay using

the *in situ*-perfusion technique with reference from Kansal and Wahie (1981) and Kim (2005).

Surgical procedure

On d 22, the fasted birds were weighed and anaesthetised with sodium pentobarbitone (40 mg/kg body weight) intraperitoneally and maintained under constant surgical general anaesthesia with sodium pentobarbitone at 20 mg/kg body weight whenever necessary. The anaesthetised birds were placed on heating pads to prevent excessive body heat loss.

A laparotomy incision was made in the mid-line of the abdomen and excess fat tissue was carefully dissected to expose the peritoneal cavity. With the caeca located, the small intestine was traced cranially for at least 50 cm to locate the Meckel's diverticulum. This section of the small intestine is referred to as the ileum. The first ligation was made at 10 cm caudal to the Meckel's diverticulum. Care was taken not to damage or occlude the mesenteric blood supply. Approximately 5 mL of warm (39°C) buffered phosphate saline were injected into the lumen of the ileum caudal to the ligation to gently wash the loop. The buffered phosphate saline was then gently persuaded along the ileum towards the ileal-caecal junction with care not to disturb the unstirred mucin-water layer lining the ileal mucosa. Another ligation was made 10 cm caudal to the first ligation forming a 10 cm isolated loop (loop A). A second loop (loop B) was made in the similar way starting about 1 cm from the previous one (Plate 5.1).

Using a 26 G needle, 1.5 mL of warm Krebs-Ringer-Bicarbonate and Krebs-Ringer-Bicarbonate containing D-glucose and L-threonine solutions were injected into loop A and B, respectively. The Krebs-Ringer-Bicarbonate solutions had an osmolality of 355 mOsm/kg water, comprising inorganic salts of 161 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 20 mM $NaHCO_3$. Since 50 mM each of L-threonine (Sigma-Aldrich) and D-glucose (Sigma-Aldrich) were added in the test Krebs-Ringer-Bicarbonate solution, the amount of sodium chloride was reduced to 111 mM so that the osmolality of the buffer did not change (Kansal and Wahie, 1981).

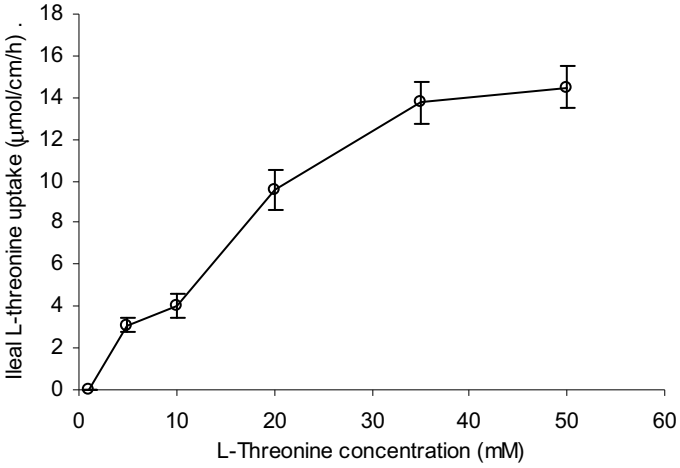


Figure 5.1 Effect of concentration on ileal L-threonine uptake during 10 min intestinal perfusion of broiler chickens at 18 days of age

Values are means ± SEM of n=3.

The concentration of the D-glucose at 50 mM was adopted from Obst and Diamond (1992). A concentration of 50 mM L-threonine was chosen because a preliminary study showed that the ileal mucosal uptake of L-threonine tended to maximise at this concentration (Figure 5.1). The exact amount of the injected incubation solutions required to make the loops just turgid were recorded. The time was also noted when loop B was half filled. The laparotomy was then closed with two pairs of Allis forceps (Plate 5.1). After 10 min, the bird was given an overdose of sodium-pentobarbitone (150 mg/kg body weight) and the loops were removed from the body as quickly as possible. The loops were wiped clean of any trace of blood with surgical gauze. The loop contents recovered were washed out with buffered phosphate saline into a small measuring cylinder and the recovered volumes were measured and recorded. For convenience and accuracy purposes, the recovered volumes of the loop contents were standardised to 3 mL by diluting with buffered phosphate saline and stored at –20°C immediately to minimise microbial activities for future D-glucose and L-threonine assays. The length and blot-dried weight of the respective loops were measured and recorded.

Glucose and threonine concentration assays

The concentration of the D-glucose was determined using Megazyme D-glucose (glucose oxidase/peroxidase; GOPOD) Assay Kit (Megazyme International Ireland Ltd., Wicklow, Ireland). D-glucose was measured colorimetrically after incubating an aliquot (0.1 mL) of the recovered perfusate sample or incubation solution (50 mM D-glucose) with 3 mL of GOPOD reagent at 50°C for 20 min. A D-glucose standard and a blank solution were prepared in a similar manner by adding 3.0 mL of GOPOD reagent to 0.1 mL of the provided D-glucose

standard solution (100 μg/0.1 mL) and 0.1 mL deionised water, respectively. The absorbance was read at 510 nm against the blank to obtain the change in absorbance in the samples (ΔA_{sample}) as well as the D-glucose standard ($\Delta A_{D\text{-}glucose\ standard}$). The concentration of D-glucose expressed as μg/0.1 mL of the sample was calculated from the following equation:

$$D-glucose = \frac{\Delta A_{sample}}{\Delta A_{D-glucose\ standard}\ (100\mu g)} \text{ x } 100$$

An aliquot of the recovered perfusate samples and the incubation solution (containing 50 mM of L-threonine) were sent to Australian Proteome Analysis Facility Ltd. (APAF, Sydney, NSW, Australia) for free L-threonine analysis using a precolumn derivatisation method (AccQTaq, Waters, Milford, MA, USA).

Calculations

The apparent ileal mucosal uptake of D-glucose or L-threonine, expressed as mmoles per cm of the ileum per hour (mmol/cm/h) or mmoles per g of the ileum per hour (mmol/g/h) was calculated based on the following equation:

$$\text{Apparent nutrient uptake} = \frac{(\text{Vol}\,I_B \text{ x Nutrient } I_B) - (\text{Vol}\,R_B \text{ x Nutrient}\,R_B)}{\text{Length}_B \text{ or Weight}_B \text{ x } 0.167}$$

where vol I_B is the volume (L) injected into loop B, vol R_B is the volume (L) recovered from loop B, nutrient I_B is the concentration (mM) of D-glucose or L-threonine injected into loop B, nutrient R_B is the concentration (mM) of D-glucose or L-threonine recovered from loop B, $length_B$ is the length (cm) of loop B, $weight_B$ is the weight (g) of loop B and 0.167 is the duration of incubation in hour.

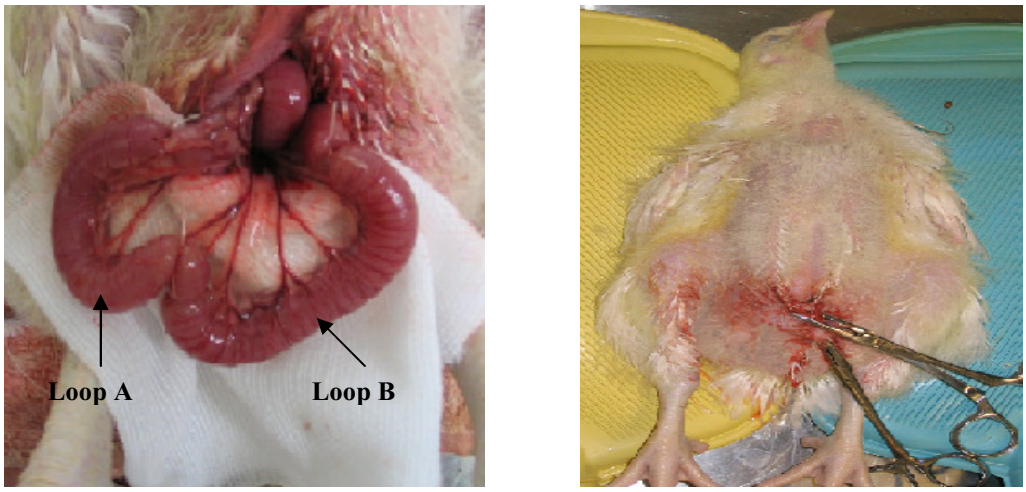
The endogenous flow of nutrient expressed in the same units as apparent nutrient uptake was calculated from the following equation:

$$\text{Endogenous nutrient flow} = \frac{(\text{Vol}\,R_A \text{ x Nutrient}\,R_A)}{\text{Length}_A \text{ or Weight}_A \text{ x } 0.167}$$

where vol R_A is the volume (L) recovered from loop A, nutrient R_A is the concentration (mM) of glucose or threonine recovered from loop A, $length_A$ is the length (cm) of loop A, $weight_A$ is the weight (g) of loop A and 0.167 is the number of incubation hour.

The true ileal mucosal uptake of D-glucose or L-threonine expressed in the same units as the apparent nutrient uptake was calculated based on the following equation:

True nutrient uptake = Apparent nutrient uptake – endogenous nutrient flow.



(a) (b)

Plate 5.1 Pictorial illustrations of the loop-gut perfusion method showing (a) the ileal loops and (b) the closure of the laparotomy for the ileal nutrient uptake assays

5.2.10 Statistical analysis

The same statistical model described in Section 3.2.10 was employed to analyse the variance of all the data with level of significance considered at $P \leq 0.05$ unless otherwise specified.

5.3 RESULTS

5.3.1 Dietary composition and treatment design

The calculated crude protein and amino acid contents of the diets and their respective analysed values are presented in Table 5.2. Generally, the analysed values were in close agreement (within 5% variation in differences) with the calculated values except for methionine values which were consistently 0.05 to 0.08% lower than the calculated values.

5.3.2 Growth performance

The effects of dietary threonine and MOS on growth performance are presented in Table 5.3. Birds deficient in threonine responded negatively in all performance criteria ($P<0.01$) except for mortality. Supplementation of threonine beyond the adequate level did not improve the growth performance but instead resulted in a decrease ($P<0.05$) in BWG, especially from 1 to 14 and 1 to 21 d of age. Addition of MOS improved ($P<0.05$) the BWG and FCR from 1 to 14 and 1 to 21 d of age. The only interaction between MOS and dietary threonine was observed on BWG from 1 to 14 d of age with the highest BWG noticed in birds given the adequate- or excess-threonine plus MOS diet. No significant effects on mortality attributable to treatments were recorded.

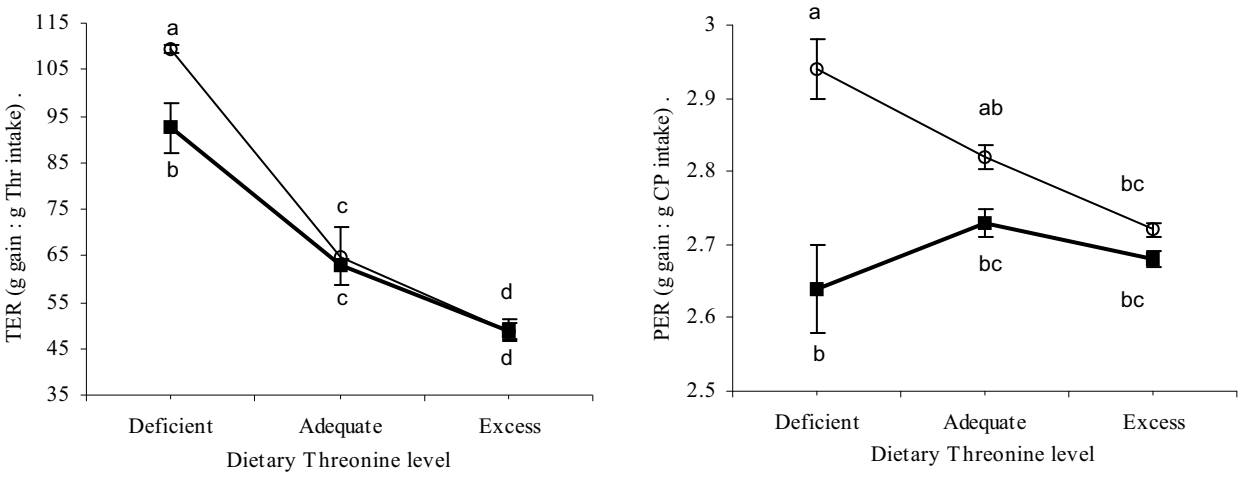
5.3.3 Protein and threonine efficiency ratios

Effects of MOS and dietary threonine on PER and TER are summerised in Figure 5.2. The interaction between MOS and dietary threonine level for PER was significant ($P<0.05$) at 0 to 3 weeks of age. In the absence of MOS, PER responded quadratically with increase in dietary threonine level. However, this response became linear with inclusion of MOS. At a deficient level of dietary threonine, supplementation of MOS significantly increased ($P<0.05$) PER. Threonine efficiency ratio was also affected by an interaction between MOS and dietary threonine. Dietary MOS increased ($P<0.01$) the TER compared with diets without MOS addition, especially at the deficient level of dietary threonine. Regardless of MOS supplementation, TER decreased as dietary threonine level increased.

Table 5.3 Effects of dietary threonine level supplemented with or without MOS on body weight gain (BWG), feed conversion ratio (FCR), feed intake (FI) and mortality rate of birds fed the experimental diets[1]

Treatment		BWG (g/bird)			FCR			Feed Intake (g/bird)			Mortality (%)		
Thr	MOS	1-7d	1-14d	1-21d	1-7d	1-14d	1-21d	1-7d	1-14d	1-21d	1-7d	1-14d	1-21d
Deficient		73^{b}	245	535^{c}	1.62^{a}	1.53^{a}	1.56^{a}	117^{b}	372^{b}	830^{b}	0.00	2.22	2.22
Adequate		127^{a}	425	922^{a}	1.24^{b}	1.34^{b}	1.37^{b}	158^{a}	569^{a}	1268^{a}	0.00	2.22	2.22
Excess		122^{a}	403	891^{b}	1.25^{b}	1.40^{b}	1.42^{b}	152^{a}	565^{a}	1261^{a}	0.00	1.11	1.11
	-	106	347	762^{b}	1.39	1.47^{a}	1.48^{a}	143	499	1109	0.00	2.22	2.96
	+	109	369	804^{a}	1.35	1.38^{b}	1.42^{b}	142	505	1130	0.00	0.74	0.74
Deficient	-	72	238^{e}	511	1.66	1.61	1.62	118	383	825	0.00	2.22	2.22
Deficient	+	74	251^{d}	559	1.58	1.45	1.49	117	363	834	0.00	2.22	2.22
Adequate	-	125	417^{b}	904	1.27	1.34	1.39	158	559	1259	0.00	2.22	4.44
Adequate	+	129	434^{a}	940	1.22	1.33	1.36	158	578	1276	0.00	0.00	0.00
Excess	-	122	385^{c}	870	1.26	1.44	1.43	153	556	1242	0.00	2.22	2.22
Excess	+	122	422^{ab}	912	1.24	1.36	1.41	151	573	1281	0.00	0.00	0.00
SEM		2.4	4.3	17.7	0.042	0.033	0.039	2.5	9.2	23.5	0.00	1.812	1.935
Source of variation		----------Probability----------											
Thr		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	NS	NS	NS
MOS		NS[2]	0.0010	0.0480	NS	0.0050	0.0057	NS	NS	NS	NS	NS	NS
Thr x MOS		NS	0.0200	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[1]Each value represents the mean of 5 replicates for each treatment group.
[2]NS=Not significant.
[a-e]Means within a continuous column with different superscripts are significantly different at $P<0.05$.



