GASTROINTESTINAL HEALTH BENEFITS OF SOY WATER-SOLUBLE CARBOHYDRATES IN YOUNG BROILER CHICKENS

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Yu Lan

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Abstract

Soybean meal is world wide used as an ingredient of poultry diets, due to both its high quality and quantity of protein content. Meanwhile soybean meal contains relatively high levels of soluble carbohydrates, known as oligosaccharides (SMO) and polysaccharides (SMP). SMO and SMP can not be digested by digestive enzymes and tend to reduce the available energy in the diet, therefore behaving as an anti-nutritional factor. However, SMO and SMP can be utilised by intestinal microflora. Thus they may act as prebiotics. They may affect gut microbial community population consistent, and promote the proliferation of gut mucosal cells including natural antibodies, especially, when SMO and SMP are used in broilers' diets during the early stage of post-hatch. As a result, SMO and SMP may affect the development of the gastrointestinal tract and furthermore on productivity. They may stimulate the growth of gut lactic acid-producing bacteria (LAB) and promote immune competence ability. To test the prebiotic and immune stimulating role of SMO and SMP on broiler chickens several experiments have been made. One *in vitro* experiment was made to study kinetics of the *in vitro* fermentation. Caecal contents were used as source of inoculum. Three animal trials (in which SMO and SMP were used as diet additives in broiler diets of the first two weeks of post-hatch) are described in this thesis. The results showed that non-digestible carbohydrates were fermented by caecal contents microbial community of broiler chickens in vitro. SMO, SMP, AMO, AMP, and STA could significantly stimulate caecal contents bacteria activity and lead to shift of caecal contents microbial community in some extent. SMO produced the lowest ammonia nitrogen and highest butyric acid in these fermentable carbohydrates after fermentation. When the diet of the first two weeks were diluted by 1% SMO and 0.5 % SMP, respectively, dietary SMO and SMP didn't affect the development of intestinal length and relative weight. However, dietary SMO and SMP decreased the villus size, and tended to increase microvilli density on villus surface; dietary SMO significantly increased LABs population in caecal contents both in healthy birds and the birds infected with *Eimeria tenella;* dietary SMO and SMP enhanced cellular but did not affect humoral immune response followed by vaccination; SMO and SMP led to an increase in immunoglobulin A, M and Y positive plasma cells (IgA^+, IgM^+ and IgY^+) in the

caecal lamina propria both in healthy birds and the birds infected by *E. tenella;* SOM and SMP led to feed restriction during its use in diets as an additive, but a complete compensatory growth took place after SMO and SMP were withdrawn from diet.

In conclusion, water-soluble carbohydrates (SMO & SMP) had prebiotic and immune stimulating effects on broiler chickens when used as feed additives in broiler diets in the early stage post-hatch period, and it may have beneficial effects on certain microbiological and immunological promotion within the GIT, might therefore be suitable additives for the promotion of GIT health. Meanwhile, it can used as a safety feed restriction additive in the diet of broiler chickens.

Keywords: soy water-soluble carbohydrates, fermentation kinetics, intestinal morphology, caecal contents lactic acid bacteria, immunoglobulin, feed restriction, broiler chickens.

Preface

A good result should start from a good begining. I would like give my first thank to my supervisor Prof. Dr Ir. Seerp Tamminga, Prof. Ga Erdi and my college classmate Zhang Renhou, for introducing me into Wageningen University.

Scientific technology is the wing of science research. It was my co-supervisor Dr. Barbara Williams gave me such wings. She taught me how to use *in vitro* gas production technique and DNA fingerprint analysis technique to assess the effect of dietary fermentable carbohydrate on the activity of intestinal microbial community. It will be to benefit for my future study. Meanwhile, I also learned more laboratory skills from Dr. Huug Boer, such as extraction oligosaccharides and water-soluble polysaccharides from plant materials. Their responsibility impressed me deeply.

Experimental results need to be expressed properly. I would like to give my special thanks to my supervisor Prof. Dr. Ir. Martin Verstegen, for giving valuable comments on this thesis started from the earliest version till the latest one, and he also translated the English Summary of this thesis into Dutch one. How many numberless days and nights he has spend on this thesis. I would express my grateful to his wife Mrs. Mariet Verstegen, a hospitable and kindly lady, for her valuable suggestion on thesis defence preparation. Her lovely cards in New Year and Chinese Spring Festival, as well her cake let me feel I was still stay in my own family. Many thanks to my supervisor Prof. Dr. Ir. Seerp Tamminga, my co-supervisor Dr. Barbara Williams, Dr. Henk Parmentier and Lu Shoufeng, for their comments on this thesis, especially Dr. Barbara Williams who corrected the thesis word by word. I would also wish to thank Dr. Ir. Rene Kwakkel for his helpful suggestion on general introduction part of this thesis.

Success cannot be achieved alone. I am very grateful to all those who have contributed to this work: the supervisors and other scientific supporters, the universities both Wageningen University and China Agricultural University, the poultry farmers. I would like to thank all the people who help me to executed experiments, including the

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At last, I would like to express my thanks to my Husband Ni Zhushan and son Nie Han for their love and support.

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Wageningen

CONTENTS

GENERAL INTRODUCTION

General Introduction

Soybean meal (SBM) is an important ingredient in poultry diets in many regions of the world. Many other grain legumes are comparable to soybeans in terms of their protein content (Petterson and Mackintosh, 1994; Mohamed and Rayas-Duarte, 1995), they do not support animal growth to the same extent as soybeans (Lacassagen, 1988; Centen *et al.,* 1990). Among protein sources, SBM has an excellent reputation for high amino acid quality and amino acid profile. The lysine content in soy proteins is higher than in other legumes, and much higher than that of cereal proteins such as wheat gluten. It is also comparable with animal proteins such as egg white (Betschart, 1978; Friedman, 1996). Although soy protein lacks significant levels of the essential amino acid L-methionine (Friedman, 1996), soybean meal is still one of the most important protein ingredients for poultry production.

Apart from its high protein content, soybean meal contains relatively high amounts of soluble carbohydrates, including sucrose, which can be digested by intestinal enzymes, and indigestible oligosaccharides (SMO) and polysaccharides (SMP) Both these components cannot be digested by animal enzymes and thus can not be digested directly by monogastric animals. They can only be digested by microbial enzymes. These carbohydrates are not eliminated by thermal processing (Leske *et al*., 1993). SMO account for approximately 4-5 % of the dry weight of SBM, and comprise mainly stachyose, raffinose and low concentrations of verbascose (Kawamura and Narasaki, 1961; Laske *et al.*, 1993; Mohamed and Rayas-Duarte, 1995; Obendorf *et al.*, 1998). Furuta (1999) showed that in soybean meal, molecular weights of the SMP (Figure. 1) and its viscosity (Figure. 2) in aqueous solution were relatively low. The molecular weight was determined to be about 114000 using a viscometric method, and the viscosity, which exhibited Newtonian behaviour, was 102 mPa s in a 10 % SMP solution in water. The viscosity of a 10 % SMP solution increased gradually with increasing pH and decreased gradually with decreasing pH (Figure 3). Meanwhile, the viscosity of SMP decreased linearly with decreasing polysaccharide concentration increased gradually with increasing pH and decreased gradually with decreasing pH (Figure 3). Meanwhile, the viscosity of SMP decreased linearly with decreasing

polysaccharide concentration.

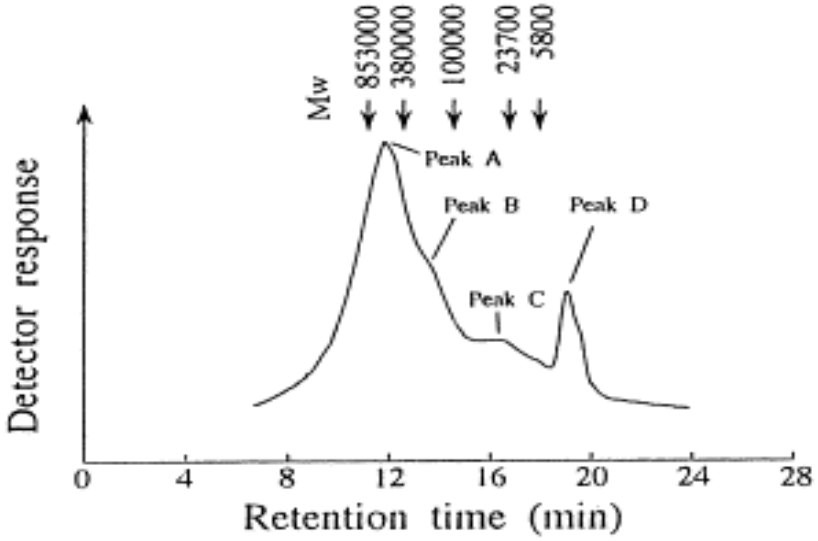

Figure 1. Molecular weight distribution of water soluble polysaccharides (SMP) in soy bean meal (Furuta, 1999). The molecular weight distribution was determined by HPLC using a gel permeation chromatographic (GPC) column. The elution conditions were as follows: column, TSK-gel G5000PWXL (7.5 mm I.D.× 30 cm, Tosoh Co., Tokyo, Japan); solvent, 0.1 M phosphate buffer (pH 6.8); detector, differential refractometer (830RI, Japan Spectroscopic Co., Tokyo,Japan); column-oven temperature, 40°C; flow rate, 0.6 ml/min; detector temperature, 40°C. The molecular weight of each peak was estimated using pullulans as standards (Showa Denko Co., Tokyo, Japan). This chromatograph shows that SSPS mainly consists of four molecular fractions. The molecular weight of each peak A, B, C, and D is 542000, 150000, 24900, and 4700, respectively

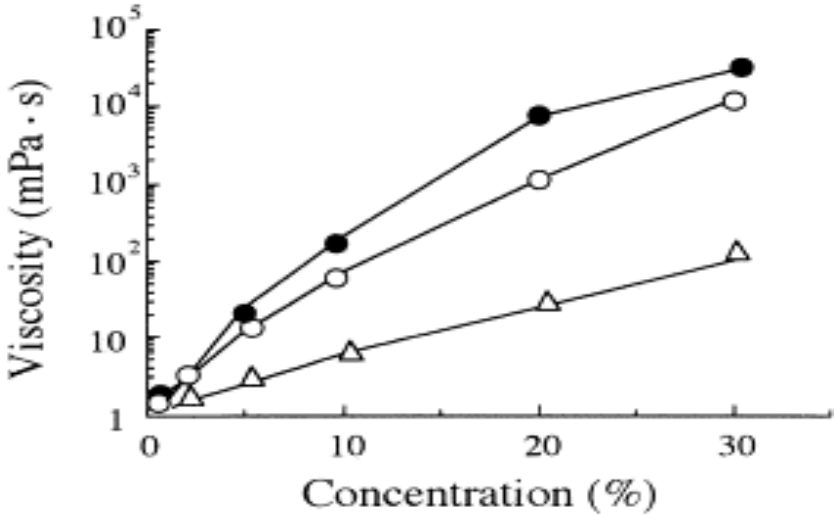

Figure 2. Effect of SMP concentration in soy bean meal on viscosity at a shear rate of 129/s and at 20°C. ○SMP; ●pulullan; △gum arabic (Furuta, 1999)

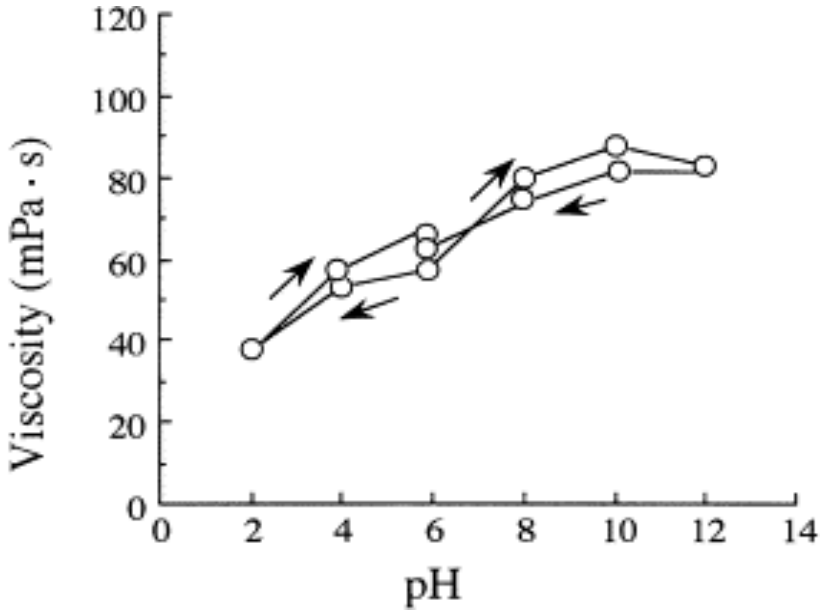

Figure 3. Effect of pH on viscosity of a 10% soy water soluble polysaccharides (SMP) solution (Furuta, 1999). 50% NaOH or 12 N hydrochloric acid was added to increase or decrease the pH of a 10% SSPS solution to pH 2.0 or 12.0 respectively. Next, the pH was returned to its original value, and the viscosity was measured at a shear rate of 129/s and at 20°C.

SMO and SMP cannot be metabolized by endogenous digestive enzymes, and are considered to be anti-nutritional factors. However, they can be fermented by microorganisms which inhabit the hindgut (Cummings, 1989 and Van Loo, 1995). As a result of this fermentation, these indigestible carbohydrates could have an effect on the development of intestinal physical structure, digestive enzyme activity and on the establishment of the gastro-intestinal microbial community. The metabolic products of these fermentable carbohydrates can nourish the intestinal cells (Mortensen and Clausen, 1996). The established microbial ecosystem can resist invasion by potential pathogens (Kubena *et al.*, 2001). It has also been observed that some types of oligosaccharides have prebiotic and immune-stimulating effects on animals, which are well documented in rats, but have not yet been well studied in broilers (Gibson, 1998). Such effects would probably depend on age of development of animals.

SCOPE OF THIS STUDY

As a popular animal feed ingredient, soybean meal contains relatively high

concentration of water-soluble non-digestible carbohydrates, known as oligosaccharides (SMO) and polysaccharides (SMP). It has been reported that SMO and SMP have anti-nutritional effect on broiler chickens. To remove these non-digestible carbohydrates from the diet would be costly. On the other hand, as a potential natural prebiotic material, the role of SMO and SMP on the health of broiler chickens requires further study. As a non-digestible, but potentially fermentable carbohydrate, they can be metabolized by the gut microbial community. Fermentation end-products, such as short chain fatty acids, especially butyric acid, may be beneficial for gastro-intestinal health. However, the fermentation kinetics and the end-products is important, particularly in relation to the longer-term effects on animal health.

Intestinal digestive enzyme activity is largely associated with mucosal cell development, and can have an affect on the animal's productivity. Non-digestible carbohydrates can affect the proliferation of gut mucosal cells and would further affect the activity of the mucosal digestive enzymes. As a result, SMO and SMP in the diet of young broilers could affect their productivity both in the short and the long-term.

After hatching, a key period starts when the young birds have to switch to external food resources that are mainly composed of carbohydrates. In order to adapt themselves to the new environment and diet, a dramatic physical development must take place, especially during the first two weeks post-hatch. In this study, it was hypothesised that the addition of SMO and SMP, to the diet for two weeks after hatching, may perform a prebiotic function in broilers, and in addition may also havean immune-stimulating nutritional effect. This would not only have an impact on the population of caecal lactic acid-producing bacteria; there may also be a carry-over effect and therefore an impact on intestinal development and productivity afterwards. Therefore, the objective of this thesis was to evaluate the fermentation characteristics of SMO and SMP by the caecal microflora of broiler chickens, and to investigate the relationship between these soy soluble carbohydrates and caecal content lactic acid bacteria population, as well as to look for any immune-stimulating effects of these additives in young birds.

Outline of this thesis

The first chapter, a literature review, deals with the development of the intestinal tract, digestive enzyme secretion and the establishment of the gut microbial ecosystem after hatching. The review provides evidence from literature for the modulatory effects of certain dietary components on the GIT microbial community. The existence of the competitive exclusion and immune response of birds and other animals and their response to the gut commensal microflora or administrated probiotics (Chapter1) are examined in detail.

The aim of the *in vitro* experiment described in Chapter 2, was to study the fermentation kinetics of four extracted water-soluble non-digestible carbohydrates (soybean meal oligo- and polysaccharides: SMO and SMP; alfalfa meal oligo- and polysaccharides: AMO and AMP) and two pure sugars (stachyose and raffinose: STA and RAFF) when inoculated with the caecal microbial community of broilers. This experiment also evaluated the effect of these non-digestible carbohydrates in terms of shifts in the gut microbial community before and after *in vitro* fermentation.

In order to investigate the effect of dietary SMO and SMP on intestinal length and weight development, the relative rate of increase in bodyweight, as well as their effects on intestinal morphology development, an experiment was conducted and the results are described in Chapter 3. Further objectives were to evaluate the lactic acid bacterial population in caecal contents as affected by dietary SMO and SMP (Chapter 4); and to investigate the effect of dietary SMO and SMP on the humoral and cellular immune ability of following vaccination (Chapter 5). This would establish a study model of a healthy broiler. To demonstrate the prebiotic role of dietary SMO and SMP, an *Eimeria tenella* infection study model was established.The competitive exclusion function of the intestinal microflora as stimulated by SMO and SMP in diets, and the survival of the lactic acid bacterial population after *Eimeria tenella* infection was also examined (Chapter 6). The numbers of caecal mucosa IgA-, IgM- and IgY-producing plasma B cells are studied in broilers affected by dietary SMO and SMP first and followed by coccidia infection (Chapter 7). Finally, the effects of dietary SMO and

SMP on the productivity of broilers in both healthy and infected animals was studied (Chapter 8). The results of the different experiments are discussed in a general discussion part of this thesis.

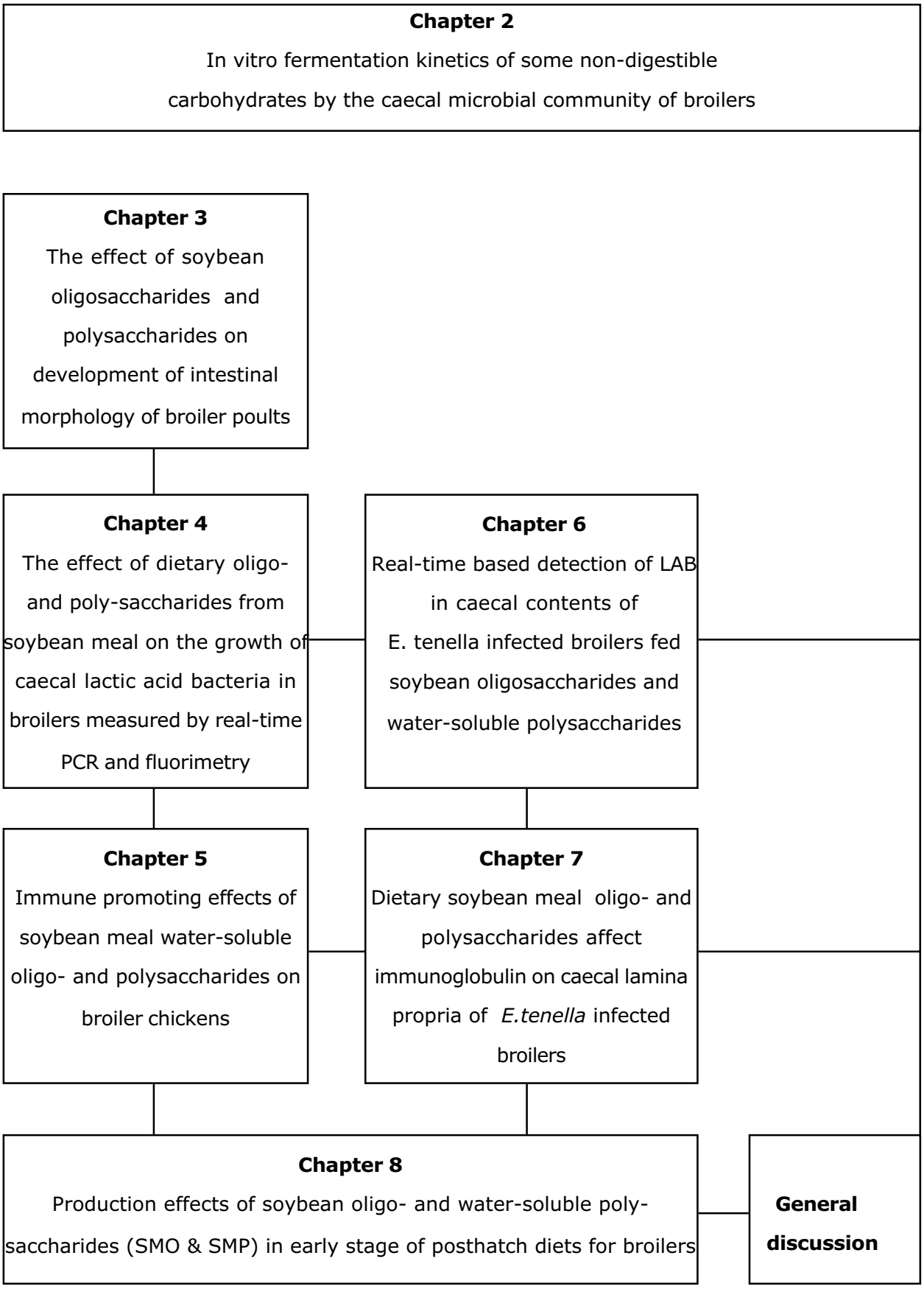

Figure 4 Schematic representation of the experiments used in thesis

REFERENCES

Betschart, A. A., 1978. Improving protein quality of bread-nutritional benefits and realities. In Nutritional Improvement of Food and Feed Proteins; Friedman, M., Ed.; Plenum: New York, pp 703-734.

Centen, C., P. Yuste, L. A. Rubio, J. Trevino and A. Brenes, 1990. Effect of including lupin (*Lupinus albus*) and flavomycin in diet for broiler chickens. Archivos de Zootecnia. 39:15-24.

Cummings, J. H., G. R. Gibson and G. T. Macfarlane, 1989. Quantitative estimate of fermentation in the hindgut of man. Acta Veterinary Scandinavia. 86: 76-82.

Friedman, M., 1996. Nutritional value of food proteins from different food sources. Journal of Agricultural Food Chemistry. 44, 6-29.

Furuta, H., 1999.Rheological properties of water-soluble soybean polysaccharides extracted under weak acidic condition. Food Hydrocolloids. 13 (3): 267-274.

Gibson, G. R., 1998. Dietary modulation of the human gut microflora using prebiotics. Brithsh Journal of Nutrition. 80(4):S209-S212.

Kawamura, S. and T. Narasaki, 1961. Study on the carbohydrates of soybean. Agricultural Biology Chemistry. 25: 527-531.

Kubena, L. F., J. A. Byrd, C. R. Young, D. E. Corrier, 2001. Effects of tannic acid on cecal volatile fatty acids and susceptibility to Salmonella typhimurium colonization in broiler chicks. Poultry Science. 80(9):1293-1298.

Lacassagen, L., 1988. Substitutes for soybean meal in the feeding of poultry. Production Animals. 1:47-57.

Leske, K. L., C. J. Jevne and C. N. Coon, 1993. Effect of oligosaccharide additions on nitrogen-corrected true metabolizable energy of soy protein concentrate. Poultry Science. 72: 664-668.

Mohamed, A. A. and P. Rayas-duarte, 1995. Composition of *Lupinus albus.* Cereal Chemistry. 72:643-647.

Mortensen, P. B. and M. R. Clausen, 1996. Short-chain fatty acids in the human colon: relation to gastrointestinal health and disease. Scandinavia Journal of Gastroenterology. Suppl.; 216:132-148.

Obendorf, R. M. Horbowicz, A. M. Dickerman, , P. Brenac and M. E. Smith, 1998. Soluble oligosaccharides and galacosyl in maturing soybean seeds. Crop Science. 38:78-84.

Petterson, D. S. and J. B. Mackintosh, 1994. The Chemical Composition and Nutritive Value of Australian Grain Legumes, Grains Reaearch and Development Corporation, Brisbane,

Australia, pp.10-13, 38-41.

Van Loo, J., P. Coussement, L. De Leenheer, H. Hoebregs, and G. Smith, 1995. On the presence of inulin and oligofructose as natural ingredients in the western diets. CRC Critic Review. Food Science Nutrition. 35: 525-552.

Chapter 1

THE ROLE OF THE COMMENSAL GIT MICROBIAL COMMUNITY IN BROILER CHICKENS

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The Role of the Commensal GIT Microbial Community in Broiler Chickens

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Abstract: To understand the relationship between the gastrointestinal inhabit microbial community and broiler health, a literature review is presented. It focused on the intestinal microbial ecosystem development in broiler chickens, to elucidate the relationship between commensal microflora and digestive enzymes and nutrient digestion, as well as on gut mucosal cell proliferation. The role of competitive exclusion for gastrointestinal tract commensal microorganisms against invading pathogens on young chicks were included. We evaluated the evidence from literature which suggest that modulation of the gut microbial community is possible by addition of prebiotics to the diet.

Keywords: gut microbial community, competitive exclusion, gut mucosal cell proliferation, prebiotic, broiler chickens.

The Role of The Commensal GIT Microbial Community in Broiler Chickens

INTRODUCTION

The selection of broilers for increased growth rate may have inadvertently resulted in changes in gastro-intestinal development during growth of the animal. Some other negative effects can also occur at times, such as metabolic disorders, poor immune competence and increased susceptibility to pathogens (Tottori *et al.*, 1997). For these animals, it was found that the use of a sub-therapeutic dose of antibiotics added to the diet led to improvements both in health and productivity. However, such additives have also sparked off some other unexpected consequences. There have been reports about increases in the incidence of antibiotic-resistant microbial pathogens (van der Waaij and Nord, 2000). Potentially, such findings could have severe negative effects on public health (Shea, 2003).

The gut lumen and mucosal surface of the intestines and cecae of broiler chickens are major sites for colonization by bacteria and other microbes. The presence of antibiotics can disturb the gastrointestinal tract (GIT) ecosystem. The use of sub-therapeutic doses of antibiotics in a broiler diet can reduce the population of Lactobacillus (Ane Knarreborg, *et al.*, 2002) in the intestines. In monogastric animals, the intestinal microbiota, the mucosa, and the immune system all provide resistance to the negative effects of enteric pathogens. Furthermore, the intestinal microbial community, in addition to colonization resistance to potential pathogens, has also been shown to affect intestinal development, gut mucosal cell proliferation, digestive enzyme secretion, and gut associated lymphoid tissue (GALT) maturation. Therefore, it is recognized that it is of great importance to establish a healthy gastrointestinal ecosystem by means of controlling diet ingredients which may in turn influence the GIT microbial community positively. This review will focus on the development of thisecosystemand, to elucidate the relationship between commensal microflora and digestive system development. It will focus on competitive exclusion and the immune responses of chickens, and will also evaluate the evidence to suggest that

modulation of the gut microbial community is possible by dietary prebiotics.

PHYSICAL GASTROINTESTINAL DEVELOPMENT CHARACTERISTICS OF BROILERS

Physical development of intestines

The intestinal development of broiler chickens starts during the embryonic (incubation) period, when the yolk supplies all the nutrients. The digestive tract will grow continuously immediately after hatching, and dramatic changes occur in both the physical size of the tract, and in the mucosal morphology (Cook and Bird, 1973; Bayer *et al.*, 1975; Uni *et al.*, 1995, 1996 and 1999). At this time, the birds must make the transition from using the energy supplied by the endogenous nutrients of the yolk, to the utilization of nutrients of an exogenous carbohydrate-rich feed. The development of the digestive tract of poultry with age has been well documented (Sklan, 2001).

Gross development. In young growing birds, the intestinal growth rate is higher than the rate of body weight gain. This is especially so in the period immediately after hatching. The process of rapid relative growth was maximal in 6-8 days old broilers (Akiba *et al.*, 1995; Noy *et al.*, 1998). After this period, the weight of the gizzard and intestines decline in relation to total body weight. Even so, the length of the small intestine continues to increase with age. The jejunum and ileum are similar in length and together account for 82.2 and 84.1% of the length of the small intestine at hatch and 21 day of age, respectively (Iji *et al.*, 2001).

The rate of development differs between duodenum, jejunum and ileum, and the most rapid one of these three is the jejunum (Iji *et al.*, 2001). Biochemical indices of cellular size and metabolic activity indicate that the gut mucosal cell population (DNA content of the intestinal mucosa) declines with age in duodenum, jejunum and ileum; the overall cellular efficiency (RNA concentration), however, increases in duodenum and ileum, and decreases in the jejunum. Intestinal cell size (Protein:DNA ratio)

increases between hatch and 21 days of age in all intestinal regions. Protein synthesis rate (Protein:RNA ratio) decreases with age in the duodenum and ileum, but increases in the jejunum. The individual cellular efficiency (RNA:DNA ratio) increases significantly with age in the duodenum and ileum, but no changes are observed in the jejunum.

Morphology development. The intestinal morphology is not yet fully developed at the time of hatching, and undergoes dramatic changes in the period after hatching. In broilers, the number of cells per crypt increases rapidly in the first 48 h post-hatch, though the rate of growth begins to decrease after this time (Geyra *et al.*, 2001). The number of crypts themselves reach a plateau between 48 and 72 h post-hatching. Crypt depth increases with age in the duodenum and the jejunum, while villus height increases significantly with age in all three regions of the small intestine. This is accompanied by an increase in the number of enterocytes in the villi (Geyra *et al.*, 2001). The villus height increases twofold in the 48 hours post-hatching and reaches a plateau at 6-8 days in the duodenum, and after 10 days or more in both the jejunum and ileum (Geyra *et al.*, 2001; Iji *et al.*, 2001). Villus width also increases slightly after hatching. This means that the villus surface area, as estimated from villus height and width, increases in all segments until day three after hatching. After this time, the jejunal surface area continues to increase more rapidly than that of the duodenum and ileum (Geyra *et al.*, 2001).

Scanning electron micrographs of the intestinal villi of broiler chickens show thatthe villi of the duodenum, jejunum and ileum already have a finger-like shape on the first day after hatching. Villi develop to a plate-like shape in the duodenum, a wave-like shape in the jejunum and a tongue-like shape in the ileum at 30 days of age from the common plate-like villi at 10 days of age. The fundamental villous shape and arrangement seem to be accomplished by 10 days of age (Yamauchi and Isshiki, 1991).

Digestive enzyme secretion after hatching

In the immediate post-hatching period, chicks must transfer from metabolic

dependence on the yolk to utilization of exogenous feed. Initial digestion of feed is performed by pepsin and pancreatic proteases. Peptidases, lipase, and amylase contribute further to breakdown (Uni *et al.*, 1999). Secretion of digestive enzymes from the pancreas and intestinal wall into the intestine is triggered by feed intake (Sklan and Noy, 2000), though pancreatic enzymes are already present in the small intestines in the late embryonic stages (Marchaim and kulka, 1967). Secretion of enzymes increases with age post-hatching (Nitsan *et al.*, 1991; Noy and Sklan, 1995), not per gram of feed intake (Uni *et al.*, 1995). Changes in the rate of secretion and in enzymatic activities differ between enzymes. In the pancreas, maximal values of enzyme activity are attained on Day 8 for amylase and lipase and on Day 11 for trypsin and chymotrypsin. In the small intestine maxima are reached at Day 4 for lipase, Day 11 for trypsin and chymotrypsin and at Day 17 for amylase (Nitsan *et al.*, 1991).