(a) (b)

Figure 5.2 Threonine efficiency ratio, TER (a) and protein efficiency ratio, PER (b) plotted as a function of dietary threonine level for broiler chickens supplemented with (—) and without (▬) MOS from 0 to 3 weeks of age

Points represent means of 5 replicates with SEM shown. Effects of MOS inclusion, dietary threonine level and MOS by threonine interaction on TER ($P < 0.01$) and PER ($P < 0.05$) were significant. a-c = values on each chart with no common letter differ significantly ($P < 0.05$).

5.3.4 Intestinal growth parameters

Intestinal growth parameters of 21 day-old broilers as affected by dietary threonine supplemented with or without MOS are shown in Table 5.4. No interaction between dietary threonine and MOS on intestinal growth parameters was observed. Deficiency in dietary threonine increased ($P<0.01$) the relative length of all the three small intestinal regions. The relative weights of the duodenum ($P<0.05$) and jejunum ($P=0.08$) as well as the whole small intestine ($P<0.05$) were also significantly higher in birds deficient in dietary threonine. Supplementation of MOS reduced ($P<0.05$) the relative length of the jejunum and the entire small intestine. However, no difference in the relative weight of any of the small intestinal regions attributable to MOS was observed.

Table 5.4 Effects of dietary threonine level and supplementation of MOS on the relative weight and length of small intestinal regions of broilers at 21 days of age[1]

Treatment		Relative regional length (cm/100 g BW)				Relative regional weight (g/100 g BW)			
Thr	MOS	Duodenum	Jejunum	Ileum	Small Intestine	Duodenum	Jejunum	Ileum	Small Intestine
Deficient		3.41[a]	7.74[a]	7.60[a]	18.74[a]	1.23[a]	1.81	1.26	4.30[a]
Adequate		2.48[b]	5.56[b]	5.61[b]	13.67[b]	1.02[b]	1.61	1.16	3.78[b]
Excess		2.56[b]	5.92[b]	6.13[b]	14.61[b]	1.10[ab]	1.83	1.29	4.22[ab]
	-	2.96	6.80[a]	6.69	16.45[a]	1.12	1.76	1.26	4.13
	+	2.66	6.04[b]	6.20	14.90[b]	1.11	1.74	1.22	4.07
Deficient	-	3.76	8.50	7.96	20.21	1.25	1.82	1.27	4.34
Deficient	+	3.05	6.97	7.24	17.27	1.21	1.79	1.25	4.25
Adequate	-	2.38	5.76	5.71	13.85	1.00	1.56	1.10	3.52
Adequate	+	2.57	5.44	5.50	13.51	1.15	1.64	1.24	3.79
Excess	-	2.74	6.15	6.40	15.28	1.22	1.89	1.22	4.53
Excess	+	2.37	5.70	5.90	13.94	1.00	1.77	1.16	3.93
SEM		0.183	0.229	0.378	0.792	0.067	0.112	0.073	0.204
Source of variation		----------Probability----------							
Thr		0.0001	0.0001	0.0001	0.0001	0.0300	0.0800	NS	0.0400
MOS		NS[2]	0.0110	NS	0.0300	NS	NS	NS	NS
Thr x MOS		NS	NS	NS	NS	NS	NS	NS	NS

[1]Each value represents the mean of 5 replicates for each treatment group.
[2]NS=Not significant.
[a,b]Means within a continuous column with different superscripts are significantly different at $P<0.05$. Probability values up to $P<0.10$ are also shown if the data suggest a trend.

5.3.5 Enteric morphometric analysis

Dietary treatments had no significant effect on either the external muscularis thickness or crypt depth measured in all the intestinal regions (Table 5.5). However, birds fed the low-threonine diets had shorter (P<0.05) villi and a lower (P<0.01) villus to crypt ratio. On the contrary, MOS-fed group tended to have longer villi (P=0.067) and a higher villus to crypt ratio (P<0.05) compared to the untreated groups. In addition, MOS also interacted with adequate feeding of dietary threonine to increase (P<0.05) the ileal villus to crypt ratio.

Table 5.5 Effects of dietary threonine level and supplementation of MOS on ileal morphometric parameters of broilers at 21days of age[1]

Treatment		Ileal morphometric parameters			
Thr	MOS	Muscularis thickness (μm)	Crypt depth (μm)	Villus height (μm)	Villus/Crypt ratio
Deficient		194	181	702[b]	3.87
Adequate		210	185	960[a]	5.31
Excess		169	160	861[ab]	5.23
	-	186	169	755	4.42
	+	196	181	927	5.20
Deficient	-	208	171	638	3.70[c]
Deficient	+	179	191	766	4.03[b]
Adequate	-	201	200	884	4.46[b]
Adequate	+	218	169	1036	6.16[a]
Excess	-	148	136	742	5.10[ab]
Excess	+	191	183	979	5.40[ab]
SEM		22.1	19.8	38.3	0.319
Source of variation		----------Probability----------			
Thr		NS[2]	NS	0.0106	0.0003
MOS		NS	NS	0.0670	0.0088
Thr x MOS		NS	NS	NS	0.0500

[1]Each value represents the mean of 5 replicates for each treatment group.
[2]NS=Not significant.
[a-c]Means within a continuous column with different superscripts are significantly different at P<0.05.
Probability values up to P<0.10 are also shown if the data suggest a trend.

5.3.6 Crude mucin concentration and output

Ileal flow of crude mucins responded quadratically (P<0.01) with graded dietary threonine levels (Table 5.6). Supplementation of MOS also increased (P<0.01) the ileal crude mucin concentration. In addition, MOS tended to interact with dietary threonine to increase (P=0.054) the ileal crude mucin output, especially at adequate level of dietary threonine.

Table 5.6 Effects of dietary threonine level and MOS supplementation on jejunal and ileal dry matter output, crude mucin concentration and output of broilers at 21 days of age[1]

Treatment		Dry matter output	Crude mucin concentration	Crude mucin output
Thr	MOS	(g/100 g fresh digesta)	(g/100g DM digesta)	(g/kg DM intake)
Deficient		21.64	2.94[b]	6.06[c]
Adequate		20.60	3.43[a]	17.21[a]
Excess		23.31	2.42[b]	11.71[b]
	-	22.07	2.73[b]	11.19
	+	21.23	3.12[a]	12.14
Deficient	-	22.03	2.69	5.72
Deficient	+	20.06	3.19	6.42
Adequate	-	22.24	3.12	15.26
Adequate	+	18.97	3.73	19.16
Excess	-	21.95	2.39	12.59
Excess	+	24.66	2.44	11.00
SEM		1.619	0.172	1.093
Source of variation		----------Probability----------		
Thr		NS[2]	0.0015	0.0001
MOS		NS	0.0014	NS
Thr x MOS		NS	NS	0.0540

[1]Each value represents the mean of 5 replicates for each treatment group.
[2]NS=Not significant.
[a-d]Means within a continuous column with different superscripts are significantly different at $P<0.05$. Probability values up to $P<0.10$ are also shown if the data suggest a trend.

5.3.7 Nutrient uptake

The intestinal uptake of D-glucose and L-threonine from the *in situ* loops of ileum is presented in Table 5.7. Dietary MOS tended to interact with threonine to increase the ileal uptake of D-glucose ($P=0.084$) and L-threonine ($P=0.077$) expressed as mmol/cm/h. Feeding of MOS or an adequate level of dietary threonine significantly ($P<0.05$) increased the segmental weight per unit length of the ileum. In comparison with threonine-deficient birds, ileal mucosal uptake of D-glucose and L-threonine, expressed as mmol/cm/h, in birds fed with adequate or excess level of threonine was significantly higher ($P<0.05$). However, the differences in ileal mucosal uptake of nutrients were not apparent when expressed relative to tissue weight of the gut. Supplementation with MOS also significantly ($P<0.05$) increased the ileal mucosal uptake of D-glucose and L-threonine when expressed relative to the unit length of the ileum.

Table 5.7 Effects of dietary threonine level and supplementation of MOS on ileal D-glucose and L-threonine uptake in broilers at 21 days of age[1]

Treatment		Segment weight/length	D-Glucose uptake		L-Threonine uptake	
Thr	MOS	g/cm	mmol/cm/h	mmol/g/h	mmol/cm/h	mmol/g/h
Deficient		0.19[b]	0.040[b]	0.213	0.027[b]	0.145
Adequate		0.29[a]	0.049[a]	0.167	0.035[a]	0.119
Excess		0.26[ab]	0.045[ab]	0.187	0.030[a]	0.122
	-	0.23	0.041[b]	0.190	0.029[b]	0.133
	+	0.27	0.048[a]	0.187	0.032[a]	0.125
Deficient	-	0.17	0.033	0.195	0.025	0.147
Deficient	+	0.22	0.046	0.230	0.029	0.144
Adequate	-	0.27	0.049	0.186	0.036	0.132
Adequate	+	0.31	0.048	0.147	0.034	0.106
Excess	-	0.25	0.040	0.190	0.026	0.120
Excess	+	0.27	0.050	0.183	0.034	0.125
SEM		0.022	0.0008	0.0308	0.0013	0.0168
Source of variation		----------Probability----------				
Thr		0.0001	0.0400	NS	0.0070	NS
MOS		0.0560	0.0160	NS	0.0480	NS
Thr x MOS		NS[2]	0.0840	NS	0.0770	NS

[1]Each value represents the mean of 3 replicates for each treatment group.
[2]NS=Not significant
[a,b]Means within a continuous column with different superscripts are significantly different at $P<0.05$. Probability values up to $P<0.10$ are also shown if the data suggest a trend.

5.3.8 Apparent and standardised ileal digestibility of amino acids

In general, no interaction between MOS and dietary threonine on apparent and standardised ileal digestibility of amino acids was detected. Both MOS and dietary threonine also had no effect on apparent digestibility of lysine and methionine (Table 5.8). However, the apparent digestibility of threonine was significantly improved ($P<0.05$) in birds raised on threonine- or MOS-supplemented diets. The basal endogenous flow of lysine, threonine and methionine averaged at 0.41, 0.60 and 0.14 g/kg DM intake, respectively (Table 5.8). As expected, the standardised ileal digestibility of threonine mirrored the apparent digestibility results ($P<0.05$). In comparison to the untreated groups, supplementation of MOS also tended to improve ($P=0.09$) the standardised ileal digestibility of lysine. However, the standardised ileal digestibility of methionine remained unaffected by treatments. It was also observed that, irrespective of MOS or dietary threonine level, the effect of endogenous correction for ileal digestibility of threonine was considerably greater (5 to 9%) than that for lysine and methionne (2 to 4%).

Table 5.8 Effects of dietary threonine level supplemented with or without MOS on basal endogenous loss and, apparent and standardised ileal digestibility of lysine, threonine and methionine in broilers at 21 days of age[1]

Treatment		Apparent ileal digestibility (%)			Standardised ileal digestibility (%)			Basal endogenous loss (g/kg DM intake)		
Thr	MOS	Lysine	Threonine	Methionine	Lysine	Threonine	Methionine	Lysine	Threonine	Methionine
Deficient		91.79	73.97^{c}	94.46	94.41	83.03^{c}	96.82	0.36	0.53	0.10
Adequate		90.84	80.58^{b}	93.03	93.92	86.94^{b}	96.22	0.42	0.54	0.15
Excess		90.69	85.28^{a}	92.70	94.16	91.69^{a}	96.08	0.47	0.75	0.16
	-	90.68	78.94^{b}	93.10	93.48	85.55^{b}	95.49	0.37	0.52	0.11
	+	91.57	80.94^{a}	93.70	94.84	88.89^{a}	97.25	0.45	0.68	0.16
Deficient	-	91.18	71.78	93.78	93.87	80.56	96.47	0.36	0.48	0.11
Deficient	+	92.39	76.18	95.13	94.95	85.49	97.34	0.35	0.57	0.10
Adequate	-	90.41	80.51	93.18	93.25	86.14	95.56	0.35	0.46	0.10
Adequate	+	91.26	80.66	92.87	94.58	87.74	96.87	0.47	0.62	0.20
Excess	-	90.47	84.54	92.31	93.34	89.97	94.63	0.39	0.63	0.13
Excess	+	90.91	86.00	93.07	94.98	93.42	97.54	0.54	0.83	0.19
SEM		0.578	1.112	0.768	0.939	2.012	1.256	0.092	0.151	0.039
Source of variation		----------Probability----------								
Thr		NS[2]	0.0001	NS	NS	0.0011	NS	NS	NS	NS
MOS		NS	0.0385	NS	0.0936	0.0490	NS	NS	NS	NS
Thr x MOS		NS	NS	NS	NS	NS	NS	NS	NS	NS

[1]Each value represents the mean of 5 replicates for each treatment group.
[2]NS=Not significant.
[a,b]Means within a continuous column with different superscripts are significantly different at $P<0.05$. Probability values up to $P<0.10$ are also shown if the data suggest a trend.

5.4 DISCUSSION

5.4.1 Dietary composition and treatment design

The analysed CP, lysine and threonine values were generally similar to the calculated values. However, the analysed methionine levels in all the experimental diets were consistently 0.05 to 0.08% lower than the calculated values. It is not clear if these variations are due to the difference in analytical methods, sampling techniques, or real differences in the ingredients used in the experiment and nutrient matrixes used to formulate the diets. The relevance of this discrepancy is important if the data are to be used for estimating methionine requirements but not for comparing nutrient digestibility as the variations were quite consistent among the treatments.

5.4.2 Growth performance

As expected, deficiency in dietary threonine decreased FI, BWG and impaired FCR. This agrees with the observations made in Chapter 3 and by others (Corzo *et al.*, 2003; Kidd *et al.*, 2003a; Çiftci and Ceylan, 2004). This underlines the importance of threonine in the process of growth. As discussed in Chapter 3, this is closely linked to its direct involvement in the synthesis of immunoglobulins (Bhargava *et al.*, 1971; Jamroz, 2005), muscle (Wang *et al.*, 2007) and more importantly mucin glycoproteins and digestive enzymes (van der Schoor *et al.*, 2002; Faure *et al.*, 2005; Hamard *et al.*, 2007).