The final stages of digestion are completed by brush border enzymes (BBE), including sucrase-*iso*-maltase, peptidases and phosphatases (Semenza, 1986). The specific activity of some BBE at all intestinal sites decreases with age and differs between intestinal sites (Ija *et al.*, 2001). The specific activities of maltase, lactase and trehalase are greatest on Day 18 of embryonic development. After hatching, maltase-specific activity gradually decreases until Day 18 (Chotinsky *et al.*, 2001). The specific activity of maltase is the highest of all enzymes examined, regardless of age and intestinal site (Ija *et al.*, 2001). After Day 7 post-hatch only traces of lactase and trehalase activity are found in the enterocytes. Significant sucrase activity has been found on Day 18 of embryonic development in chicks, and the activity of this enzyme increased two-fold after hatching, then remained generally constant up to Day 35, after which the enzyme specific activity gradually declined (Chotinsky *et al.*, 2001). The brush border enzyme (BBE) activity is present over a large proportion of the villus. Activity per unit villus surface area is similar between ages, except for jejunal alkaline phosphatase (AP). The digestive capacity of older birds may be sustained by an increase in total enzyme activity related to the increased surface area of the jejunum. (Ija *et al.*, 2001).

Gut microorganisms development

The intestinal microflora comprises a diverse collection of microbial species in broiler chickens. Until recently, the assessment and identification of commensal bacteria has been based on traditional culturing techniques. Recently, molecular techniques (MT) are also being used to analyze commensal ecosystems, so as to characterize community profiles (Tannock, 2001).

Earlier culture-based studies found that host factors such as age influence the composition of the cecal microbiota (Mead and Adams, 1975). Knowledge based on these methods indicates that the normal intestinal microflora of the small intestine, caecum and large intestine in chickens develop after hatching. Bacteria have not been detected in any of the sites at hatching (Day 1), but by Day 3, significant numbers of faecal streptococci and coliforms have been isolated from all sites (Barnes *et al.*, 1980; Coloe *et al.*, 1984). Theecosystem of the microbial community is established in the small intestine within approximately two weeks. The community of the small intestine is limited to faecal streptococci and coliforms for the first 40 days, and then lactobacilli become established and dominant (Smith, 1965; Coloe *et al.*, 1984). In contrast, the cecal ecosystem is established later than that in the small intestine. The establishment in the cecum needs 6-7 weeks (Barnes *et al.*, 1972; Coloe, *et al.*, 1984). A large variety of both facultative and strictly anaerobic organisms colonize the caecum. Fecal streptococci, clostridia, enterobacteria, pediococci, and occasionally *Pseudomonas aeruginosa* have all been isolated. After the chick has been fed for one day, the numbers of lactobacilli in the ceca are quite variable; by the third day, however, large numbers of bacteria are present throughout the alimentary tract. Many of these species are only transient; after 40 days the flora becomes stable and consists predominantly of fecal streptococci, *Escherichia coli*, Bacteroides *spp*. and Lactobacillus *spp.* (Barnes, *et al.*, 1980; Coloe, *et al.*, 1984).

Molecular assessment results are in agreement with the observations obtained from culture techniques, with regard to population changes which occur within one week after hatching. Molecular research indicated that samples from 1-day-old birds

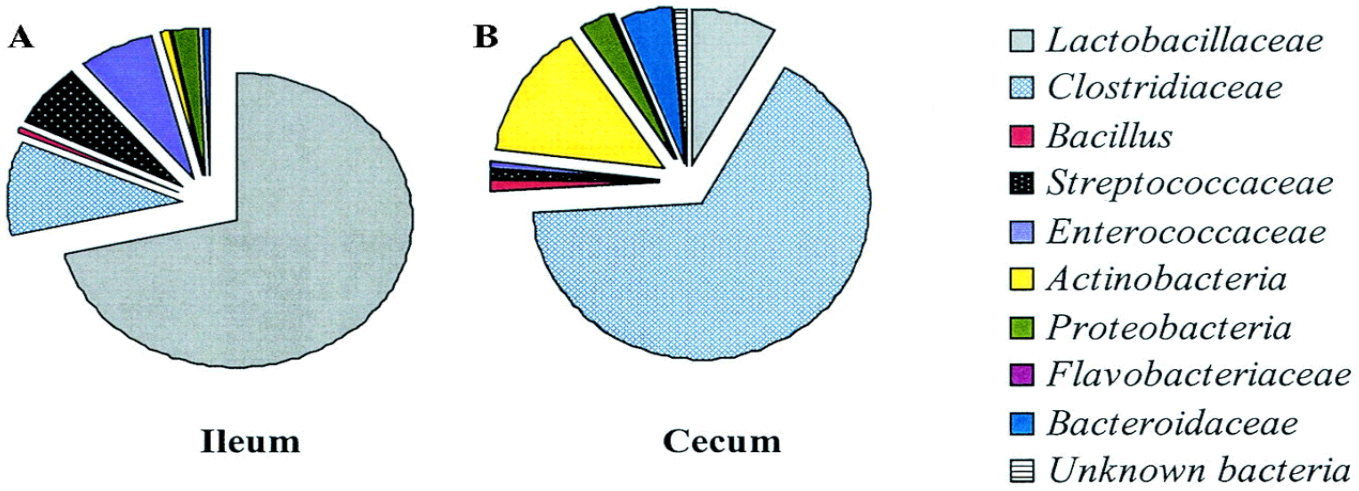

Figure 1. Composition of the bacterial floras of the ilea and caeca of broiler chickens as determined by sequencing of 1,230 clones from a 16S rDNA community DNA library. (A) Bacterial composition of the ileum. (B) Bacterial composition of the caecum (Jiangrang *et al.,* 2003).

revealed the lowest number of bacteria. There are however, quite small differences in the number of major bacteria visible in the samples between 1-day-old birds and the older birds. Analysis of cecal contents based on molecular techniques showed that the microbial community is somewhat different to the contents based on culture analyses. As for adult broilers, *Clostridiaceae*-related sequences (65%) were the most abundant group detected in the cecum, with the other most abundant sequences being related to *Fusobacterium* (14%), *Lactobacillus* (8%), and *Bacteroides* (5%). Sequences of members of the Bacteroides group, the *Bifidobacterium infantis* subgroup, and of Pseudomonas *spp*. each accounted for less than 2% of the total. Microflora also harbor in ileum. Nearly 70% of sequences from the ileum were related to those of *Lactobacillus*, with the majority of the rest being related to *Clostridiaceae* (11%), *Streptococcus* (6.5%), and *Enterococcus* (6.5%) (Xiang *et al.*, 2002; Jiangrang *et al.,* 2003). Microbiota from the ileal and cecal contents of broiler chickens analyzed by 16S rRNA-based technique are shown in Figure 1 (Jiangrang *et al.,* 2003).

In summary, the early stage of post-hatch is a critical period for broiler chickens. The birds undergo not only changes in nutrient supply and living environment, but also a dramatic physical development of the gastro-intestinal tract takes place**.** The development of the gut commensal microorganisms is particularly worthy of notice. The microbial community must become established and reach an ecological balance starting from a nearly sterile gastrointestinal environment at the moment of hatching. This event is affected both by diet and environmental conditions of the birds. Therefore, it should be possible to manipulate the gut microbial community by dietary ingredients, such as some non-digestible but fermentable carbohydrates, starting from the very first meal post-hatch.

RELATION OF COMMENSAL GUT MICROORGANISMS WITH NUTRIENT DIGESTION, DIGESTIVE ENZYMES AND THE GUT MUCOSAL CELL PROLIFERATION

Intestinal microbial community and nutrient digestion

The microbial community is distributed along the entire gastrointestinal tract of broiler chickens, and the cecum is the major region for anaerobic bacteria (Barnes, *et al.*, 1980; Coloe, *et al.*, 1984). The fermentative part of the microbial activity yields mainly lactic acid and short chain fatty acids (SCFA). The microbial populations in the ceca stimulate reabsorption of water. Here also, non-protein nitrogen may be present in the ceca by means of retrograde peristalsis. Such compounds are degraded by and incorporated in caecal microorganisms (Braun and Campbell, 1989; Mead, 1989), and urinary nitrogen is likely to be recovered (Mattocks, 1971).

The bacterial activities of the GIT can affect the nutrient utilization of birds. The large numbers of GIT microbes need and consume energy. This normally comes from feed and they may therefore use some of the energy available for the chicken. The microflora can also benefit the host bird by producing energy in the form of SCFA. This release of SCFA from fermentation of non-hydrolyzable oligo- and poly-saccharides, may provide extra energy to the bird, which was otherwise unavailable, and this can mean a better feed conversion ratio. This would be particularly so when the birds are fed a high-fiber diet which may otherwise be limiting in energy (Hegde *et al.*, 1982; Muramatsu *et al.*, 1991). However, when dietary energy is supplied in the form of substrates which are easily digestible by the animal itself, the efficiency of energy utilization may be reduced due to the presence of the GIT microflora, which competes with the host enzymes for substrates (Muramatsu *et al.*, 1994). Therefore, the presence of microorganisms can either improve or limit metabolizable energy (ME) values of the diets. Not all energy digested in conventional chicks is necessarily utilized for growth, because the microflora will use a part of it for its own metabolism (Furuse and Yokota, 1984b and 1985). This suggests that although the gut microflora may have beneficial effects on the digestion of certain dietary components, it may

also have a negative effect on utilization of ME by the host. Thus, chicks that harbor an active GIT microflora apparently have an increased energy requirement for maintenance.

Meanwhile, the intestinal microbial community also affects dietary protein digestion. Conventional birds, which receive a diet with low digestibility, have a lower body weight than their germ-free (GF) counterparts, because the microflora uses the dietary protein as an energy source. Conventional birds have been shown to excrete higher amounts of endogenous amino acids than germ-free birds when fed high-fiber diet. This indicates that there is a substantial microbial synthesis of amino acids in the gut (Parsons *et al.*, 1983).

Intestinal microbial community and digestive enzymes

The presence of the GIT bacteria can affect the activity of digestive enzymes. This influence relates to the bacterial metabolic end-products, and their effect on gut mucosal morphology and on intestinal cell proliferation in rat (Palmer and Rolls, 1983). For example, the activity of alkaline phosphatase is greater in the mucosal homogenates of conventional chicks compared with the GF birds. The concentrations of acid phosphatase and *iso*-citric dehydrogenase are however similar in the two groups (Palmer and Rolls, 1983). There is a tendency that proteolytic activity is increased and the amylase activity is decreased in conventional chicks compared with GF animals (Philips and Fuller, 1983). On the other hand the numbers of gut beneficial bacteria are increased by the administration of prebiotics in the diet. This may improve the activities of amylase and total protease (Xu *et al.,* 2003).

Intestinal microbial community and gut mucosal proliferation

The dietary composition, and microflora, as well as the interaction between the diet and microflora can affect the intestinal development, mucosal architecture and the mucus composition of the GIT. Therefore, it appears that the functional characteristics of the intestinal tract are altered by the microflora. The absolute and

relative weight (weight/kg body-weight) of all intestinal sections are greater in conventional birds compared with their GF counterparts, when chickens receive average and higher concentrations of protein in the diet (Furuse and Yokota, 1984a; Muramatsu *et al.*, 1988; Muramatsu *et al.*, 1993). Protein synthesis in the intestines is enhanced by the presence of the gut microflora in terms of both fractional (%/d) and absolute rates [mg/(100g BW.d)] (Muramatsu *et al.*, 1988). Fermentable carbohydrates can increase the weight of the gut, this is associated with their stimulating role on a normal microflora (Komai and Kimura, 1980; Goodlad *et al.*, 1989; Muramatsu *et al.*, 1993).

Considerable amounts of SCFA are produced by microbial fermentation in the hindgut. Most of the anions in the cecal contents are SCFA, mainly acetate, propionate and butyrate. It has been suggested that SCFA can accelerate gut epithelial cell proliferation, thereby increasing intestinal tissue weight, which will result in changes of mucosal morphology (Sakata and von Engelhardt, 1983; Lupton *et al.*, 1985a; Sakata, 1986; Kripke *et al.*, 1989; Furuse *et al.*, 1991; Sakata *et al.*, 1995; Chu and Montrose, 1995; Reilly *et al.*, 1995; Wilson and Gibson, 1997; Ichikawa and Sakata, 1998; Le Blay *et al.*, 2000; Fukunaga *et al.*, 2003). Several mechanisms are involved in the growth-stimulating role of SCFA on animal intestines. Luminal and systemic SCFA stimulate mucosal proliferation by increasing plasma glucagon-like peptide 2 (GLP-2) and ileal pro-glucagon mRNA, glucose transporter (GLUT2) expression and protein expression, which are all signals which can potentially mediate SCFA-induced mucosal proliferation (Tappenden and McBurney, 1998). The trophic effects of SCFA infused in the colon on the jejunum are mediated in part by gastrin (Reilly *et al.*, 1995). SCFA infused into the colon act systemically to improve jejunal structure and increase jejunal gastrin concentrations in rats. Gastrin receptor blockade abolished the effects of SCFA on jejunal DNA, protein, crypt cell proliferation, and gastrin. Gastrin blockade does not reduce SCFA- induced augmentation of villous height or crypt depth. Le Blay *et al.*(2000) reported that at a low concentration (0.05 to 0.5 mM),of butyrate in the lumen significantly stimulates the proliferation of ileal and colonic myocytes of rats in culture, though an inhibition is observed at high concentrations. Collagenous and non-collagenous protein synthesis in mucosa areis enhanced by both stimulated

by butyrate. Thus butyrate, which is produced by dietary fiber fermentation, may affect intestinal muscles by directly acting at the molecular level on myocytes. Luminal pH has a significant inverse correlation with the percentage of cells in the S phase (a cell activity phase in which DNA is actively synthesized) in the cecum, proximal colon,and distal colon (Lupton *et al.*, 1985b). Transepithelial gradients of SCFA generate pH gradients across the colonic epithelium. Extracellular pH regulation occurs in two separate microdomain surroundings: (a) colonic crypts: the crypt lumen and (b) the subepithelial tissue adjacent to crypt colonocytes. Physiologic SCFA gradients cause polarized extracellular pH regulation because epithelialarchitecture and vectorial transport work together to establish properly regulated microenvironments (Chu and Montrose, 1995).

As living beings, the GIT bacteria need nutrients for maintenance and development, and therefore compete with the host animal for some available nutrients. However, their unique role of metabolizing non-digestible carbohydrates cannot be substituted by intestinal enzymes, and the fermentation end-products, such as some SCFA, can also be of benefit for the host animal.

THE ROLE OF SHORT CHAIN FATTY ACIDS (SCFA)

Fermentation, the process whereby anaerobic bacteria break down carbohydrates and protein to short-chain fatty acids (SCFAs), exerts an important function in the hindgut. The GIT bacteria of broiler chickens can also convert fermentable carbohydrates into SCFAs (Carré *et al.,* 1995). Recently, it has become evident that colonal metabolism of monogastric animals is more complex than previously thought. Luminal SCFAs are recognized as an essential source of energy for the colonocytes. Gastrointestinal functions and beneficial effects in the gut wall are associated with these acids (Rabassa and Rogers, 1992; Mortensen and Clausen, 1996). On the other hand, a lack of SCFA can lead to endogenous starvation of enterocytes, and may be one of the contributing factors to the development of ulcerative colitis and other inflammatory conditions of the GIT (Wachtershauser and Stein, 2000).

The studies with rats indicate that absorption of SCFAs is rapid, and large quantitiesare metabolized by the large intestinal epithelium before reaching the portal blood. Part of the butyrate is converted to ketone bodies or CO_2 by the epithelial cells, the remainder reaches the liver via the portal vein and is removed by the liver. The concentration of butyrate in the portal vein is strongly influenced by the production rate in the large intestine; increased gut production of butyrate further raises the circulating level of butyrate (Bergman, 1990; Bach Knudsen *et al.*, 2003). Propionate is similarly removed by the liver, but is largely converted to glucose. Although host species differences exist, acetate is used principally by peripheral tissues, especially fat and muscle (Bergman, 1990). SCFA are absorbed by colonocytes in the proximal colon by both passive diffusion and active transport mechanisms, which are linked to various ion exchange transporters. In the distal colon, the main mechanisms of absorption are passive diffusion of the lipid-soluble form (Velazquez *et al.*, 1997). There is evidence to show that dietary propionic acid can be absorbed rapidly in the intestine of broiler chickens (Hume *et al.*, 1993).

SCFAs can benefit animals in several ways. SCFA have been shown to promote calcium absorption from the large intestine *in vitro* (Mineo *et al.*, 2001). SCFA have also been shown to stimulate the local release of mucus from the hindgut mucosa (Sakata and Setoyama, 1995). Acetate and butyrate increase colonic blood flow, enhance ileal motility and may enhance colonic anastomosis healing (Scheppach, 1997; Velazquez *et al.*, 1997). Butyric acid at physiological concentrations can induce multiple and reversible biological effects. Butyrate may influence lipid metabolism in Caco-2 cells, suggesting a potential regulatory role of butyrate for intestinal fat absorption and circulation of lipoproteins (Marcil *et al.*, 2002). Butyrate has also been shown to have beneficial effects against some colonic pathologies. This fatty acid may be protective against colorectal neoplasia. Butyrate may also reduce the symptoms of ulcerative colitis and diversion colitis, and it may prevent the progression of colitis in general (Velazquez *et al.*, 1997).

In humans, it is also thought that SCFA are also involved in preventative mechanisms for large bowel cancer. The presence of naturally occurring differentiating

agents, such as *n*-butyrate, may modify the patterns of growth and differentiation ofgastrointestinal tumours (Jass, 1985). Evidence indicates that acetic, propionic and butyric acids can inhibit the promotion of colonic carcinogenesis at physiological concentrations, but of the three, butyrate is thought to be the most effective (Newmark and Lupton, 1990; Hague *et al.*, 1995; Lupton, 1995; Hassig *et al.*, 1997; Krithevsky, 1998; Fuchs *et al.*, 1999 and Potter, 1999). In particular, butyric acid leads to the arrest of cell reproduction and induces terminal cellular differentiation of colonic adenoma and cancer cells presumably because it inhibits histone deacetylase (Archer *et al.*, 1998; Hassig *et al.*, 1997; Sakata, 1987; Singh *et al.*, 1997 and Whiteheah *et al.*, 1986). In colonic adenoma or carcinoma cell lines, butyric acid induces apoptosis (Hague *et al.*, 1996, 1997a and 1997b; Singh *et al.*, 1997). Consequently, it is considered to be of interest to use butyric acid for therapeutic purposes in the treatment of colorectal cancer and hemoglobinopathies (Scheppach *et al.*, 1997; Pouillart, 1998).

To summarize, short chain fatty acids (SCFAs) are the metabolic product of mainly fermentable carbohydrates by GIT bacteria. Their beneficial role for health is becoming clearer, especially in the human, rat, and in other omnivorous animals. Indigestible, but fermentable, carbohydrates can be fermented by GIT bacteria of broiler chickens, even though the intestinal tract is relatively short, and cannot be compared with that of a ruminant or even other monogastric animals, such as the pig. The potential fermentability of various carbohydrates normally present in different ingredients of a poultry diet, needs further investigation.

MODULATION OF THE INTESTINAL MICROBIAL COMMUNITY BY DIETARY INGREDIENTS

The intestinal ecosystem contains a large diversity of bacterial cells that perform different functions for the host. Some specific components of the intestinal microflora, including lactobacilli and bifidobacteria, have been associated with beneficial effects for the host, such as: (1) promotion of gut maturation, (2) gut integrity, (3) antagonisms against pathogens, and (4) immune modulation (Carter and Pollard, 1971; Berg and Savage, 1975; Tlaskalova-Hogenova *et al.*, 1980; Tlaskalova-

Hogenova *et al.*, 1983; Tlaskalova-Hogenova *et al.*, 1994). In addition, the microflora seems to play a significant role in the maintenance of intestinal immune homeostasis and prevention of inflammation (Schwartz, 1989; Beattie, 1994; Powrie, 1995). Young birds obtain microorganisms from the outside environment via feed and water, and this microflora is constantly, though indirectly, exposed to the external environment. Therefore, it is logical that one can modulate the microbial community by external factors, such as nutrition (Gibson and Roberfroid, 1995; Gibson, 1998; Collins and Gibson, 1999; McBain and MacFarlane, 2001; Glunder, 2002; Schiffrin and Blum, 2002).

"Prebiotics" are defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and /or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health (Gibson, 1995). Any food ingredient that enters the large intestine is therefore a potential prebiotic. However, to be effective, selectivity of fermentation is essential. Most current attention and successes have been derived using non-digestible oligosaccharides, especially those that contain fructose, xylose, galactose, glucose and mannose (Gibson and Roberfroid, 1995; Gibson, 1998). It has been reported that oligosaccharides and polysaccharides are specifically utilizable by bifidobacteria (Yazawa *et al.*, 1978). And the prebiotic characteristics of fructooligosaccharide (FOS) in this regard for humans, have been well demonstrated (Gibson, 1998).

In poultry diets, the beneficial effects of synthetic oligosaccharides, such as FOS, have also been shown (Unno *et al*., 1993; Durst, 1996). The natural oligosaccharides, mainly coming from grain legumes and oil seeds, seem to have some negative effects on broiler chickens due to the incidence of gut flatulence (Rackis, 1975), and reduced available energy in the diet (Coon et al., 1990; Leske et al., 1991, 1993). However, there are still many unknown prebiotic effects of oligosaccharides in poultry diets, and further research is needed.

THE COMPETITIVE EXCLUSION ROLE OF GIT COMMENSAL MICROORGANISMS AGAINST INVADING PATHOGENS

In this review, "competitive exclusion" is defined as any action by the GIT commensal microorganisms which resists colonization by invading pathogens, suchas occupation of attachment sites on the intestine mucosal surface, competition for nutrients, or the release of bacteriocins.

The gastrointestinal system consists of a myriad of small ecological "niches" within which most bacteria grow in glycocalyx-enclosed microcolonies that form highly structured consortia in biofilms on the surfaces of both tissue and digesta (Costerton *et al.*, 1983). Newly hatched chicks are particularly vulnerable to invasion by pathogens before their intestinal microbial ecosystem becomes established. Therefore, administration of an autochthonous intestinal microflora to chicks during the early period after hatching (CE) is applied worldwide as a prophylactic method to control some pathogens infection in poultry. This practice was introduced some decades ago (Snoeyenbos *et al.*, 1978 and 1979; Weinack *et al.*, 1979). The microbiological strategies using adult intestinal microflora are such that they may increase resistance to pathogens in young chicks, but may be ineffective in older hens (Corrier *et al.*, 1993a). In young chicks, CE products have been shown to be effective for several pathogen species, such as *Yersinia enterocolitica* (Soerjadi-Liem *et al.*, 1984a), *Campylobacter fetus subsp. jejuni* (Soerjadi *et al.*, 1982; Soerjadi-Liem *et al.*, 1984b; Schoeni and Wong, 1994; Stern, 1994), *Salmonella typhimurium* (Impey *et al.*, 1982; Bailey *et al.*, 1988; Baba *et al.*, 1991; Salvat *et al.*, 1992; Corrier *et al.*, 1993b; Kogut *et al.*, 1994; Hume *et al.*, 1996; Bailey *et al.*, 1998; Mead, 2000), *Salmonella kedougou* (Ferreira *et al.*, 2003), *Salmonella infantis* (Goren *et al.*, 1984), *Escherichia coli* (Weinack *et al.*, 1981; Snoeyenbos *et al.*, 1982) and *Listeria monocytogenes* (Hume *et al.*, 1998). The safety and efficacy of competitive exclusion products have been proven in large-scale trials (Snoeyenbos *et al.*, 1985; Salvat *et al.*, 1992). However, the practice has not been shown to be effective for the practical control of fowl typhoid (Silva *et al.*, 1981). It is effective to use CE products as a prophylactic treatment ratherthan as therapeutic agents (Watkins and Miller, 1983).

CE products

Resistance of young chicks to pathogenic colonization can be increased by treatingthem with either a suspension or anaerobic cultures of gut contents from healthy, adult birds. Such treatments have an unknown bacterial composition and are therefor not acceptable for regulatory agencies in some countries (Stavric, 1992). Efforts are being made to establish defined colonization resistance products either alone or in mixtures. Experiments demonstrate that mixtures containing a large number of strains are protective (Impey *et al.*, 1982; Schoeni and Wong, 1994). A single bacterial genus, such as Lactobacillus or Bifidobacterium has more controversial effects (Soerjadi *et al.*, 1981; Watkins and Miller, 1983). The preparation method can affect the efficiency of CE products. Less effective methods of preparation are to include oral applications to chicks of fresh faeces or lyophilised faeces (Hoszowski and Truszynski, 1997). Maintenance of intestinal isolates on laboratory media, as well in storage at –70° C, leads to a progressive decrease in their protective abilities (Bailey *et al.*, 1988).

Several methods are used to administer colonization resistance products. The basic and most effective method of administration is by crop gavage (Impey *et al.*, 1982; Corrier *et al.*, 1994a; Corrier *et al.*, 1994b; Hollister *et al.*, 1994). Chicks treated with cecal cultures in the drinking water or by spray application show rable protection and a significantly decreased number of colonized pathogens (Corrier *et al.*, 1994a; Goren *et al.*, 1988; Corrier *et al.*, 1994b). The consumption of caecal bacteria which are encapsulated and lyophilized in alginate beads can be effective, allthough results differ between trials (Hollister *et al.*,1994; Corrier *et al.*, 1994a). Studies show that cecal bacteria from adult chickens may become rapidly established in the ceca of newly hatched chicks following contact with the vent lips (Corrier *et al.*, 1994b). Furthermore, a combination method, such as first spraying the product on chicks in the hatchery, followed by administration in the first drinking water, can serve as a useful means to reduce pathogen contamination (Blankenship *et al.*, 1993).

Material which can promote competitive exclusion

The establishment of the commensal microflora occurs simultaneously with the development of competitive exclusion ability in young chicks. The SCFA concentrationof cecal contents is related to the establishment of an active anaerobic microflora and may indicate the level of protection present against colonization of thececae by pathogens. Dietary ingredients, such as some fermentable carbohydrates, can stimulate the growth of the intestinal microflora and possibly affect the competitive exclusion abilities of chicks. Feeding fructooligosaccharide (FOS) in the diet of chickens may lead to a shift in the intestinal gut microflora, and under some circumstances may result in reduced susceptibility to Salmonella and Campylobacter colonization. The effect is dose dependent (Bailey *et al.*, 1991; Schoeni and Wong, 1994). Feeding of FOS, tannic acid or mannose in the diet of chicks with a CE treatment may result in a greatly reduced susceptibility to Salmonella colonization (Bailey *et al.*, 1991; Schoeni and Wong, 1994; Fukata *et al.*, 1999; Kubena *et al.*, 2001). Propionic acid used as feed additive has not yet been shown to be effective in reducing Salmonella infection in the crop and ceca (Champ *et al.*, 1981).

Possible mechanisms of CE

Prevention of colonization by pathogens in the GIT by a commensal or administrated microflora can be achieved through several different mechanisms. Firstly, pathogens that invade the gastrointestinal tract of chicks must penetrate the epithelial layer before infecting the birds. Administration of CE products to newly hatched chicks can lead to early colonization of adherent bacteria on the gut mucosal surface, forming a mat of microflora and occupying the adhering niches (Soerjadi *et al.*, 1982; Stavric *et al.*, 1991). Furthermore, undefined microbial populations and strains freshly isolated from washed ceca, show hydrophobic properties, which improves the efficacy of protective mixtures (Stavric *et al.*, 1991). Secondly, competition for nutrients between native bacteria and invading pathogens is a limiting factor for the colonization of pathogens. Thirdly, some commensal bacteria produce bactericidal or bacteriostatic products. Finally, the endogenous microflora seems to have a capacity to modulate

cellular mechanisms related to host defenses and homeostasis. Of these possible mechanisms, only the first one is tested and proven in chicks, while the others need further research.

The competitive exclusion role of either gut native bacteria or administrated bacteria has been accepted as a way to limit colonization by several pathogenic bacterial species. However, whether CE products and gastrointestinal microflora will have an exclusion effect for a wider range of pathogens still needs to be evaluated.

IMMUNESTIMULATORY ROLE OF LACTIC ACID-PRODUCING BACTERIA

Lactic acid bacteria (LAB) are present in the intestines of most animals. The beneficial role played by these microorganisms in humans and other animals, including the effect on the immune system (Haller, *et al.*, 1999), has been reported extensively. Therefore, Lactobacilli derived from the endogenous flora of normal donors are being increasingly used as probiotics in functional foods and as vaccine carriers. Gut mucosal surfaces are habitats for the commensal microflora that is closely related to the mucosal immune compartment (mucosa-associated lymphoid tissue, MALT). These commensal bacteria influence the development of the mucosal immune system via direct contact of LAB or bacterial product (cell wall or cytoplasmic component) with immune cells in the intestine (Pulverer *et al.*, 1993; Takahashi *et al.,* 1993).

Strain dependent characteristics of the immunostimulatory activity of the commensal microorganisms

The surface of the intestinal mucosa is colonized by the commensal microflora that is composed of very high numbers of bacteria. The distal small intestine, caecum and colon are the most densely populated. These non-pathogenic bacteria may play a modulation role on the physiology and immunology of the host. It has been found that at least two different patterns exist for the innate response to gram-negative and gram-positive bacteria. Especially for the latter group, big differences have been observed between bacterial species (Blum, *et al.*, 1999). A variety of studies carried

out using distinct strains of lactobacilli have suggested that despite similar in vitro probiotic properties, distinct Lactobacillus strains may colonize the gut differently and generate divergent immune responses (Perdigon *et al.*, 2000; Ibnou-Zekri, et al 2003). Different Lactobacillus strains induce specific mucosal cytokine profiles and possess differential intrinsic adjuvanticity (Maassen *et al.*, 2000). Some strains of LABs have more immunopotentiating activity than others (Nagafuchi, *et al.*, 1999).

Role in immune enhancement by native and administered LABs

The gut-associated lymphoid tissue (GALT) plays an essential role in both the development and maintenance of immunity. Secretory IgA (S-IgA), the major immunoglobulin of the GIT, is induced by GALT-derived B cells with the help of Th1- and Th2-type CD4(+) T lymphocytes. Cytotoxic T-lymphocytes (CTL) in the mucosal epithelium, and a sub-population of intra-epithelial lymphocytes (IELs), also help to maintain the mucosal barrier. The bacteria, either native or administered, can interact with GALT, and therefore leads to an immune modulating effect on animals, although the effect is not as powerful as that of pathogens (Haller, *et al.*, 1999). Studies have demonstrated that both the cell wall and cytoplasmic fractions, as well as killed LABs are able to have a stimulatory role on the immune system of animals (Hacher and Lambrecht, 1993; Tejada-Simon and Pestka, 1999; Gill and Rutherfurd, 2001; Murosaki *et al.*, 2000)

Effect of indigenous microorganisms and administered LABs on natural immunity of animals. It has been reported that some lactic acid bacterial strains, such as *L. casei*, *L. bulgaricus*, *Streptococcus thermophilus* and *L. acidophilus* administered orally are able to activate macrophages in mice, and in this way they significantly enhance the enzymatic and phagocytic activity of peritoneal macrophages compared with controls. They also accelerate the phagocytic function of the reticulo-endothelial system as revealed by the carbon clearance test (Perdigon *et al.*, 1986; Perdigon *et al.*, 1987; Gill *et al.*, 2000). Two types of indigenous microbes, segmented filamentous bacteria (SFB) and clostridia, whose habitats are the small and large intestines, respectively, can promote the development of intraepithelial lymphocytes

in the small and large intestine, suggesting the occurrence of compartmentalization of the immunological responses to the indigenous bacteria between the small and large intestines (Umesaki *et al.*, 1999). *L. acidophilus* and *L. casei*, when administered for two weeks by a gastric tube, promoted the migrating capacity of lymphocytes from the germinal center into the inter-epithelial spaces and into the lamina propria of the mucosa of the gut (Mihal *et al.*, 1990).

Modulation of humoral immune response through the native intestinal microflora and probiotic intake. The implantation of the intestinal microflora plays an important role in the development of the intestinal IgA immune system (Moreau *et al.*, 1982) in mice. It has been shown that probiotics can enhance the IgA secreting B cells in both the small and large intestines (Perdigon *et al.*, 1994; Herias *et al.*, 1999; Umesaki *et al.*, 1999), and increase specific immunoglobulin secretion after both vaccine and infection (Kostiuk *et al.*, 1993; Benyacoub *et al.*, 2003). This increase would strengthen the host's defence mechanisms in the intestinal mucosa against pathogens and also systematically enhance the immune system.

Effect of native gut associated microflora and administered LABs on cell-mediated immunity. Modulation of intestinal epithelial cell cytokine production has the potential to profoundly affect the mucosal microenvironment. This would influence the immune response to pathogens and other ingested antigens. Different Lactobacilli strains are frequently used in consumer food products and sometimes in feed for chickens. Different Lactobacillus strains induce distinct mucosal cytokine profiles (Maassen *et al.*, 1998; Maassen *et al.*, 2000; Wallace *et al.*, 2003). Meanwhile, the mucosa-associated lactobacilli can be potent stimulators of some cytokines, and thus potentially of cell-mediated immunity, if they pass over the gut epithelial barrier and interact with cells of the gut immune system (Hassle *et al.*, 1999).

Effect of butyric acid on the immune response.

Some of the SCFAs produced by anaerobic bacteria, namely propionic, butyric and iso-butyric acids, exhibit a dose-dependent inhibition of both phytohemagglutinin-

induced blastogenesis and mixed lymphocyte culture in the millimolar range of SCFA concentrations. Furthermore, butyric acid displayed an interesting biphasic stimulation of monocyte interleukin-1 beta production, a cytokine with a powerful bone-resorbing activity (Eftimiadi *et al.*, 1991). When *Salmonella typhimurium* was cultured in Luria Bertani broth supplemented with acetate, propionate, butyrate, or a mixture of the three SCFA, there was a decrease in cell adhesion and in its ability to invade cultured HEp-2 cells (Durant *et al.*, 1999).

Possible mechanisms involved in the mucosal immune system activation by lactic acid bacteria.

Gut mucosal surfaces are habitats of some of the commensal and administrated microorganisms and are closely related to the mucosal immune compartment (mucosa-associated lymphoid tissue, MALT). Several possible mechanisms are involved in the immune-stimulatory role of the gut commensal microflora and administered probiotics. One of the important mechanisms is association with cell wall components. LABs are gram-positive bacteria with cell wall components such as peptidoglycans, polysaccharide and teichoic acid, all of which have been shown to have immunostimulatory properties (Takahashi *et al.,* 1993). Peptidoglycan and muramyl dipeptide (MDP), that make up 30%-70% of the LABs cell wall (Rook *et al.,* 1989), can be released by lysozyme (Peeters *et al.,* 1975). Peptidoglycans were known to have adjuvant effects on the immune response (Stewart-Tull, 1980), especially on the intestinal mucosal surface (Link-Amster *et al.,* 1994). Binding sites for peptidoglycans were identified on lymphocytes and macrophages (Dziarski, 1991). Another important mechanism is the secretion of substrates by the microflora. Considerable evidence has been accumulated showing that various members of the microflora liberate low molecular weight peptides which are apparently essential for an adequate immune response by the host. Antibiotic decontamination (e.g. of the BALB/c-mouse intestinal tract) results in a lack of generation of immuno-priming microbial peptides, which can lead to immunosuppression. Biochemical analysis of the peptides revealed reproducible chromatographic fractions which selectively influence maturation, proliferation, and activation of lymphatic cells (Pulverer *et al.*, 1993).

In conclusion, the gastro-intestinal tract is an area by which the host animal is in very close contact with its environment. Therefore, the gut-associated lymphoid tissue (GALT) is the first line of defense by which animals resist invasion of pathogens from that environment. GALT is distributed along the entire intestine of broilers. It plays an important role in making specific antibodies for broilers (Bryant *et al.,* 1973; Befus *et al.,* 1980). Microbial communities, which inhabit the intestinal tract, can stimulate these immune responses and therefore act to strengthen the host's defense mechanisms. This intestinal microbial community is distributed along the gastrointestinal tract of broiler chickens, and the caeca are the major region where anaerobic bacteria are located (Coloe, *et al.*, 1984). The intestinal microflora community can be modulated by dietary ingredient, such as prebiotics. Therefore, an increased lactic acid bacterial population by means of using prebiotics in the diet of broiler chickens would enhance the natural immune competence ability of the animals by increasing the natural antibody levels, and further to promote the immune defense responsibility. The possible effect of prebiotics on natural immune competence ability and the possibility of alternating antibiotics with prebiotics in diet of broiler chickens need further study.

REFERENCES

Akiba, Y. and H. Murakami, 1995. Partitioning of energy and protein during early growth of broiler chickens and contribution of vitteline residue. Proceeding of the 10th European Symposium on Poultry Nutrition, Antalia, Turkey.

Ane Knarreborg, M. A. Simon, R. M. Engberg, B. B. Jensen, and G. W. Tannock, 2002. Effects of Dietary Fat Source and Subtherapeutic Levels of Antibiotic on the Bacterial Community in the Ileum of Broiler Chickens at Various Ages. Applied and Environmental Microbiology. 68: 12. 5918-5924.

Archer, S. Y., S. Meng, A. Shei and R. A. Hodin, 1998. P21 (WAF1) is required for butyrate-mediated growth inhibition of human colon cancer cells. Proceedings of the National Academy of Sciences of the United States of America. U.S.A. 95:6791-6796.

Baba, E., S. Nagaishi, T. Fukata, A. Arakawa, 1991. The role of intestinal microflora on the prevention of Salmonella colonization in gnotobiotic chickens. Poultry Science. 70(9): 1902-1907.

Bach Knudsen, K. E., A. Serena, N. Canibe and K. S. Juntunen, 2003. New insight into butyrate metabolism. The Proceedings of the Nutrition Society. 62(1):81-86.

Bailey, J. S., J. A. Cason, and N. A. Cox, 1998. Effect of Salmonella in young chicks on competitive exclusion treatment. Poultry Science. 77(3):394-399.

Bailey, J. S., L. C. Blankenship, and N. A. Cox, 1991. Effect of fructooligosaccharide on Salmonella colonization of the chicken intestine. Poultry Science. 70(12):2433-2438.

Bailey, J. S., L. C. Blankenship, N. J. Stern, N. A. Cox, and F. McHan, 1988. Effect of anticoccidial and antimicrobial feed additives on prevention of Salmonella colonization of chicks treated with anaerobic cultures of chicken feces. Avian Disease. 32(2):324-329.

Barnes, E. M., C. S. Impey, and D. M. Cooper, 1980. Manipulation of the crop and intestinal flora of the newly hatched chick. American Journal of Clinical Nutrition. Nov;33 (11 Suppl): 2426-2433.

Barnes, E. M., G. C. Mead, D. A. Barnum, and E. G. Harry, 1972. The intestinal flora of the chicken in the period 2 to 6 weeks of age, with particular reference to the anaerobic bacteria. British Poultry Science. 13(3):311-326.

Bayer, R. R., C. B. Chawan, F. H. Bird and S. D. Musgrave, 1975. Characteristics of the absorptive surface of small intestine of the chicken from 1 day to 14 weeks of age. Poultry Science 54:155-159.

Beattie, R. M., E. J. Schiffrin, A. Donnet-Highes, A. C. Huggett, P. Domizio, T. T. MacDonald and J. Walker-Smith, 1994. Polymeric nutrition as the primary therapy in the children with small bowel Crohn's disease. Alimentary Pharmacology and Therapeutics. 8:609-615.

Befus, A. D., N. Johnston, G. A. Leslin and J. Bienenstock, 1980. Gut-associated lymphoid tissue in the chicken. I. Morphology, ontogeny, and some functional characteristics of Peyer's patches. The Journal of Immunology. 125(6):2626-2632.

Benyacoub, J., G. L. Czarnecki-Maulden, C. Cavadini, T. Sauthier, R. E. Anderson, E. J. Schiffrin, and T. von der Weid, 2003. Supplementation of food with Enterococcus faecium (SF68) stimulates immune functions in young dogs. Journal of Nutrition. 133(4):1158-1162.

Berg, R. D. and D. C. Savage, 1975. Immune responses of specific germ-free and gnotobiotic mice to antigens of indigenous and nonindigenous microorganisms. Infection Immunology. 11:320-329.

Bergman, E. N., 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiology Review. 70(2):567-590.

Blankenship, L. C., J. S. Bailey, N. A. Cox, N. J. Stern, R. Brewer, and O. Williams, 1993. Two-step mucosal competitive exclusion flora treatment to diminish salmonellae in commercial broiler chickens. Poultry Science. 72(9):1667-1672.

Blum, S., S. Alvarez, D. Haller, P. Perez, ad E. J. Schiffrin, 1999. Intestinal microflora and the interaction with immunocompetent cells. Antonie Van Leeuwenhoek 76(1-4):199-205.

Braun, E. J., and C. E. Campbell, 1989. Uric acid decomposition in the lower gastrointestinal tract. The Journal of Experimental Zoology. Supplement. 3:70-74.

Bryant, B. J., H. E. Adler, D. R. Cordy, M. Shifrine and A. J. DaMassa, 1973. The avian bursa-independent humoral immune system: serological studies. European Journal of Immunology. 3:9.

Carré, B., J. Gomez and A. M. Chagneau, 1995. Contribution of oligosaccharide and polysaccharide digestion, and excreta losses of lactic acid and short chain fatty acids, to dietary metabolisable energy values in broiler chickens and adult cockerels. British Poultry Science. 36:611-629.

Carter, C. P. and M. Pollard, 1971. Host responses to "normal" microbial flora in germ-free mice. Journal of the Reticuloendothelial Society. 9:580-587.

Champ, M., O. Szylit, and D. J. Gallant, 1981. The influence of microflora on the breakdown of maize starch granules in the digestive tract of chicken. Poultry Science. 60(1):179-187.

Chotinsky, D, E, Toncheva and Y. Profirov, 2001. Development of disaccharidase activity in the small intestine of broiler chickens. British Poultry Science. 2001. 42(3):389-933.

Chu, S. and M. H. Montrose, 1995. Extracellular pH regulation in microdomains of colonic crypts: effects of short-chain fatty acids. Proc Natl Acad Sci U S A. 11;92 (8):3303-3307.

Collins, M. D., and G. R. Gibson, 1999. Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. American Journal of Clinical Nutrition. 69(5): 1052S-1057S.

Coloe, P. J., T. J. Bagust, and L. Ireland, 1984. Development of the normal gastrointestinal microflora of specific pathogen-free chickens. The Journal of Hygiene. (Lond). 92(1):79-87.

Cook, R. H. and F. H. Bird, 1973. Duodenal villus area and epithelial cellular migration in conventional and germ-free chickens. Poultry Science. 52:2276-2280.

Coon, C. N., K. L. Leske, O. Akavanichan and T. K. Cheng, 1990. Effect of oligosaccharide free soybean meal on true metabizable energy and fiber digestion in adult roosters and broilers. Poultry Science. 69(5) 787-793.

Corrier, D. E., A. G. Hollister, D. J. Nisbet, C. M. Scanlan, R. C. Beier, and J. R. DeLoach, 1994a. Competitive exclusion of Salmonella enteritidis in Leghorn chicks: comparison of treatment by crop gavage, drinking water, spray, or lyophilized alginate beads. Avian Disease. 38(2): 297-303.

Corrier, D. E., B. M. Hargis, A. Jr Hinton, and J. R. DeLoach, 1993a. Protective effect of used

poultry litter and lactose in the feed ration on Salmonella enteritidis colonization of leghorn chicks and hens. Avian Disease. 37(1):47-52.

Corrier, D. E., D. J. Nisbet, A. G. Hollister, C. M. Scanlan, B. M. Hargis, and J. R. DeLoach, 1993b. Development of defined cultures of indigenous cecal bacteria to control salmonellosis in broiler chicks. Poultry Science. 72(6):1164-1168.

Corrier, D. E., D. J. Nisbet, A. G. Hollister, R. C. Beier, C. M. Scanlan, B. M. Hargis, and J. R. Deloach, 1994b. Resistance against Salmonella enteritidis cecal colonization in Leghorn chicks by vent lip aplication of cecal bacteria culture. Poultry Science. 73(5):648-652.

Costerton, J. W., K. R. Rozee, and K. J. Cheng, 1983. Colonization of particulates, mucous, and intestinal tissue. Progress in Food and Nutrition Science. 7(3-4):91-105.

Durant, J. A., V. K. Lowry, D. J. Nisbet, L. H. Stanker, D. E. Corrier, and S. C. Ricke, 1999. Short-chain fatty acids affect cell-association and invasion of HEp-2 cells by Salmonella typhimurium. Journal of Environmental Science Health B. 34(6):1083-1099.

Durst, L., 1996. Inclusion of fructo- and galacto-oligosaccharides in broiler diets. Archiv fur Geflugelkunde. 60:160-164.

Dziarski, R., 1991. Demonstration of peptigolycan-binding sites on lymphocytes and macrophages by photoaffinity cross-linking. Journal of Biology Chemistry. 266:4713-4718.

Eftimiadi, C., P. Stashenko, M. Tonetti, P. E. Mangiante, R. Massara, S. Zupo, and M. Ferrarini, 1991. Divergent effect of the anaerobic bacteria by-product butyric acid on the immune response: suppression of T-lymphocyte proliferation and stimulation of interleukin-1 beta production. Oral Microbiology Immunology. 6(1):17-23.

Ferreira, A. J., C. S. Ferreira, T. Knobl, A. M. Moreno, M. R. Bacarro, M. Chen, M. Robach, and G. C. Mead, 2003. Comparison of three commercial competitive-exclusion products for controlling Salmonella colonization of broilers in Brazil. Journal of Food Protection. 66(3): 490-492.

Fleming, S.E., 1981. A study of relationship between flatus potential and carbohydrate distribution in legume seeds. Journal of Food Science. 46-794-798.

Fuchs, C. S., E. L. Giovannucci, G. A. Colditz, D. J. Hunter, M. J. Stampfer, B. Rosner, F. E. Speizer and W. C. Wellett, 1999. Dietary fiber and the risk of colorectal cancer and adenoma in women. N. Engl. Journal of Medicine. 340:169-176.

Fukata, T., K. Sasai, T. Miyamoto, and E. Baba, 1999. Inhibitory effects of competitive exclusion and fructooligosaccharide, singly and in combination, on Salmonella colonization of chicks. Journal of Food Protection. 62(3):229-233.

Fukunaga, T., M. Sasaki, Y. Araki, T. Okamoto, T. Yasuoka, T. Tsujikawa, Y. Fujiyama and T. Bamba, 2003. Effects of the soluble fibre pectin on intestinal cell proliferation, fecal short chain Fatty Acid production and microbial population. Digestion. 67(1-2):42-49.

Furuse, M. and H. Yokota, 1984a. Effect of the gut microflora on the size and weight of organs of chicks fed diets of different protein content. British Poultry Science. 25(3):429-439.

Furuse, M. and H. Yokota, 1985. Effect of the gut microflora on chick growth and utilisation of protein and energy at different concentrations of dietary protein. British Poultry Science. 26(1):97-104.

Furuse, M., and H. Yokota, 1984b. Protein and energy utilization in germ-free and conventional chicks given diets containing different levels of dietary protein. British Journal of Nutrition. 51(2):255-264.

Furuse, M., S. I. Yang, N. Niwa and J. Okumura, 1991. Effect of short chain fatty acids on the performance and intestinal weight in germ-free and conventional chicks. British Poultry Science. 32(1):159-165.

Geyra, A., Z. Uni, and D. Sklan, 2001. Entrocyte dynamics and mucosal development in the post hatch chicken. Poultry Science. 80:776-782.

Gibson, G. R, and M. B. Roberfroid, 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. Journal of Nutrition. 125(6):1401-1412.s

Gibson, G. R., 1998. Dietary modulation of the human gut microflora using prebiotics. Brithsh Journal of Nutrition. 80(4):S209-S212.

Gibson, G. R., 1999. Dietary modulation of the human gut microflora using the prebiotics oligofructose and inulin. Journal of Nutrition. 129(7 Suppl):1438S-1441S.

Gill, H. S., K. J. Rutherfurd, 2001. Viability and dose-response studies on the effects of the immunoenhancing lactic acid bacterium Lactobacillus rhamnosus in mice. British Journal of Nutrition. 86(2):285-289.

Gill, H. S., K. J. Rutherfurd, J. Prasad, P. K. Gopal, 2000. Enhancement of natural and acquired immunity by Lactobacillus rhamnosus (HN001), Lactobacillus acidophilus (HN017) and Bifidobacterium lactis (HN019). British Journal of Nutrition. 83(2):167-176.

Glunder, G. 2002. Influence of diet on the occurrence of some bacteria in the intestinal flora of wild and pet birds. Deutsche tierarztliche Wochenschrift. 109(6):266-270.

Goodlad, R. A., B. Ratcliffe, J. P. Fordham and N. A. Wright, 1989. Does dietary fibre stimulate intestinal epithelial cell proliferation in germ free rats? Gut. 30(6):820-825.

Goren, E., W. A. de Jong, P. Doornenbal, J. P. Koopman, and H. M. Kennis, 1984. Protection of chicks against Salmonella infantis infection induced by strict anaerobically cultured intestinal microflora. The Veterinary Quarterly. 6(1):22-26.

Goren, E., W. A. de Jong, P. Doornenbal, N. M. Bolder, R. W. Mulder, and A. Jansen, 1988. Reduction of salmonella infection of broilers by spray application of intestinal microflora: a longitudinal study. The Veterinary Quarterly. 10(4):249-255.

Hague, A., A. J. Butt and C. Paraskeva, 1996. The role of butyrate in human colonic epithelial

cells: an energy source or inducer of differentation and apoptosis?. The Proceedings of the Nutrition Society.55:937-943.

Hague, A., B. Singh and C. Paraskeva, 1997b. Butyrate acts as a survival factor for colonic epithelial cells: Further fuel for the in vivo versus in vitro debate. Gastroenterology. 112: 1036-1040.

Hague, A., D. J. Elder, D. J. Hicks and C. Paraskeva, 1995. Apoptosis in colorectal tumour cells: induction by the short chain fatty acids butyrate, propionate and acetate and by the bile salt deoxycholate. International journal of cancer. Journal International du Cancer. 27;60 (3):400-406.

Hague, A., G. D. Diaz, D. J. Hicks, S. Krajewski, J. C. Reed and C. Paraskeva, 1997a. Bcl-2 and Bak may play a pivotal role in sodium butyrate-induced apoptosis in colonic epithelial cells: However overexpression of bcl does not protect against Bak-mediated apoptosis. International journal of cancer. Journal International du Cancer. 72:898-905.

Haller, D., C. Bode, and W. P. Hammes, 1999. Cytokine secretion by stimulated monocytes depends on the growth phase and heat treatment of bacteria: a comparative study between lactic acid bacteria and invasive pathogens. Microbiology Immunology. 43(10):925-935.

Hassig C. A., J. K. Tong and S. L. Schreiber, 1997. Fiber-derived butyrate and the prevention of colon cancer. Chemistry and Biology. 4:783-789.

Hessle, C, L. A. Hanson and A. E. Wold, 1999. Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL-12 production. Clinical Experimental Immunology. 116(2):276-282.

Hatcher, G. E., and R. S. Lambrecht, 1993. Augmentation of macrophage phagocytic activity by cell-free extracts of selected lactic acid-producing bacteria. Journal of Dairy Science. 76(9):2485-2492.

Hegde, S. N., B. A. Rolls, and M. E. Coates, 1982. THe effects of the gut microflora and dietary fibre on energy utilization by the chick. British Journal of Nutrition. 48(1):73-80.

Herias, M. V., C. Hessle, E. Telemo, T. Midtvedt, L. A. Hanson, and A. E. Wold, 1999. Immunomodulatory effects of Lactobacillus plantarum colonizing the intestine of gnotobiotic rats. Clinical Experiment Immunology. 116(2):283-290.

Hessle, C., L. A. Hanson, and A. E. Wold, 1999. Lactobacilli from human gastrointestinal mucosa are strong stimulators of IL-12 production. Clinical Experiment Immunology. 116(2):276-282.

Hollister, A. G., D. E. Corrier, D. J. Nisbet, and J. R. Deloach, 1994. Effect of cecal cultures encapsulated in alginate beads or lyophilized in skim milk and dietary lactose on Salmonella colonization in broiler chicks. Poultry Science. 73(1):99-105.

Hoszowski, A., and M. Truszczynski, 1997. Prevention of Salmonella typhimurium caecal colonisation by different preparations for competitive exclusion. Comp Immunology Microbiology Infection Disease. 20(2):111-117.

Hume, M. E., A. G. Hollister, D. J. Nisbet, D. E. Corrier, and J. R. DeLoach, 1996. Effect of a characterized continuous-flow culture of cecal bacteria on Salmonella typhimurium crop colonization in broiler chicks. Avian Disease. 40(2):391-397.

Hume, M. E., D. E. Corrier, G. W. Ivie, and J. R. Deloach. 1993. Metabolism of [14C]propionic acid in broiler chicks. Poultry Science. 72(5):786-793.

Hume, M. E., J. A. Byrd, L. H. Stanker, and R. L. Ziprin, 1998. Reduction of caecal Listeria monocytogenes in Leghorn chicks following treatment with a competitive exclusion culture (PREEMPT). Letter Applied Microbiology. Jun;26(6):432-426.

Ibnou-Zekri, N., S. Blum, E. J. Schiffrin, and T. Von Der Weid, 2003. Divergent patterns of colonization and immune response elicited from two intestinal Lactobacillus strains that display similar properties in vitro. Infection Immunology. 71(1):428-436.

Ichikawa, H. and T. Sakata, 1998. Stimulation of epithelial cell proliferation of isolated distal colon of rats by continuous colonic infusion of ammonia or short-chain fatty acids is nonadditive. Journal of Nutrition. 128(5):843-7843.

Ija, P. A., A. Saki, and D. R. Tivey, 2001. Body and intestinal growth of broiler chicks on a commercial starter diet. 1. Intestinal weight and mucosal development. British Poultry Science. 42(4):505-513.

Impey, C. S., G. C. Mead, and S. M.George, 1982. Competitive exclusion of salmonellas from the chick caecum using a defined mixture of bacterial isolates from the caecal microflora of an adult bird. The Journal of Hygiene. 89(3):479-490.

Jass, J. R., 1985. Diet, butyric acid and differentiation of gastrointestinal tract tumours. Medical Hypotheses.18(2):113-118.

Jiangrang, L., U. Idris, B. Harmon, C. Hofacre, J. J. Maurer, M. D. Lee, 2003. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. Applied Environmental Microbiology. 69(11):6816-6824.

Kogut, M. H., T. Fukata, G. Tellez, B. M. Hargis, D. E. Corrier, J. R. DeLoach, 1994. Effect of Eimeria tenella infection on resistance to Salmonella typhimurium colonization in broiler chicks inoculated with anaerobic cecal flora and fed dietary lactose. Avian Disease. 38(1): 59-64.

Komai, M. and S. Kimura, 1980. Gastrointestinal responses to graded levels of cellulose feeding in conventional and germ-free mice. Journal of Nutrition Science Vitaminology (Tokyo).

26(4):389-399.

Kostiuk, O. P., L. I. Chernyshova, and I. I. Slukvin, 1993. Protective effect of Lactobacillus acidophilus on development of infection, caused by Klebsiella pneumoniae. Fiziologicheskii zhurnal. 39(4):62-68.

Kripke, S. A., A. D. Fox, J. M. Berman, and R. G. Settle, 1989. Rombeau JL.Stimulation of intestinal mucosal growth with intracolonic infusion of short-chain fatty acids. JPEN J Parenter Enteral Nutrition. 13(2):109-116. Kritchevsky, D., 1998. Cereal fibres and colorectal cancer: a search for mechanisms. European Journal of Cancer Prevention. Suppl. 2:S33-S39.

Kubena, L. F., J. A. Byrd, C. R. Young, and D. E. Corrier, 2001. Effects of tannic acid on cecal volatile fatty acids and susceptibility to Salmonella typhimurium colonization in broiler chicks. Poultry Science. 80(9):1293-1298.

Le Blay, G., H. M. Blottiere, L. Ferrier, E. Le Foll, C. Bonnet, J. P. Galmiche and C. Cherbut, 2000. Short-chain fatty acids induce cytoskeletal and extracellular protein modifications associated with modulation of proliferation on primary culture of rat intestinal smooth muscle cells. Digestive Disease Science. 45(8):1623-1630.

Leske, K. L., C. J. Jevne and C. N. Coon, 1993. Effect of oligosaccharide additions on nitrogen-corrected true metabolizable energy of soy protein concentrate. Poultry Science. 72:664-668.

Leske, K. L., O. Akavanichan, T. K. Cheng and C. N. Coon, 1991. Effect of ethanol extract on nitrogen corrected true metabolizable energy for soybean meal with broilers and roosters. Poultry Science. 70:892-895.

Link-amster, H., F. Rochat, K. Y. Saudan, O. Mignot and J. M. Aeschlimann, 1994. Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intakes. FEMS Immunology and Medical Microbiology. 10:55-64.

Lupton, J. R., 1995. Butyrate and colonic cytokinetics: differences between in vitro and in vivo studies. European Journal of Cancer Prevention. 4:373-378.

Lupton, J. R., D. M. Coder and L. R. Jacobs, 1985a. Influence of luminal pH on rat large bowel epithelial cell cycle. American Journal of Physiology. 249(3 Pt 1):G382-G328.

Lupton, J. R., D. M. Coder, and L. R. Jacobs, 1985b. Influence of luminal pH on rat large bowel epithelial cell cycle. American Journal of Physiology. 249(3 Pt 1):G382-G388.

Maassen, C. B., J. C. van Holten, F. Balk, M. J. Heijne den Bak-Glashouwer, R. Leer, J.D. Laman, W. J. Boersma, and E. Claassen, 1998. Orally administered Lactobacillus strains differentially affect the direction and efficacy of the immune response. The Veterinary quarterly. 20 Suppl 3:S81-83.

Maassen, C. B., van C. Holten-Neelen, F. Balk, den M. J. Bak-Glashouwer, R. J. Leer, J. D. Laman, W. J. Boersma, and E. Claassen, 2000. Strain-dependent induction of cytokine profiles in the gut by orally administered Lactobacillus strains. Vaccine. 22;18(23):2613-2623.

Marchaim, U. and R. G. Kulka, 1967. The non-parallel increase of amylase, chymotrypsinogen and procarboxypeptidase in the developing chick pancreas. Biochimia Biophysica Acta. 146:553-559.

Marcil, V., E. Delvin, E. Seidman, L. Poitras, M. Zoltowska, C. Garofalo and E. Levy, 2002. Modulation of lipid synthesis, apolipoprotein biogenesis, and lipoprotein assembly by butyrate. American Journal of Physiology Gastrointestine Liver Physiology. 283(2):G340-346.

Mattocks, J. G., 1971. Goose feeding and celluose digestion. Wild life. 22:107-113.

McBain, A. J., and G. T. MacFarlane, 2001. Modulation of genotoxic enzyme activities by non-digestible oligosaccharide metabolism in in-vitro human gut bacterial ecosystems. Journal of Medical Microbiology. 50(9):833-842.

Mead, G. C., 2000. Prospects for 'competitive exclusion' treatment to control salmonellas and other foodborne pathogens in poultry.Veterinary Journal. 159(2):111-123.

Mead, G. C, and B. W. Adams, 1975. Some observations on the caecal microflora of the chick during the first two weeks of life. British Poutry Science. 16(2):169-176.

Mead, G. C., 1989. Microbes of the avian cecum: types present and substrates utilized. Journal of Experimental Zoology. Suppl;3:48-54.

Mihal, V., V. Lackovic, M. Plockova, and P. Brezina, 1990. Immunobiologic properties of lactobacilli. Cesk Pediatr. 45(10):587-590.

Mineo, H., H. Hara, and F. Tomita, 2001. Short-chain fatty acids enhance diffusional ca transport in the epithelium of the rat cecum and colon. Life Science. 69(5):517-526.

Moreau, M. C., P. Raibaud, and M. C. Muller, 1982. Relationship between the development of the intestinal IgA immune system and the establishment of microbial flora in the digestive tract of young holoxenic mice. Annual Immunology (Paris). 133D(1):29-39.

Mortensen, P. B. and M. R. Clausen, 1996. Short-chain fatty acids in the human colon: relation to gastrointestinal health and disease. Scandinavia Journal of Gastroenterology. Suppl.; 216:132-148.

Muramatsu, T., H. Kodama, T. Morishita, M. Furuse, and J. Okumura, 1991. Effect of intestinal microflora on digestible energy and fiber digestion in chickens fed a high-fiber diet. American Journal of Veterinary Research. 52(7):1178-81.

Muramatsu, T., O. Takasu and J. Okumura, 1993. Research note: fructose feeding increases lower gut weights in germ-free and conventional chicks. Poultry Science. 72(8):

1597-1600.

Muramatsu, T., O. Takasu, M. Furuse and J. Okumura, 1988. Effect of diet type on enhanced intestinal protein synthesis by the gut microflora in the chick. Journal of Nutrition. 118(9): 1068-1074.

Muramatsu, T., S. Nakajima, and J. Okumura, 1994. Modification of energy metabolism by the presence of the gut microflora in the chicken. British Journal of Nutrition. 71(5):709-717.

Murosaki, S., K. Muroyama, Y. Yamamoto, and Y. Yoshikai, 2000. Antitumor effect of heat-killed Lactobacillus plantarum L-137 through restoration of impaired interleukin-12 production in tumor-bearing mice. Cancer Immunology Immunother. 49(3):157-164.

Nagafuchi, S., T. Takahashi, T. Yajima, T. Kuwata, K. Hirayama, and K. Itoh, 1999. Strain dependency of the immunopotentiating activity of Lactobacillus delbrueckii subsp. bulgaricus. Bioscience Biotechnology Biochemistry. 63(3):474-479.

Newmark, H. L. and J. R..Lupton, 1990. Determinants and consequences of colonic luminal pH: implications for colon cancer. Nutrition and Cancer. 14:161-173.

Nitsan, Z., G. Ben-Avraham, Zipora Zoref and I. Nir, 1991. Growth and development of the digestive organs and some enzymes in broiler chicks after hatching. British Poultry Science. 32:515-523.

Noy, Y. and D. Sklan, 1995. Digestion and absorption in the young chick. Poultry Science 74: 366-373.

Noy, Y. and D. Sklan, 1998. Yolk utilization in the newly hatched poult. British Poultry Science. 39:446-451.