As highlighted in Chapters 3 and 4, the significant effect of MOS and its interaction with dietary threonine on growth response is presumably associated with a series of interrelated enhancement in intestinal mucosal integrity, gut microflora and local intestinal immunoresponse (Spring *et al.*, 2000; Iji *et al.*, 2001b; Uni and Smirnov, 2006).

5.4.3 Protein and threonine efficiency ratio

As the dietary threonine level increased, PER in groups without MOS supplementation responded in a similar manner as the BWG. This agrees with the data of Emmert *et al*. (2000), Çiftci and Ceylan (2004) and Mandal *et al*. (2006). In fact, it was concluded by Emmert *et al*. (2000) that chicks fed on amino acid-adequate diets showed higher PER than those on threonine-deficient diets. As previously reported, these results indicate that relative amino acid deficiency or excess, created by a dietary threonine imbalance, decreases protein efficiency (Çiftci and Ceylan, 2004). The decrease in PER as the result of excessive dietary

threonine level may have arisen from decreases in efficiency of protein digestion and (or) amino acid and peptide absorption. The uptake of free amino acids and peptides into the enterocyets is an active process involving a multiplicity of transport systems (Webb, 1990). Therefore, it is possible that over-supply of dietary threonine may have "flooded" the active transport sites and thus, minimising the efficiency of absorption of other amino acids. However, under such conditions, inclusion of MOS markedly increased the PER, especially at low-threonine level. The actual underlying mechanisms remain unclear and are mostly to be associated with the improvement in nutrient utilisation induced by MOS.

In contrast to PER values, TER decreased as dietary threonine increased irrespective of MOS supplementation. This may indicate a difference in the utilisation of individual amino acids for protein synthesis due to a relative deficiency of threonine. Gahl *et al.* (1996) also noted a greater increase in the efficiency of threonine utilisation for protein synthesis in rats when threonine was first limiting in the diet compared with when other amino acids are more limiting. On the other hand, Edwards *et al.* (1997) suggested otherwise that the contribution of threonine to protein accretion is fairly constant at all levels of threonine intake. The variations in the whole body protein deposition as influenced by dietary threonine level, if any, might explain the disparity between the current finding and literature data.

5.4.4 Intestinal growth parameters

The significant increase in the relative regional length and weight of the small intestine in birds deficient in threonine is in accordance with the observations made in a previous experiment (Chapter 3). The same explanation can be applied here where, during dietary threonine restriction, an increase in mucosal degradation (Visek, 1978; Law *et al*., 2007) and decrease in mucosal synthesis rate (Faure *et al.*, 2005; Wang *et al.*, 2007; Nichols and Bertolo, 2008) may have caused more wastage of net energy and amino acids (Stoll *et al.*, 1997; Bruins *et al.*, 2000; Wang *et al.*, 2007), resulting in a poorer BWG and thus significantly higher relative gut weight and length. Conversely, a decrease in the relative length of small intestine following adequate feeding of threonine or MOS is presumably related to faster growth rate due to more efficient and rapid digestion of nutrients (Marquardt, 1996, Yang *et al*., 2007a).

5.4.5 Enteric morphometric analyses

The negative effect of threonine deficiency on intestinal villus height was again observed in the present investigation and is in good agreement with the observation of Hamard *et al*.

(2007). Spring *et al*. (1996), Iji *et al*. (2001b), and Baurhoo *et al*. (2007) also reported similar findings on the increase in villus height through MOS supplementation. As the measurements of villus height and villus to crypt ratio generally give an indication of the likely maturity and functional capacity of the enterocytes (Hampson, 1986), it can be deduced that MOS significantly accelerates the maturation of the gut function. As explained in Chapter 3, it is likely that threonine might have a direct effect on the intestinal morphology, mediated through its obligatory involvement in the fractional as well as absolute synthesis of intestinal mucosa and mucins (Faure *et al.*, 2005; Wang *et al.*, 2007; Nichols and Bertolo, 2008), whereas MOS might exert its main influence on villus morphology via its modulating effects on intestinal mucin dynamics and microbial-mucosal interactions (Uni and Smirnov, 2006).

5.4.6 Ileal flow of crude mucins

Intestinal flow of crude mucins is an indicator of total mucin dynamics in the gastrointestinal tract. It is obvious that there exists a link between dietary threonine level and ileal crude mucin flow. This finding tallies with the quadratic relationship between the intestinal adherent mucous layer and the dietary threonine observed in Chapter 3. This indicates that dietary threonine is in high demand for the synthesis of intestinal mucin glycoproteins (Faure *et al.*, 2005; Law *et al.*, 2007; Nichols and Bertolo, 2008). The mechanism by which MOS exerts its influence on the optimisation of ileal crude mucin flow or mucous layer thickness is unknown. It may be closely associated with the microbial-mucin interaction noted in Chapter 4. As indicated in Chapter 3, it is possible that this interaction is highly dependent on the availability of dietary threonine to act as the substrate for the completion of mucin synthesis.

Alteration in intestinal mucin secretion could affect the thickness, viscosity, and integrity of the unstirred mucous-water layer lining the intestinal mucosa (Todd, 2008). Thus, a compromised or over-expressed intestinal mucous layer may have significant implications on the gut protective functions and nutrient utilisation (Hariharan *et al.*, 2004; Smirnov *et al.*, 2005; Todd, 2008). Although intestinal mucous layer generally does not disturb nutrient absorption (Morita *et al.*, 2006), excessive mucin secretion may increase the endogenous nutrient loss and impair nutrient absorption (Jeurissen *et al.*, 2002). As both MOS and dietary threonine exert positive effects on mucin dynamics, there is a concern that these supplementation could have negative impact on the digestibility and intestinal uptake of other nutrients. As such, this concern is best assessed in conjunction with the findings on nutrient utilisation which will be highlighted in later discussion.

5.4.7 Ileal nutrient uptake

Several techniques have been devised to measure patterns of mucosal absorption *in vitro*, but for most of them, the confidence with which findings can be related to the actual physiological condition of the intact live birds is open to question. This criticism is particularly important when it comes to assessing intestinal nutrient uptake in relation to the alteration in mucous layer thickness. Use of an *in situ*-gut perfusion technique has the advantage of not only ensuring a continuous normal blood supply to the gut but also any damages to the mucosa or mucous layer during expressing of the luminal content of the gut are obviated. In addition, this technique also enables the endogenous secretion of nutrients to be measured simultaneously and corrected for the determination of true ileal nutrient uptake.

The initial concentration of D-glucose (50 mM) was that used in earlier studies of D-glucose absorption in chickens and was determined to be within the physiological range that saturated or nearly saturated the carriers for D-glucose (Obst and Diamond, 1989, 1992). Similarly, a pre-optimised L-threonine concentration of 50 mM was selected on the basis of measurements of the uptake as a function of the concentration, indicating that this concentration saturated or nearly saturated the transporters for L-threonine (Figure 5.1). Thus, the mucosal uptake for D-glucose and L-threonine determined herein measured the combined effects of the active transport systems and passive diffusion. Any difference between treatments in the proportions contributed by these two systems would also be masked.

The average intestinal uptake rate of D-glucose at 0.19 mmol/g/h is in good agreement with those of 0.18 mmol/g/h determined by Obst and Diamond (1992) for 21 days old chicken. However, when expressed in per unit length, the intestinal uptake of D-glucose in the range of 0.033 to 0.055 mmol/cm/h is well above that of 0.008 mmol/cm/h determined in the jejunum (Yokota and Coates, 1982). The difference in site of study (Fearon and Bird, 1967) and use of radioactive glucose molecule of 3-*O*-methyl[^{11}C]glucose and/or continuous-perfusion method by Yokota and Coates (1982) as opposed to the loop-perfusion method adopted in this study may have accounted for this discrepancy. The average mucosal uptake rate of 0.031 mmol/cm/h for L-threonine could not be justified as, to the best of our knowledge, no previous study on this measurement for broiler chickens has been reported.

These findings raise the possibility that ileal absorption may impose limits to the availability of nutrients, which in turn, constrained the growth rate as observed in the threonine-deficient birds. The ileal contribution to a bird's mucosal uptake capacity largely depends on both the

ileum's level of nutrient specific Na^+-dependent transporters (SGLT-1 and transport system B^0 for D-glucose and L-threonine, respectively) and the existence of a diffusion barrier between the lumen and the brush-border membranes. Not surprisingly, the significant increase in the mucosal absorptive area (non-specific adaptive mechanisms) through the increase in ileal villus height and villus to crypt ratio induced by MOS, dietary threonine and their interaction may have contributed to the increase in brush-border nutrient uptake (Ferraris and Diamond, 1997). Previous studies by Iji *et al.*(2001b) in chickens also yielded a similar conclusion that increase in jejunal uptake of L-tryptophan by MOS is closely linked to the improvement in gut development.

The contribution from specific adaptive mechanisms mediated through an increase in site density of the transporters and in affinity constants is also possible (Ferraris and Diamond, 1997). For example, increase in intestinal volatile fatty acid concentrations, which is frequently associated with MOS supplementation (Ao, 2004; Santos and Teixeira, 2004; Yang *et al.*, 2007a), is capable of up-regulating the gene expression of glucose transporters resulting in an increase in glucose uptake (Drozdowski *et al.*, 2002). In fact, recent findings have shown that broiler diet supplemented with MOS significantly elevated the gene expression and activity of several major brush border enzymes and transporters, including peptide transporter (PepT1), y-Glutamyl transferase (yGT) and sodium/glucose cotransporter (SGLT-1) (Uni, 2008).

On the contrary, the increase in intestinal adherent mucous thickness posed by these supplementations may also hinder the absorption of nutrients across the brush-border membranes (Jeurissen *et al.*, 2002). However, as observed in this case, the magnitude of the effect of an increase in mucosal absorptive area is more likely to outweigh an expected increase in mucosal diffusion barrier. At the least, the results presented herein agree with the finding of Morita *et al*. (2006) that mucous layer does not disturb nutrient absorption.

The lack of change in the rates of mucosal uptake for D-glucose and L-threonine when normalised to wet tissue mass is not totally surprising. This is because the weight per unit length of the gut segment was significantly higher in birds supplemented with adequate threonine or MOS. Increase in tissue weight per unit length may be directly connected to the improvement in mucosal development and thus, nutrient uptake. However, increase in connective tissue which is not directly associated with nutrient uptake may also account for quite a substantial increase in the intestinal wall thickness or absolute weight (Yokota and

Coates, 1982). Therefore, the transport rates based on per unit intestinal length appear to reflect more of a true representation of the physiological differences attributable to the treatments than values based on intestinal weight (Fearon and Bird, 1967; Yokota and Coates, 1982).

5.4.8 Apparent and standardised ileal digestibility of amino acids

The term, true ileal digestibility, has been used interchangeably with standardised ileal digestibility leading to some confusion about the interpretation of ileal digestibility values (Stein *et al.*, 2007). However, standardised ileal digestibility is a more appropriate term and should be used for the interpretation of experimental observations (Stein *et al.*, 2007). Therefore, for the purpose of this discussion, results on ileal digestibility found in the literature data were interpreted based on the definition rather than the terminology used.

A significant increase in the apparent and standardised ileal digestibility of threonine by MOS is compatible with the finding of Biggs *et al.* (2007), although improvements in lysine and methionine digestibilities were also observed by the same authors. The specific effect of graded levels of threonine on ileal digestibility of amino acids has not been reported.

The significant increase in apparent ileal digestibility of threonine by MOS and dietary threonine may have arisen from a suppression of diet-specific endogenous losses and/or an increase in digestible dietary threonine. In the context of this experiment, contribution from the suppression of diet-specific endogenous losses may not likely be the case. This is because both MOS and dietary threonine increased the ileal flow of crude mucins where threonine constitutes a large proportion of its amino acid contents (Lien *et al.*, 1997; Ravindran *et al.*, 2004b). Although mucins may represent a relatively small proportion (5 to 11%) of the total ileal endogenous protein loss (both diet-specific and non-specific), the contribution of threonine from its glycoproteins can be as high as 28 to 35% of the total ileal amino acid flow (Hoskins, 1984; Montagne *et al.*, 2004).

Therefore, through these supplementation, one might anticipate an increase in diet-specific endogenous protein loss (Montagne *et al.*, 2004). This contradicts the finding on the improvement in apparent ileal digestibility of threonine. However, it is possible that the increase in mucin endogenous loss is less significant when compared to the overall improvement in the digestion and mucosal uptake of nutrients mediated through the modulating effect of intestinal mucus on the mucosal development. This is substantiated by

the earlier finding on the improvement in villus morphology as well as by other authors on the increase in brush-border membrane enzymatic response (Iji *et al.*, 2001b; Hamard *et al.*, 2007; Yang *et al.*, 2007a) and mucosal fractional synthesis rate (Faure *et al.*, 2005; Wang *et al.*, 2007) associated with such supplementation.

This view is further reinforced by the suggestion that the mucous layer actually plays an important role in the digestive processes by creating a digestion zone comprising of a network of glycoprotein filters called glycocalyx (Moran, 1985; Uni, 2008). It is in this unstirred mucin-water layer that the digestive enzymes are immobilised near the epithelium surface (Mizuno *et al.*, 1982). This mucin-water complex prevents the rapid removal of enzymes by peristalsis and places them in a more favourable position for the hydrolysis as well as absorption of nutrients. Nutrients derived in this manner have the advantage of not readily been swept into the luminal contents but are held there by the unstirred water layer to accrue near the active transport sites (Levin, 1976; Uni, 2008). The unstirred mucin-water layer is also envisaged to protect enzymes immobilised on the enterocyte surface from degradation by pancreatic proteases in the lumen (Levin, 1976; Uni, 2008). In addition, the mucous layer also acts as a selective diffusion barrier that filters the nutrients able to be absorbed and prevents larger compounds from reaching the epithelium (Nimmerfall and Rosenthaler, 1980).

Interestingly, graded levels of dietary threonine had no effect on the apparent digestibility of lysine and methionine. It is possible that owing to their sharing of the same transport systems, low luminal threonine may have relatively increased the concentration of other amino acids and thus, facilitated the mucosal uptake of these amino acids. The opposite may also be true where excessive luminal threonine may have "flooded" the active transport sites and thus, decreased the intestinal uptake of these amino acids (Wu, 1998; Wang *et al.*, 2007). Since the relative uptake of these amino acids as influenced by the changing concentration of luminal threonine was not assessed in the current study, the aforementioned statement may warrant future research to further explore these interactive effects.

Different methods are available to assess the basal (non-specific) endogenous amino acid losses. Feeding a protein-free diet is the most common method but has been criticised for not reflecting the true physiological condition (Bryden and Li, 2004; Ravindran *et al.*, 2004a). The regression method gave similar results but is more complicated to perform (Donkoh *et al.*, 1995). Therefore, the proposed alternative method of utilising highly digestible enzymatically hydrolysed casein treated by ultrafiltration to remove small peptides has

proven to be more suitable for avian species (Moughan and Rutherfurd, 1990; Ravindran *et al.*, 2004a).

Previously, endogenous amino acid flows at the terminal ileum of chickens fed an EHC diet have been determined (Bryden and Li, 2004; Ravindran *et al.*, 2004b; Cowieson and Ravindran, 2007). On average, the flows determined in the study reported here were lower than those found in the previous work (Bryden and Li, 2004; Cowieson and Ravindran, 2007), but were quite similar to those reported by Ravindran *et al*. (Ravindran *et al.*, 2004a) for 14-day old birds. It would appear that there is a relatively large amount of variation between endogenous flows determined in different laboratories which may largely arise from either the use of different sources and dosage of EHC (Rutherfurd *et al.*, 2004) or individual variation (Hess and Seve, 1999). Other findings have also indicated that body weight and/or feeding level may have influence on basal endogenous losses (Furuya and Kaji, 1991; Hess and Seve, 1999). Although the provision of equal adjacent replicates for the determination of basal endogenous losses in the current study may have improved the endogenous correction of apparently digestible amino acids for the calculation of standardised ileal digestibility, no significant differences in individual variation on basal endogenous losses were detected. As such, the significant improvement in standardised ileal digestibility of threonine is likely to be extended from the improvement in apparent ileal digestibility of threonine substantiated by an overall improvement in intestinal mucosal development induced by MOS or adequate feeding of dietary threonine.

5.5 CONCLUSIONS

It can be deduced that the modulating effect of MOS and dietary threonine on intestinal mucin dynamics and mucosal development leads to the maximisation of digestive and absorptive functions of the gut. This, in turn, accentuates the improvement in the utilisation of nutrients and thus, a better growth performance. However, owing to its visco-elastic and lubricative properties, intestinal mucin flow may also likely have an effect on the feed passage rate. Further studies are needed to elucidate the effect of these supplementations, in association with the intestinal mucin output, on the gut motility and intestinal transit time.

CHAPTER 6
IMPACT OF MANNOOLIGOSACCHARIDES AND DIETARY THREONINE ON INTESTINAL MUCIN TURNOVER, MICROBIAL ACTIVITIES AND FEED PASSAGE RATE IN BROILER CHICKENS

6.1 INTRODUCTION

It was postulated in Chapter 3 that the decrease in thickness and relative weight of intestinal external muscularis in relation to the feeding of MOS is likely to be associated with an overall improvement in gut viability where there is a reduced need for gut motility to control gut microbial activities. The role of interdigestive gut motility in nutrient digestion and absorption as well as intestinal clearance and prevention of bacterial overgrowth has long been recognised (Vantrappen *et al.*, 1977; Choct *et al.*, 1996). However, the close relationship between feed passage rate and modulation of intestinal commensal microflora and mucin dynamics induced by pro- and prebiotics has not been fully explored. More recently, Lesniewska *et al.* (2006) demonstrated that bifidobacteria and lactobacilli are not able to only increase the intestinal motility but also the intestinal contractile force in rats. This mechanism is especially important for a proper mixing of the residual food with the enzymatic secretions and also enabling a more effective propulsion of luminal content, debris and bacterial cell distally over a longer distance.

In addition, results from previous studies also showed that bacterial fermentation products such as short chain fatty acids (SCFAs) can regulate gastric (Code and Marlett, 1975), ileal (Cuche *et al.*, 2000) and colonic (Kamath *et al.*, 1988) motility. Peristaltic movement plays an integral role in the rapid expulsion of microorganisms from the small intestine through the formation of antigen-antibody complexes in the mucous coat (Cherbut *et al.*, 1998). This suggests that the binding of pathogens by luminally available MOS could also be beneficial in the same manner. Furthermore, intestinal mucus also acts as a lubricant that aids the passage of digesta along the digestive tract, thus preventing stagnation and bacterial overgrowth.

Therefore, the objective of this study was to evaluate the interactive effects of MOS and dietary threonine on feed passage rate by focusing on their modulating effects on gut microflora composition, SCFA profiles and intestinal mucin turnover.

6.2 MATERIALS AND METHODS

6.2.1 Bird management and experimental diets

The basal experimental diets used were the same as described in Section 5.2.1 and the same six treatments were allocated to the experimental diets. Ninety six day-old male Cobb broiler chicks (Baiada Poultry Pty. Ltd., Kootingal, NSW, Australia), weighing 47.55 ± 0.25 g, were group-brooded according to treatment in a multi-tier brooder measuring 120 x 90 x 23 cm for 14 days. At d 15, 72 and 24 birds were reallocated and randomly assigned to the six treatment groups kept in metabolic (60 x 33 x 33 cm) and individual (45 x 36 x 24 cm) cages, respectively. Birds kept in the metabolic cages, comprising 4 replicates per treatment with 3 birds per replicate, were used for the assessment of feed passage rate using cumulative and non-cumulative TiO_2 excretion methods. The individually caged birds with 4 replicates per treatment were used for the evaluation of feed passage rate based on first excreta appearance and mean retention time assessed by intestinal region. All the brooding cages were housed in environmentally controlled rooms with initial room temperature of 34°C maintained for the first 5 days and then gradually decreased to 21°C by the end of the experiment at week 3. Birds were provided *ad libitum* access to feed and water except for the periods required for the assessment of feed passage rate. Artificial lighting of the room was continuous to minimise development of a diurnal rhythm of feeding.

6.2.2 Animal ethics

All experimental procedures were approved by the Animal Ethics Committee of the University of New England with the approval number AEC 07/156.

6.2.3 Feed passage rate and sample collection

Excreta first appearance and regional mean retention time

At d 16, birds caged individually were fasted for 12 h before being fed with the marker diets. The time the bird first began to eat and the time of first appearance of solid excreta was recorded. Total tract transit time was calculated as the difference between these 2 times (Washburn, 1991). Finally, excreta samples were collected from each cage and processed immediately for determination of excreta dry matter content.

The birds were then allowed to continue to consume the marker diets for 5 days. It was assumed that the birds were already in a steady state at the end of d 21 with respect to the

intake and excretion of TiO_2. The quantity of the consumed feed was determined for each individual bird. After live weight was recorded, the birds were euthanised using CO_2 asphyxiation method and quickly dissected, to obtain the separate regions of duodenum, jejunum and ileum as described in Section 3.2.5. Digesta of the respective intestinal regions was squeezed out, frozen, and stored at –20°C for further analysis of gut microflora (without freezing), mucous extraction, organic acids and TiO_2 assays. Titanium dioxide contents in the digesta samples were treated and processed in the same manner as described in Section 5.2.6.

Regional mean retention time was calculated from the ratio between TiO_2 present in the respective regions ($TiO_{2\ in\ region}$) of the small intestine and the daily TiO_2 intake ($TiO_{2\ intake}$) based on the equation described by van der Klis *et al.* (1990):

$$MRT = 1440 \text{ x } \frac{TiO_{2\ in\ region\ (mg)}}{TiO_{2\ intake\ (mg/day)}}$$

where MRT is the mean retention time (min) and 1440 is the number of minutes per 24 h.

The remaining parts of the respective intestinal regions were collected for the measurement of intestinal growth parameters and enteric morphometric analysis as described in Section 3.2.5.

Cumulative and non-cumulative TiO_2 excretion study

At d 21, the birds kept in the metabolic cages were fasted for 12 h, but were provided with water *ad libitum.* At the end of the fasting period, the birds were given their respective TiO_2 marked diets. After 1 h, feed intake was recorded, and the marked feed was replaced with the normal experimental diets. Excreta were collected hourly for the first 8 h and at 12 and 24 h after the birds received the marked feed. The excreta collected from each cage were kept in a separate resealable plastic bag and stored at –20°C before being freeze-dried, ground to pass through 1 mm sieve, and kept in tightly closed 100 mL plastic containers until used for the analyses. A sample of each of the marked diet was also obtained and processed through the same procedure. The TiO_2 contents of the samples were measured according to the procedure described in Section 5.2.6.

The TiO_2 excreted was expressed as cumulative fraction of the total amount of the TiO_2 determined at various sampling times during the 24-h collection period. The cumulative

excretion curve of each diet was plotted and fitted to the Hill equation (Almirall and Esteve-Garcia, 1994) of:

$$y = \frac{t^n}{\left(t^n + k\right)}$$

where t is the time (h) and y is the excreted TiO_2. In order to determine n (the slope of the line), k (the constant of the equation) and the correlation coefficient, curves were transformed into a linear equation (Ferrando *et al.*, 1987) as follows:

$$\mathrm{In}\left(\frac{y}{1-y}\right) = n\,\mathrm{In}(t) - \mathrm{In}\,k \Rightarrow Y = AX + B$$

where Y=In [y/(1-y)], A= n, X = In (t), and B= -In k.

Times of 1% (T_1) and 50% (T_{50}) excretion, which were the times required to excrete 1 and 50% of the marker administered, respectively, were determined for each replicate (Ferrando *et al.*, 1987). T_1 is regarded as the time (h) taken for the first appearance of TiO_2 and determined from the transformed equation as:

$$y = 0.01 = \left(\frac{T_1^{\,n}}{T_1^{\,n} + k}\right) \Rightarrow T_1 = \left(\frac{k}{99}\right)^{\frac{1}{n}}$$

T_{50} is regarded as the total tract mean retention time (h) and calculated as:

$$y = 0.50 = \left(\frac{T_{50}{}^{n}}{T_{50}{}^{n} + k}\right) \Rightarrow T_{50} = k^{\frac{1}{n}}$$

The non-cumulative excretion data were analysed in which the amount of excreta TiO_2 content from each cage was calculated as a percentage of total amount of TiO_2 excreted during the 24-h period and presented in a graph form (Vergara *et al.*, 1989).

6.2.4 Excreta dry matter content

Excreta dry matter content was determined by drying the excreta samples at 105°C overnight to a constant weight. Difference in weight before and after drying was regarded as the dry matter content.

6.2.5 Crude mucin turnover and dry matter output

Crude mucins were isolated from both jejunal and ileal contents as described in Section 5.2.7.

6.2.6 Enumeration of the gut bacteria

In addition to the ileal contents, ileal mucosal samples were harvested as described in 4.2.4. Intestinal bacteria were cultured and analysed as described in Section 4.2.5.

6.2.7 Enteric morphometric and gross morphology parameters

Intestinal gross- and histo-morphological studies were assessed as described in Sections 3.2.5 and 3.2.7, respectively.

6.2.8 Measurement of organic acids

The analytical method described by Jensen *et al*. (1995) was adopted with modifications for the analysis of organic acid (lactic and volatile fatty acids (VFA)) concentrations. Briefly, frozen ileal digesta samples were thawed and homogenised by vigorous shaking. About 2-3 g (wet weight) of the homogenised digesta sample were weighed and 1 mL internal standard (0.01 M ethylbutyric acid) was added. After the samples were centrifuged (12,000 *g*, 15 min), approximately 1 mL of the supernatant was taken out and mixed with 0.5 mL of concentrated hydrochloric acid and 2 mL of ether. An internal standard solution and a blank were also prepared in the same manner by replacing the supernatant with 1 ml of the standard acid mixture and 1 mL of water, respectively. Again, the mixture was centrifuged (8,000 *g*, 15 min at 5°C) and 400 μL of the supernatant were transferred to a gas chromatograph vial and mixed with 40 μL of *N*-tert-butyldimethylsilyl-*N*-methyltrifluoro-acetamide. Before running on a Varian CP3400 CX gas chromatograph (Varian Analytical Instruments, Palo Alto, CA, USA), the vials were kept in a heating block (80 °C) for 20 min and left at room temperature for 48 h.

6.2.9 Statistical analysis

The same statistical model as described in Section 3.2.10 was employed to analyse the variance of all the data. The level of significance was considered at $P \leq 0.05$ unless otherwise specified.

6.3 RESULTS

6.3.1 Dietary composition and treatment design

The calculated crude protein and threonine contents of the diets and their respective analysed values were as described in Section 5.3.1.

6.3.2 Intestinal growth parameters

Intestinal growth parameters of 21 day-old broilers as affected by dietary threonine supplemented with or without MOS are shown in Tables 6.1 and 6.2. The small intestinal regions were consistently longer ($P<0.01$) in threonine-deficient birds. Diets deficient in threonine also significantly increased ($P<0.05$) the relative weights of the ileum as well as the whole small intestine. However, dietary threonine had no effect on the relative weights of muscularis externa of any of the small intestinal regions. In addition, no significant effect on the intestinal growth parameters attributable to MOS and its interaction with dietary threonine was observed.

Table 6.1 Effects of dietary threonine level and MOS supplementation on relative length of small intestinal regions of broilers at 21 days of age[1]

Treatment		Relative length (cm/100g body weight)			
Thr	MOS	Duodenum	Jejunum	Ileum	Small Intestine[2]
Deficient		4.55[a]	9.16[a]	8.62[a]	22.33[a]
Adequate		3.30[b]	6.59[b]	6.43[b]	16.33[b]
Excess		3.65[b]	7.36[b]	7.55[b]	18.57[b]
	-	3.86	7.70	7.38	18.94
	+	3.81	7.70	7.69	19.21
Deficient	-	4.56	9.43	8.98	22.98
Deficient	+	4.55	8.88	8.66	22.10
Adequate	-	3.49	6.48	6.24	16.23
Adequate	+	3.10	6.71	6.61	16.43
Excess	-	3.77	7.52	8.21	19.50
Excess	+	3.52	7.20	6.90	17.63
SEM		0.202	0.455	0.568	1.010
Source of variation		----------Probability----------			
Thr		0.0001	0.0001	0.0031	0.0001
MOS		NS[3]	NS	NS	NS
Thr x MOS		NS	NS	NS	NS

[1]Each value represents the mean of 4 replicates for each treatment group.
[2]Small intestine=summation of duodenum, jejunum and ileum.
[3]NS=Not significant.
[a,b]Means within a continuous column with different superscripts are significantly different ($P<0.05$).