Palmer, M. F., and B. A. Rolls, 1983. The activities of some metabolic enzymes in the intestines of germ-free and conventional chicks. British Journal of Nutrition. 50(3):783-790.

Parsons, C. M., L. M. Potter and R. D. Jr. Brown, 1983. Effects of dietary carbohydrate and of intestinal microflora on excretion of endogenous amino acids by poultry. Poultry Science. 62(3):483-489.

Peeters, T., and G. Vantrappen, 1975. The paneth cell: a source of intestinal lysozyme. Gut. 16:553-558.

Perdigon, G., M. Medina, E. Vintini, and J. C. Valdez, 2000. Intestinal pathway of internalisation of lactic acid bacteria and gut mucosal immunostimulation. International Journal of Immunopathology Pharmacology. 13(3):141-150.

Perdigon, G., M. Rachid, M.V. De Budeguer, and J. C. Valdez, 1994. Effect of yogurt feeding on the small and large intestine associated lymphoid cells in mice. Journal of Dairy Research. 61(4):553-562.

Perdigon, G., M. E. de Macias, S. Alvarez, G. Oliver, and A. A. de Ruiz Holgado, 1986. Effect of

perorally administered lactobacilli on macrophage activation in mice. Infection Immunology. 53(2):404-410.

Perdigon, G., M. E. Nader de Macias, S. Alvarez, G. Oliver, and A. A. Pesce de Ruiz Holgado, 1987. Enhancement of immune response in mice fed with Streptococcus thermophilus and Lactobacillus acidophilus. Journal Dairy Science. 70(5):919-926.

Philips, S. M. and R. Fuller, 1983. The activities of amylase and a trypsin-like protease in the gut contents of germ-free and conventional chickens. British Poultry Science. 24(1):115-121.

Potter, J. D., 1999. Fiber and colorectal cancer. New England Journal of Medicine. 340:223-224.

Pouillart P.R., 1998. Role of butyric acid and its derivatives in the treatment of colorectal cancer and hemoglobinopathies. Life Science. 63(20):1739-1760.

Powrie, F., 1995. T cells in inflammatory bowel disease: protective and pathogenic roles. Immunity. 3:171-174.

Pulverer, G., H. L. Ko, and J. Beuth, 1993. Immunomodulating effects of antibiotics influencing digestive flora. Pathology Biology (Paris). 41(8 Pt 2):753-758.

Rabassa, A. A. and A. I. Rogers, 1992. The role of short-chain fatty acid metabolism in colonic disorders. American Journal of Gastroenterology. 87(4):419-423.

Rackis, J. J, D. H. Honig, D. J. Sessa, and F. R Steggerda, 1975. Flavor and flatulence factors in soybean protein products. Journal of Agricultural Food Chemistry. 18: 977-82.

Reilly, K. J., W. L. Frankel, A. M. Bain and J. L. Rombeau, 1995. Colonic short chain fatty acids mediate jejunal growth by increasing gastrin. Gut. 37(1):81-86.

Rook, G., 1989. Immunity to bacteria. In: Roitt I, Brostoff J, Male D. Eds. Immunology. St Louis: Mosby.

Sakata, T. and H. Setoyama, 1995. Local stimulatory effect of short-chain fatty acids on the mucus release from the hindgut mucosa of rats (Rattus norvegicus).Comp Biochem Physiol A Physiol. Jul;111(3):429-432.

Sakata, T. and W. von Engelhardt, 1983. Stimulatory effect of short chain fatty acids on the epithelial cell proliferation in rat large intestine. Comp Biochem Physiol A. 74(2):459-462.
Sakata, T., 1986. Effects of indigestible dietary bulk and short chain fatty acids on the tissue weight and epithelial cell proliferation rate of the digestive ract in rats. Journal of Nutrition Science Vitaminol (Tokyo) 32(4):355-362.

Sakata, T., 1987. Stimulatory effect of short-chain fatty acids on epithelial cell proliferation in the rat intestine: a possible explanation for trophic effects of fermentable fibre, gut microbes and luminal tropic factors. British Journal of Nutrition. 58:95-103.

Sakata, T., M. Adachi, M. Hashida, N. Sato, T. Kojima, 1995. Effect of n-butyric acid on epithelial cell proliferation of pig colonic mucosa in short-term culture. Dtsch Tierarztl Wochenschr. 102(4):163-164.

Salvat, G., F. Lalande, F. Humbert, and C. Lahellec, 1992. Use of a competitive exclusion product (Broilact) to prevent Salmonella colonization of newly hatched chicks. International Journal of Food Microbiology. 15(3-4):307-311.

Scheppach, W., S. U. Christl, H.P. Bartram, F. Richter and H. Kasper, 1997. Effects of short-chain fatty acids on the inflamed colonic mucosa. Scandinavia Journal of Gastroenterology Suppl.222:53-57.

Schiffrin, E. J., and S. Blum, 2002. Interactions between the microbiota and the intestinal mucosa. European Journal of Clinical Nutrition. 56 Suppl 3:S60-S64.

Schoeni, J. L,, and A. C. Wong, 1994. Inhibition of Campylobacter jejuni colonization in chicks by defined competitive exclusion bacteria. Applied Environmental Microbiology. 60(4):1191-1197.

Schwartz, R. B., 1989. Acqustion of self-tolerance. Cell. 57:1073-1081.

Semenza, G., 1986. Anchoring and biosynthesis of stalked brush border membrane protein: glycosidases and peptidases of enterocytes and of renal tubuli. Annual Review of Cell Biology. 2:255-313.

Shea, K. M., 2003. Antibiotic resistance: what is the impact of agricultural uses of antibiotics on children's health? Pediatrics. 112(1 Pt 2):253-258.

Silva, E. N., G. H. Snoeyenbos, O. M. Weinack, and C. F. Smyser, 1981. The influence of native gut microflora on the colonization and infection of Salmonella gallinarum in chickens. Avian Disease. 25(1):68-73.

Singh, B., A. Halestrap and C. Paraskeva, 1997. Butyrate can act as a stimulator of growth or inducer of apoptosis in human colonic epithelial cell lines depending on the presence of alternative energy sources. Carcinogenesis. 18:1265-1270.

Sklan D and Y. Noy, 2000. Hydrolysis and absorption in the small intestines of posthatch chicks. Poultry Science. 79(9):1306-10.

Sklan, D. 2001. Development of the digestive tract of poultry. World's Poultry Science Journal. 57: 415-428.

Smith, H. W., 1965. The development of the flora of the alimentary tract in young animals. Journal of Pathology and Bacteriology. 90:495-513.

Snoeyenbos, G. H., A. S. Soerjadi, and O. M. Weinack, 1982. Gastrointestinal colonization by salmonellae and pathogenic Escherichia coli in monoxenic and holoxenic chicks and poults. Avian Disease. 26(3):566-575.

Snoeyenbos, G. H., O. M. Weinack, A. S. Soerjadi-Liem, B. M. Miller, D. E. Woodward, and C. R. Weston, 1985. Large-scale trials to study competitive exclusion of salmonella in chickens. Avian Disease. 29(4):1004-1011.

Snoeyenbos, G. H., O. M. Weinack, and C. F. Smyser, 1978. Protecting chicks and poults from Salmonellae by oral administration of "normal" gut microflora. Avian Disease. 22(2):273-287.

Snoeyenbos, G. H., O. M. Weinack, and C. F. Smyser, 1979. Further studies on competitive exclusion for controlling Salmonellae in chickens. Avian Disease. 23(4):904-914.

Soerjadi, A. S., G. H. Snoeyenbos, O. M.Weinack, 1982. Intestinal colonization and competitive exclusion of Campylobacter fetus subsp. jejuni in young chicks. Avian Disease. 26(3):520-524.

Soerjadi, A. S., R. Rufner, G. H. Snoeyenbos, and O. M. Weinack, 1982. Adherence of salmonellae and native gut microflora to the gastrointestinal mucosa of chicks. Avian Disease. 26(3): 576-584.

Soerjadi, A. S., S. M. Stehman, G. H. Snoeyenbos, O. M. Weinack, and C. F. Smyser, 1981. The influence of lactobacilli on the competitive exclusion of paratyphoid salmonellae in chickens. Avian Disease. 25(4):1027-1033.

Soerjadi-Liem, A. S., G. H. Snoeyenbos, and O. M. Weinack, 1984a. Establishment and competitive exclusion of Yersinia enterocolitica in the gut of monoxenic and holoxenic chicks. Avian Disease. 28(1):256-260.

Soerjadi-Liem, A. S., G. H. Snoeyenbos, and O. M. Weinack, 1984b.Comparative studies on competitive exclusion of three isolates of Campylobacter fetus subsp. jejuni in chickens by native gut microflora. Avian Disease. 28(1):139-146.

Stavric, S., 1992. Defined cultures and prospects. International Journal of Food Microbiology. 15(3-4):245-263.

Stavric, S., T. M. Gleeson, and B. Blanchfield, 1991. Effect of avian intestinal microflora possessing adhering and hydrophobic properties on competitive exclusion of Salmonella typhimurium from chicks. Journal of Applied Bacteriology. 70(5):414-421.

Stern, N. J., 1994, Mucosal competitive exclusion to diminish colonization of chickens by Campylobacter jejuni. Poultry Science. 73(3):402-407.

Stewart-Tull, D. E. S., 1980. The immunological activities of bacterial peptidoglycans. Annual Review of Microbiology. 34:311-340.

Takahashi, T., T. Oka, H. Iwana, T. Kuwata and Y. Yamamoto, 1993. Immune response of mice to orally administered lactic acid bacteria. Bioscience, Biotechnology, and Biochemistry 57:1557-1560.

Tannock, G. W., 2001. Molecular assessment of intestinal microflora. American Journal of Clinical Nutrition. 73(suppl):410S-414S.

Tappenden, K. A., and M. I. McBurney, 1998. Systemic short-chain fatty acids rapidly alter gastrointestinal structure, function, and expression of early response genes. Digestive Diseases and Sciences. 43(7):1526-1536.

Tejada-Simon, M. V., and J. J. Pestka, 1999. Proinflammatory cytokine and nitric oxide induction in murine macrophages by cell wall and cytoplasmic extracts of lactic acid bacteria. Journal of Food Protection. 62(12):1435-1444.

Tlaskalova-Hogenova, H. and R.Stepankova, 1980. Development of antybody formation in germ-free and conventionally reared rabbits: the role of intestinal lymphoid tissue in antybody formation to E. Coli antigens. Folia Biology (Praha). 26:81-93.

Tlaskalova-Hogenova, H., J. Sterzl, and R.Stepankova, 1983. Development of immulogical capacity under germfree and conventional conditions. Annual N Y Academic Science. 409:96-113.

Tlaskalova-Hogenova, H., L. Mandel, I. Trebichavsky, F. Kovaru, R. Barot and J. Sterzl, 1994. Development of immune responses in early pig ontogeny. Veterinary Immunology and Immunopathology. 43:135-142.

Tottori, J., R. Yamaguchi, Y. Murakawa, M. Sato, K. Uchida, and S. Tateyama, 1997. The use of feed restriction for mortality control of chickens in broiler farms. Avian Disease. 41(2):433-4377.

Umesaki, Y., H. Setoyama, S. Matsumoto, A. Imaoka, and K. Itoh, 1999. Differential roles of segmented filamentous bacteria and clostridia in development of the intestinal immune system. Infection Immunology. 67(7):3504-3511.

Uni, Z., Y. Noy, and D. Sklan, 1995. Post hatch changes of morphology and function of the small intestins in heave and light strain chicke. Poultry Science. 74:1622-1629.

Uni, Z., Y. Noy, and D. Sklan, 1996. Developmental parameters of the small intestine of heavy and light train chicks pre- and post-hatch. British Poultry Science. 36:63-71.

Uni, Z., Y. Noy, and D. Sklan, 1999. Posthatch development of small intestinal function in the poult. Poultry Science. 78:215.

Unno, T., M. Sugawara, T. Nakakuki and G. Okada, 1993. Effect of â-gluco-oligosaccharides on the human intestinal microflora. Journal of Starch Related Carbohydrates Enzymes. 40:21-27.

van der Waaij, D. and C. E. Nord, 2000. Development and persistence of multi-resistance to antibiotics in bacteria; an analysis and a new approach to this urgent problem. International J Antimicrobial Agents. 16(3):191-197.

Velazquez, O. C., H. M. Lederer, and J. L. Rombeau, 1997. Butyrate and the colonocyte. Production, absorption, metabolism, and therapeutic implications. Advances in Experimental Medicine and Biology. 427:123-134.

Wachtershauser, A. and J. Stein, 2000. Rationale for the luminal provision of butyrate in intestinal diseases. European Journal of Nutrition. 2000 39(4): 164-171.

Wallace, T. D., S. Bradley, N. D. Buckley, and J. M. Green-Johnson, 2003. Interactions of lactic acid bacteria with human intestinal epithelial cells: effects on cytokine production. Journal of Food Protection. 66(3):466-472.

Watkins, B. A., and B. F. Miller, 1983. Competitive gut exclusion of avian pathogens by Lactobacillus acidophilus in gnotobiotic chicks. Poultry Science. 62(9):1772-1979.

Weinack, O. M., G. H. Snoeyenbos, and C. F. Smyser, 1979. A supplemental test system to measure competitive exclusion of Salmonellae by native microflora in the chicken gut. Avian Disease. 23(4):1019-1030.

Weinack, O. M., G. H. Snoeyenbos, C.F. Smyser, and A.S. Soerjadi, 1981. Competitive exclusion of intestinal colonization of Escherichia coli in chicks. Avian Disease. 25(3):696-705.

Whiteheah, R. H., G. P. Young and P. S. Bhathal, 1986. Effect of short chain fatty acids on the new human colon carcinoma cell line (LIM1215). Gut. 27:1457-1463.

Wilson, A. J. and P. R. Gibson, 1997. Short-chain fatty acids promote the migration of colonic epithelial cells in vitro.Gastroenterology 113(2):487-496.

Xiang, Y. Z., Z. Tanya, P. Yoga and R. D. Joerger, 2002. 16S rRNA-Based Analysis of Microbiota from the Cecum of Broiler Chickens. Applied and Environmental Microbiology. 68(1):124-137.

Xu, Z. R., C. H. Hu, M. S. Xia, X. A. Zhan, and M. Q. Wang, 2003. Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. Poultry Science. 82(6):1030-1036.

Yamauchi, K. and Y. Isshiki, 1991. Scanning electron microscopic observation on the intestinal villi in growing white Leghorn and broiler chickens from 1 to 30 days of age. British Poultry Science. 32(1):67-70.

Yazawa, K., K. Imai, and Z. Tamura, 1978. Oligosaccharides and polysaccharides specifically utilizable by bifidobacteria. Chemical Pharmacological Bulletin (Tokyo). 26(11):3306-3311.

Chapter 2

IN VITRO FERMENTATION KINETICS OF SOME NON-DIGESTIBLE CARBOHYDRATES BY THE CAECAL MICROBIAL COMMUNITY OF BROILERS

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In vitro fermentation kinetics of some non-digestible carbohydrates by the caecal microbial community of broilers

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Abstract: Fermentable carbohydrates can lead to changes in the gut microflora, which may have positive consequences for health. However, too often, ingredients are added to diets without first investigating their potential fermentability within the target animal. This experiment was conducted to investigate the fermentation kinetics of some non-digestible carbohydrates (NDC) by the caecal microbial community of broiler chickens by an assessment of the cumulative gas production during fermentation of each substrate. It also aimed to study changes in the microbial community, following fermentation of these non-digestible carbohydrates by use of polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Four extracted non-digestible carbohydrates (ENC): soybean meal oligosaccharides (SMO), soybean meal water-soluble polysaccharides (SMP), alfalfa meal oligosaccharides (AMO) and alfalfa meal water-soluble polysaccharides (AMP) were studied. Two pure sugars, raffinose (RAFF) and stachyose (STA) were also included. To assess the fermentability of the substrates, cumulative gas pressure was monitored continuously, for 72 hours, and at the end of fermentation, pH, and volatile fatty acid (VFA) and ammonia (NH_3) concentrations were measured in the fermentation solution. The PCR-DGGE technique was applied to compare microbial DNA fingerprints between substrates at the end of fermentation. The inoculum for the *in vitro* gas production was obtained from a mixture of caecal contents of 40 81 day-old broiler birds.

Results showed that SMO had the highest total sugar (720 g/kg in DM) content and SMP had the highest protein concentration (504.6 g/kg in DM) among the ENC. Experimental results suggested that the amount of gas and VFA produced as well as the decrease in pH of the fermentation fluid were positively related to the sugar content in ENC. SMO led to significantly more butyric acid production ($P<0.05$) after fermentation compared with other ENC. The production of butyric acid was

155.2 mg/g DM, 100.3 mg/g DM, 84.5 mg/g DM and 71.8 mg/g DM for SMO, SMP, AMP and AMO, respectively. SMO had the lowest pH (5.5) and ammonia nitrogen concentration (199.3 mg/l) after fermentation. This was significantly different from other ENCs and the pure sugars. DGGE analysis revealed that the fingerprint of caecal bacterial communities showed some variation (C, value >60 %-80 %) between some of the substrates.

It was concluded that mixed oligosaccharides and water-soluble polysaccharides extracted from soybean meal (SMO and SMP) and alfalfa meal (AMO and AMP), as well as the pure oligosaccharides raffinose (RAFF) and stachyose (STA) were significantly different both in terms of their fermentation kinetics and end-products using caecal contents from adult broilers. The extent to which these non-digestible, but fermentable, carbohydrates could change the microbial community of the broiler caecum, either in terms of the species detected, or its activity, needs to be investigated further, and then related to its effect on gut health in the animal itself.

key words: caecum, fermentability, gas production kinetics, VFA, DGGE, microbial DNA fingerprint, broiler chickens.

INTRODUCTION

The gastrointestinal tract is a complex ecosystem. It is inhabited by a diverse and highly evolved microbial community composed of hundreds of different microbial species (Dunne, 2001). It is generally accepted that the bacterial community of the intestinal tract has a major impact on gastrointestinal function, and can thereby also influence animal health and productivity. The large number of bacterial species, the complex nature of their interactions and the end-products of their fermentation processes are all likely to have effects on animal health. Considerable efforts have been made to modulate the intestinal microorganisms by dietary means in such a way that the health of the host is beneficially affected (Cavazzoni *et al.,* 1998; Jenkins, *et al.,* Buddington, *et al.,* Rao, Gibson, Gallaher, *et al.,* and Reddy, 1999; Sanders, Menne *et al.,* Brady *et al.,* 2000).

Prebiotics are food ingredients which are supposed to stimulate the activity and potentially alter the composition of the gut flora, by providing energy to selected species of the microbial community. For a food ingredient to be classified as a prebiotic it must fulfil the following criteria: 1) Neither be hydrolyzed, nor absorbed in the upper part of the gastrointestinal tract. 2) Be selectively fermented by one or a limited number of potentially beneficial bacteria commensal to the colon, e.g. bifidobacteria and lactobacilli, which are stimulated to grow and/or become metabolically activated. 3) Prebiotics must be able to alter the colonic microflora towards a healthier composition (Gibson, 1998). Attempts have been made to increase numbers of *Bifidobacterium* and *Lactobacillus* bacterial strains which are considered to have health-promoting properties. These strains are also assumed to be stimulated by various types of non-digestible carbohydrates (NDC).

In the poultry industry, there are two categories of material which are claimed to be potentially prebiotic. They are the synthetic and natural oligosaccharides (Iji and Tivey, 1998). There has been evidence of negative effects on animal health and productivity from the use of the raffinose series of oligosaccharides. Due to the absence of a-galactosidase in the upper gastrointestinal tract of broiler chickens, the

raffinose series of oligosaccharides reaches the lower gut undigested. Such substrates in the large intestine are then potentially digestible by the enzymes of microorganisms. This fermentation can lead to the release of gases which have been associated with flatulence (Rackis, 1975; Fleming, 1981; Saini and Knights, 1984). Another potentially negative effect is that the water-soluble indigestible carbohydrates in poultry diets tend to reduce the availability of dietary energy (Coon *et al*., 1990; Leske *et al*., 1991, 1993). In contrast to this, the synthetic oligosaccharides in poultry diets are considered beneficial for the animals (Unno *et al*., 1993; Durst, 1996). VFA are a major end-product of fermentation of the carbohydrates and other substrates in the large intestine of animals. It appears that VFA, particularly butyric acid, can contribute significantly to the health of the colon mucosa. All VFA can contribute to the energy supply of the host. By modification of the diet e.g. addition of appropriate prebiotic ingredients, one can potentially modify the amount and ratios of VFA which are produced and absorbed from the gut (Ruppin *et al.,* 1980). In some cases, the effects of diet on the microorganisms and their capacity to produce VFA can be estimated using *in vitro* techniques (Williams *et al.,* 2000).

Legumes, especially the grain legumes, are the most common natural sources of oligosaccharides, they also contain some water-soluble polysaccharides (Petterson and Mackintosh, 1994). For most grain legumes, the most common oligosaccharide is stachyose, followed by raffinose and verbascose, though their distribution varies in difference grain legumes (Petterson and Mackintosh, 1994; Mohamed and Rayas-Duarte, 1995; Saini and Gladstones, 1986; Reddy and Salunkhe, 1980; Rao and Belavady, 1978; Saini and Knights, 1984). Alfalfa meal is sometimes added to the poultry diet for the sake of yolk and skin color (Sunde, 1992). Consequently, it would be useful to know how fermentation of its carbohydrate fraction may affect the microbial community of broilers.

Soybean meal (SBM) is widely used in poultry production, mainly for its protein content, though it actually contains about the same amount of carbohydrates as protein. The latter contain a relatively large amount of soluble indigestible carbohydrates, both oligosaccharides and polysaccharides. Oligosaccharides account

for approximately 4-5 % of the dry weight of SBM (Kawamura and Narasaki, 1961, Obendorff *et al*., 1998). It consists mainly of stachyose [STA: ß-D-fructofuranosyl-*O*-α-D-galactopyranosyl-(1→6)- α-D-galactopyranosyl-(1→6)- α-D-glucopyranoside] and raffinose [RAFF: ß-D-fructofuranosyl-*O*-α-D-galactopyranosyl-(1→6)-α-D-glucopyranoside]. Soy oligosaccharides cannot be digested by the endogenous digestive enzymes of the broiler. However, they can be fermented by microorganisms inhabiting the hindgut (Cummings, 1989; Van Loo, 1995).

The aim of this experiment was therefore, to evaluate the fermentability of some natural sources of water-soluble indigestible carbohydrates using the cumulative gasproduction technique (Davies *et al.* 1995). Changes in the microbial communities before and after *in vitro* fermentation of these substrates were then compared, using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) (Vaughan *et al.* 1999). Variation in the fermentation kinetics and gut microbial community of the natural (soybean meal oligo- and water-soluble polysaccharides, alfalfa meal oligo- and water-soluble polysaccharides) and synthetic (raffinose and stachyose) non-digestible carbohydrates was examined.

Isolation of oligosaccharide and water-soluble polysaccharides from soybean meal and alfalfa meal

Soybean meal oligosaccharides (SMO) and water-soluble polysaccharides (SMP), as well as alfalfa meal oligosaccharides (AMO) and water-soluble polysaccharides (AMP) were extracted from soybean meal and alfalfa meal as follows: soybean meal or alfalfa meal was dried overnight at 45 °C and ground through a 1 mm sieve. 1kg sample was refluxed twice in 10 l alcohol (80 % (v/v)) at 75 °C for 1 h, then filtrated using a filter paper (Schleicher & Schuell, 0859). The liquid part (Part 1) was used for oligosaccharides isolation and the solid residue (Part 2) for water-soluble polysaccharides isolation.

For oligosaccharides (SMO and AMO) isolation, the alcohol of Part 1 was evaporated using a rotavapor (Büchi RE 120), and the residue was dissolved in 500ml

demineralised water. Protein was then removed by adding an equal volume of 10 % trichloroacetic acid (TCA). After standing for 2 h, the protein was separated by centrifugation at 2500 g for 20 min. The supernatant containing oligosaccharides was kept at 4 °C for further use. In order to remove TCA and amino acids from the supernatant, two different kinds of ion-exchange resins were used. The first one was Amberlite 200, Na^+ form (Fluka, 06437). Before use, the column with 800 g Amberlite 200 was washed with an excess 3 M HCl solution, then washed with de-mineralised water till no H^+ comes out (detected by pH test paper: pH=7). Next the oligosaccharide solution was run through the column with Amberlite 200. The column was washed three times with an equal volume of water. The oligosaccharide solution and the wash liquid were combined (solution A).

The second resin was Amberlite IRA-900, Cl^- form (Fluka, 06461). A column was filled with 800 g Amberlite IRA-900 and washed with an excess 3 M NaOH solution, then washed with de-mineralised water till no OH^- comes out (detected by pH test paper: pH=7). Solution A was passed through the column, collecting the solution. The column was washed three times with equal volumes of water. The oligosaccharide solution and the washing liquid were combined, frozen and freeze-dried, giving the oligosaccharide product.

Polysaccharides (SMP and AMP) were obtained by an alkaline extraction procedure. Part 2 was hydrolysed with 10 l of a 0.2 M NaOH solution at 80 °C for 2 h and filtrated using cheesecloth. The filtrate was neutralised with 30 % acetic acid and freeze-dried. The freeze-dried sample was dissolved in 400 ml water. Protein was removed by adding an equal volume of 10% TCA. After 1 h, the supernatant was centrifuged at 13000 g for 30 min. Polysaccharides was precipitated by adding alcohol to the supernatant till the alcohol concentration was 80 % (v/v) and kept at 4 °C over night. The polysaccharide precipitate was centrifuged at 2500 g for 10 min. The polysaccharide product was freeze-dried.

Replicate products extracted at different times were mixed together and stored at -20 °C .

Test substrates

Six products were tested for their fermentability. Four extracted non-digestible carbohydrates (ENC): soybean meal oligosaccharides (SMO), soybean meal polysaccharides (SMP), alfalfa meal oligosaccharides (AMO) and alfalfa meal polysaccharides (AMP). Two pure sugars which are known to exist within the soybean oligosaccharide fraction: raffinose (RAFF) and stachyose (STA)(Pfanstiehl Laboratories, Inc. USA) were also studied. Total sugar contents of the ENC were determined by the phenol-sulphuric acid assay method (Dubois *et al.,* 1956) and crude protein was analyzed by a semi-automated Kjeldahl technique (ISO, 1997).

Individual sugars in ENC were analyzed based on the Seaman hydrolysis method (Garleb *et al*., 1989) with some modifications. Briefly, samples (40 mg) of each ENC were hydrolyzed by weighing into Kimax tubes. The tubes were put in an ice-bath and 0.45 ml 72 % (w/w) H_2SO_4 added. The tubes were then put in a heating block at 30 °C for 1 h. After this pretreatment, distilled water (4.95 ml) was added to the mixture and mixed well using a vortex mixer. The tubes were heated at 100 °C for 3h in the heating block and then allowed to cool to room temperature. After cooling, the hydrolysates (120 µ l) were added to the tubes with water (2.4 ml) and neutralized with 1.0M $Ba(OH)_2$ (480 µ l), before being filtered through a 0.2 µm nylon filter. Samples were stored at 4°C for sugar composition analysis. Rhamnose, arabinose, galactose, glucose, mannose and xylose were used as standard samples. HPAEC of the samples was performed in a Dionex DX-500 Bio-LC system (Dionex Corp., Sunnyvale, CA), using a CarboPac PA 10 column (250 x 4 mm), and detection was made using a pulsed amperometric detector (Dionex Corp., Sunnyvale, CA). Data were collected and analyzed on a computer equipped with the Dionex PeakNet software.

In vitro Fermentability

In vitro gas production was measured using the automated system of Davies *et al*. (2000). Approximately 1.0 g of extracted carbohydrates (SMO; SMP; AMO and AMP) and 0.5 g (RAFF and STA) from each pure sugar were weighed into 100 ml

serum bottles and dissolved in 82 ml medium B (Lowe *et al.* 1985) overnight. Two negative control bottles were included (Blank before fermentation (BLAN-B) and blank after fermentation (BLAN-A), which contained the same medium and inoculum but no substrate. Three replicates were used for each of the six substrates. All bottles were inoculated with 5ml of inoculum and incubated at 39 °C for 72 h, BLAN-B were kept at -20 °C pending further analysis, immediately following inoculation.

Caecal contents were collected from forty 81-day old broilers within 15 minutes after slaughter. As inoculum, these birds had been grown under "organic" conditions, which meant that they were a slow-growing breed, and had been given a diet free of added copper and antibiotics. The diet was based on corn and soybean meal. At the abattoir, caecal contents from 40 birds was added to a pre- warmed thermos flask, which had been filled with CO_2 gas. Upon arrival at the laboratory, (~1 hour post-collection) the caecal contents were homogenized using a hand-held mixer for 60 seconds, diluted 1:4 with saline (9 g/l NaCl), and filtered through a double-layer of cheesecloth under a constant stream of CO_2.

At the end of fermentation, the pH of fermented solution in each bottle was recorded using a pH meter. The solid residue remaining at the end of fermentation was separated by centrifugation at 10,000 rpm for 10 min. Samples were taken of the remaining supernatant and stored pending analysis for VFA and ammonia. VFA analysis was determined using GLC (Fisons HRGC Mega 2: CE Instruments, Milan, Italy- glass column filled with Chromosorb 101, carrier gas N_2 saturated with methanoic acid, 190 ºC with *iso*-caproic acid as the internal standard). Total VFA concentration (mMTot) in millimoles, total VFA production (Atot) in units of acetic acid equivalents, and the branched-chain ratio (BCR) were calculated according to Bauer *et al*. (2001). Acetic acid equivalents (Henry, 1981) allow a comparison to be made between acids, based on their carbon content.

Ammonia-N content was determined according to the method described by Scheiner (1976). Supernatants were deproteinized using 10 % trichloric acetic acid.

Curve fitting gas production profiles

Cumulative gas production (ml / g DM) was fitted to a multi-phasic Michaelis-Menten equation (Groot *et al.,* 1996). The mono-phasic model was used unless the diphasic model significantly improved the fit (Motulsky and Ransnas, 1987). The model equation was:

$$Y = \sum_{i=1}^{n} \frac{Ai}{1+\left(\frac{Ci}{t}\right)^{Bi}}$$

Where Y = the cumulative gas production (ml / g DM); A_i = asymptotic gas production for phase i (ml); B_i = smoothness factor for phase i; C_i = time at which half of the asymptotic gas has been produced for phase i (h); i = number of phase in gas production; t = time (h).

The maximum rate of gas production (R_{max}) and the time at which it occurs (T_{Rmax}) were calculated according the following equations (W. J. J., Gerrits, Animal Nutrition group, Wageningen University-personal communication):

$$R_{max} = (A \cdot (C^B) \cdot B \cdot (T_{Rmax}^{(-B-1)})) / (1 + (C^B) \cdot (T_{Rmax}^{(-B)}))^2$$

$$T_{Rmax} = C \cdot (((B-1) / (B+1))^{(1/B)})$$

PCR/DGGE

The solid residue remaining at the end of fermentation and after centrifugation was re-suspended in phosphate buffer solution (PBS) and stored at -20 ^{o}C for further use. Of the stored solution, 1ml was used for microbial DNA fingerprint analysis by the PCR technique (Vaughan *et al.* 1999). Total DNA was extracted from samples and used as templates to amplify fragments of the 16S rDNA gene. PCR products of V6 to V8 regions were analyzed by DGGE following the same procedure as described by Konstantinov *et al*., (2003). This technique allows genes of different bacteria to be separated from each other, and then the DGGE profiles can be analyzed for

similarities between the samples by a computer program (Molecular Analyst version 2.15).