Table 6.2 Effects of dietary threonine level and MOS supplementation on relative weight of whole tissue and muscularis in different regions of the small intestine of broilers at 21 days of age[1]

Treatment		Relative segmental weight (g/100 g BW)				Relative muscularis weight (g/100 g BW)			
Thr	MOS	Duodenum	Jejunum	Ileum	Small Intestine	Duodenum	Jejunum	Ileum	Small Intestine
Deficient		1.33	1.91	1.54[a]	4.77[a]	0.63	0.98	0.99	2.60
Adequate		1.25	1.79	1.33[b]	4.38[b]	0.52	1.03	0.83	2.38
Excess		1.21	1.78	1.32[b]	4.32[b]	0.51	0.91	0.97	2.39
	-	1.30	1.79	1.36	4.44	0.54	0.93	0.95	2.42
	+	1.23	1.87	1.44	4.54	0.57	1.01	0.92	2.51
Deficient	-	1.26	1.82	1.44	4.52	0.57	0.90	0.92	2.43
Deficient	+	1.40	2.00	1.63	5.03	0.69	1.06	1.02	2.78
Adequate	-	1.40	1.82	1.36	4.58	0.56	1.04	0.92	2.52
Adequate	+	1.10	1.75	1.32	4.17	0.49	1.00	0.76	2.25
Excess	-	1.23	1.72	1.28	4.22	0.49	0.84	0.98	2.30
Excess	+	1.20	1.84	1.37	4.40	0.53	0.98	0.97	2.48
SEM		0.080	0.088	0.062	0.181	0.067	0.102	0.081	0.179
Source of variation		----------Probability----------							
Thr		NS[2]	NS	0.0030	0.0420	NS	NS	NS	NS
MOS		NS	NS	NS	NS	NS	NS	NS	NS
Thr x MOS		NS	NS	NS	NS	NS	NS	NS	NS

[1]Each value represents the mean of 4 replicates for each treatment group.
[2]NS=Not significant.
[a,b]Means within a continuous column with different superscripts are significantly different ($P<0.05$).

6.3.3 Enteric morphometric analysis

The effects of dietary treatments on the microscopic structures of the small intestine are summarised in Table 6.3. No significant differences in either the external muscularis thickness or crypt depth measured in all the intestinal regions were detected among the treatments. However, birds fed the threonine-supplemented diets had longer ileal villi ($P<0.05$) and higher ileal villus to crypt ratio ($P<0.05$) compared to those deficient in threonine. Similar positive effects on villus height and villus to crypt ratio ($P<0.05$) were also observed in birds supplemented with MOS compared to their untreated counterparts. In addition, MOS also interacted with dietary threonine to increase ($P<0.05$) the jejunal and ileal villus to crypt ratio, especially in birds fed the threonine and MOS-supplemented diets.

6.3.4 Characterisation of intestinal microflora by plate counts

The bacterial counts in the ileal contents as well as the ileal mucosal samples collected at d 21 are shown in Table 6.4. Birds fed the diet deficient in threonine harboured less ($P<0.05$) lactobacilli, lactic acid bacteria and coliforms in the ileal content than those fed with adequate threonine. An interaction between MOS and dietary threonine on coliform counts was observed. MOS-supplemented birds fed with adequate threonine also had fewer coliforms in the ileal content and mucosa. However, the opposite was true for MOS-supplemented birds fed the threonine-excess diet.

6.3.5 Lactate and volatile fatty acids

Birds fed with adequate or excess threonine tended ($P=0.10$) to have a higher ileal lactic acid concentration (Table 6.5). With respect to the ileal VFA concentrations, no significant changes were detected among the treatment groups. However, birds supplemented with MOS had numerically higher concentrations of VFAs (formic and acetic acids) in the ileum compared to those in the untreated group.

6.3.6 Crude mucin concentration and output

Since the intestinal dry matter output did not correlate significantly with the intestinal flow of crude mucin, both crude mucin concentration and output were analysed independently of the dry matter output (Table 6.6). Flow of crude mucins in the duodenum was not determined due to insufficient digesta obtained. No significant differences in jejunal flow of

Table 6.3 Effects of dietary threonine level and MOS supplementation on jejunal and ileal morphometric parameters of broilers at 21days of age [1]

Treatment		Jejunum				Ileum			
Thr	MOS	Muscularis thickness (μm)	Crypt depth (μm)	Villus height (μm)	Villus/Crypt ratio	Muscularis thickness (μm)	Crypt depth (μm)	Villus height (μm)	Villus/Crypt ratio
Deficient		285	245	1314	5.49	250	186	735^{b}	4.16
Adequate		331	260	1376	5.50	266	183	994^{a}	5.72
Excess		310	271	1515	5.63	269	170	923^{a}	6.01
	-	296	258	1301^{b}	5.16	265	195	853^{b}	4.50
	+	322	259	1502^{a}	5.93	258	164	915^{a}	6.09
Deficient	-	269	260	1229	4.74^{bc}	261	183	748	4.41^{bc}
Deficient	+	301	230	1398	6.26^{ab}	239	188	722	3.91^{c}
Adequate	-	336	267	1178	4.68^{c}	271	200	938	4.79^{bc}
Adequate	+	327	252	1574	6.32^{a}	261	165	1041	6.66^{ab}
Excess	-	283	245	1497	6.04^{abc}	264	201	862	4.31^{bc}
Excess	+	337	296	1534	5.21^{abc}	274	139	984	7.71^{a}
SEM		21.8	24.0	104.2	0.529	22.2	20.1	38.3	0.742
Source of variation		----------Probability----------							
Thr		NS[2]	NS	NS	NS	NS	NS	0.0001	0.0452
MOS		NS	NS	0.0298	0.0885	NS	NS	0.0500	0.0162
Thr x MOS		NS	NS	NS	0.0500	NS	NS	NS	0.0495

[1]Each value represents the mean of 4 replicates for each treatment group.
[2]NS=not significant.
[a-c]Means within a continuous column with different superscripts are significantly different at $P<0.05$. Probability values up to $P<0.10$ are also shown if the data suggest a trend.

Table 6.4 Effects of dietary threonine level and MOS supplementation on the counts of luminal and mucosa-associated bacteria in the ileum of broiler chickens at 21 days of age[1]

Treatment		Total anaerobes		Lactobacilli		Lactic-acid		Lactose-negative enterobacteria		Coliforms		*C. perfringens*	
		Log_{10} CFU/g digesta or mucosa											
Thr	MOS	Digesta	mucosa	Digesta	mucosa	Digesta	mucosa	Digesta	mucosa	Digesta	mucosa	Digesta	mucosa
Deficient		8.23	6.77	8.27^{b}	6.65	8.36^{b}	6.59	5.46	4.47	5.37	4.26	7.10	6.19
Adequate		8.12	7.11	8.61^{a}	6.40	8.73^{a}	7.01	5.44	4.14	6.17	4.46	6.57	5.78
Excess		8.40	7.32	8.48^{ab}	6.43	8.82^{a}	7.20	5.31	4.27	6.12	4.11	6.84	5.95
	-	8.15	7.14	8.39	6.60	8.61	6.96	5.81	4.38	5.90	4.21	7.00	6.25
	+	8.30	6.99	8.51	6.40	8.60	6.91	5.66	4.21	5.88	4.34	6.66	5.71
Deficient	-	8.35	6.88	8.29	6.64	8.38	6.64	5.28	4.79	5.30^{b}	4.18^{abc}	7.58	6.45
Deficient	+	8.12	6.64	8.25	6.68	8.34	6.53	5.65	4.14	5.45^{b}	4.40^{abc}	6.66	5.93
Adequate	-	7.65	7.35	8.53	6.65	8.58	7.04	5.72	4.21	6.84^{a}	4.97^{a}	6.47	6.10
Adequate	+	8.05	6.87	8.68	6.09	8.69	6.98	5.16	4.07	5.55^{b}	3.94^{bc}	6.66	5.47
Excess	-	8.44	7.18	8.38	6.54	8.85	7.21	6.42	4.12	5.47^{b}	3.55^{c}	6.96	6.20
Excess	+	8.40	7.46	8.59	6.32	8.80	7.20	6.19	4.41	6.69^{a}	4.17^{abc}	6.72	5.71
SEM		0.227	0.310	0.118	0.256	0.165	0.311	0.584	0.367	0.288	0.342	0.251	0.335
Source of variation		Probability											
Thr		NS[2]	NS	0.0253	NS	0.0300	NS	NS	NS	0.0167	NS	NS	NS
MOS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Thr x MOS		NS	NS	NS	NS	NS	NS	NS	NS	0.0010	0.0200	NS	NS

[1]Each value represents the mean of 4 replicates for each treatment group.
[2]NS=Not significant.
[a,b]Means within the same column with different superscripts are significantly different at $P<0.05$.

Table 6.5 Effects of dietary threonine level and MOS supplementation on lactic and volatile fatty acid concentrations in the ileum of broilers at 21 days of age[1]

Treatment		Organic acid concentration (μmol/g digesta)					Molar ratio (%/total organic acid)			
Thr	MOS	Formic	Acetic	Total VFA	Lactic	Total organic	Formic	Acetic	Total VFA	Lactic
Deficient		1.05	1.49	2.55	12.03	14.38	9.30	1.20	21.36	78.64
Adequate		0.86	1.34	2.17	16.05	18.09	4.86	7.55	12.41	87.59
Excess		0.83	1.33	2.16	16.00	17.12	6.29	8.60	14.90	85.11
	-	0.82	1.35	2.18	14.85	17.02	5.56	8.37	13.93	86.07
	+	0.98	1.43	2.41	14.54	16.80	8.07	10.44	18.52	81.08
Deficient	-	0.96	1.45	2.46	11.90	14.36	7.26	10.75	18.00	81.99
Deficient	+	1.15	1.50	2.64	12.16	14.80	11.30	13.38	24.71	75.29
Adequate	-	0.91	1.37	2.27	17.75	20.17	4.70	6.98	11.68	88.32
Adequate	+	0.75	1.32	2.06	14.36	16.25	5.02	8.12	13.14	86.86
Excess	-	0.59	1.20	1.80	14.89	16.08	4.72	7.37	12.09	87.91
Excess	+	1.05	1.50	2.52	17.12	17.68	7.87	9.83	17.70	82.30
SEM		0.353	0.202	0.421	3.179	3.582	3.192	2.642	5.169	5.781
Source of variation		----------Probability----------								
Thr		NS[2]	NS	NS	0.1000	NS	NS	NS	NS	0.1000
MOS		NS	NS	NS	NS	NS	NS	NS	NS	NS
Thr x MOS		NS	NS	NS	NS	NS	NS	NS	NS	NS

[1]Each value represents the mean of 4 replicates for each treatment group.
[2]NS=Not significant.
[a,b]Means within the same column with different superscripts are significantly different at $P<0.05$. Probability values up to $P<0.10$ are also shown if the data suggest a trend.

Table 6.6 Effects of dietary threonine level and MOS supplementation on jejunal and ileal dry matter output, crude mucin concentration and output of broilers at 21 days of age[1]

Treatment		Dry matter output[2]		Crude mucin concentration		Crude mucin output	
Thr	MOS	Jejunum	Ileum	Jejunum	Ileum	Jejunum	Ileum
		g/100g fresh digesta		g/100g DM digesta		g/kg DM intake	
Deficient		22.39	20.17	2.49	1.81	15.10	4.81^{c}
Adequate		21.87	20.81	2.33	3.32	17.01	17.78^{a}
Excess		22.17	22.44	2.32	2.74	18.21	12.92^{b}
	-	22.19	22.00	2.30	2.28	16.45	11.04
	+	22.08	20.27	2.32	2.96	17.09	12.63
Deficient	-	21.83	21.27	2.47	1.63^{d}	14.54	4.82
Deficient	+	22.94	19.08	2.58	1.98^{cd}	15.74	4.88
Adequate	-	22.74	22.91	2.33	2.70^{b}	16.76	15.00
Adequate	+	20.99	18.71	2.43	3.94^{a}	17.20	20.57
Excess	-	22.02	21.85	2.16	2.50^{bc}	16.86	13.30
Excess	+	22.32	23.03	2.49	2.97^{b}	19.57	12.53
SEM		1.125	1.737	0.318	0.208	2.341	1.724
		----------Probability----------					
Thr		NS[3]	NS	NS	0.0001	NS	0.0001
MOS		NS	NS	NS	0.0080	NS	NS
Thr x MOS		NS	NS	NS	0.0400	NS	0.0680

[1]Each value represents the mean of 4 replicates for each treatment group.
[2]The dry matter output did not correlate significantly with crude mucin concentration and output measured in both the jejunum and ileum.
[3]NS=Not significant.
[a,b]Means within a continuous column with different superscripts are significantly different at $P<0.05$. Probability values up to $P<0.10$ are also shown if the data suggest a trend.

crude mucins were detected between the treatments. However, MOS interacted with dietary threonine to increase the ileal crude mucin concentration ($P<0.05$) and output ($P=0.07$) with the highest values noticed in birds given the adequate-threonine plus MOS diet (Table 6.6). Graded levels of dietary threonine produced a quadratic response in the ileal flow of crude mucins. Threonine-deficient birds had a lower ($P<0.01$) ileal crude mucin concentration and output compared to the adequately fed birds. Supplementation with excess threonine beyond the adequate level did not result in any further increase in crude mucin concentration or output; instead resulted in a reduction ($P<0.01$) in these values. In comparison to the untreated groups, addition of MOS also significantly increased ($P<0.01$) the ileal crude mucin concentration.

Table 6.7 Effects of dietary threonine level and MOS supplementation on total intestinal tract transit time and excreta dry matter (DM) content of birds at 16 days of age[1]

Treatment		Total tract transit time (h)	Excreta DM content (%)
Thr	MOS		
Deficient		2.37	73.49
Adequate		2.15	75.35
Excess		2.12	75.48
	-	2.21	75.44
	+	2.20	74.11
Deficient	-	2.33	76.66
Deficient	+	2.40	70.32
Adequate	-	2.21	74.74
Adequate	+	2.01	75.96
Excess	-	2.10	74.91
Excess	+	2.12	76.04
SEM		0.154	2.423
Source of variation		----------Probability----------	
Thr		NS[2]	NS
MOS		NS	NS
Thr x MOS		NS	NS

[1]Each value represents the mean of 4 replicates for each treatment group.
[2]NS=Not significant.

6.3.7 Feed intake, feed passage rate and excreta characteristics

Total tract transit time and excreta moisture content

The excreta dry matter contents and total tract transit times of birds fed the different diets are shown in Table 6.7. The total tract transit times as indicated by first excreta appearance averaged at 2.2 h. However, neither the total tract transit time nor the excreta dry matter content was affected by the dietary treatments.