Data analysis

Data were analyzed statistically using SAS analysis software (Statistical Analysis Systems 6.11, 1995). The General Linear Models procedure was used for analysis of variance, and Tukey's studentized range test of multiple comparisons (Steel and Torrie, 1980) was used to test for significantly different treatment means.

RESULTS AND DISCUSSION

Fermentation substrate characteristics:

Total sugar, crude protein, and raffinose (RAFF) and stachyose (STA), contents of extracted non-digestible carbohydrate (NEC) were analyzed, and the results are shown in Table 1. There were 23.5 g/kg oligosaccharide (AMO) and 21.5 g/kg polysaccharide (AMP) extracted from alfalfa meal, and 88.5 g/kg oligosaccharide (SMO) and 40.5 g/kg polysaccharide (SMP) extracted from soybean meal. Of the AMO, 58.3 % were recovered as individual sugars. For AMP and SMP these figures were 61.3 and 72.7 % respectively. Unfortunately, individual sugars could not be measured in SMO due to unexpected polymerization of the sample. Galactose and raffinose were the major ingredients of SMP. AMO consisted mainly of glucose. A greater diversity of sugars was in AMP. There was a higher protein content in the polysaccharide compared with the oligosaccharide fractions. The ENC content of soybean meal was relatively higher both in oligosaccharides and polysaccharides (SMO and SMP) compared with alfalfa meal (AMO and AMP). SMO had the highest total sugar content of the four ENC samples (AMO, AMP, SMO and SMP).

Fermentation kinetics

The parameters describing cumulative gas production are shown in Table 2.

Table 1. Chemical component of extracted non-digestible carbohydrate (ENC) (g/kg)

	DM	Protein	Total sugar	Rhamnose	Arabinose	Galactose	Glucose	Mannose	Xylose
AMO	980.5	59.6	326	10	10	10	160	0	0
AMP	962.7	146.5	408	50	10	60	60	20	50
SMO	985.8	15.7	724	_[1]	_[1]	_[1]	_[1]	_[1]	_[1]
SMP	965.5	504.6	275	50	10	120	10	10	0

[1] Not analyzed

Table 2. Mean parameters of in vitro gas production for six fermentation substrates

Sample	C(h)	Trmax(h)	Rmax(ml/h)	DMCV(ml/gDM)	pH	NH_3(mg/l)
Alfala meal						
AMO	4.7	1.9	20.4 [c]	152.9 [c]	6.3 [a]	286.3 [bc]
AMP	3.7	1.7	23.7 [c]	96.8 [c]	6.5 [a]	348.5 [b]
Soybean meal						
SMO	3.3	2.6	58.7 [b]	245.7 [b]	5.5 [b]	199.3 [d]
SMP	5.5	4.2	17.7 [c]	122.8 [c]	6.5 [a]	679.3 [a]
Sugar						
STA	3.5	2.9	77.548 [a]	327.1 [a]	6.3 [a]	252.8 [d]
RAFF	3.7	3.0	67.6 [ab]	299.6 [ab]	6.4 [a]	258.1 [cd]
SEM	1.7	0.7	3.5	11.3	0.1	21.2

Means within columns without common superscripts are significantly different ($P<0.05$). C represented the time (h) when there was half gas produced. T_{Rmax} represented the time (h) when there was the max rate of gas production. R_{max} represented the max rate of gas production. DMCV represented gas production of per gram dry matter (ml/g DM).

There were no significant differences between any of the substrates in terms of the half-time C and the $T_{Rmax.,}$ which are both indicators of fermentation kinetics after incubation However, for the maximum rate of gas production, both STA, RAFF and SMO were high. AMO, AMP, and SMP were all much lower. In terms of total gas production, this pattern was similar for DMCV, whereby STA, RAFF and SMO had high gas productions, and AMO, AMP, and SMP were comparatively lower. Cumulative gas production curves (Figure 1) showed that STA, RAFF and SMO had a higher cumulative gas production than AMO, AMP, and SMP. SMO had a different gas production pattern compared with both pure sugars and other extracted carbohydrates. SOM had a slower fermentation speed than the pure sugars in the initial period and did not differ from other extracted carbohydrates. In the later fermentation period SMO continued to produce gas when other carbohydrate had stopped producing gas (Figure 1).

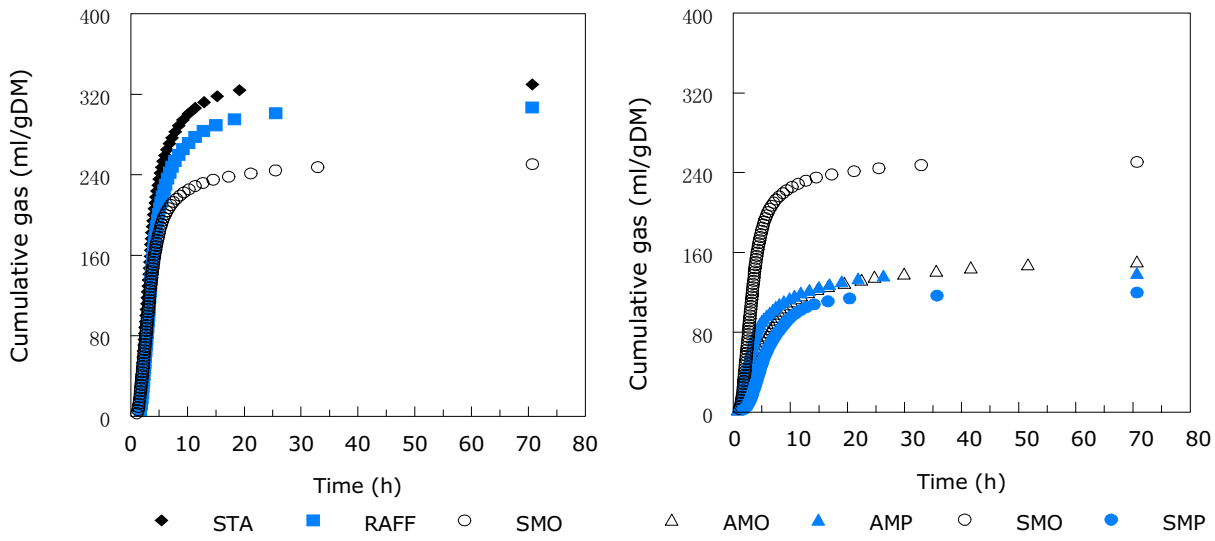

Figure 1. Gas production profile from fermentation of soybean meal oligosaccharides and water-soluble polysaccharides (SMO &SMP); alfalfa meal oligosaccharidesand water-soluble polysaccharides (AMO & AMP); pure sugars stachyose and raffinose (STA & RAFF)

Fermentation end-products

Values for VFA concentrations after 72 h fermentation are shown in Table 3, and these data are also shown graphically in Figures 2 and 3.

Table 3. VFA produced as mg per g dry matter disappeared during fermentation (mg/g DM)

Sample	ACC	APR	ABU	Atot(mmol/gD)	Atotal	BCR
Alfalfa meal						
AMO	223.3 ^{b}	167.5 ^{ab}	71.8 ^{d}	6.4 ^{c}	490.5 ^{d}	0.1 ^{b}
AMP	226.0 ^{b}	138.8 ^{c}	84.5 ^{cd}	6.7 ^{c}	510.0 ^{cd}	0.1 ^{b}
Soybean meal						
SMO	261.8 ^{b}	187.2 ^{a}	155.2 ^{b}	8.0 ^{b}	640.3 ^{b}	0.1 ^{b}
SMP	240.2 ^{b}	128.6 ^{c}	100.3 ^{c}	6.9 ^{c}	563.2 ^{c}	0.2 ^{a}
Sugar						
STA	374.0 ^{a}	160.4 ^{b}	191.7 ^{a}	10.0 ^{a}	778.6 ^{a}	0.1 ^{b}
RAFF	378.8 ^{a}	137.0 ^{c}	191.7 ^{a}	10.0 ^{a}	759.4 ^{a}	0.1 ^{b}
SEM	7.9	7.4	7.1	0.2	14.9	0.01

Means within columns without common superscripts are significantly different ($P<0.05$). AAC, APR, ABU and Atotal represented the disappeared material produced acetic, propionic, butyric and total VFA (mg/g DM). Total branch in total straight chain VFA (BCR) was calculated by (iso-butyric acid + iso-valeric acid + valeric acid)/(acetic acid + propoinic acid + butyric acid)

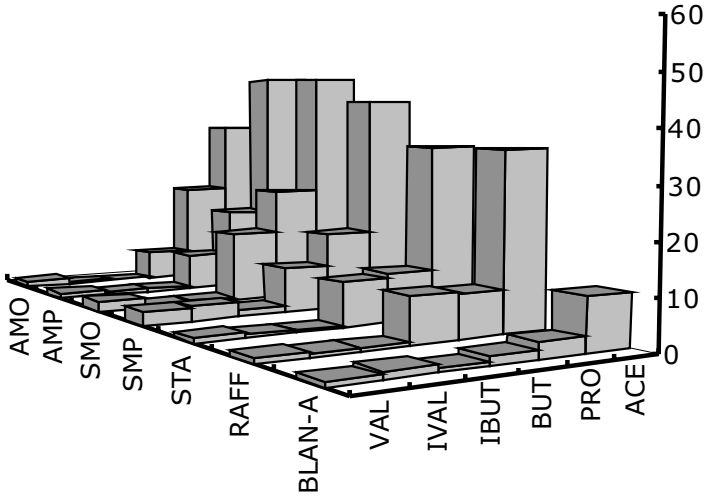

Figure 2.VFA concentration (mMol)(y) in solutions after 72 h fermentation. Each pillar represented a mean of one fermentation acid. The SEM was 1.74 (acetic acid, ACE), 1.04 (propionic acid, PRO), 0.07 (iso-butyric acid, IBUT), 0.55 (butyric acid, BUT), 0.15 (iso-valeric acid, IVAL) and 0.11 (valeric acid, VAL).. VFA concentration of four extracted non-digestible carbohydrates: soybean meal oligosaccharides (SMO) and water-soluble polysaccharides (SMP), alfalfa meal oligosaccharides (AMO) and water-soluble polysaccharides (AMP); two pure sugar: raffinose (RAFF) and stachyose (STA), as well as the blank incubation (BLAN-A) were shown in this Figure.

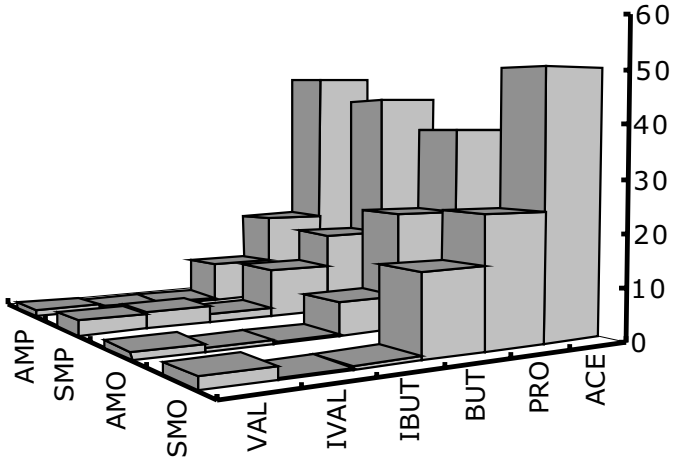

Figure 3. VFA concentration (mMol)(y) in solutions of four extracted non-digestible carbohydrates after 72 h fermentation. Each pillar represented a mean of one fermentation acid. The SEM was 1.74 (acetic acid, ACE), 1.04 (propionic acid, PRO), 0.07 (iso-butyric acid, IBUT), 0.55 (butyric acid, BUT), 0.15 (iso-valeric acid, IVAL) and 0.11 (valeric acid, VAL). VFA concentration of four extracted non-digestible carbohydrates: soybean meal oligosaccharides (SMO) and water-soluble polysaccharides (SMP), alfalfa meal oligosaccharides (AMO) and water-soluble polysaccharides (AMP) were shown in this Figure.

Fermentation of the two pure sugars (STA and RAFF), resulted in significantly higher total VFA ($P<0.05$), and also, more specifically, in higher acetic and butyric acid concentrations. Compared with SMP, pure sugars also had significantly lower values of NH_3 in the final medium. Generally speaking, there was no difference between the AMO and AMP in terms of the fermentation end-products. Fermentation of SMO led to a significantly increased butyric and total acid production ($P<0.05$) compared with the other ENC, and the lowest ammonia concentration in all test material. SMP, on the other hand, had the highest ammonia concentration, and the highest BCR. These higher values were most likely related to the presence of a higher protein content for this substrate (Figure 4).

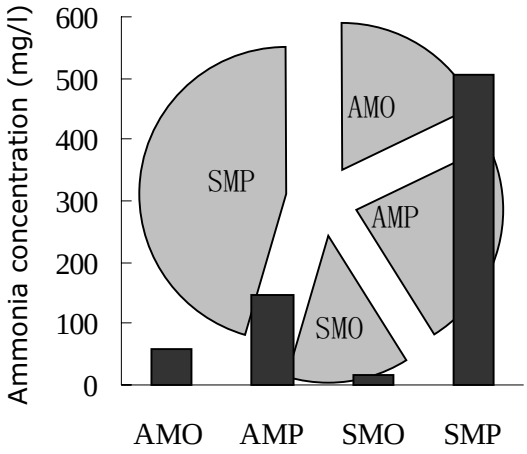

Figure 4 Protein content (ml / g DM) in extracted carbohydrates (pies) and ammonia concentration (mg / l) (columns) in fermentation solutions.

SMO = soybean meal oligosaccharids

SMP = soybean meal water-soluble polysacchars

AMO = alfalfa meal oligosaccharids

AMP = alfalfa meal water-soluble polysaccharides

If proteins are used as a source of energy by microorganisms, this will result in different end-products compared to carbohydrates (McDonald *et al.,* 1995). Firstly, they are hydrolyzed to peptides and amino acids by microorganisms, and some amino acids are then degraded further, to branched-chain VFA and ammonia-N during anaerobic fermentation (Wright, 1971). Some of these products (e.g amino acids and peptides) can also be incorporated into microbial protein (MCP). It has also been suggested that fermentation of protein produces less gas per g of N-containing matter

compared to carbohydrates (Pell *et al.,* 2000, Cone *et al.,* 2000). In this research, SMP had a significantly higher amount of protein (Table 1), a relatively lower DMCV, a later C and T_{Rmax}, lower VFA production and a higher ammonia concentration. This suggests that of all the substrates tested, SMP was least fermentable by the bacteria from the caecal content which was used as inoculum. This tends to confirm the relation between fermentation and high protein content postulated by others.

SMO showed the lowest ammonia concentration in all tested substrates, and even lower than the blank control after fermentation (373.4 mg/l, data not shown in table). This means that SMO has probably resulted in more biomass (Lee, 1977). It has been reported that caecal bacteria can incorporate ammonia nitrogen and produce microbial protein (Karasawa, 1999). If fermentation of SMO had resulted in increased biomass (which could not be measured here), it could be expected that it would mainly be produced towards the end of the GI tract. As a result, less N would be absorbed and would therefore not be excreted as uric acid following processing by the kidneys. It could also be expected that in that case it would be less harmful to the external environment, because ammonia production from biomass in excreta would occur at a slower rate than from uric acid. Therefore, in theory, by addition of a suitable fermentable carbohydrate in poultry diets, air pollution by ammonia could be reduced, as shown by van Berlo *et al*. (1988) for pigs. Whether this is of significance for broilers still needs to be investigated.

VFA are the end products of both carbohydrate and protein fermentation. It is known that VFA make a physiologically significant contribution to the health of the colonic mucosa, as well as to the energy supply of the host (Fleming *et al.,* 1986). Total VFA production in this experiment was positively related with the sugar content of tested substrates (Figure 5). It seems that significant protein fermentation took place in the SMP group, given the higher BCR and ammonia compared with the other substrates ($p<0.05$). Undoubtedly, this is also related to protein content (Figure 3). Compared with other ENC however, fermentation of the SMO produced higher total VFA, especially butyric acid ($p<0.05$). Data obtained from several *in vitro* studies strongly suggest that butyrate could potentially act as a therapeutic agent by inhibiting

the growth of some cancer cells (Basson *et al.,*1996; Hague *et al., et al.,*1997; Velazquez *et al.,* 1996; 1997, Medina *et al.,*1998; D'Argenio *et al.,*1999). Furthermore, butyrate is more effective than acetate or propionate in enhancing sodium (Na) absorption. This capacity is of considerable importance on limiting fluid losses in those acute diarrhoeal illnesses that are primarily characterized by small intestinal fluid secretion (Williams *et al*., 2001).

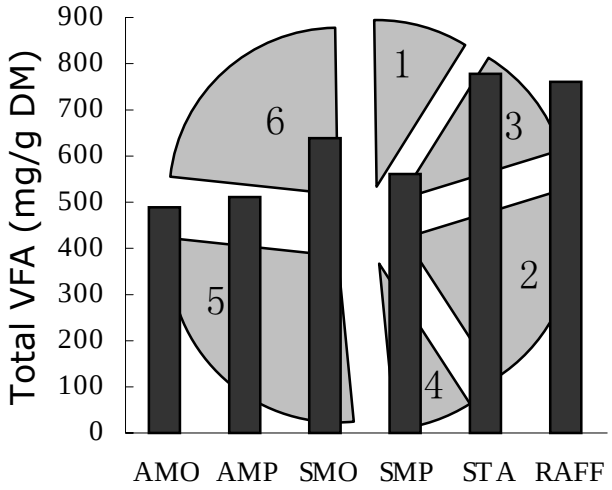

Figure 5. Total VFA(mg /g DM) (colums) in fermentation solutions and sugar content (g/kg DM) (pies) in extracted carbohydrates and pure sugars.

SMO (2) = soybean meal oligosaccharides

SMP (4) = soybean meal water-soluble polysaccharides

AMO (1) = alfalfa meal oligosaccharides

AMP (3) = alfalfa meal water-soluble polysaccharides

STA (5) = stachyose

RAFF (6) = raffinose

According to these results relating to microbial activity *in vitro*, in terms of VFA and ammonia production, it would appear that SMO are the most promising for addition to poultry diets. VFA can benefit the host in several ways, such as an energy supply to intestinal cells, and by limiting pathogenic infection by decreasing chyme pH. Furthermore, the increased microbial population, which could potentially attach to the gut mucosal surface, could also lead to the competitive exclusion of potential pathogens. The decreased fecal ammonia-N would be helpful for reducing aerial ammonia concentration in nursery poultry facilities.

Similarity index of bacterial community

Following fermentation for 72 hours, samples were collected, and analyzed for bacterial 16S rDNA (V6-V8 regions) by DNA extraction procedures, PCR amplification and the DGGE technique. DGGE profiles were analyzed for similarities between samples using a computer program (Molecular Analyst version 2.15) (Figure 6).

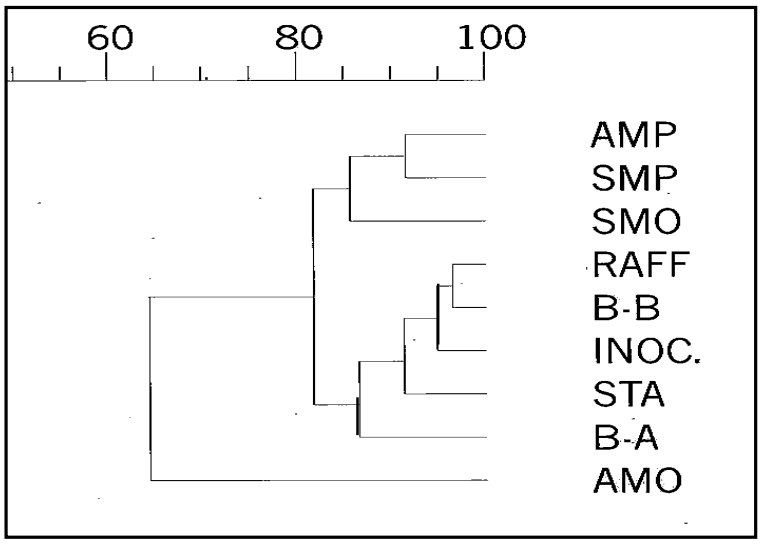

Figure 6. Similarity index of bacterial fingerprints. The ruler on top is similarity as %. The substrates analyzed here are alfalfa meal oligosaccharides(AMO) and polysaccharides (AMP); soybean meal oligosaccharides (SMO) and polysaccharides (SMP); raffinose (RAFF) and stachyose (STA); blank control before (B-B) and after fermentation (B-A), and inoculum (INOC)

DGGE analysis results revealed that fingerprints of the caecal bacterial communities before and after fermentation of the different substrates had a similarity of > 80 % between AMP, SMO, and SMP. Also, STA, RAFF, and BLAN-A had very similar microbial profiles. The original inoculum (INOC) also had a very similar profiles (C, value > 80%) compared with the rest. Only the AMO group had a profile which was quite different from the other substrates (C, value >60%). In general, the intestinal microbial ecosystem is considered to be quite stable, once established (Mead, 1989). In this experiment, the caecal contents came from 81-day old broilers, and the microbial population in the ceca was fully developed at that age (Barnes *et al.,* 1972). Also, there were balanced amounts of nutrients in the basal medium to enable survival or

maybe development for a wide range of microbial species. However, apart from raffinose, it can still be seen that within different substrates, some selection of species must occur (Figure 7). This was most markedly for AMO, though interestingly, the AMO did not show significant differences in terms of microbial activity, compared with AMP. However, it must also be remembered that a change in microbial activity, may not necessarily result in a dramatic change in the microbial profile as such.

It must also be remembered that the *in vitro* fermentation procedure is somewhat different from fermentation *in vivo*. In the caecum of broiler chicken, chyme will flow in and flow out continuously. Therefore, those microorganisms which are unattached will be mobile. In contrast, gas is the only material which can escape from the reaction medium in the *in vitro* gas production measurement system, while bacteria remain in the incubation solution throughout the experimental period. Therefore, the bacteria which were not stimulated by those fermentable carbohydrates, and even the dead bacteria remained in the culture. As a result, both living and dead bacterial DNA may have been present in the fermentation solution, and their DNA fingerprint will also be found in the DGGE profile. In other words, the selective properties of those non-digestible carbohydrates on the caecal content microbial community would be underestimated in this experiment and further experiments are required.

In terms of broiler gut health (and potential environmental pollution due to the release of ammonia from poultry waste), it would appear that SMO was the most promising feed ingredient given its higher VFA and lower NH_3 production *in vitro*. However, whether this difference would also be reflected in the animal, still remains to be tested.

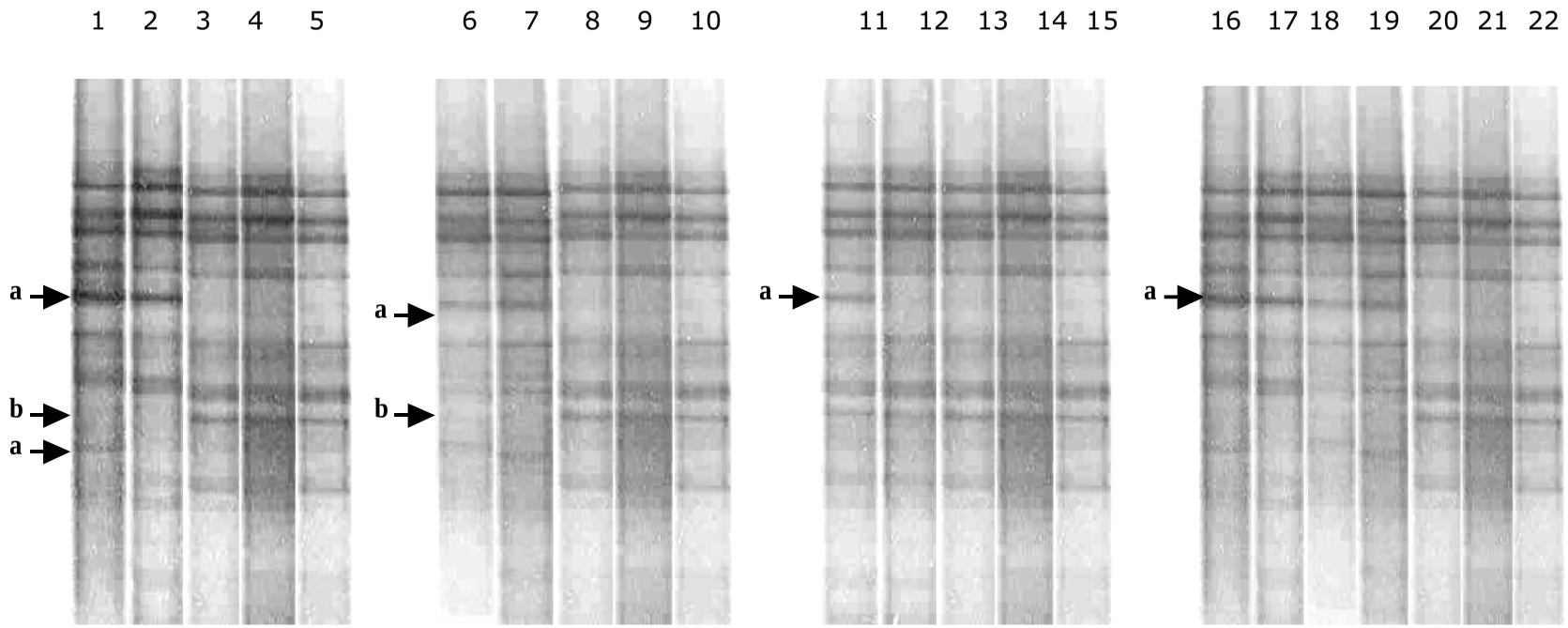

Figure 7. DGGE band pattern of bacteria community profiles of samples containing the extracted non-digestible carbohydrates from soybean meal and alfalfa meal. DGGE profile lanes: 1 and 16: alfalfa meal oligosaccharides (AMO); 2 and 17: soybean meal oligosaccharides (SMO); 6 and 18: alfalfa meal water-soluble polysaccharides (AMP); 7 and 19 soybean meal water-soluble polysaccharides (SMP); 11: stachyose (STA); 12: raffinose (RAFF); 3, 8, 13 and 20: blank before incubation; 4, 9, 14 and 21: blank after incubation; 5, 10, 15 and 22: inoculum. Solid arrowheads indicate the position where the band become predominant (a) or disappear (b). as compared with inoculum and blank.

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REFERENCES

Barnes, E. M., G. C. Mead, D. A. Barnum, and E. G. Harry, 1972. The intestinal flora of the chicken in the period 2 to 6 weeks of age, with particular reference to the anaerobic bacteria. Competitive exclusion of Salmonellas from the newly hatched chicken. British Poultry Science. 12: 311-326.

Basson, M. D., G. A. Turowski, Z. Rashid, F. Hong, J. A. Madri, 1996. Regulation of human colonic cell line proliferation and phenotype by sodium butyrate. Digital Dissertation Science. 41(10): 1989-1993.

Bauer, E., B. A. Williams, C. Voigt, R. Mosenthin, and M. W. A. Verstegen, 2001. Microbial activities of faeces from unweaned and adult pigs, in relation to selected fermentable carbohydrates. Animal Science 73: 313-322.

Brady, L. J., D. D. Gallaher and F. F. Busta, 2000. The role of probiotic cultures in the prevention of colon cancer. Journal of Nutrition. 130: 410S-414S.

Buddington, R. K., and Evan Weiher, 1999. The application of ecological principles and fermentable fibers to manage the gastrointestinal tract ecosystem. Journal of Nutrition. 129: 1446S-1450S.

Cavazzoni, V., A. Adami and C. Castrovilli, 1998. Performance of broiler chickens supplemented with Bacillus coagulans as probiotic. British Poultry Science. 39: 526-529.

Cone, J. W. and A. H. Van Gelder, 2000. Influence of protein fermentation on gas production profiles. An EAAP Satellite Symposium: Gas production: Fermentation kinetics for feed evaluation and to assess microbial activity. Wageningen, The Netherlands. P: 23-24. .

Coon, C. N., K. L. Leske, O. Akavanichan and T. K. Cheng, 1990. Effect of oligosaccharide free soybean meal on true metabizable energy and fiber digestion in adult roosters and broilers. Zootecnica International September, 44-50.

Cummings, J. H., G. R. Gibson and G. T. Macfarlane, 1989. Quantitative estimate of fermentation

in the hindgut of man. Acta Veterinarica Scandinavica. 86: 76-82.

D'Argenio, G, G. Mazzacca, 1999. Short-chain fatty acid in the human colon. Relation to inflammatory bowel diseases and colon cancer. Advances in Experimental Medical Biology, 472: 149-58.

Davies, D. R., M.K. Theodorou, J. Baughan, A. E. Brooks and J. R. Newbold, 1995. An automated pressure evaluation system (APES) for determining the fermentation characteristics of ruminant feeds. Annales. de Zootechie. 44: Suppl. 36.

Davies, Z. S., D. Mason, A. E. Brooks, G. W. Griffith, R. J. Merry and M. K. Theodorou, 2000. An automatic system for measuring gas production from forages incubated with rumen fluid and it use in determinating the effect of enzymes. Animal Feed Science and Technology. 83(3-4):205-221.

Dubois, M., K. A. Gilles, J. K. Hamilton, D. A. Rebers and F. Smith, 1956. Analytical Chemistry. 28:350-356.

Dunne, C., 2001. Adaptation of bacteria to the intestinal niche: probiotics and gut disorder. Inflammatory Bowel Diseases. 7(2):136-45.

Durst, L., 1996. Inclusion of fructo- and galacto-oligosaccharides in broiler diets. Archiv fur Geflugelkunde. 60:160-164.

Fleming, S. E., 1981. A study of relationship between flatus potential and carbohydrate distribution in legume seeds. Journal of Food Science. 46-794-798.

Fleming, S. E., D. S. Arce, 1986. Volatile fatty acids: their production, absorption, utilization, and roles in human health. Clinical Gastroenterology. 15(4): 787-814.

Gallaher, D. D., 1999. The effect of symbiotic on colon carcinogenesis in rats Journal of Nutrition 129: 1483S-1487S.

Garleb, K. A., L. D. Bourquin and G. C. Jr., Fahey, 1989. Neutral monosaccharide composition of various fibrous substrates: A comparison of hydrolytic procedures and use of anion-exchange high-performance liquid chromatography with pulsed amperometric detection of monosaccharides. Journal of Agricultural and food Chemistry. 37(5): 1287-1293.

Gibson, G. R., 1998. Dietary modulation of the human gut microflora using prebiotics. British Journal of Nutrition. 80(4):S209-212.

Gibson, G. R., 1999. Dietary modulation of the human gut microflora using prebiotics oligofructose and lnulin. Journal of Nutrition. 129: 1438S-1441S.

Groot, J. C. J., B. A. Williams, F. M. A. Debersaques and E. A. Lantinga, 1996. Multiphasic analysis of gas production kinetics for *in vitro* fermentation of ruminant feeds. Animal Feed Science and Technology. 64:77-99.

Hague, A., B. Singh, and C. Paraskeva, 1997. Butyrate acts as a survival factor for colonic epithelial cells: further fuel for the in vivo versus in vitro debate. Gastroenterology. 112 (3): 875-81.