Cumulative excretion curves and feed intake

The excretion patterns of birds fed the experimental diets were similar and sigmoidal in shape (Figure 6.1). Their linear representations are shown in Figure 6.2. Table 6.8 presents all the parameters that define the cumulated excretion patterns of the different treatments. Comparison of the slopes of the six treatment lines showed no significant difference between them. Estimations of the first appearance time as indicated by the excretion time for 1% TiO_2 (T_1) ranged from 2.08 to 2.58 h and are shown in Table 6.8. No significant differences in the first appearance time attributable to the treatments were observed. In contrast, MOS

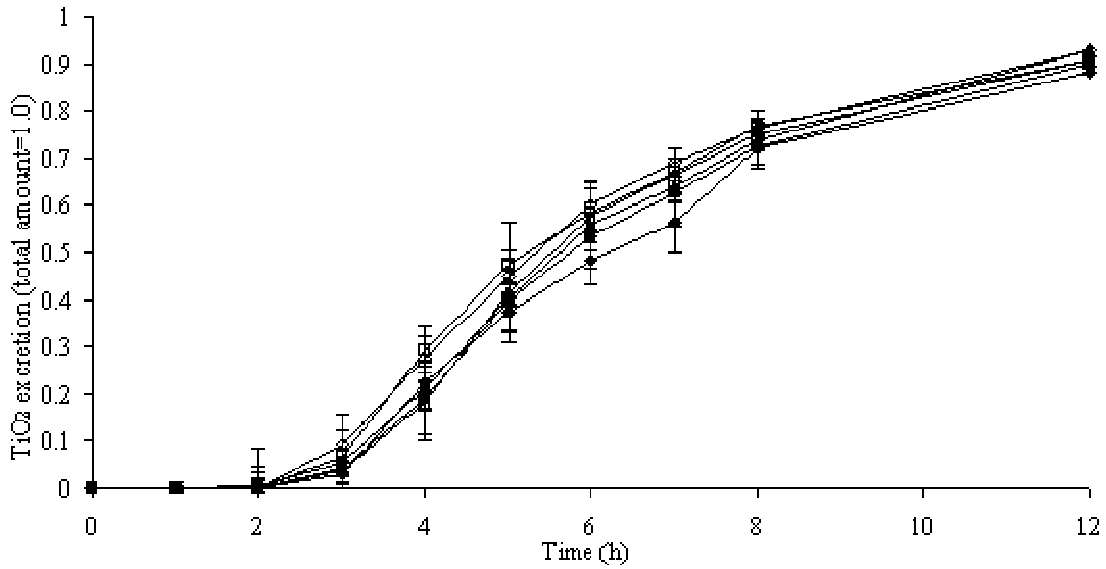


Figure 6.1 Cumulative TiO_2 excretion curves of broilers fed different treatment diets at 21 days of age

Traetment diets: deficient Thr without MOS (♦); adequate Thr without MOS (■); excess Thr without MOS (▲); deficient Thr with MOS (◊); adequate Thr with MOS (□), and excess Thr with MOS (△). Curve equation $y=t^n/(t^n + k)$, where y is the amount of TiO_2 excreted, t is the time (in h), n is the slope of the line, and k is the constant from the equation. Values are means ± SEM of n=4.

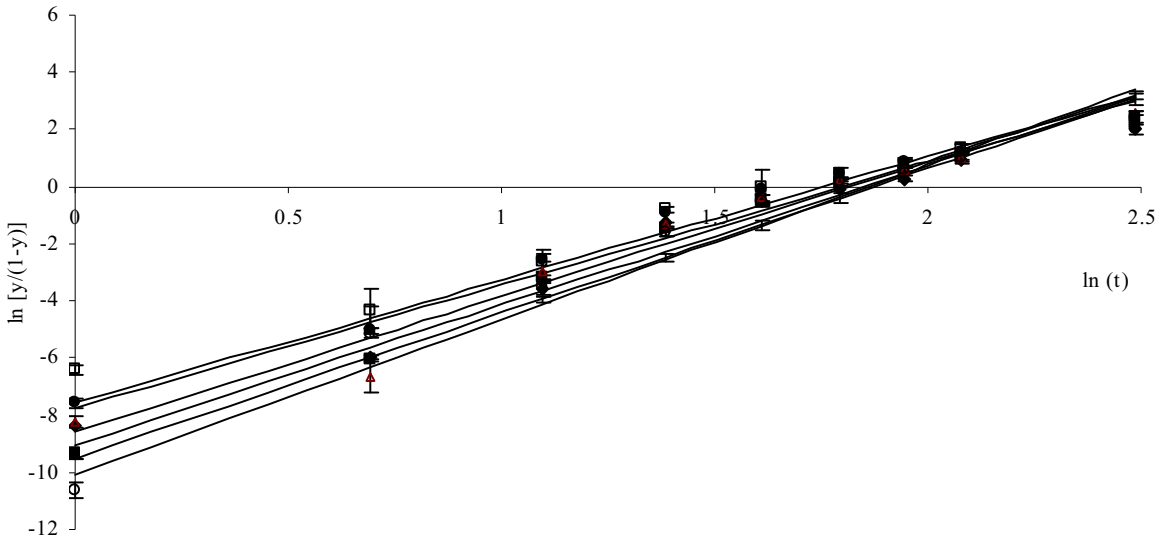


Figure 6.2 Linear representations of cumulative TiO_2 excretion curves of broilers fed different treatment diets at 21 days of age

Treatment diets: deficient Thr without MOS (♦); adequate Thr without MOS (■);excess Thr without MOS (▲); deficient Thr with MOS (◊); adequate Thr with MOS (□), and excess Thr with MOS (△) at day 21. Curve equation $In\ [y/(1-y)]=nIn\ (t) - In\ k$, where y is the amount of TiO_2 excreted, t is the time (in h), n is the slope of the line, and k is the constant of the equation. Values are means±SEM of n=4.

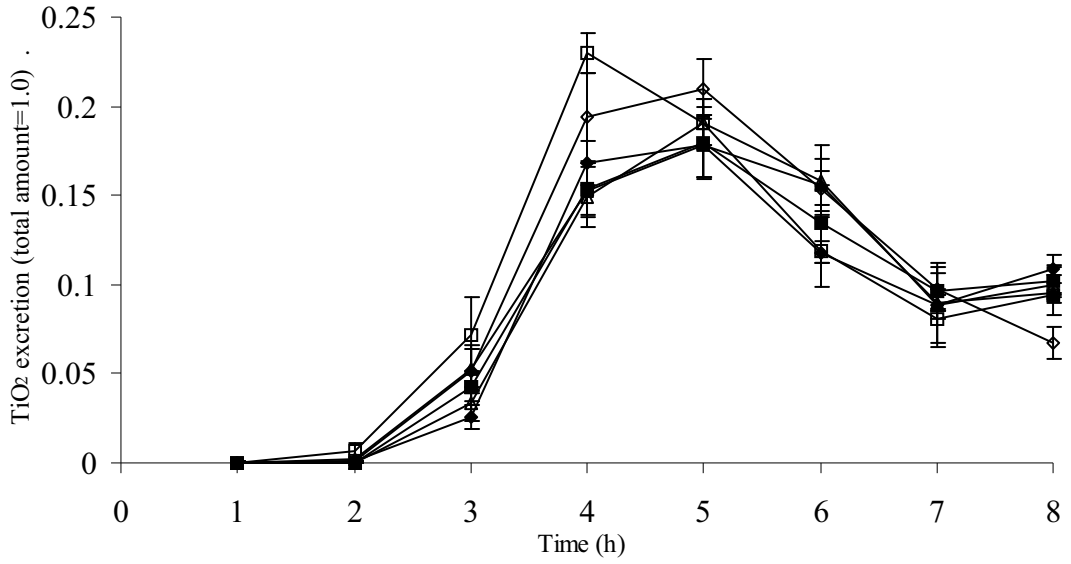


Figure 6.3 Non-cumulative TiO_2 excretion curves of broilers fed different treatment diets at 21 days of age

Treatment diets: deficient Thr without MOS (♦); adequate Thr without MOS (■);excess Thr without MOS (▲); deficient Thr with MOS (◊); adequate Thr with MOS (□), and excess Thr with MOS (△). Values are means ± SEM of n=4.

tended (P=0.08) to interact with dietary threonine to reduce the time taken to excrete 50% of the TiO_2 (T_{50} values) with the shortest transit time noted in birds given the adequate-threonine plus MOS diet (Table 6.8). T_{50} values represent the mean retention time of the feed particles in the whole gastrointestinal tract. For the feed intake, birds deficient in threonine consumed significantly less (P<0.01) feed than those supplemented with adequate or excess threonine. Dietary MOS had no significant effect on the feed intake of the birds.

Noncumulative excretion of TiO_2

Excretions of TiO_2 in a noncumulative manner by the chickens are shown in Figure 6.3. Irrespective of the treatments, all the birds started excreting TiO_2 between 2 to 3 h after the administration of the marker. However, in MOS-treated birds fed with adequate threonine, maximum excretion occurred at 4 h after the introduction of the marker diet compared to those in the other treatment groups which occurred at 5 h after the marker administration.

Table 6.8 Parameters to define the cumulative excretion curves and the determined feed intake (FI) and excretion times of broilers fed diets with different dietary threonine levels and with or without MOS supplementation at 21 days of age[1]

Treatment		Parameter[2]			FI[3](g)	Excretion time[4] (h)	
Thr	MOS	*n*	*k* x10^3	*r*	Per cage	T_1	T_{50}
Deficient					46.7[b]	2.33	6.30
Adequate					69.4[a]	2.22	6.07
Excess					73.0[a]	2.43	6.27
	-				65.8	2.45	6.34
	+				60.3	2.20	6.09
Deficient	-	5.07	65.99	0.9173	49.6	2.58	6.53
Deficient	+	4.33	3.28	0.9124	43.9	2.08	6.06
Adequate	-	4.88	2.10	0.9683	71.7	2.45	6.35
Adequate	+	4.32	3.24	0.9551	67.2	2.00	5.80
Excess	-	4.73	6.04	0.9450	76.3	2.32	6.13
Excess	+	5.41	34.43	0.9574	69.8	2.54	6.40
SEM					5.19	0.188	0.161
Source of variation		----------Probability----------					
Thr					0.0001	NS	NS
MOS					NS	NS	NS
Thr x MOS		NS[5]	NS	NS	NS	NS	0.080

[1]Each value represents the mean of 4 replicates for each treatment group.
[2]*n*=the slope of the line; *k*=the constant from equation $y=t^n/(t^n + k)$, and *r*=correlation coefficient.
[3]Feed intake of diets containing 0.5% TiO_2.
[4]T_1=excretion time for 1% of TiO_2 in excreta; T_{50}=excretion time for 50% of TiO_2 in excreta.
[5]NS=Not significant.
[a,b]Means within a continuous column with different superscripts are significantly different at $P<0.05$. Probability values up to $P<0.10$ are also shown if the data suggest a trend.

Mean retention time in various regions of the small intestine and feed intake

The mean retention time of digesta in the individual regions and the whole small intestine did not correlate with the body weight or the intestinal length (data not shown). The mean retention time in the different regions is shown in Table 6.9. MOS interacted with dietary threonine to shorten ($P<0.01$) the mean retention time in the ileum, especially in those MOS-fed birds on diets that were deficient or adequate in threonine. However, no main effects of dietary threonine or MOS on regional mean retention time were observed.

Dietary deficiency in threonine significantly reduced ($P<0.01$) the feed intake of the birds compared to those fed with adequate or excess level of threonine. Supplementation of MOS did not have any significant effect on feed intake.

Table 6.9 Effects of dietary threonine level supplemented with or without MOS on the feed intake (FI) and regional mean retention time of birds at 21 days of age[1]

Treatment		FI[2] (g)	Mean retention time (min)		
Thr	MOS	Per cage	Duodenum	Jejunum	Ileum
Deficient		100.0[b]	3.17	62.31	111.89
Adequate		123.4[a]	3.22	61.28	108.01
Excess		114.4[ab]	2.89	57.98	106.48
	-	117.0	2.74	61.42	112.59
	+	107.7	2.90	59.64	105.02
Deficient	-	103.8	3.20	65.58	121.69[a]
Deficient	+	96.2	3.14	59.05	102.09[bc]
Adequate	-	127.6	2.62	62.07	115.80[ab]
Adequate	+	119.1	1.83	60.49	100.21[c]
Excess	-	120.8	3.41	56.59	100.46[c]
Excess	+	107.9	2.96	59.36	112.49[abc]
SEM		6.78	0.722	2.749	4.690
Source of variation		----------Probability----------			
Thr		0.0071	NS	NS	NS
MOS		NS[3]	NS	NS	NS
Thr x MOS		NS	NS	NS	0.0070

[1]Each value represents the mean of 4 replicates for each treatment group.
[2]Feed intake of diets containing 5 g/kg TiO_2.
[3]NS=Not significant.
[a-c]Means within a continuous column with different superscripts are significantly different ($P<0.05$).

6.4 DISCUSSION

6.4.1 Dietary composition and treatment design

As discussed in Chapter 5, the high degree of consistency in the calculated and analysed CP and threonine values in all the experimental diets validates the treatment effects as the source of variations in this experiment.

6.4.2 Intestinal growth parameters

The increase in the relative intestinal length and weight of birds fed the threonine-deficient diet agrees with the findings in the preceding experiments (Chapters 3 and 5). The same explanations given in Chapters 3 and 5 can be applied here, where deficiency in dietary threonine may have caused an increase in mucosal degradation (Visek, 1978) and a reduction in mucosal protein and mucin synthesis (Faure *et al.*, 2005; Wang *et al.*, 2007; Nichols and Bertolo, 2008). This, in turn, may have caused more wastage of net energy and

amino acids (Stoll *et al.*, 1997; Bruins *et al.*, 2000; Wang *et al.*, 2007) and resulted in a poorer BWG that significantly increased the relative gut weight and length.

The thinning effect that MOS had on the intestinal muscularis externa of 14 day-old birds as noted in Chapter 3 and by Ferket *et al*. (2002) was not detected in the current study. This inconsistency may be due to differences in age and would suggest that MOS effect is lost with age. It is possible that older birds with more mature gut microflora are less likely to depend on gut motility to control the gut microbial activities.

6.4.3 Enteric morphometric analyses

The negative effect of threonine deficiency on intestinal villus height was again observed in the present investigation and is in full agreement with those reported by Hamard *et al*. (2007). Spring *et al*. (1996), Iji *et al*. (2001b), and Yang *et al.* (2007a) also reported similar findings on the effect of MOS in increasing the intestinal villus height. The measurements of villus height and villus to crypt ratio generally give an indication of the likely maturity and functional capacity of the enterocytes (Hampson, 1986). Therefore it can be deduced that MOS significantly accelerated the maturation of gut function. As explained in Chapter 3, it is likely that threonine might have a direct effect on the intestinal morphology mediated through its obligatory involvement in the fractional as well as absolute synthesis of intestinal mucosa and mucins (Faure *et al.*, 2005; Wang *et al.*, 2007; Nichols and Bertolo, 2008). On the other hand, MOS might exert its main influence on villus morphology via its modulating effects on intestinal mucin dynamics and microbial-mucosal interactions (Uni and Smirnov, 2006).

The lack of any significant effect of MOS or dietary threonine on intestinal external muscularis thickness correlates well with the negative finding on the relative weight of the intestinal muscularis externa. Again, as explained in Section 6.4.2 that older birds with more mature gut microflora are less likely to depend on gut motility to control the in gut microbial activities.