Henry, D. P., 1981. Studies on Canadian ingens a potential feed and food yeast. Ph.D thesis. University of Queensland.

Iji, P. A. and D. R. Tivey, 1998. Natural and synthetic oligosaccharides in broiler chicken diets. World's Poultry Science Journal. 54:129-143.

ISO (International Organization for Standardization), 1997. Animal Feeding stuffs-Determination of nitrogen content and calculation of crude protein content. (ISO 5983), Genéve, Switzerland.

Jenkins, D. J. A., C. W. C. Kendall and V. Vuksan, 1999. Inulin, oligofructose and intestinal function. Journal of Nutrition 129: 1431S-1433S.

Karasawa, Y., 1999. Significant role of the nitrogen recycling system through the ceca occurs in protein-depleted chickens. Journal of Experimental Zoology. 283:418-425.

Kawamura, S. and T. Narasaki, 1961. Study on the carbohydrates of soybean. Agricultural. Biological. Chemistry. 25: 527-531.

Konstantinov, S. R., W-Y. Zhu, B. A.Williams, S.Tamminga, W.M.de Vos, and A. D. L. Akkermans, 2003. Effect of fermentable carbohydrates on piglet faecal bacterial communities as revealed by denaturing gradient gel electorphoresis analysis of 16S ribosomal DNA. FEMS Microbial Ecology 43:225-235.

Lee, D. J., 1977. Urease activity in the digestive tract of the chick and its role in the utilisation of urea as a source of non-amino nitrogen. Brithish Poultry Science. 18:449-458.

Leske, K. L., C. J. Jevne and C. N. Coon, 1993. Effect of oligosaccharide additions on nitrogen-corrected true metabolizable energy of soy protein concentrate. Poultry Science. 72:664-668.

Leske, K. L., O. Akavanichan, T. K. Cheng and C. N. Coon, 1991. Effect of ethanol extract on nitrogen corrected true metabolizable energy for soybean meal with broilers and roosters. Poultry Science. 70:892-895.

Lowe, S. E., M. K. Theodorou, A. P. J. Trinci, and R. B. Hespell, 1985. Growth of anaerobic rumen fungi on defined and semi-defined media lacking rumen fluid. Journal of General Microbiology. 131: 2225-2229.

McDonald, P., R. A. Edwards, J. F. D. Greenhalgh and C. A. Morgan, 1995. Animal Nutrition (Fifth Edition). Produced by Longman Singapore Publishers (Pte) Ltd. p116.

Mead, G. C., 1989. Microbes of the avian cecum: types present and substrates utilized. The Journal of Experimental Zoology (Suppl.) 3: 48-54.

Medina, V., J. J. Afonso, H. Alvarez-Arguelles, C. Hernandez, and F. Gonzalez, 1998. Sodium butyrate inhibits carcinoma development in a 1,2-dimethylhydrazine-induced rat colon cancer. Journal of Parenterol Enteral Nutrition. 22(1): 14-7.

Menne, E., and N. Guggenbuhl, 2000. Fn-type chicory inulin hydrolysate has a prebiotic effect in humans. Journal of Nutrition 130: 1197-1199.

Mohamed, A. A. and P. Rayas-Duarte, 1995. Composition of *Lupinus albus.* Cereal Chemistry. 72:643-647.

Motulsky, H. J. and L. A. Ransnas, 1987. Fitting curve to data using nonlinear regresion: A practical and nonmathematical review. Faseb J. 1: 365-374.

Obendorf, R. M. Horbowicz, A. M. Dickerman, P. Brenac and M. E. Smith, 1998. Soluble oligosaccharides and galactosyl in maturing soybean seeds. Crop Science. 38:78-84.

Pell, A. N., D. O. Molina and P. Schofield, 2000. Measurement gas production in vitro. An EAAP Satellite Symposium: Gas production: Fermentation kinetics for feed evaluation and to assess microbial activity. Wageningen, The Netherlands. P: 1-12.

Petterson, D. S. and J. B. Makintosh, 1994. The Chemical Composition and Nutritive Value of Australian Grain Legumes, Grains Research and Corporation, Brisbane, Australia, pp.10-13, 38-41.

Rackis, J. J, D. H. Honig, D. J. Sessa, and F. R Steggerda, 1975. Flavor and flatulence factors in soybean protein products. Journal of Agricultural Food Chemistry. 18: 977-82.

Rackis, J. J., 1981. Flatulence caused by soy and its control through processing. Journal of the American Oil Chemists Society. 58: 503-9.

Rao, A. V., 1999. Dose-response effects of inulin and oligofructose on intestinal bifidogenesis effects. Journal of Nutrition. 129: 1442S-1445S.

Rao, P. U. and B. Belavady, 1978. Oligosaccharides in pulses: varietal differences and effect of cooking and germination. Journal of Agriculture Food Chemistry. 26:316-319.

Reddy, B. S., 1999. Possible mechanisms by which pro- and prebiotics influence colon carcinogenesis and tumor growth. Journal of Nutrition.129: 1478S-1482S.

Reddy, N. R., and D. K. Salunkhe, 1980. Changes in oligosaccharides during germination and cooking of black gram and fermentation of black gram/rice blend. Cereal Chemistry. 57: 356-360.

Ruppin, H., S. Bar-Meir, K. H. Soergel, C. M. Wood and Jr. M. G. Schmitt, 1980. Absorption of short chain fatty acids by the colon. Gastroenterology 78: 1500-1507.

Saini, H. S. and E. J. Knights, 1984. Chemical constitution of starch and oligosaccharide component of "Desi" and "Kabuli" chickpea (*Cicer arietinum*) seed types. Journal of Food Chemistry. 32:940-944.

Saini, H. S. and J. S. Gladstones, 1986. Variability in the total and component galactosyl sucrose oligosaccharides of *Lupinus* species. Australian Journal of Agricultural Research. 37:157-166.

Sander, M. E., 2000. Considerations for use of probiotic bacteria to modulate human health. Journal of Nutrition. 130: 348S-390S.

Scheiner, D., 1976. Determination of ammonia and Kjeldahl nitrogen by indophenol wethod. Water Research. 10:31-36.

Steel, R. G. D. and J. H.Torrie, 1980. Principles and procedures of statistics: a biometrical approach, second edition. McGraw-Hill International Book Company, Singapore.

Sunde, M. L., 1992. The scientific way to pigment poultry products. Introduction to the symposium. Poultry Science. 71(4):709-710.

Unno, T., M. Sugawara, T. Nakakuki and G. Okada, 1993. Effect of b-gluco-oligosaccharides on the human intestinal microflora. Journal of Starch Related Carbohydrates Enzymes. 40: 21-27.

van Berlo, C. L., P. A. van Leeuwen, and P. B. Soeters, 1988. Porcine intestinal ammonia liberation. Influence of food intake, lactulose and neomycin treatment. Journal of Hepatology. 7(2):250-257.

Van Loo, J., P. Coussement, L. De Leenheer, H. Hoebregs and G. Smith, 1995. On the presence of inulin and oligofructose as natural ingredients in the western diets. CRC Critical Reviews on Food Science and. Nutrition. 35: 525-552.

Vaughan, E. E., G. H. J. Heilig, E. G. Zoetendal, R. Satokari, J. K. Collins and A. D. L. Akkermans, 1999. Molecular approaches to study probiotic bacteria. Trends Food Science and Technology. 10:400-404.

Velazquez, O. C., H. M. Lederer, and J. L. Rombeau, 1996 Butyrate and the colonocyte. Implications for neoplasia. Digital Dissertation Science. 41(4): 727-39.

Velazquez, O. C., H. M. Lederer, and J. L. Rombeau, 1997. Butyrate and the colonocyte. Production, absorption, metabolism, and therapeutic implications. Advances in Experimental Medical Biology, 427: 123-34.

Williams B. A., M. W. A. Verstegen and S. Tamminga, 2001. Fermentation in the large intestine of single stomached animals and its relationship to animal health. Nutrition Research Reviews, 14: 207-227.

Williams B. A., S. Tamminga and M. W. A. Verstegen, 2000. Fermentation kinetics to assess microbial activity of gastro-intestinal microflora. An EAAP Satellite Symposium. Gas production: fermentation kinetics for feed evaluation and to assess microbial activity. Published by the British Society of Animal Science. P97-100.

Wright, P. L., 1971. Protein and carbohydrate utilization by rumen microorganisms maintained in continuous in vitro culture. Dairy Science. 54(5):688-692.

Chapter 3

THE EFFECT OF SOYBEAN OLIGOSACCHARIDES AND POLYSACCHARIDES ON DEVELOPMENT OF INTESTINAL MORPHOLOGY OF BROILER POULTS

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The Effect of Soybean Oligosaccharides and Polysaccharides on Development of Intestinal Morphology of Broiler Poults

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Abstract: This experiment was conducted to investigate the effect of dietary water-soluble soybean meal polysaccharides (SMP) and oligosaccharides (SMO) on intestinal growth and mucosal morphology development in broiler chickens. SMO and SMP were used in a soybean meal-free diet for 14 days. In addition, the subsequent development of the intestines was examined for a period of two weeks after withdrawal at the diet. There were four dietary treatments in this experiment: a commercial diet as positive control (CON), which contained soybean meal and antibiotics. A soybean meal-free and antibiotic-free basal diet served as negative control (Diet NSM). The two other diets were obtained by diluting the NSM diet by either 1 % SMO or 0.5 % SMP (SMO and SMP). Intestinal weight and length were recorded of four intestinal segments (duodenum, jejunum, ileum and caecum) at 1, 3, 6, 9, 12, 15 and 28 days of age. The digestive tract-free body weight was recorded at 15 and 28 days of age. Intestinal morphology of duodenum, jejunum, ileum and caecum of broilers were investigated by scanning electron microscopy at 15 days of age. The results indicated that intestinal weight as a percentage of the digestive tract-free body weight(IW/ DTFBW × 100) showed that the birds fed the soybean meal-free diet had a higher IW/DTFBW of duodenum, jejunum and ileum than the CON group at 15 and 28 days of age. Birds in the SMO and SMP groups had a higher relative duodenal weight and a thinner caecal wall than the NSM group at Day 28 ($p<0.05$). Compensatory duodenal and ileal growth was noticed in the SMO and SMP birds after SMO and SMP were withdrawn from the diet. Birds in the SMO and SMP group had a significantly higher IW/DTFBW for duodenum than those in the CON and the NSM group ($P<0.05$) and a higher value for ileal relative weight than CON animals ($P<0.05$) at 28 days of age. Birds in the SMP and SMO groups had a smaller size of villi in duodenum, jejunum and ileum than in the NSM animals. A higher density and undeveloped micro-villi were observed in duodenum and ileum of the birds of the SMO and SMP groups

compared with the birds of the NSM group. Attached microorganisms were observed on the caecal mucosal surface for birds receiving the SMO diet. It was concluded that SMO and SMP administered in the early stage of post-hatch do not affect the development of the intestines relative weight and length when compared with the soybean meal-free fed animals at 15 days of age. However, SMO and SMP diets can significantly affect the morphological development of the gut mucosa. The SMO diet promoted the attached population of microorganisms on the caecal wall. Compensatory intestinal growth took place during 14 to 28 days of age after SMO and SMP were withdrawn from the diet.

Keywords: Soybean meal, Oligosaccharides, Polysaccharides, intestinal development, morphology, and broiler poults

INTRODUCTION

The period immediately after chickens hatch is critical for the animal's survival. During this time, birds must adapt to changes of nutrient source, and consequently, dramatic changes occur in size, as well as in morphology, immunology and function of the intestine (Cook and Bird, 1973; Bayer *et al.,* 1975; Uni *et al.,* 1996). Development of the digestive tract of poultry has been well documented (Sklan, 2001). There is a relatively rapid increase in gastrointestinal weight as a proportion of the whole body mass, which reaches a peak at 6-8 days, and subsequently declines (Ija *et al* 2001, Uni. *et al* 1999). Morphological observations of the small intestinal mucosa in chickens have indicated that the villus height increases two-fold in the first 48 hours after hatching, reaches a plateau at 6-8 days in the duodenum, but only reaches a similar plateau after ten days or more in both the jejunum and ileum. The width of the villi also increase slightly. The intestinal mucosal surface area can be estimated from height and width data, and a parallel increase is observed up until three days after hatching for the duodenum, jejunum and ileum, after which the jejunum increases considerably in absorptive area, whereas the duodenum and ileum increase more slowly (Geyra *et al.,* 2001). Meanwhile, the villus size and the number of enterocytes per villus also increase (Geyra *et al.,* 2001; Uni. *et al* 1995, 1996). The villus shape changes dramatically during the early stage of post-hatch. Villus shape and arrangement is completed by 10 days of age (Yamauchi *et al*, 1991).

The intestinal mucosal morphology may be related to body weight of chickens (Uni et al., 1999; Noy and Sklan, 1999). This is due to the mucosal enzyme activity per mass of intestine, which is responsible for the final stage of nutrient hydrolysis, and is closely correlated with the number of enterocytes per villus in all regions of the intestine after 2 days of age (Uni. *et al* 1999). The interactions between intestinal tract growth, mucosal cell formation, digestive function and diet, is critical during the post-hatch period when broilers switch from yolk as a source of nutrients to external nutrient sources from the offered diets. Some non-digestible carbohydrates could affect the development of the intestinal mucosal morphology (Langhout *et al*., 1999, Tellez *et al*., 1993). The objectives of this experiment were to investigate the effect

of dietary water-soluble soybean meal oligosaccharides (SMO) and polysaccharides (SMP) on intestinal development. The development of the duodenum, jejunum, ileum and caecum were studied in terms of their weight, length and morphology when SMO and SMP were added to a soybean meal-free diet during 1-14 days of age. Subsequent development of the intestines was also measured after SMO and SMP had been withdrawn from the diet.

MATERIAL AND METHODS

Animal and diets

Two-hundred and forty Arbor Acres male broilers were obtained from a commercial hatchery at 3h post hatching. Four groups of 60 birds each were allocated to one of four dietary treatments. Each group comprised four cages with 15 birds per cage. Each group had free access to water and feed throughout the experimental period of 28 days. The experiment were designed to have two control diets. A commercial diet containing both soybean meal and antibiotics served as a positive control (CON). A soybean and antibiotic-free diet served as negative control (NSM). One test diet contained SMO and the other contained SMP, each added to the NSM basal diet. Thus, there were three dietary groups free of soybean meal (NSM, SMO and SMP). SMO and SMP were used for the period 1-14 days of age only. They were made by diluting the NSM diet by 1 % SMO and 0.5 % SMP respectively. After Day 14, the experimental diets were substituted by the basal diet (NSM) within three days as follows: ratio basal diet to experimental diet = 30 to 70 at Day 15; 60 to 40 at Day 16; 90 to 10 at Day 17 and 100 to 0 at Day 18. After 14 days, the birds in the SMO and SMP groups received the NSM diet until sampling at 28 days of age. The CON group was fed with Maduramicin (5 ppm) and Aureomycin (0.22 %) in the diet. The other treatment groups did not receive antibiotics. All diets were fed as ground, and the particle size was around 1 mm^3. The diet formulations are shown in Table 1.

Table 1. The diet formulations

Ingredient	NSM (% as fed)	CON(% as fed)
Ground corn	47.90	60.25
Soybean meal	-	11.00
Corn germ meal	15.00	-
Corn gluten meal	24.0	17.00
Fish meal	7.00	7.00
$CaHPO_4$	0.70	1.25
Limestone	1.60	1.20
Salt	0.30	0.30
Sunflower oil	2.50	1.00
Vitamin-mineral mixture*	1.00	1.00
Crude protein	20.21	20.22
Metabolizable energy (MJ/Kg)	12.63	12.59
Ca	1.00	1.01
P	0.64	0.64

* Provide vitamins and minerals as previously described (Lee et al., 2001)

Extraction of SMO and SMP

The extraction of SMO and SMP from soybean meal has bean described previously as follows: 1 kg soybean meal was refluxed twice in 10 l alcohol (80% (v/v)) at 75 °C for 1 h. This supernatant was then used for SMO isolation and the residue for SMP isolation. The supernatant was condensed by rotavapor (Büchi RE 120) and protein was subsequently removed with 5 % trichloroacetic acid (TCA). The liquid was freeze-dried after passing a column filled with Amberlite 200 and Amberlite IRA-900 for removing TCA and amino acids from the supernatant. The residue was hydrolyzed in a 0.2 M NaOH solution at 80 °C for 2 h and filtrated using cheesecloth. The filtrate was neutralised with 30% acetic acid and freeze-dried. The freeze-dried sample was dissolved in 400 ml water. Protein was removed with 5 % TCA. After

1h, the supernatant was centrifuged at 13000 g for 30 min. SMP was precipitated by adding alcohol to the supernatant until the alcohol concentration was 80 % (v/v) and kept at 4 °C over night. The polysaccharide precipitate was centrifuged at 2500 g for 10 min. The polysaccharide product was freeze-dried. Replicate products extracted at different times were mixed together and stored at –20 °C .

Method of sampling

A total of 112 birds with four broilers from each group (one per cage) were sacrificed on Day 1, 3, 6, 9, 12, 15 and 28, respectively. Immediately after sacrifice, the entire gastro-intestinal tract was removed, and the length and empty weight of the intestines recorded. The empty weight of the intestines was measured by cutting the intestines longitudinally after the contents were gently rinsed out with distilled water. Samples (1 cm intestinal segments) were taken from the duodenum, jejunum, ileum and caecae on Day 15 of post-hatch immediately after cutting the intestines. The sampling sites were: 1) the apex of the duodenum, 2) midway between the point of entry of the bile ducts and the Meckel diverticulum (jejunum), 3) 10 cm proximal to the caecal junction (ileum), and 4) the middle part of both caecae. The intestinal samples for morphology analysis were flushed with a cold phosphate buffer solution (PBS), and weighed together with the other parts that originally connected with the sample used for morphology analysis. In preparation for scanning electron microscopy, the fresh samples were cut into 5 x 5 mm pieces which were fixed in a solution of 0.1 M sodium cacodylate buffer containing 25 g/L glutaraldehyde and 25g/L paraformaldehyde of 0.35 osmol/L with a pH of 7.4. Samples were processed routinely (Anderson, 1969) and examined under a HITACHI S-530 scanning electron microscope.

Data analysis

Each dietary treatment was considered as an experimental unit. The results were compared as means using a one-way ANOVA model, the significant difference between groups were analysed by LSD t test, performed using SPSS 8.0 (Statistical Package

for the Social Science, 1997) statistical software.

RESULTS

Change in length and weight of intestines

Changes of weight and length at the four intestinal sites are shown in Figure 1 (1A-1H). Intestinal length of duodenum, jejunum, ileum and caecum increased with time post-hatch (Figure 1A-1D). The commercial diet animals (CON) reached their plateau values around 12 days of age in all the four intestinal segments thereafter they remained at the plateau value or even declined. Intestinal length continued to increase slowly for the three soybean meal-free groups (NSM, SMO and SMP). However, intestinal length of each of the four segments was longer in the CON group at day 12 compared with the other groups.

Intestinal weight increased continuously until Day 15 in the different treatments (Figure. 1E-1H). The birds of the CON group had a rapid intestinal weight increase of the duodenum, jejunum and ileum until Day 12 and of the caecum until 15 days of age. In contrast with this, the birds in the NSM, SMP and SMO groups had a faster duodenal and caecal weight increase until Day 9 (Figure.1E and 1H), but for the jejunum and ileum a similar pattern of development was observed compared with that in the CON group (Figure.1F and 1G). Birds which consumed the soybean meal-free basal diet, had a relatively lower intestinal weight in the four sites compared with those in the CON group. There were no significant differences in weight and length of the four intestinal segment sites between the three non-soybean meal groups at 15 days of age ($P>0.05$).

The intestinal weights as percentages of the digestive tract-free body weight (IM/DTFBW × 100), representing the relative intestinal weight, at 15 and 28 days of age are shown in Table 2. The birds that had consumed soybean meal-free diets had a higher IW/DTFBW of duodenum, jejunum and ileum compared with the birds of theCON group, and showed a significant increase of the jejunum at the

Day 15($P<0.05$). Birds of the SMO and NSM groups had a higher relative ileal weight compared with that of CON ($P<0.05$). There was no significant difference in the relative intestinal weight at the four sites between the NSM, SMP and SMO groups of birds at Day 15 ($P>0.05$). However, in 28 day-old birds of the SMO and SMP groups, the IW/DTFBW were significantly higher compared with the CON and NSM groups for the duodenum ($P<0.05$), and higher than in CON for the ileum ($P<0.05$). Meanwhile, birds of the NSM group had a higher relative caecal weight compared with that of the CON group ($P<0.05$). Data in Table 2 indicate that in growing birds, the IW/DTFBW had a tendency to decline for the duodenum, jejunum and ileum for all treatments. However, only a very small decrease of IW/DTFBW was noticed for the caecum of the SMO and SMP groups, and there was even an increase in the NSM group.

The intestinal weight over length ratio (IW/L), which represents the thickness of the intestinal wall, at Days 15 and 28, is shown in Table 3. Except for the duodenum of the CON group, intestinal wall thickness increased with age. Moreover, IW/L of duodenum and caecum increased faster in the non-soybean meal diet groups (NSM, SMO and SMP) than for the CON group. The CON group had a significantly higher IW/L compared with the SMP and SMO groups in the caecum at Day 15 ($p<0.05$), and there seemed to be a higher IW/L for the duodenum than in other groups of birds, though this was not statistically significant. There was no difference between jejunum and ileum ($p>0.05$). However, the data of IW/L at Day 28 indicated that birds in the NSM, SMP and SMO groups had a thicker duodenum and caecum than birds of the CON group (Table 3). Analysis of the differences between the three non-soybean meal groups indicated that the SMO and SMP groups had a higher relative duodenum weight and a thinner caecal wall than the NSM group at 28 days of age ($P <0.05$). There were no statistical differences in IW/DTFBW and IW/L between the SMP and SMO groups for the four intestinal segments at 15 and 28 days of age ($P>0$ 05).

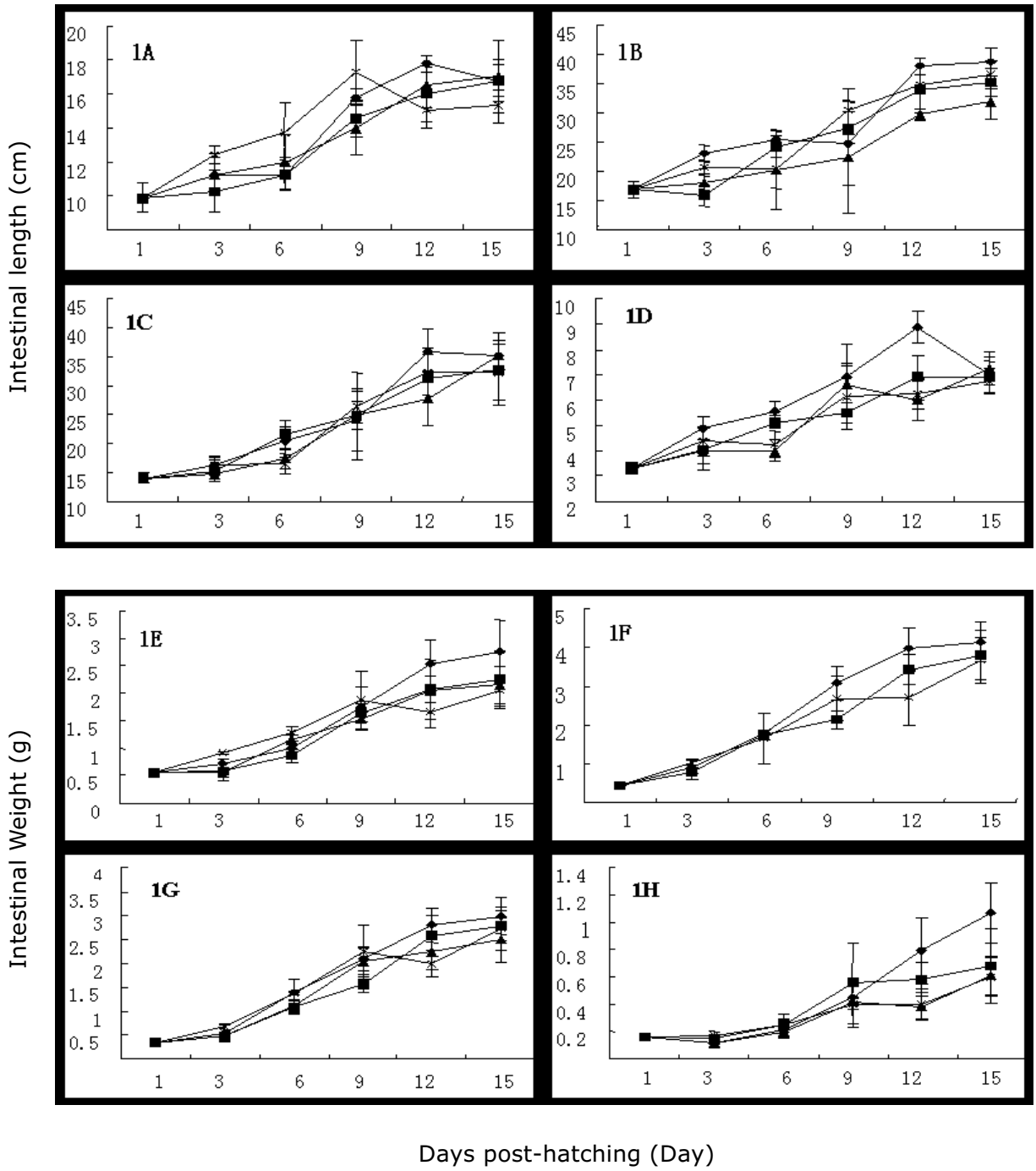

Figure 1. Mass and length change of intestines at four sites during 1-15 day. Figures 1A for duodenum length; 1B for jejunum length; 1C for ileal length; 1D for caecal length; 1E for duodenal weight; 1F for jejunal weight; 1G for ileal weight and 1H for caecal weight. There are four lines in each Figure Which represent different treatments. ◆,■, ▲ and **X** symbols line represent the commercial diet control group (CON), the soybean meal-free control group (NSM), and the SMP or SMO used group (SMO and SMP), respectively.

Table 2. Intestinal weight as % of digestive tract-free body weight (IW/DTFBW) at Days 15 and 28 in different treatments

Treatment	Duodenum	Jejunum	Ileum	Caecum
Day 15				
CON	1.29	1.96 ^{b}	1.41 ^{b}	0.51
NSM	1.37	2.37 ^{a}	1.73 ^{a}	0.40
SMP	1.40	2.34 ^{a}	1.63 ^{ab}	0.40
SMO	1.32	2.39 ^{a}	1.78 ^{a}	0.39
SEM	0.03	0.06	0.06	0.02
Day 28				
CON	0.69 ^{b}	1.71	1.05 ^{b}	0.31 ^{b}
NSM	0.71 ^{b}	1.69	1.25 ^{ab}	0.45 ^{a}
SMP	0.98 ^{a}	1.78	1.39 ^{a}	0.36 ^{ab}
SMO	0.91 ^{a}	1.95	1.39 ^{a}	0.38 ^{ab}
SEM	0.04	0.05	0.04	0.02

Mean came from four repeats. Means within columns followed by different letters were significant ($P<0.05$).

Effect of SMO and SMP on the development of intestinal mucosal surface micro-villi and attached microorganisms

Scanning electron microscopy on the villi of the duodenum, jejunum, ileum and caecum was conducted on the Day 15 and the scanning pictures are shown in Figure 2, 3, 4, and 5. The NSM, SMP and SMO groups had tongue-like villi in the duodenum and jejunum (Figures 2C, 2D, 2E and 2F). The CON group had leaf-like villi in the duodenum and plate-like villi in the jejunum (Figures 2A, and 2B). The villus sizes of the mucosa in the duodenum (Figure 2D), in the jejunum (Figure 2F) and in the ileum (Figures 3B and 3C) were smaller and thinner for the SMO and SMP birds respectively compared with the NSM birds (Figures 2C, 2E and 3A). Also, there seem to have been more attached materials on the ileal villi surface of the birds in the SMO and SMP groups (Figures 3E and 3F) compared to the birds in the NSM group (Figure 3D). The SMO and SMP groups seem to have a higher density of micro-villi when compared

with the NSM groups in the duodenum and ileum (Figures 4A, 4C, 4E and 4G). However, there were no apparent differences between the birds of the NSM, SMO and SMP groups in terms of micro-villi of the jejunum (Figures 4B and 4F). Furthermore, attached microorganisms were observed on the caecal mucosal surface of the SMO group (Figures 4H and 5C). Meanwhile, it was noticed that there were a few microorganisms attached to the caecal wall of the birds in the CON group (Figure 5A).

Table 3. Intestinal weight over intestinal length ratio (g/cm) at Day 15 and 28 in different treatments

	Duodenum	Jejunum	Ileum	Caecum
Day 15				
CON	0.16	0.11	0.08	0.15 ^{a}
NSM	0.13	0.11	0.09	0.10 ^{ab}
SMP	0.13	0.11	0.07	0.08 ^{b}
SMO	0.13	0.10	0.09	0.09 ^{b}
SEM	0.006	0.003	0.004	0.01
Day 28				
CON	0.14 ^{b}	0.16	0.12	0.16 ^{ab}
NSM	0.16 ^{ab}	0.16	0.13	0.21 ^{a}
SMP	0.20 ^{a}	0.15	0.13	0.15 ^{b}
SMO	0.17 ^{ab}	0.16	0.13	0.14 ^{b}
SEM	0.007	0.007	0.005	0.011

Means within columns followed by different letters was significant ($P<0.05$).

Each mean in table came from four repeats.

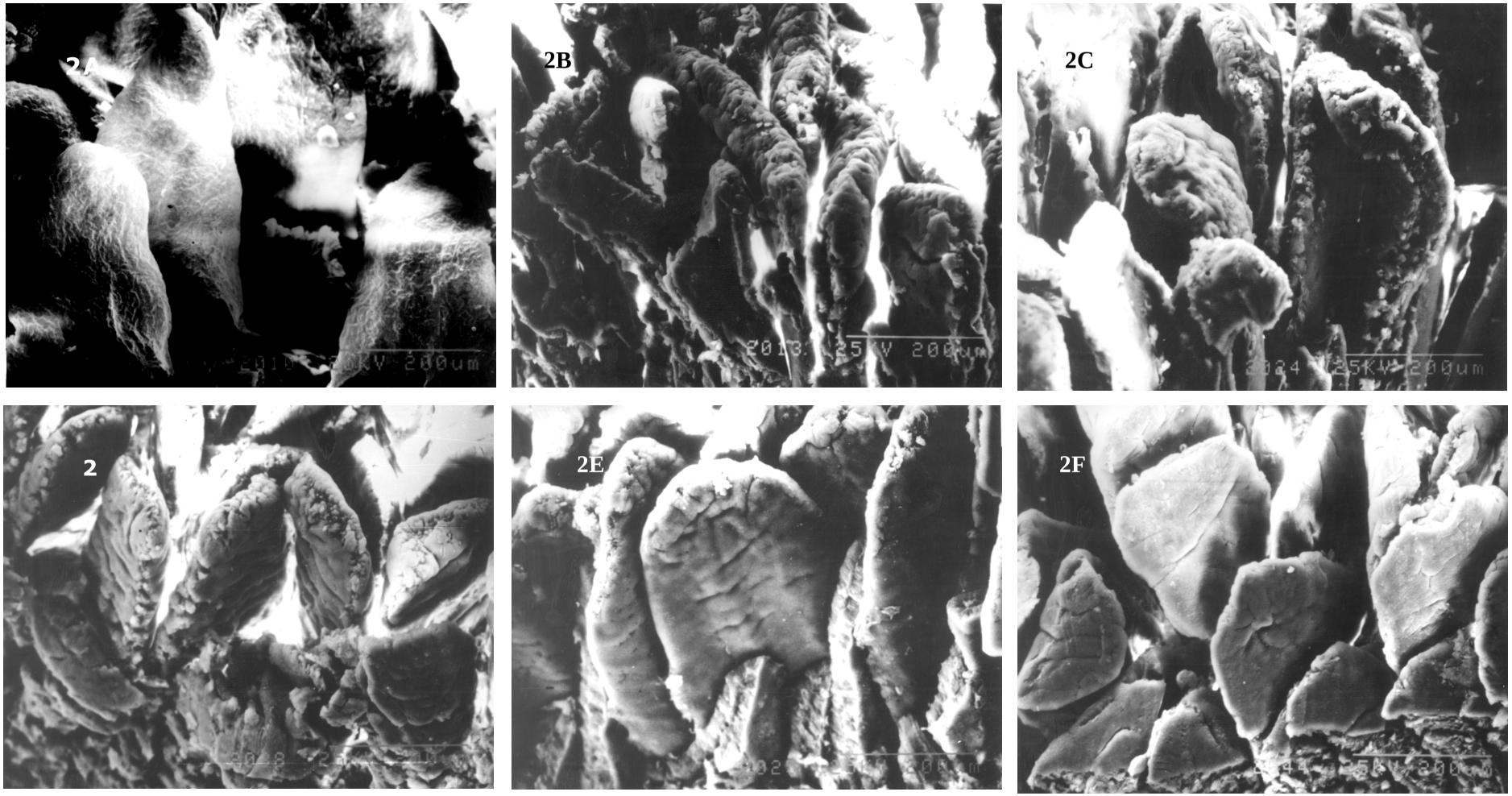

Figure 2. Observations of scanning electron microscopy on the villi of the intestines at 15 days of age in the commercial control group (CON), non-soybean meal control diet group (NSM), the soybean meal oligo- and water soluble polysaccharides used diets groups (SMO and SMP) (2A-2F). The bar in all pictures was 200 μm. 2A and 2B were duodenum, jejunum surface of the birds in CON group. 2C and 2D were the duodenum surface of the birds in NSM and SMO groups respectively. 2E and 2F were the jejunum surface of the birds in NSM and SMO groups respectively.

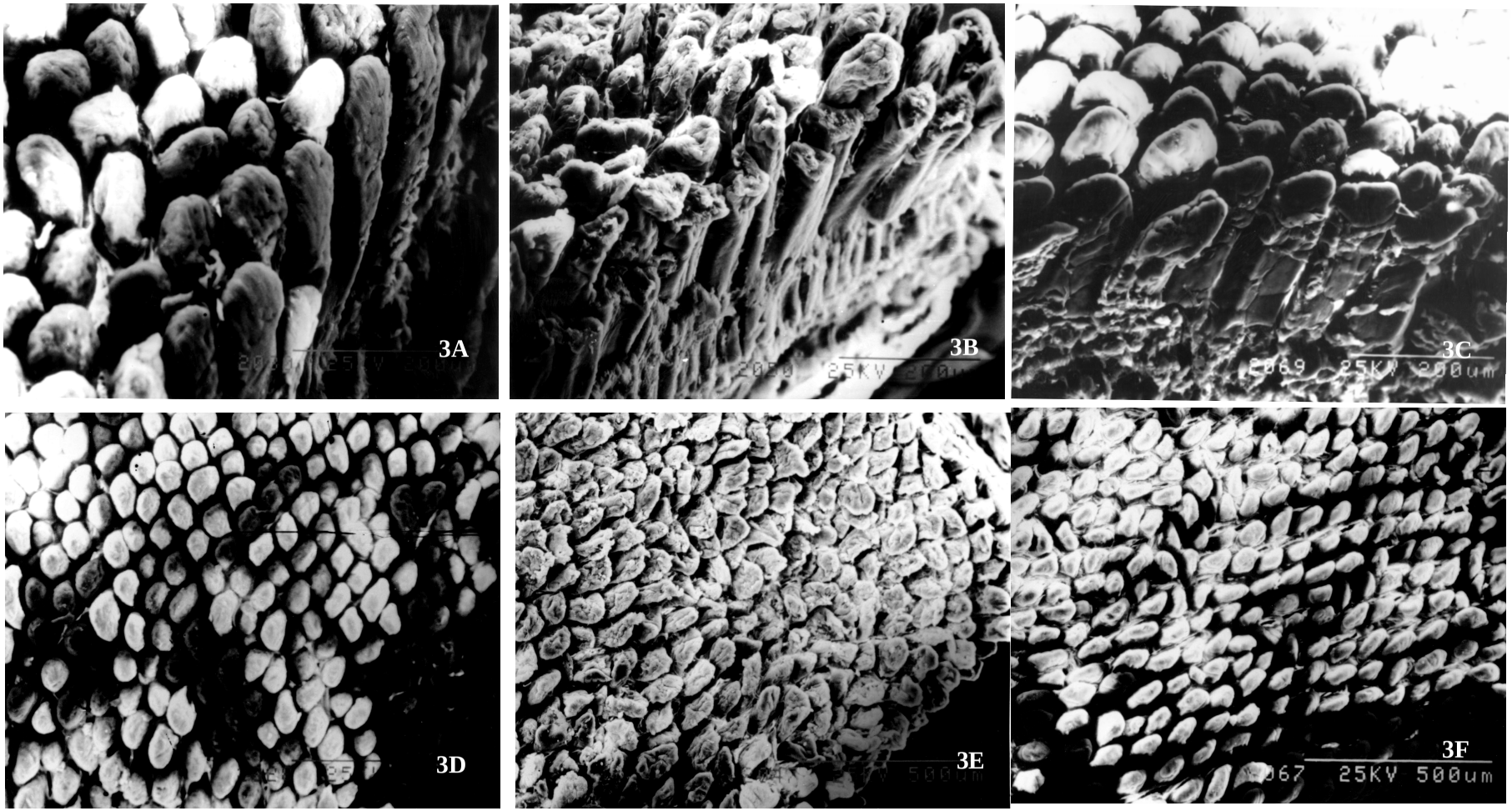

Figure 3 Observations of scanning electron microscopy on the villi of ileum at 15 days of age in the non-soybean meal control diet group (NSM), the soybean meal oligo- and water soluble polysaccharides used diets groups (SMO and SMP) (3A-3F). The bar in was 200 μm and 500μm for the pictures of first and second line respectively. 3A and 3B was the ileum surface of the birds in NSM group, 3B and 3E was SMO group's and 3C and 3F was SMP group's.

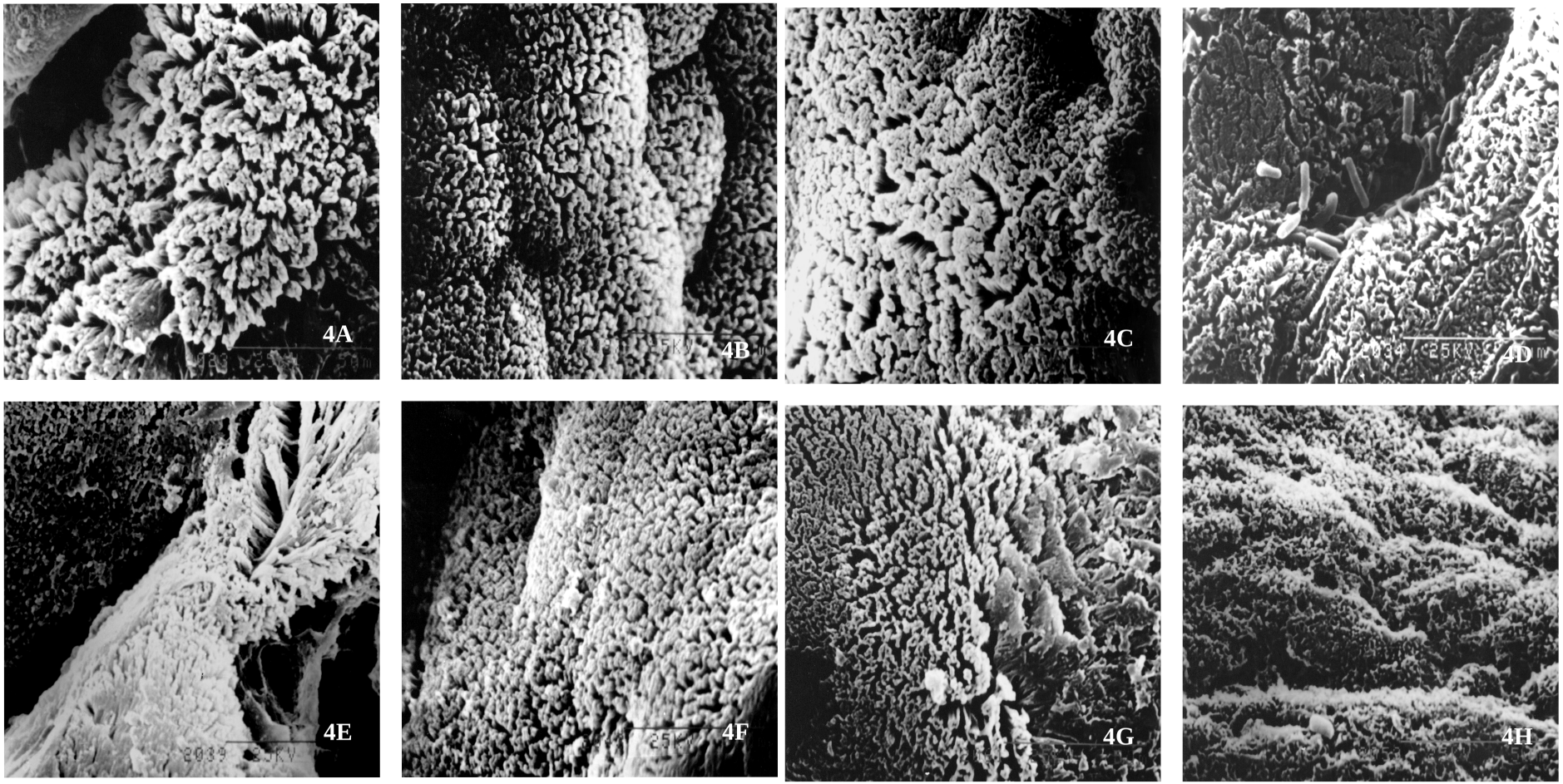

Figure 4. Observations of scanning electron microscopy on the villi of the intestines at 15 days of age in the non-soybean meal control diet group (NSM), the soybean meal oligo- and water soluble polysaccharides used diets groups (SMO and SMP) (4A-2H). The bar in all pictures was 5 μm. 4A, 4B, 4C and 4D were were duodenum, jejunum, ileum and caecum surface of the birds in NSM group, respectively. 4E, 4F and 4H were the duodenum, jejunum and caecum surface of the birds in SMO group, respectively. 4G was the ileum surface of birds in SMP group.

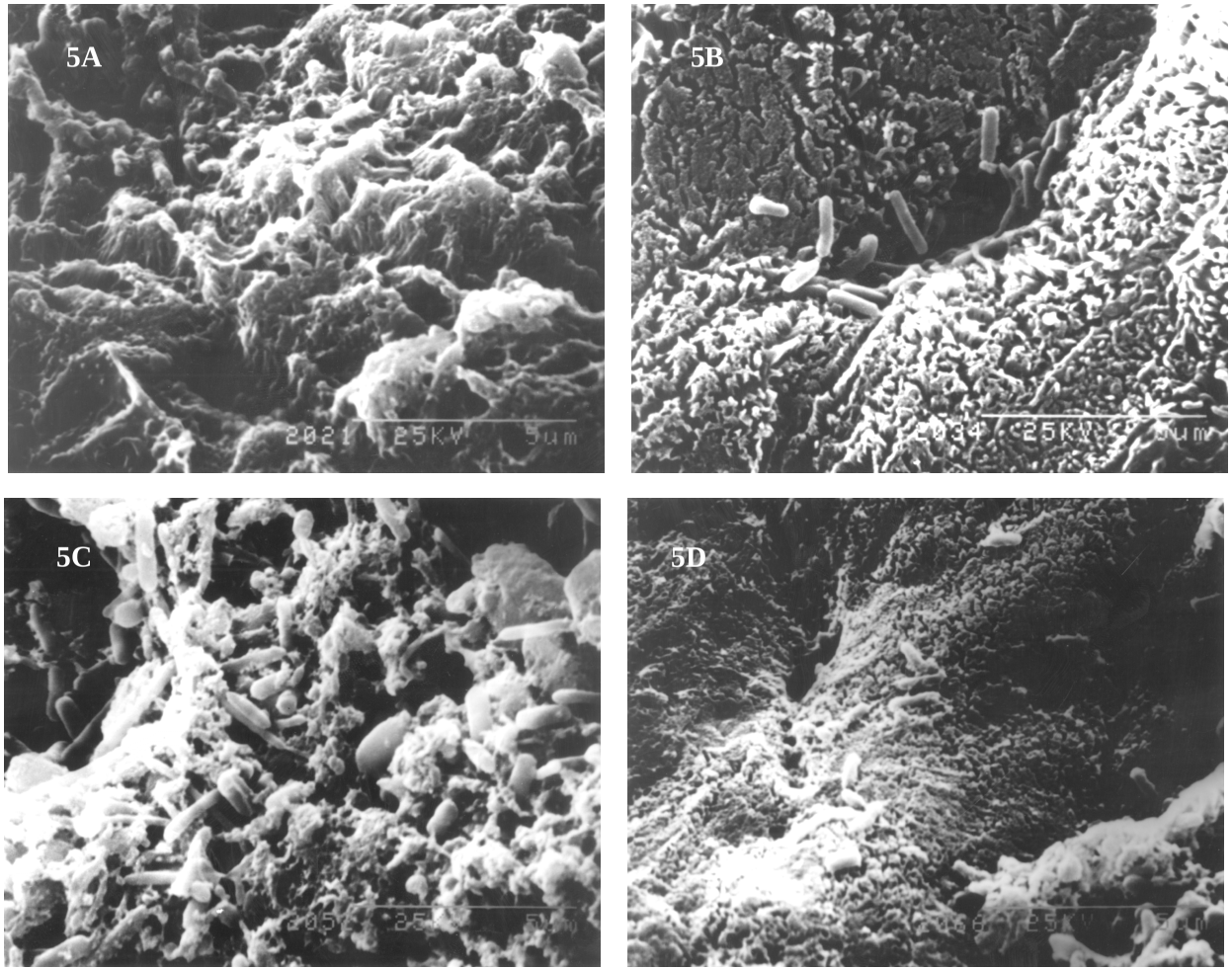

Figure 5. Observations of scanning electron microscopy on the villi of caecum at 15 days of age in the commercial control group (CON) (5A), non-soybean meal control diet group (NSM) (5B), the soybean meal oligo- and water soluble polysaccharides used diets group (SMO and SMP) (5C and 5D). The bar in all pictures was 5μ m.

DISCUSSION

This experiment was conducted to investigate the effect of water-soluble soybean meal polysaccharides (SMP) and oligosaccharides (SMO) on the intestinal growth and mucosal morphology development in broiler chickens. SMO and SMP were used in a soybean meal-free diet during the early period post-hatch. The sequential development of the intestines in birds was also measured after SMO and SMP had been withdrawn from the diet. The results indicated that birds in the soybean meal-free diet groups (NSM, SMO and SMP) had a lower intestinal weight and length at 9 and 15 days of age. This might contribute to the lower body weight in these groups (Chapter 8).

Undoubtedly, the intake as well as the composition of the diets will have contributed to the differences in relative development of the gut segments. Lower group feed consumption was noticed in SMO and SMP groups compared with the commercial diet control and NSM groups at 15 days old birds. The birds in NSM group, however, had feed intake as same as the birds in CON group (Yu Lan *et al.,* unpublished data). Both soybean meal and fishmeal were the main protein sources in the diet of the CON group, however, corn gluten meal, fishmeal and corn germ meal were the major ingredients in the diet of the three soybean meal-free groups (NSM. SMO and SMP). Due to the different protein quality between soybean and corn as protein sources, the diet of the CON group had a higher nutritional value than that of the soybean meal-free diets, even though they had the same crude protein and metabolizable energy concentrations. Furthermore, SMP and SMO contain stachyose and raffinose, which are not hydrolysed by the birds' enzymes nor absorbed in the intestinal tract of broilers. It has been reported that non-digestible carbohydrates, such as SMO and SMP have anti-nutritional properties and could have adverse effects on broiler productivity (Irish and Balnave 1993; Coon *et al*., 1990; Leske 1991). A significant growth depression was noticed for the SMO and SMP consuming birds (Chapter 8).

Statistical analysis of the results of the relative intestinal weight, showed that the

percentage of duodenum, jejunum and ileum in digestive tract-free body weight (IW/DTRBW × 100), was higher in the birds of the NSM, SMP and SMO groups, compared with those in the CON group on Day 15 (Table 2). Furthermore, the SMO and SMP diets showed a growth stimulating effect on the duodenum and ileum by Day 28 compared with the CON and NSM diets (Table 2). The results suggest that diet in this experiment and the use of SMO and SMP during the early stage of post-hatch had not led to significant changes in the relative intestinal growth. This might be because in early development there is a primary emphasis on growth of the organs of supply (e.g. intestine, pancreas, liver) and subsequently on that of the carcass (organs of demand-e.g. muscles, fat) (Kantanbaf et al., 1988). However, this needs to be investigated further.

The effects of SMO and SMP on the morphological development of the intestine were also investigated by means of scanning electron microscopy at 15 days of age. There was a significant difference in villus shape and size, not only between the commercial diet (positive control, CON) group and the three other treatment groups, which were free of soybean meal, (NSM, SMO, and SMP), but also between the soybean meal-free negative control group (NSM) and the and the animals fed the non-digestible carbohydrate containing diets (SMO and SMP). The SMP and SMO groups, especially the SMO group, had slimmer villi than the NSM group (Figures 2 and 3) for the duodenum, jejunum and ileum. This effect was more obvious in the duodenum, ileum and caecum, compared with jejunum. The pictures of duodenum and ileal surface at 8000 times magnification, showed that the density of the micro-villi seemed higher and less developed in the SMO and SMP animals compared with the NSM animals (Figure 4). This could possibly be attributed to the potentially anti-nutritional effects of SMO and SMP and the microbial activity promoting role of these non-digestible carbohydrates (Choct et al., 1996). The increased gut microbial population and their fermentative activity was also be responsible for increasing nutritional requirements, which would be used to supply the maintenance needs of the gut microbial population. This could delay the development of the villi size.

In this experiment, SMO and SMP were included in the diet up to 14 days of age,

after which the birds received a soybean meal-free basal diet (NSM) until additional sampling took place at 28 days of age. The NSM and CON groups did not experience any change in diet throughout the experimental period of 28 days. A noticeably faster relative growth of the duodenum had occurred in the SMO and SMP groups compared with the NSM group at Day 28 (Table 2). The relative intestinal growth showed no statistical difference between SMO, SMP and NSM groups at Day 15, and there were no significant differences in weight and length of the four sites between the three non-soybean meal groups at Day 15 and 28 ($P>0.05$). The birds fed SMO and SMP diets, however, had a smaller villi size (Figure 2) compared with that of the NSM group. These results suggest that dietary SMO and SMP can increase the gut mucosa micro-villi density when used in early post-hatch diets. Due to their chemical composition SMP and SMO will pass intact into the lower portions of the intestine and caecum. This will stimulate fermentation by microorganisms which inhabit the caecum and possibly also those in the ileum. Consequently, the pH of chyme will be lower due to the accumulation of microbial fermentation end-products, such as acetic, propionic, butyric and lactic acids. Microbial fermentation can also take place in the crop. It has been reported that changes of intracellular pH often parallel the changes of extra-cellular pH and may trigger off cell division. A lower luminal pH has been associated with an increase in the numbers of cells that are actively synthesizing new DNA in the caecum of rats (Jacobs and Lupton, 1984; Lupton and Jacobs, 1987). It is also known that short-chain fatty acids, especially butyric acid can be absorbed and utilized by intestinal mucosal cells (McNeil et al., 1978; Ruppin et al., 1980). On the other hand, SMO and SMP significantly promote the growth of bacteria in the caecum (Yulan *et al.,* unpublished). In this experiment, attached microorganisms were observed apparently in the caecal surface of the SMO group (Figure 5). This interaction between the bacteria and the mucosal cells is most likely to affect development of the intestinal morphology.

The caecum is known to be a major site of fermentative activity, but bacterial activities have also been detected in the ileum and crop. SMO and SMP can be fermented by microorganisms inhabiting crop and hind-gut (Cummings, 1989; Van Loo, 1995). Therefore, the effects of SMO and SMP on the morphology of the gut

would be expected to be mainly apparent in the lower parts of the digestive tract. The lower caecal weight per length unit (centimetre) (see Table. 3) in the SMP and SMO groups may have resulted from the expansion function of the gas that is produced from the fermentation process of SMO and SMP. Experimental results of Leske and Coon (1999) indicate that chicks intubated with extracted soybean meal (ESBM) with alpha-galactosides added to the levels of SBM (ESBMG) produced 2.2 times the amount of total hydrogen gas than chicks intubated with ESBM. The research indicates that alpha-galactoside oligosaccharides are a major cause of hydrogen gas production from SBM in poultry. In a separate experiment, a large gas production was noticed when SMO and SMP were fermented by bacteria of broiler caecal content (Chapter 2) in vitro. Furthermore, during this experiment, a flatulent caecum was occasionally noticed.

In conclusion, water-soluble soybean meal oligo- and polysaccharides (SMO and SMP) do not seem to affect the development of the intestines when used in broilers' diet in the early stage of post-hatch. However, they appear to significantly affect the morphological development of gut mucosa, SMO and SMP consumed birds had smaller villi size in duodenum, jejunum and ileum, and seems had higher density and undevelopment microvilli. These non-digestible carbohydrates, especially the SMO can apparently promote the attached population of microorganisms at caecal wall. These microflora can competitively exclude the infection of pathogens.

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REFERENCES

Anderson, T. F., 1969. Techniques for the preservation of three dimensional structure in preparing specimens for the electron microscope. Trans. N.Y. Acad. Science. 13:130-134.

Bayer, R. R., C. B. Chawan, F. H. Bird and S. D. Musgrave, 1975. Characteristics of the absorptive surface of small intestine of the chicken from 1 day to 14 weeks of age. Poultry Science 54:155-159.

Choct, M., R. J. Hughes, J. Wang, M. R. Bedford, A. J. Morgan, G. Annison, 1996. Increased small intestinal fermentation is partly responsible for the anti-nutritive activity of non-starch polysaccharides in chickens. British Poultry Science. 37(3):609-621.

Cook, R. H. and F. H. Bird, 1973. Duodenal villus area and epithelial cellular migration in conventional and germ-free chickens. Poultry Science. 52:2276-2280.

Coon, C. N., K. L. Leske, O. Akavanichan and T. K. Cheng, 1990. Effect of oligosaccharides-free soybean meal on true metbolizable energy and fiber digestion in adult roosters. Poultry Science. 69: 787-793.

Cummings, J. H., G. R. Gibson and G. T. Macfarlane, 1989. Quantitative estimate of fermentation in the hindgut of man. Acta Veterinaria Scandinavica. 86: 76-82.

Geyra, A., Z. Uni, and D. Sklan, 2001. Enterocyte dynamics and mucosal development in the post hatch chicken. Poultry Science. 80:776-782.

Ija, P. A., A. Saki, and D. R. Tivey, 2001. Body and intestinal growth of broiler chicks on a commercial starter diet. 1. Intestinal weight and mucosal development. British Poultry Science. 42(4):505-513.

Irish, G. G. and D. Balnave, 1993. Non-starch oligosaccharides and broiler performance on diets containing soybean meal as the sole protein concentrate. Australian Journal of Agricultural Research. 44: 1483-1499.

Jacobs, L. R., and J. R. Lupton, 1984. Effect of dietary fibres on rat large bowel mucosal growth and cell proliferation. American Journal of Physiology. 264:378-385.

Katanbaf, M. N., P. B. Siegel and E. A. Dunnington, 1988. Organ growth of selected lines of chickens and their F1 crosses of chickens selected over 27 generations for high or low body weight. Growth Development and Aging. 52:11-22.

Langhout, D. J., J. B. Schutte, P. Van Leeuwen, J. Wiebenga and S. Tamminga, 1999. Effect of dietary high- and low-methlated citrus pectin on activity of the ileal microflora and morphology of the small intestine wall of broiler chicks. British Poultry Science. 40(3):340-347.

Lee, K. H. and S. Leeson, 2001. Performance of broilers fed limited quantities of feed or nutrients during seven to fourteen days of age. Poultry Science. 80:446-454.

Leske, K. L., C. N.Coon, 1999. Hydrogen gas production of broiler chicks in response to soybean meal and alpha-galactoside free, ethanol-extracted soybean meal. Poultry Science.78(9): 1313-1316.

Leske, K. L., T. K. Akavanichan, Cheng and C. N. Coon, 1991. Effect of ethanol extract on nitrogen-corrected true metabolizable energy for soybean meal with broilers and roosters. Poultry Science. 70:892-895.

Lupton, J. R. and L. R. Jacobs, 1987. Fibre supplementation results in expanded proliferative zones in rat gastric mucosa. American Journal of Clinical Nutrition, 46:980-984.

McNeil, N. I., J. H. Cummings, and W. P. T. James, 1978. Short chain fatty acid absorption by the human large intestine. Gut 19: 819-822.

Noy, Y. and D. Sklan, 1999. Energy utilization in newly hatched chicks. Poultry Science 78: 1750-1756.

Ruppin, H., S. Bar-Meir, K. H. Soergel, C. M. Wood, and M. G. Jr. Schmitt, 1980. Absorption of short-chain fatty acids by the colon. Gastroenterology 78: 1500-1507.

Sklan, D., 2001. Development of the digestive tract of poultry. World's Poultry Science Journal. 57: 415-428.

SPSS, Inc. 1997. SPSS for windows graduate pack advanced version, Version 8.0. Chicago, IL: SPSS Inc.

Tellez, G., C. E. Dean, D. E. Corrier, J. R. Deloach, L. Jaeger, and B. M. Hargis, 1993. Effect of dietary lactose on cecal morphology, pH, organic acids, and *Salmonella enteritidis* organ invasion in Leghorn chicks. Poultry Science. 72:636-642.

Uni, Z., Y. Noy, and D. Sklan, 1995. Post hatch changes of morphology and function of the small intestines in heave and light strain chick. Poultry Science. 74:1622-1629.

Uni, Z., Y. Noy, and D. Sklan, 1996. Developmental parameters of the small intestine of heavy and light train chicks pre- and post-hatch. British Poultry Science. 36:63-71.

Uni, Z., Y. Noy, and D. Sklan, 1999. Posthatch development of small intestinal function in the poult. Poultry Science. 78:215

Van Loo, J., P. Coussement, L. De Leenheer, H. Hoebregs, and G. Smith, 1995. On the presence of inulin and oligofructose as natural ingredients in the western diets. CRC Critical Reviews on Food Science and Nutrition, 35: 525-552.

Yamauchi, K. and Y. Isshiki, 1999. Scanning electron microscopic observation on the intestinal villi in growing white Leghorn and broiler chickens from 1 to 30 days of age. British Poultry Science. 32(1):67-70.

Chapter 4

THE EFFECT OF DIETARY OLIGO- AND POLY-SACCHARIDES FROM SOYBEAN MEAL ON THE GROWTH OF CAECAL LACTIC ACID BACTERIA IN BROILERS MEASURED BY REAL-TIME PCR AND FLUORIMETRY

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The Effect of Dietary Oligo- and Poly-Saccharides from Soybean Meal in The Growth of Caecal Lactic Acid Bacteria in Broilers Measured by Real-Time PCR and Fluorimetry

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Abstract: This study was undertaken to determine whether soybean meal water-soluble oligosaccharides (SMO) and polysaccharides (SMP) can affect the growth of caecal lactic acid bacteria (LAB) in the early stage of post-hatch in chickens fed a soybean meal-free basal diet. The experiments consisted of four dietary treatments: two control treatments CON and NSM and two dietary treatments containing specific soybean carbohydrates. A commercial diet, mainly comprising of corn, soybean meal, corn gluten, and fishmeal, served as a positive control group (CON). Maduramicin (5ppm) and Aureomycin (0.22 %) were also included in the CON Diet. A soybean meal-free control diet (NSM) comprising mainly corn, corn gluten meal, fish meal and corn germ meal, with no added antibiotics, served as a negative control. In the SMO and SMP groups, SMO and SMP were fed to the birds from 1-15 days of age, by diluting the NSM diet with 1 % and 0.5 % SMO and SMP respectively. On Day 15, eight birds from each treatment (32 in total), were sacrificed by cervical dislocation. Caecal contents was taken from each bird and mixed per treatment group. Total DNA was extracted from the samples. LABs of the genera Lactobacillus, Pediococcus, Weissella and Leuconostoc in caecal contents of broilers was detected with Light Cycler real-time PCR and fluorescence (SYBR Green I), followed by melting curve analysis of specific primer PCR products, which are supposed to represent the features of the LAB group. The results of melting curve analysis showed that amplification melting peaks (Tm) appeared at 91.1 °C for SMO, 90.85 °C for SMP, 91.05 °C for NSM, and 90.50 °C for CON respectively. A single predominant peak also appeared for each group. Real-time PCR results showed that SYBR Green I labeling stranded DNA was detected at the amplification cycles 13.36, 15.24, 16.11 and 18.00 for the four groups respectively. The cycle number is an indication of target DNA concentration in the PCR reaction solution, and the two are inversely proportional. These results suggested that SMO and SMP stimulated LAB growth in the caecal contents during

the early stage of post-hatch of broiler chickens. In conclusion, soybean meal oligosaccharides (SMO) significantly increased the population size of a group of lactic acid bacteria (genera Lactobacillus, Pediococcus, Weissella and Leuconostoc) when SMO was added to the broilers' diet for two weeks post-hatch. Although the selective stimulating role of SMO on LAB could not be certified by the current experimental results, SMO may assist in the pathogenic competitive exclusion capability. Therefore, SMO could then act as an antibiotic substitute in young broiler chicks.

Keywords: lactic acid bacteria (LAB), real-time PCR, melting curve analysis, soybean meal oligo- and polysaccharides (SMO and SMP), caecal contents, broilers.

INTRODUCTION

The gastrointestinal tracts of animals harbor a complex microbial community of possibly hundreds of different bacterial species (Tannock, 1995). In recent years, increasing interest has focused on the lactic acid producing bacteria (LAB). These are commonly present as members of a large microbial community and are of interest because of their supposed health-promoting properties (Gibson and Fuller. 2000). Considerable efforts have been made to increase selectively, the relative activity of these lactic acid producing intestinal microorganisms by dietary ingredients such as oligosaccharides (Cavazzoni *et al.,* 1998; Jenkins *et al.,* 1999; Buddington *et al.,* 1999; Rao, 1999; Gibson, 1999; Reddy, 1999; Sanders, 2000; Menne *et al.,* 2000; Brady *et al.,* 2000).