6.4.4 Intestinal microflora and lactic and volatile fatty acids

In accordance with the findings of Stanley *et al.* (1996) , MOS-supplemented birds fed with adequate threonine had a lower number of intestinal coliforms compared to those in the non-supplemented groups. In addition, Song and Li (2001) and Jamroz *et al*. (2003) also reported

that MOS significantly decreased the number of *E. coli* in either the small intestine or the excreta of chickens.

It is suggested that MOS, as a receptor analogue, can act as a decoy for those pathogenic or potentially pathogenic bacteria possessing type-1 fimbriae (mannose-sensitive lectins) (Spring *et al.*, 2000). This, in turn, prevents them from attaching to the gut mucosa to colonise the gut and cause disease (Yang *et al.*, 2007a). 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 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 lumen of the ileum of birds supplemented with MOS and excess threonine. One possible reason for the increase in the count of coliform bacteria might be that MOS not only binds but also displaces those type-1 fimbriae bacteria attached to the gut wall (Yang *et al.*, 2007b). As indicated in an *in vitro* study, attached *E. coli* could be displaced from the epithelial cells within 30 minutes when exposed to a mannan derivative (Ofek and Beachey, 1978). This is likely to happen in the presence of a high luminal mucin concentration that constantly draws the displaced coliforms away from the epithelium into the luminal contents (Gill, 2003). Similar results were found when using the same commercial MOS product as in the present study (Peuranen *et al.*, 2006).

The increase in numbers of ileal lactobacilli, lactic acid bacteria and coliforms in birds fed with adequate threonine agrees with the finding of Faure *et al.* (2006) indicating a close relationship between gut microflora, intestinal mucin dynamics and mucosal development. This close relationship has been critically reviewed by Deplancke and Gaskins (2001).

Both lactate and VFA being the major end products of bacterial fermentation are the two most common indicators of microbial activity. In the current investigation, dietary threonine did not influence the concentrations of individual VFAs but tended to elevate the ileal lactic acid concentration. This is closely linked to the increase in the numbers of ileal lactobacilli and lactic acid bacteria. Mannooligosaccharides only increased the concentration of individual VFAs numerically, suggesting that the overall gut microflora activities, particularly those of the lactobacilli and lactic acid bacteria, were not extensively altered by MOS addition. As indicated in Chapter 4, it is possible that MOS may have altered the gut

microbial composition without having any significant effect on the overall gut microflora activities.

6.4.5 Crude mucin concentration and output

Evidence of the obligatory role played by threonine in mucin synthesis has already been demonstrated in the previous study (Chapter 3) and by others (Fuller *et al.*, 1994; Stoll *et al.*, 1998; Wang *et al.*, 2007). The influence of threonine on the ileal flow of crude mucin observed in the present study further supports the aforementioned statement. A significant increase in the ileal flow of crude mucins attributable to MOS and its interaction with threonine also unequivocally confirms the modulating effect of MOS on intestinal mucin dynamics.

It has been postulated in Chapter 3 that the increase in intestinal goblet cell density and its staining intensity by MOS and dietary threonine may potentially increase the mucin secretion capacity of the mucosa (Piel *et al*., 2005). This hypothesis is supported by the concomitant increase in the ileal flow of crude mucins as demonstrated in the current study. The reason why this effect was not observed in the jejunum remains unclear. However, this is not totally surprising as luminal flow of mucin provides information on the dynamic nature of the cumulative net output of the nondigested fraction of mucins produced in the proventiculus as well as all along the subsequent regions of the small intestine with ileum being the end collector and most active site for bacterial mucin degradation (Lien *et al.*, 2001).

6.4.6 Feed intake, feed passage rate and excreta characteristic

Total tract transit time and mean retention time

In the present study, the estimations of feed passage rate based on first excreta appearance correlate well with the T_1 values of the cumulative excretion curves. T_1 value is defined as the first appearance of TiO_2 in a significant amount of more than 1%. Although these variables, ranging from 2.01 to 2.61 h, agree with those reported by Ferrando *et al.* (1987), they were not significantly modified by MOS or dietary threonine. In fact, it has been pointed out that first appearance of excreta materials or marker is not a good method to evaluate the feed passage rate as transit of feed particles is a sigmoidal function. As in other physiological processes, when the parameter studied is situated on the asymptote of the curve, variations in the parameter are of little significance (Ferrando *et al.*, 1987).

As opposed to T_1 values, estimation of feed passage rate based on T_{50} values are more reliable as calculations are placed on the linear component of the sigmoidal curve. As there were no significant changes in the T_{50} values, dietary threonine did not exert any influence on the feed passage rate. Intriguingly, MOS tended to interact with dietary threonine to shorten the total tract mean retention time as revealed by a decrease in the T_{50} values. The mean T_{50} value of 6.22 h reported herein agrees with that reported by Sieo *et al.* (2005) at 6.86 h for 21 day-old birds but were slightly longer than 5.14 h noted by Hetland and Svihus (2001) for broilers at 17 days of age. This inconsistency is presumably related to the difference in the age of birds studied. It is known that the passage rate of digesta through the entire GIT is slower in adult than in younger birds (Hillerman *et al.*, 1953; Hurwitz and Bar, 1966; Sklan *et al.*, 1975).

Regional mean retention time

The mean retention time increased linearly along the small intestine. The average mean retention times for duodenum, jejunum and ileum estimated at 3, 61 and 109 min were in good agreement with those reported in the literature (Hurwitz and Bar, 1966; van der Klis *et al.*, 1990). Taken as a whole, the reduction in the ileal mean retention time as influenced by the interaction between MOS and dietary threonine was paralleled by a significant increase in the ileal flow of crude mucin. This apparent relationship between the two variables is likely to be mediated through the lubricating effect of crude mucins (Forstner and Forstner, 1985; Forstner *et al.*, 1995; Montagne *et al.*, 2000). On the contrary, the visco-elastic nature of crude mucins (Allen, 1981, 1984) may not seem to support such phenomenon as any increase in intestinal viscosity will unavoidably increase the feed retention time (Cherbut *et al.*, 1990; Almirall and Esteve-Garcia, 1994). However, it is likely that the lubricating effect of crude mucins may have overridden its rheological effect on the passage of intestinal digesta. Furthermore, the increase in intestinal water retention which is frequently associated with increased intestinal viscosity (Leterme *et al.*, 1998; Sieo *et al.*, 2005) was not detected in this study. This is because the intestinal dry matter output and excreta moisture content remained unaffected in spite of an increase in the ileal crude mucin concentration. At the least, it appears that some level of mucin synthesis is crucial to facilitate the passage of luminal contents, although the optimal amount of mucin synthesis for this function is yet to be determined.

Despite the significant increase in the ileal flow of crude mucins, the main effect of dietary threonine or MOS supplementation did not result in a significant reduction in the ileal mean

retention time. It is likely that the small data set used in this study may have reduced the significance of the results. It also indicates that the quantity of mucus might not be the sole contributing factor to the increased feed passage rate. The quality of the mucus may also count as the physicochemical property of the mucins, which is highly dependent on the glycoprotein composition, changed according to the dietary effects. For example, it is known that acidic mucins are more viscous than neutral mucins (Allen *et al.*, 1982) and that the glycosylation profile of the luminal mucins varies with intestinal glycosidases and microbial activities (Montagne *et al.*, 2000). Since the glycosylation profile of the luminal mucins was not analysed in the current study, its significance on the feed passage rate cannot be deduced here.

There is no clear indication as to what extent the intestinal musculature may influence the feed passage rate as no significant differences in external muscularis thickness and weight attributable to the treatments were observed. As suggested earlier, this effect may be of more significance to younger birds as older birds may have sufficiently well established gut microflora and thus, are less likely to depend on gut motility to control the intestinal microbial activities. The difference in mean retention time attributable to volatile fatty acids or gut microflora (Kamath *et al.*, 1988; Richardson *et al.*, 1991; Lesniewska *et al.*, 2006) is also not conclusive. It should only be taken as a rough indication as MOS only increased the ileal volatile fatty acid concentration numerically. Recent evidence suggests the possible effect of volatile fatty acids on motility at a distance from their site of production (Cherbut *et al.*, 1997). If this is established, then it follows that the findings of others (Ao, 2004; Santos and Teixeira, 2004; Yang *et al.*, 2007a) on the increase in the caecal volatile fatty acid concentration through MOS supplementation may also play an integral role in shortening the ileal transit time.

Similarly, it has not been determined yet whether feeding activity also influenced the feed passage rate as reported by Vergara *et al.* (1989) and Hetland and Svihus (2001). This is because an increase in voluntary feed intake due to adequate feeding of dietary threonine did not result in any significant reduction in intestinal retention time. The influence of the digestive tract volume on feed intake and feed passage rate is less likely to happen in this case as all diets were prepared and given in the same physical form. In fact, Hetland and Svihus (2001) suggested that the increase in passage rate is due more to the physical structure of the feed ingredients than to the actual increase in feed intake *per se*. Moreover,

Washburn (1991) also found that there were no significant correlations between feed passage rate and feed consumption.

The increase in ileal transit time induced by MOS and threonine interaction is somewhat unexpected and contradicts with the postulations made by Biggs and Parsons (2007) and Middelbos *et al.* (2007). However, it is noteworthy to point out that the biological relevance of an increased feed passage rate is dependent on the location of the GIT. A more rapid passage of feed in the anterior digestive tract may not likely to have any beneficial effect on nutrient digestion due to a lesser grinding activity of gizzard and less homogenous mixing of chyme with the digestive enzymes in the duodenum and jejunum (Svihus *et al.*, 2002).

An increase in ileal feed passage rate is more arguable. The ileum is the location where the digesta stays for a relatively long time to increase the contact time between nutrients and digestive enzymes. As such, a more rapid feed passage has been suggested to reduce the time available for digestion (Krogdahl, 1986; Choct, 1997). Consequently, the reduction in movement of nutrients from the lumen to the mucosal epithelium may reduce the mucosal uptake of nutrients (Ellis *et al.*, 1996). On the other hand, it can also be argued that an increased feed passage rate through the ileum may also reduce the time available for microbial fermentation and thus, more nutrients could potentially be made available for the growth of the birds (Hetland and Svihus, 2001). It is also certain that a fast moving digesta provides a more dynamic environment in the ileum that can better resist bacterial stagnation and thus, the proliferation and colonisation of gut microflora (Wagner and Thomas, 1978; Svihus *et al.*, 2002). Increased movement of digesta also plays an integral role in the rapid expulsion of microorganisms from the small intestine through the formation of antigen-antibody complexes in the mucous coat (Cherbut *et al.*, 1998). This suggests that the binding of pathogens by luminally available MOS could also be beneficial in the same manner. Furthermore, a shortened ileal emptying time may also protect the ileal mucosa against the potentially harmful effects of the reflux of colonic contents (Kamath *et al.*, 1988; Cherbut *et al.*, 1997).

6.5 CONCLUSIONS

In conclusion, the results of this study demonstrated that MOS interacted with dietary threonine to reduce the ileal mean retention time. Correspondingly, a tendency for the reduction in total tract mean retention time through the interaction of MOS and dietary

threonine interaction was also recorded. These interactive effects of MOS and dietary threonine are likely to be mediated through the lubricating effect of the increased ileal flow of crude mucins. The influential roles of volatile fatty acids, gut microflora and voluntary feed intake on feed passage rate attributable to dietary threonine or MOS supplementation were not conclusive. The effects of intestinal musculature on feed passage rate were not detected and are likely to be age-dependent.

CHAPTER 7
GENERAL DISCUSSION

7.1 INTRODUCTION

As reviewed in Chapter 2, the search for effective alternatives to antibiotics demands a great knowledge of the multi-disciplinary nature of nutritional science, microbiology, gut physiology, immunology and disease. However, previous investigations on alternatives to antibiotics have yielded mixed results with varying levels of success, both in terms of growth performance and the mechanisms involved. There are a number of important impediments to finding highly effective alternatives to antibiotics. Firstly, the interaction between these alternatives and dietary nutrients on growth performance is not fully understood; secondly, only a small fraction of the over 600 intestinal microbial species are elucidated with respect to their activities and contributions to the well-being of the birds, and thirdly, there has been a lack of knowledge on the interrelationship between intestinal mucins, gut microflora and these alternatives on the growth of the birds. Therefore, four experiments were conducted in the present work to examine the interactive effects of MOS and dietary threonine on the growth performance, mucin dynamics, gut microflora, gut morphology, feed passage rate, and nutrient utilisation of birds. The major findings of the work and possible correlations among them are further discussed in the following sections.

7.2 GENERAL PERFORMANCE

Positive interactions between MOS and threonine supplements on BWG were apparent in all phases of growth (Chapter 3). Graded levels of dietary threonine produced a quadratic response in growth performance (Chapters 3 and 5). This is in good agreement with results reported by Kidd and Kerr (1997), Corzo *et al*. (2003), and Kidd *et al*. (2003b). Dietary MOS also increased the growth performance of the birds (Chapters 3, 4 and 5) with more pronounced effects observed in early life (Chapters 3 and 4). These observations generally agree with those in the literature (Jamroz *et al.*, 2003; Tucker *et al.*, 2003; Yang *et al*, 2007a). However, this age-dependent growth-promoting effect of MOS is also threonine-sensitive, especially at the later stage of growth. Thus, feeding of MOS with threonine supplemented to meet the extra amount demanded by MOS resulted in additional BWG and better FCR and was economically justified based on the improved net return.

7.3 INTESTINAL MUCIN DYNAMICS AND GENE EXPRESSION

Intestinal adherent mucus thickness and ileal flow of crude mucins responded quadratically with graded dietary threonine levels (Chapters 3, 5 and 6). Generally, dietary threonine had no effects on all types of goblet cell densities but significantly increased their staining intensities (Chapter 3). In addition, an increase in dietary threonine level did not result in any change in the jejunal MUC2 expression (Chapter 3). Collectively, these findings indicate that: i) dietary threonine is in high demand for the synthesis of intestinal mucin glycoproteins and mucosal development, ii) threonine is involved directly in intestinal mucin synthesis but only at the translational stage, and iii) the importance of optimising dietary threonine and intestinal mucin dynamics for the maximisation of growth.

The positive effects of MOS and its interaction with dietary threonine on intestinal adherent mucus thickness and ileal crude mucin flow were also noted (Chapters 3, 5 and 6). Dietary MOS increased the goblet cell "cup" size and selectively enhanced the differentiation of acidic mucin goblet cells, especially those of the sulphated subtype (Chapters 3 and 4). Supplementation of MOS also resulted in a constant up-regulation of jejunal MUC2 expression and was independent of dietary threonine level (Chapter 3). Thus, it can be deduced that the interaction between MOS and threonine on growth performance is partially linked to its modulating effect on mucin dynamics. Under such conditions, a constant up-regulation of MUC2 mRNA expression without adequate supply of threonine would not translate into an overall improvement in mucin production. The increase in sulphated-acidic mucins induced by MOS also indicates a better gut protection against bacterial translocation. This is because more mature sulphomucins are highly viscous and are less degradable by bacterial glycosidases and host proteases (Robertson and Wright, 1997; Fontaine *et al.*, 1998).

7.4 GUT DEVELOPMENT

Deficiency in dietary threonine consistently decreased the villus height and increased the relative length and total weight of the small intestine (Chapters 3, 5 and 6), although the effect on relative weight of muscularis externa was inconsistent (Chapters 3 and 6). This effect is likely the result of an increase in mucosal degradation rate (Visek, 1978) and a reduction in mucosal fractional synthesis rate (Wang *et al.*, 2007; Nichols and Bertolo, 2008). Consequently, it may have caused more wastage of net energy and amino acids (Stoll

et al., 1997; Bruins *et al.*, 2000; Wang *et al.*, 2007), resulting in a poorer BWG and thus, significantly higher relative gut tissue weight and length.

Inclusion of MOS induced mixed modulating effects on the gross development of the small intestine. The reduction in relative muscularis weight of all regions of the small intestine was consistently noted at 14 d of age (Chapter 3) but not at 21 d of age (Chapters 3, 5 and 6). The mucosal architecture also changed as a result of MOS addition. Increase in villus height and villus to crypt ratio associated with MOS supplementation were common findings in the present work (Chapters 3, 4, 5 and 6) as well as by other authors (Spring, 1996; Iji *et al.*, 2001b; Baurhoo *et al.*, 2007). However, none of the above experiments showed any significant effect of MOS on crypt depth. Unlike mammals, in which enterocyte proliferation only occurs in the crypts, enterocyte proliferation occurs in the crypts as well as the villus region in birds (Uni *et al.*, 2000). Therefore, villus to crypt ratio appears to be a better indication for the efficacy of energy utilisation for gut development. Thus, the growth-promoting effects of MOS can also partly be explained by its energy-and-nutrient-saving contributions at the level of gut development.

7.5 NUTRIENT UTILISATION AND FEED PASSAGE RATE

The importance of feeding adequate threonine was directly supported by improved ileal digestibility and mucosal uptake of threonine and glucose (Chapter 5). No main effect of dietary threonine on feed passage rate was observed. Comparatively, the effects of MOS on intestinal nutrient utilisation (TER and PER, ileal amino acid digestibility, and mucosal nutrient uptake) were more intense and even noticeable at suboptimal threonine level (Chapter 5). This is likely to be linked to the positive modulating effects of these supplements on intestinal mucosal development and mucin dynamics. An improvement in gut morphology (Chapters 3, 4, 5 and 6) as well as in the gene expression and specific activity of brush-border enzymes and transporters (Iji *et al.*, 2001b; Hamard *et al.*, 2007; Uni, 2008) may suggest a relatively higher proportion of mature enterocytes (Hampson, 1986; Uni, 2008) and absorptive area (Yasar and Forbes, 1999), which would be expected to improve the capacity for digestion and mucosal nutrient absorption.

In addition, MOS also interacted with dietary threonine to reduce the ileal mean retention time, especially in those birds on diets that were deficient or adequate in threonine (Chapter 6). This interactive effect of MOS and dietary threonine is likely to be mediated through the

lubricating effect of the increased ileal flow of crude mucins (Chapter 6). The effects of volatile fatty acids, gut microflora and voluntary feed intake on feed passage rate attributable to dietary threonine or MOS supplementation were not conclusive (Chapter 6). The effects of intestinal musculature on feed passage rate were not detected and are likely to be age-dependent (Chapters 3 and 6). This is because older birds may have sufficiently well established gut microflora (Chapters 4, and 6) and thus, are less likely to depend entirely on intestinal peristaltic movement to control the intestinal microbial activities. As a whole, an increased passage rate through the distal part of the digestive tract may favour more the control of intestinal microbial activities (Svihus *et al*., 2002) than the reduction of time available for digestion (Krogdahl, 1986; Choct, 1997).

7.6 THE DEVELOPMENT OF GUT MICROFLORA

Both zinc bacitracin and MOS have the ability to stabilise and modulate the gut microflora towards a balance that will be beneficial to the development of gut function and body growth. Enumeration of bacteria by culture demonstrated that MOS accelerated the maturation of gut microflora by promoting the growth of lactobacilli in the ileal mucosa and at the same time inhibiting the proliferation of coliforms and *C. perfringens* in both the ileum and caeca (Chapter 4). At adequate level of threonine, MOS also reduced the populations of coliforms in the ileal digesta and mucosa (Chapter 6). The inhibitory effect of MOS on intestinal *Clostridium* population is particularly intriguing as this group of bacteria do not possess type-1 fimbriae as is common in coliforms. Similar results were reported in turkeys on MOS treatment (Sims *et al.*, 2004; Zdunczyk *et al.*, 2005). It is speculated that MOS indirectly inhibits the growth of intestinal clostridium by modulating the immune system (Savage *et al.*, 1996; Cotter, 1997).

Unlike MOS, zinc bacitracin suppressed the number of intestinal bacteria, especially those of the lactobacilli and clostridia (Chapter 4). Use of T-RFLP further revealed the complexity of the mechanisms adopted by MOS and zinc bacitracin in modulating the gut microflora. Dietary MOS increased the diversity of lactobacilli in the ileal mucosa and ileum but the opposite was true for zinc bacitracin (Chapter 4). Despite these differences, both MOS and zinc bacitracin increased the proportional abundance of ileal lactobacilli at the expense of the clostridia (Chapter 4). It also appears that MOS and zinc bacitracin possessed a common ability to differentially favour the growth of certain *Lactobacillus* species (Chapter 4). However, the beneficial significance of these differentially selected *Lactobacillus* species in

relation to nutrient utilisation such as fat digestion could not be established as ileal bile salt dehydrolysing activities were not determined in this study. Additionally, the increase in homogeneity of the gut microflora induced by MOS and zinc bacitracin may link directly to the improvement in flock uniformity frequently observed with the dietary inclusion of these additives (Ao, 2004; Yang, 2007).

7.7 CONCLUSIONS

The current studies clearly demonstrated the importance of optimising dietary threonine and intestinal mucins for the maximisation of growth. The results also imply that, by modulating the intestinal mucin dynamics, gut microflora and mucosal development, MOS improved the digestive and absorptive functions of the gut and thus, leading to a better growth performance. However, this age-dependent growth-promoting effect of MOS was also demonstrated to be threonine sensitive, especially at the later stage of growth. Therefore, optimisation of dietary threonine in conjunction with MOS usage may help to improve its efficacy. Collectively, these results point to the practical importance of assessing the interactions between dietary nutrients and additives. Investigations into the interactive effects of MOS with other nutrients may need to be elucidated in future work. Furthermore, future experiments conducted under germ-free environment are also warranted to determine whether MOS has any direct effect on intestinal mucin dynamics. Use of advanced molecular technique such as the T-RFLP is also equally important for providing a more comprehensive profile of gut bacteria and thus, will facilitate in the search for more effective alternatives to antibiotics.

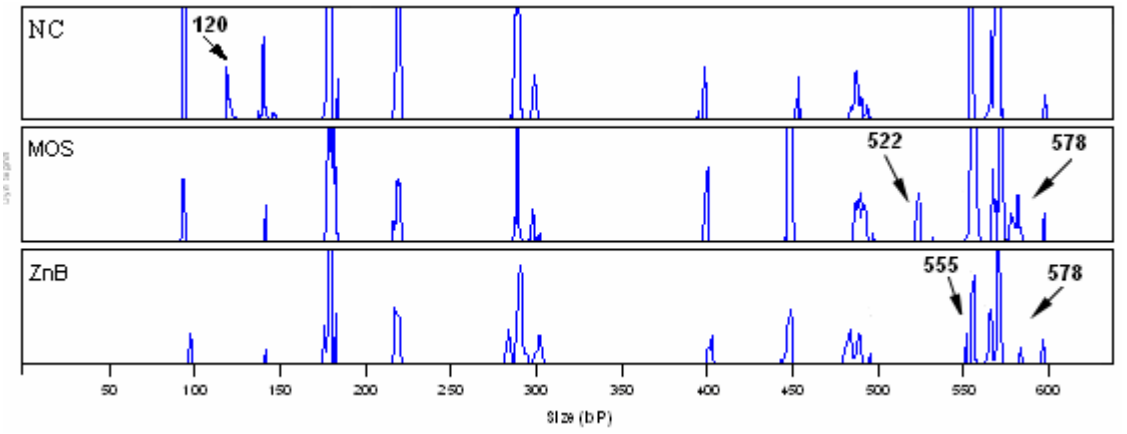
APPENDICES

Appendix 1 List of theoretically identified major bacterial species based on *Hha*I and *Msp*I *in silico* restriction detected in the ileal digesta, ileal mucosa and caeca of NC, MOS and ZnB-treated broiler chickens at 21 days of age

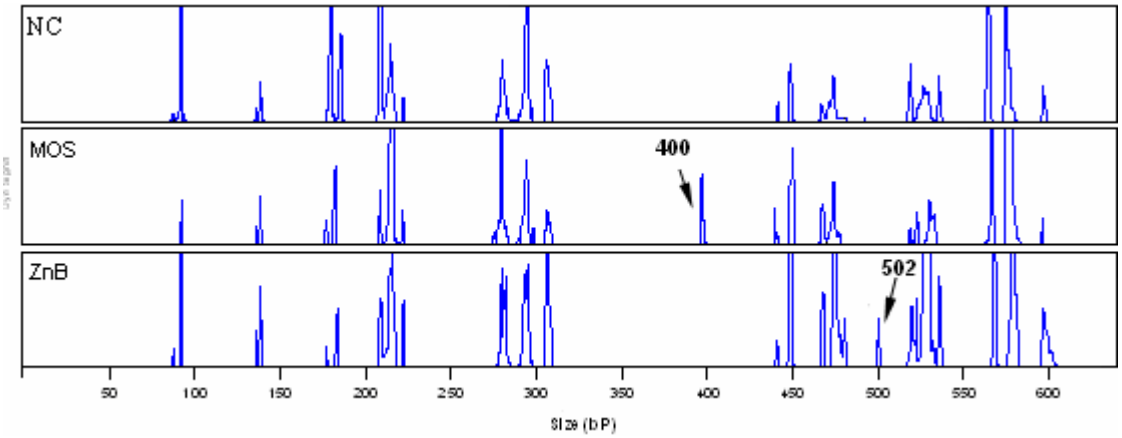
Bacterial identification		T-RF length (bp)	
Bacterial groups	Compatible bacteria	*Hha*I	*Msp*I
Lactic acid bacteria	*L. vaginalis*	60	29[a]
	L. fructivorans	69	582
	L. delbrueckii subsp. *lactis*	224	179
	L. delbrueckii subsp. *delbrueckii*	254	179
	L. mucosae, L. ingluviei	266, 267	29[a]
	L. agilis, L. aviarius	393, 394	568, 569
	L. maltaromicus	587	561
	L. acetotolerans	595	180
	L. acidophilus group	596, 597	181, 182
	L. plantarum and L. casei group	598, 599	572, 573
	L. sakei subsp. *sakei*	604	578
	L. salivarius, L. ruminus	1122[1], 1123[1]	565, 566
	Enterococcus spp.	172, 216, 218	521, 565, 566
	Bacillus subtilis	238	143
	Lactococcus spp.	583	555
Clostridium and relatives	*Clostridium* subcluster XIV_a	186,187	297, 298
	Clostridium clostridioforme	190	222
	Clostridium ramosum	228, 229	295, 520
	Clostridium cochlearium	362	522
	Clostridium butyricum	545	520
	C. colinum	897[1]	478
	Eubacterium. Spp.	245, 377	194, 222, 224, 296
Bacteroides and relatives	*Cytophaga, Nitrospiro &* *Flexibacter* spp.	99,100 248	91, 93, 143, 203, 93, 97, 477, 487
Fibrobacter	*Fibrobacter succinogenes*	138	164[1]
Proteobacteria	*Escherichia* spp.	369, 371,373	97, 492, 497, 496, 495
Other bacterial groups	Other enteric bacteria (*Salmonella, Citrobacter,*	367,370,371, 372,373, 570	97, 344, 492, 493, 495, 496
	Norcardiopsis spp./	355	141
	Gammaproteobacteria	369	143
	Kitasatosphora *spp*	438	158
	Chloroflexi bacterium	531, 551	95, 478
	Thermoanaerobactriacease	620	595

[1]T-RF peaks not detected or beyond detection limit of DNA sequencer but listed out based on *in silico* restriction using Ribosomal Database Project II and public database (http://mica.ibest.uidaho.edu/).

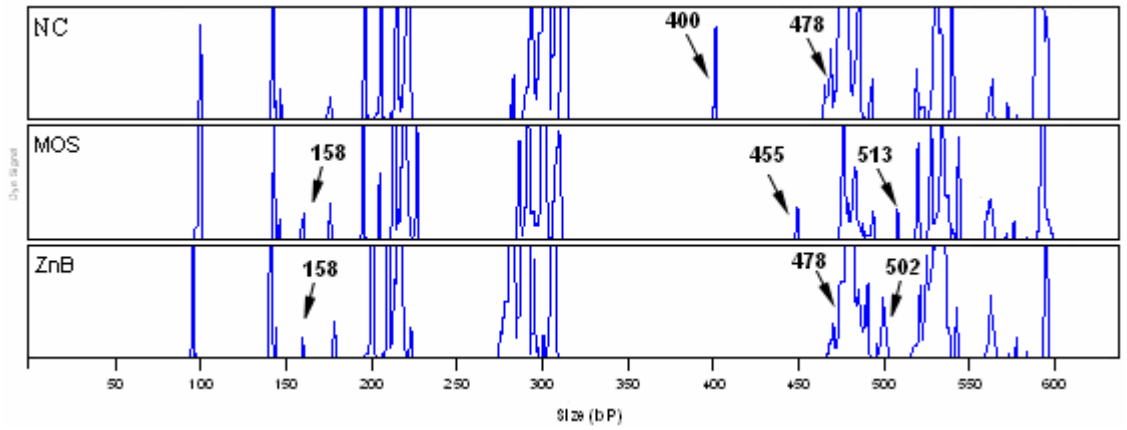
Appendix 2 Representative T-RFLP patterns of bacterial communities present in the (a) ileal digesta, (b) ileal mucosa and (c) caeca after digestion with *Msp*I in NC, MOS and ZnB-supplemented broiler chickens at 21 days of age. Arrows indicate presence of major T-RF peaks with fragment lengths in bp.



(a)



(b)



(c)

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