Several methods have been employed to measure microbial community population shifts by means of plate counts, molecular techniques, fluorescence and immunological techniques (Charteris *et al.*, 1997). Different techniques will result in different results, especially in the detection of groups or species that are not present in abundant numbers (Hugenholtz *et al.* 1998, Pacher *et al.* 1996), or which are unculturable. A quick and economical technique based on denaturing gradient gel electrophoresis (DGGE) of 16S ribosomal DNA (rDNA) amplicons has proved to be practical for the analysis of microbial communities (Muyzer *et al.,* 1993). While such quantitative techniques as PCR and fluorescence *in situ* hybridization (FISH) can be used to detect DNA and RNA. It is difficult with these techniques to measure and compare the exact amounts of amplified products (Kim, 2001). Therefore, quantitative, fluorescence-based real-time PCR assays have been developed (Heid *et al.,* 1996; Wittwe *et al.,* 1997). This technique can be used for a large dynamic range of quantification, yet requires no post-PCR sample handling and only very small amounts of starting material (De Preter *et. al,* 2002), so that it is applicable in many research areas (Knutsson *et al. and* Weghofer *et. al.,* 2001; Aldea *et. al.,* Yamaguchi *et. al.,* Wong *et. al. and* De Preter *et. al.,* 2002; Oleastro *et. al.,* 2003).

The aim of this study, was to detect and evaluate LAB of the genera *Lactobacillus,*

Pediococcus, Weissella and *Leuconostoc* in caecal contents of young broiler chickens and to determine whether these populations were affected by dietary soybean meal oligosaccharides (SMO) or polysaccharides (SMP). Real-time PCR and fluorimetry techniques were used.

MATERIALS AND METHODS

Isolation of oligo- and water-soluble polysaccharides from soybean meal

Soybean meal oligosaccharides (SMO) and water-soluble polysaccharides (SMP), were extracted from soybean meal has bean described previously as follows: 1 kg soybean meal was refluxed twice in 10 l alcohol (80% (v/v)) at 75 °C for 1 h. This supernatant was then used for SMO isolation and the residue for SMP isolation. The supernatant was condensed by rotavapor (Büchi RE 120) and protein was subsequently removed with 5 % trichloroacetic acid (TCA). The liquid was freeze-dried after passing a column filled with Amberlite 200 and Amberlite IRA-900 for removing TCA and amino acids from the supernatant. The residue was hydrolyzed in a 0.2 M NaOH solution at 80 °C for 2 h and filtrated using cheesecloth. The filtrate was neutralised with 30 % acetic acid and freeze-dried. The freeze-dried sample was dissolved in 400 ml water. Protein was removed with 5 % TCA. After 1 h, the supernatant was centrifuged at 13000 g for 30 min. SMP was precipitated by adding alcohol to the supernatant until the alcohol concentration was 80 % (v/v) and kept at 4 °C over night. The polysaccharide precipitate was centrifuged at 2500 g for 10 min. The polysaccharide product was freeze-dried. Replicate products extracted at different times were mixed together and stored at – 20 °C.

Animals and diet

Two hundred and forty 1-day-old male Arbor Acres broilers were used. Four groups of 60 birds each were randomly allocated to one of four diets. Each group was divided at random into four subgroups (15 per group), and each sub-group was housed in one cage. Each group had free access to water and one of the four diets.

The experiment consisted of two control diets and two carbohydrate test diets: 1) a commercial diet, mainly composed of corn, soybean meal, corn gluten and fishmeal served as a positive control diet (CON); 2) a negative control diet which was soybean meal-free (NSM) and was mainly composed of corn, corn gluten, fish meal and corn germ meal; the two experimental test diets 3) SMO and 4) SMP were composed by diluting the NSM diet by 1 % and 0.5 % of SMO and SMP respectively. All four diets contained about 12.5 MJ/Kg of metabolizable energy (ME) and 20.2 % of crude protein (CP). The diets were fed for 15 days. The CON group contained two antibiotics: Maduramicin (5 ppm) and Aureomycin (0.22 %). The three diets, NSM, SMO and SMP, were antibiotic-free throughout the study.

Sampling and DNA extraction

At Day 15, a total of 32 birds, eight birds from each group (two from each cage), were used to detect LAB in their caecal contents. After the animals were sacrificed by cervical dislocation, the caecal contents were collected from the birds. In order to minimize the difference within diet treatment group, samples from animals in the same treatment group were mixed in sterilized bottles.

The samples were kept at – 80 °C pending further analysis. To extract total DNA from the caecal contents, the frozen samples were thawed in ice water and then homogenized. One hundred mg of sample was weighed into a sterilized tube. Bacterial DNA was separated from feed particles (Wang *et al.,* 1996) and bacterial DNA was then extracted (Lewington *et al.,* 1987). Briefly, 100 mg sample was suspended in 900 ml 0.05 M cold PBS, vortexed for 5-10 min. and then centrifuged at 200 g for 5 min. This procedure was repeated three times. The bacteria remained in the supernatant. All the supernatant was collected in a tube and centrifuged at 9000g for 3 min. The deposit was resuspended and centrifuged at 9000 g for 3 min., this procedure was repeated three times with cold PBS and once with water. This final deposit was then used for total DNA extraction (Lewington *et al.,* 1987). Agarose gel (1.2 %) electrophoresis was performed using 5 ml of the resultant DNA solution to estimate the amount and integrity of the DNA.

Primers

PCR primers specific for LAB of the genera *Lactobacillus, Pediococcus, Weissella* and *Leuconostoc* were used in this research (Walter *et al.,* 2001). The primers were obtained from Shanghai Biochemicals Co., China and included forward primer Lac1 (5'-AGC AGT AGG GAA TCT TCC A-3') and reverse primer Lac2GC (5'-GC-Clamp-ATT YCA CCG CTA CAC ATG-3'). In this way, a 340-bp fragment of the V3 region of the 16S rRNA gene was obtained.

Real-time quantitative PCR

The PCR technique described in this paper was employed by means of a Light Cycler, which allows real time detection of amplification products through fluorescence of SYBR Green I dye on the double stranded DNA (Wittwer *et al.,* 1997). Real-time PCR was performed using a Light Cycler rapid thermal cycler system (BIORAD icycler, USA). Reactions were performed in a 20 µ l volume with 2.5 nM of Lac1 and Lac2GC primers, 2.5 nM Mg^{++}, 20 µM dNTP, 0.2 µ l Taq DNA polymerase, 1 µ l SYBR Green I and 1 µ l extracted DNA. The amplification program included an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s; and finally 72 °C for 2 min. Fluorescence was measured through the slow heating phase. The specificity of the amplified product was then determined by melting curve analysis. Immediately after the PCR program was completed, melting curves were obtained by heating at 95 °C for 2 s, cooling to 65 °C for 40 s and re-heating slowly by 0.2 °Cs until 95 °C with continuous recording of fluorescence. Melting curves were recorded by plotting fluorescence signal intensity versus temperature, and amplicon melting temperature (Tm) was determined by calculating the derivatives of the curve with the Light Cycler software and visualized by plotting the negative derivative against the temperature (Komurian-Pradel *et al.,* 2001). Samples of each treatment were run three times in the Light Cycler. Again, the PCR products were confirmed using agarose gel electrophoresis: the gel was stained with ethidium bromide and viewed by UV transillumination after running the PCR product.

Statistical analysis

All data statistical analysis were performed by SPSS 8.0 statistical software (Statistical Package for the Social Science, 1997). Each dietary treatment was considered as an experimental unit. The results were compared by means of one-way ANOVA. The significant difference between each treatment was analysed by the LSD t test, with significance set at $p<0.05$.

RESULTS AND DISCUSSION

Melting curve analysis of PCR products from different experimental groups

Melting curve analysis results showed a single predominant peak for each group. The commercial control diet (CON) had Tm at 91.05 °C. The negative control diet (NSM) had a Tm at 90.50 °C. Groups that had consumed soybean meal oligosaccharide (SMO) and polysaccharide (SMP) had Tm at 91.16 °C and at 90.85°C respectively (Figure 1a). There were no significant differences for the Tm ($p>0.05$) for different dietary treatments (SEM 0.10). Meanwhile, a unique band appeared in the electrophoresis results of the different groups (Figure 1b). The curve demonstrated that the primers involved were specific for LAB of the genera *Lactobacillus, Pediococcus, Weissella* and *Leuconostoc,* in that they amplified one major specific product at a distinct Tm. The shape and position of a DNA melting curve are characterized by its GC/AT ratio, sequence and length (Ririe *et al.,* 1997). The difference between Tm values of the PCR products indicates the different character of the DNA template. All Tm values in this experiment were around 91 °C with differences between them of not more than 0.66 °C. Meanwhile, the Tm values were significantly distinguished from other small peaks with lower Tm values. This demonstrated that there was a large amount of PCR products solved around 91°C. These results suggest that a LAB specific gene was amplified. During PCR amplification, there are products which can be bound by SYBR Green I, which are non-specific. These include the primer-dimers, non-target amplicons, and even a fold of Lac2GC itself can form double stranded DNA that can be bound by SYBR Green I. Therefore,

analysis of the melting curves provides a check to ensure that specific amplification has occurred, and distinguishes products from non-specific primer-dimers, and therefore highlights any contamination (Ririe *et al.,* 1997, Pietilä *et al* 2000). For this melting curve, which was specific for LAB DNA, there were some waves with Tm values under 86 °C, which were apparently distinguishable from the LAB-specific PCR products. These waves can probably be attributed to DNA originating from other bacteria present within the caecal contents. In addition, the Lac2GC reverse primer, which was originally designed for DGGE analysis and was therefore relatively longer (68bp) because of the 40bp GC clamp (Wu *et al.,* 1998), may fold during the real-time PCR procedure. No primer-dimer or other DNA products electrophoresic bands were visible in the gel of the same samples, indicating that LAB specific amplicons were dominant in the PCR products. Electrophoresis results coincided with that for the melting curve analysis.

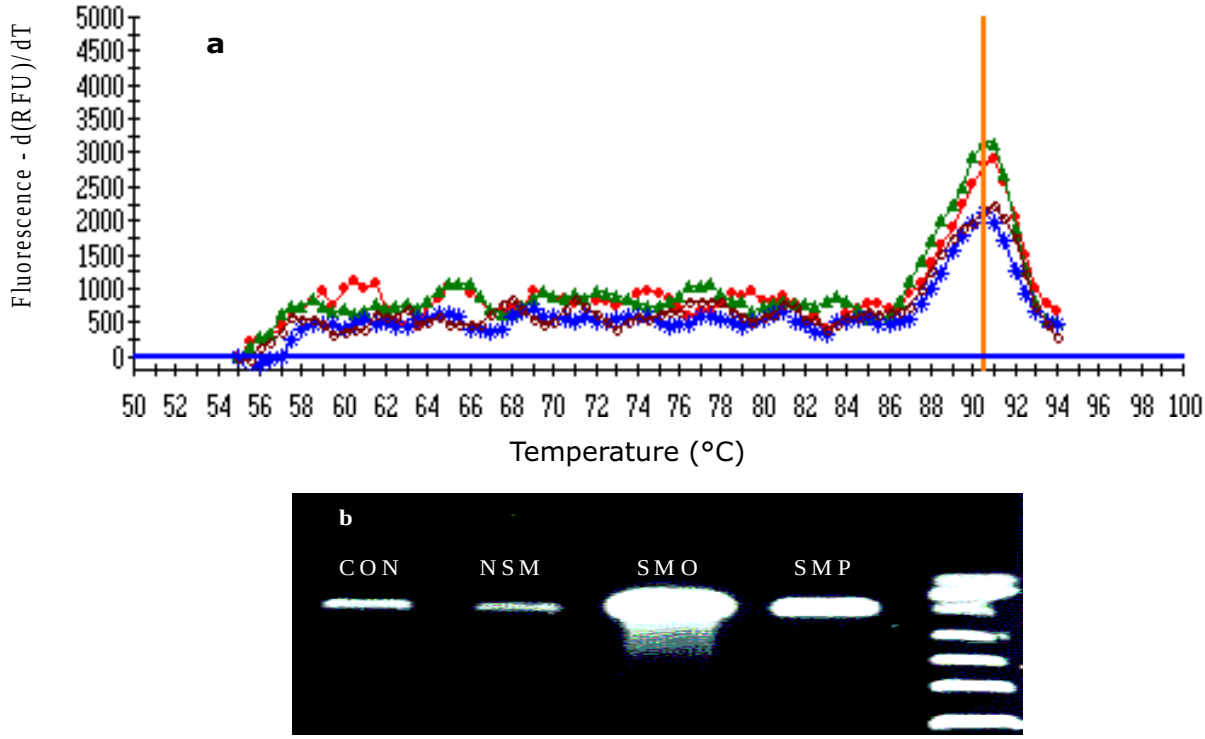

Figure 1. Plot of the negative derivative (-d*F* / d *T*) of the melting curves versus temperature. (a). Tm at 91.05 °C, 90.50°C, 91.16 °C and 90.85 °C for commercial diet control group (CON), soybean meal-free control group (NSM), soybean meal ligosaccharides treatment group (SMO) and soybean meal water-soluble polysaccharides treatment group (SMP) respectively. Agarose gel electrophoresis analysis (b) demonstrated that these peaks correspond to a single band of the position of about 340 bp.

Real-time PCR amplification

Real-time PCR amplification of the LAB V3 region gene of the genera *Lactobacillus, Pediococcus, Weissella* and *Leuconostoc* was carried out using a real-time PCR technique performed with a Light Cycler instrument, coupled with staining with SYBR Green I which is a nucleic acid dye (Weinbauer *et al.,* 1998). During real-time PCR, amplicon synthesis was monitored by SYBR Green I binding to amplified products. Figure 2 shows a plot of fluorescence versus cycle number. SYBR Green I was detected at the amplification cycle numbers 13.36, 15.24, 16.11 and 18.00 for SMO, SMP, NSM and CON groups respectively (SEM = 0.48). Fluorescence-labeled stranded DNA of the SMO groups was identified at earlier cycles than that of the other groups ($p<0.05$). The SMP and NSM diets showed a lower cycle number than the CON diet (CON) ($p<0.05$). The SMP diet, in turn, had a lower cycle number than NSM, though the difference was not significant ($p>0.05$). These results would suggest that the SMO and SMP diets could both stimulate LAB growth in the caecae of broilers during the early stage of post-hatch.

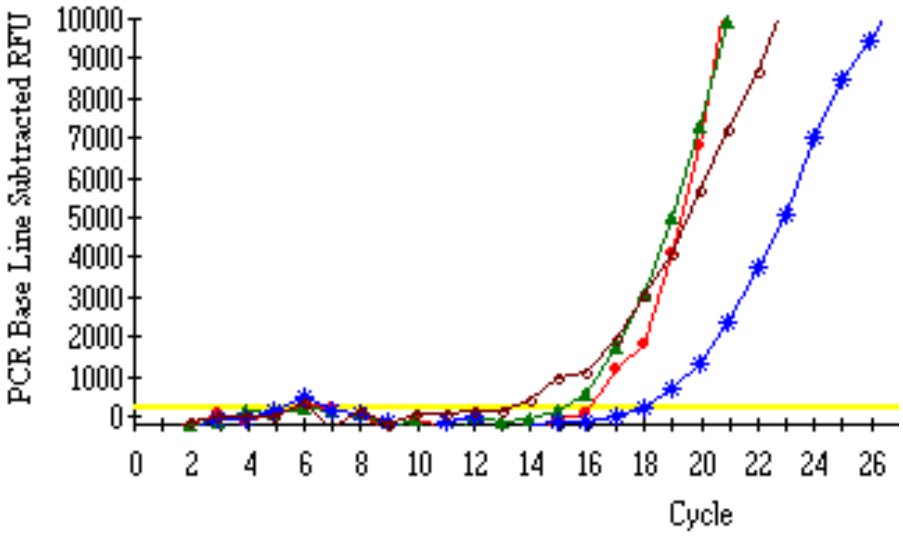

Figure 2. A plot of fluorescence (y) against cycle number (x).

O = soybean meal oligosaccharides diet treatment group (SMO);
▲ = soybean meal water-soluble polysaccharides diet treatment group SMP;
● = soybean meal–free control group (NSM);
✱ = commercial diet control group (CON)

Real-time PCR allows continuous detection of PCR products by monitoring SYER Green I from the initial synthesis procedure. The sensitivity of SYBR Green I analysis is more than adequate for the majority of research applications, and its quantitative nature is demonstrated by a linear relationship between the log of the template concentration and the cycle number at which fluorescence rises above the baseline (Simpson *et al.,* 2000; Bates *et al.,* 2001). These results indicate that the LAB template concentration in the samples was highest for the SMO-fed group. This means that for the caecal contents of SMO fed birds, there was the largest LAB population, compared with birds fed the other diets.

The exact population of LAB can be calculated by making a standard curve by DNA coming from the pure LAB culture. With regard to this research, the PCR product was a mixture of several species lactic acid bacteria. The proportion of each specie was unknown. It is difficult to decide the LAB specie with which to make standard curve. Therefore, there is not a corresponding standard curve to the population of LAB available yet in this experiment. From these results, it can only be stated that the amplified DNA consisted of several genera of LAB. However, the actual proportion of each specific genera for each diet group cannot be estimated from these data. Accurate quantification will therefore be necessary in subsequent research.

The aim of this study was to determine whether soybean meal water-soluble oligosaccharides (SMO) and polysaccharides (SMP) can effect the development of caecal lactic acid bacteria (LAB) in the early stage of post-hatch in a soybean meal-free basal diet. LAB group specific primers, real-time PCR and a fluorimetric technique, as well as melting curve analysis were used to study this development. The results suggest that soybean meal non-digestible carbohydrates do indeed play a role in stimulating the growth of LAB in the caecal contents of chickens. However, according to the strict definition of prebiotics proposed by Gibson and Fuller (2000), it is not possible to state clearly that SMO and SMP have prebiotic effects in broilers because while the LAB genera were clearly stimulated, it is unclear whether or not other bacterial species were also stimulated as these were not studied. Scanning electron micrographs of the caecal lumen surface have shown that there was a denser bacteria.

population on the caecal mucosal surface of birds fed SMO, SMP and NSM diets compared with the CON diet (Figure. 3). Even so, it is still impossible to define the exact prebiotic characteristics of SMO and SMP on broilers from this current study. The PCR amplification product was a gene mixture of the genera *Lactobacillus, Pediococcus, Weissella* and *Leuconostoc*, and as they are impossible to differentiate using this technique, it is still impossible to determine which were most stimulated by either SMO or SMP. Further studies are required to elucidate this further

The real-time PCR results suggest that the population of caecal contents LAB was lower in antibiotic-containing diet (CON), compared with the other groups (SMO, SMP and NSM). The antibiotic Aureomycin was included in the CON diet throughout the study. It belongs to a group of broad-spectrum antibiotics, which not only prohibit pathogens but also gut inhabiting bacteria including LAB. Subtherapeutic dose of antibiotics, as a health and growth promote substrate, routinely use in broilers' diet at most part of the world. This is potentially harmful to human beings. The ban of using antibiotics in diet of animals stimulate more studies to focus on funding some safety substrates to increase the immune competence ability of animal selves and finally to substitute antibiotics in animal feed.

In conclusion, soybean meal oligosaccharides (SMO) can significantly increase the population of a group of lactic acid bacteria (genera *Lactobacillus, Pediococcus, Weissella* and *Leuconostoc*) when SMO is an ingredient of a broiler diet of two weeks post-hatch. Although selective stimulation of SMO on LAB could not be certified by the current experimental results, SMO does show promise for use as a product which may promote competitive exclusion of potential pathogens. Pending further confirmation, SMO may therefore, be a suitable substitute for dietary antibiotics in young broiler chickens in the future.

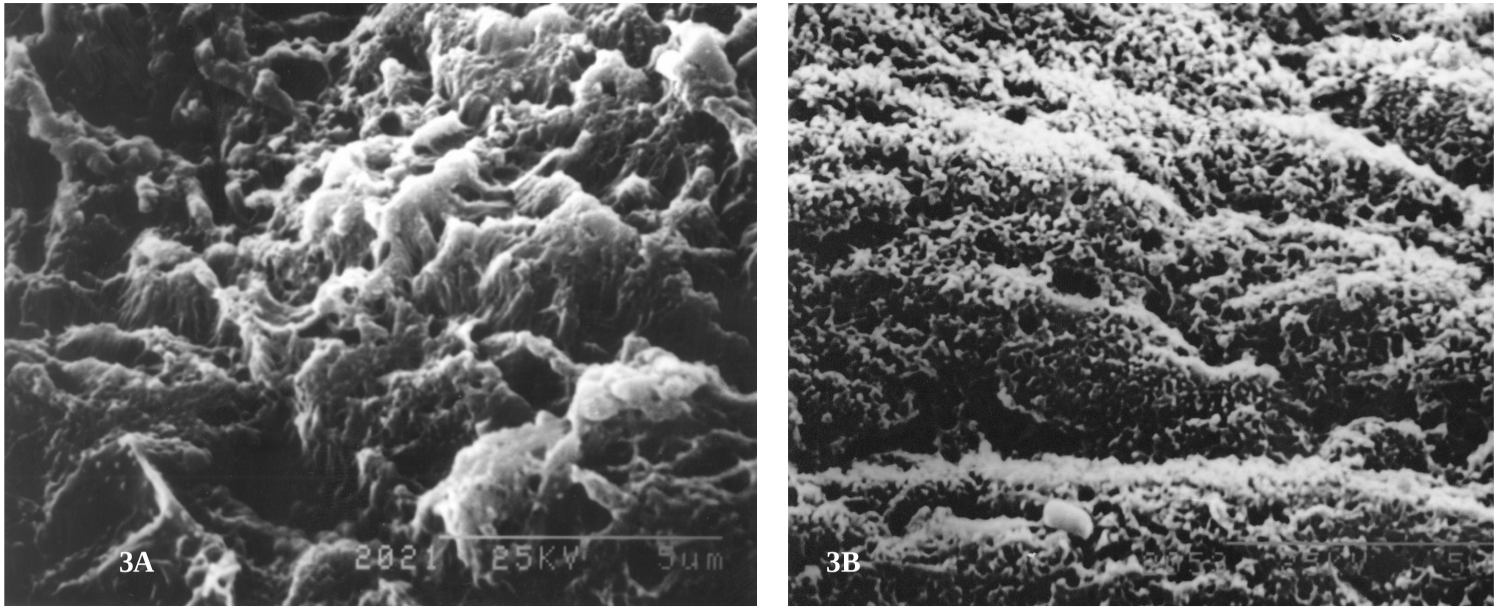

Figure 3. Pictures of scanning electron microscope on caecal surface (×8000. Bar=5 μm.). 3A and 3B is the caecal mucosal surface of commercial control group (CON) and soybean meal oligosaccharides treatment group (SMO), respectively.

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REFERENCES

Aldea, C., C. P Alvarez, L. Folgueira, R. Delgado, J. R. Otero, 2002. Rapid Detection of Herpes Simplex Virus DNA in Genital Ulcers by Real-Time PCR Using SYBR Green I Dye as the Detection Signal. Journal of Clinical Microbiology, Mar; 40(3):1060-1062.

Bates. J. A., E. J. A. Taylor, D. M. Kenton and J. E. Thomas, 2001. The amplication of real-time PCR to the identification, detection and quantification of *Pyrenophora* species in barley seed. Molecular Plant Pathology. 2(1):49-57.

Brady, L. J., Daniel, D. Gallaher and Frank F. Busta, 2000. The role of probiotic cultures in the prevention of colon cancer. Journal of Nutrition 130:410S-414S.

Buddington, R. K., and Evan Weiher, 1999. The application of ecological principles and fermentable fibers to manage the gastrointestinal tract ecosystem. Journal of Nutrition 129: 1446S-1450S.

Cavazzoni, V., A. Adami and C. Castrovilli, 1998. Performance of broiler chickens supplemented with *Bacillus coagulans* as probiotic. British Poultry Science 39:526-529.

Charteris, W. P., P. M. Kelly, L. Morelli, J. K. Collins, 1997. Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. International Journal of Food Microbiology. 35:1-27.

De Preter, K., F. Speleman, V. Combaret, J. Lunec, G. Laureys, B. H. Eussen, N. Francotte, J. Board, 2002. Quantification of MYCN, DDX1, and NAG gene copy number in neuroblastoma using a real-time quantitative PCR assay. : Modern Pathology. 2002 Feb;15(2):159-66.

Gibson, G. R. and R. Fuller, 2000. Aspects of in vitro and in vivo research approaches directed toward identifying probiotics and prebiotics for human use. Journal of Nutrition, 130(2S Suppl): 391S-395S.

Gibson, G. R., 1999. Dietary modulation of the human gut microflora using prebiotics oligofructose and lnulin. Journal of Nutrition 129:1438S-1441S.

Heid, C. A., J. Stevens, K. J. Livak, P. M. Williams, 1996. Real-time quantitative PCR. Genome Research. 6:986-994.

Hugenholtz, P., B. M. Goeble, and N. R. Pace, 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. Journal of Bacteriology. 180:4765-4774.

Jenkins, DJ. A., W. Cyril, C. Kendall and Vladimir Vuksan, 1999. Inulin, oligofructose and intestinal function. Journal of Nutrition 129:1431S-1433S.

Kim, D. W., 2001. Real time quantitative PCR. Experimental Molecular Medical. Apr 21;33(1 Suppl):101-109.

Knutsson, R., Fontanesi M, Grage H, Radstrom, 2001. Development of a PCR-compatible enrichment medium for *Yersinia enterocolitica*: amplification precision and dynamic detection range during cultivation. International Journal of Food Microbiology. Feb 5;72(3):185-201.

Komurian-Pradel, F., G. Paranhos-Baccalà, M. Sodoyer, P. Chevallier, B. Mandrand, V. Lotteau and P. André, 2001. Quantitation of HCV RNA using real-time PCR and fluorimetry. Journal of Virology Methods. 95:111-119.

Lewington, J., S. D. Greenaway and B. J. Spilline, 1987. Rapid small scale preparation of bacterial genomic DNA, suitable for cloning and hybridization analysis. Letters in Applied Microbiology. 5:51-53.

Menne, E., and N. Guggenbuhl, 2000. Fn-type chicory inulin hydrolysate has a prebiotic effect in humans. Journal of Nutrition. 130:1197-1199.

Muyzer, G., E. de Waal, A. Uitterlinden, 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain-reaction-amplified genes coding for 16S rRNA. Applied Environmental Microbiology. 59:695-700.

Oleastro, M., A. Menard, A. Santos, H. Lamouliatte, L. Monteiro, P. Barthelemy, F. Megraud, 2003. Real-Time PCR assay for rapid and accurate detection of point mutations conferring resistance to clarithromycin in helicobacter pylori. Journal of Clinical Microbiology. Jan; 41 (1):397-402.

Pacher, B., W. Kneifel, 1996. Development of a culture medium for the detection and enumeration of bifidobacteria in fermented milk products. International Dairy Journal. 6:43-64.

Pietilä, J., Q. He, J. Oksi and M.K. Viljanen, 2000. Rapid differentiation of *borrelia garinii* from *Borrelia afzelii* and *Borrelia burgdorferi*sensu stricto by Lightcycler fluorescence melting curve analysis of PCR product of the *recA* gene. Journal of Clinical Microbiology. 38(7): 2756-2759.

Rao, A. V., 1999. Dose-response effects of inulin and oligofructose on intestinal bifidogenesis effects Journal of Nutrition. 129:1442S-1445S.

Reddy, B. S., 1999. Possible mechanisms by which pro- and prebiotics influence colon carcinogenesis and tumor growth. Journal of Nutrition 129:1478S1482S.

Ririe, K. M., R. P. Rasmussen,and C. T. Wittwer, 1997. Product differentiation by analysis of DNA melt curves during the polymerase chain reaction. Analytical. Biochemistry. 245:154-160.

Sander, M. E., 2000. Considerations for use of probiotic bacteria to modulate human health. Journal of Nutrition 130:348S-390S.

Simpson, D. A. C., S. Feeney, C. Boyly and A. W. Stitt, 2000. Retinal VEGF mRNA measured by SYBR Green I fluorescence: A versatile approach to quantitative PCR. Molecular Vision,. 6: 178-183.

SPSS, Inc. 1997. SPSS for windows graduate pack advanced version, Version 8.0. Chicago, IL: SPSS Inc.

Tannock, G. W., 1995. Normal microflora. An introduction to microbes inhabiting the human body. Chapman and Hall, London, United Kingdom.

Walter, J., C. Hertel, G. W. Tannock, C. M. Lis, K. Munro and W. P. Hammes, 2001. Detection of *Lactobacillus,Pediococcus, Weissella* and *Leuconostoc* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Applied and Environmental Mirobiology. June, 2578-2585.

Wang, R. f., W. W. Cao and C. E. Cerniglia, 1996. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Applied and Environmental microbiology. 62;1242-1247.

Weinbauer, M. G., C. Beckmann and M. Höfle, 1998. Utility of green fluorescent nucleic acid dyes and aluminum oxide membrane filters for rapid epifluorescence enumeration of soil and sediment bacteria. Applied and Environmental Microbiology. Dec. 5000-5003.

Wittwer, C. T., M. G. Herrmann, A. A. Moss, R. P. rasmussen, 1997. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques. 22:130-138.

Wong, Y. W., G. M. Sia, H. P. Too, 2002. Quantification of mouse glial cell-line derived neurotrophic factor family receptor alpha 2 alternatively spliced isoforms by real time detection PCR using SYBR Green I. Neuroscience letters. Mar 8;320(3):141-5.

Wu, Y., V. M. Hayes, J. Osinga, I. M. Mulder, M. W. G. Looman, C. H. C. M. Buys, and R. M. W. Hofstra, 1998. Improvement of fragment and primer selection for mutation detection by denaturing gradient gel electrophoresis. Nucleic Acids Research. 54:32-40.

Yamaguchi, A., N. Hashimoto, W. Tsutae, K. Seino, Y. Ebina, T. Tokino, N. Sato, K. Kikuchi, 2002. Detection of human papillomavirus DNA by PCR/microfluorometry for screening of cervical cancer. Clinica Chimica Acta., 318(1-2):41

Chapter 5

IMMUNE PROMOTING EFFECTS OF SOYBEAN MEAL WATER-SOLUBLE OLIGO- AND POLYSACCHARIDES ON BROILER CHICKENS

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Immune-Promoting Effects of Soybean Meal Water-Soluble Oligo- and Polysaccharides on Broiler Chickens

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Abstract: This experiment was conducted to investigate the effects of SMO and SMP (oligo- and polysaccharides extracted from soybean meal) on the immune responses of broilers during 42 days of life. Soybean meal-free basal diets were diluted by 1% SMO and 0.5% SMP and fed during Days 1 to 14 post-hatch. From Day 14 to Day 30 of age, all animals were fed a soybean-free diet and from Day 30 to 42 of age they received a commercial diet containing soybean meal. Animals were vaccinated against avian infectious bronchitis (ABV) on Day 1, against infectious bursa (IBDV) on Day 7 and 20, and against Newcastle disease virus (NDV) on Day 14 and 28. Several aspects of humoral, cellular and mucosal immunity were measured at 7, 14, 28 and 42 days of age. The experimental results indicated that SMO and SMP led to an increase in immunoglobulin A, M and Y positive plasma cells (IgA^+, IgM^+ and IgY^+) within the caecal mucosa. There was also a tendency for increased T lymphocyte transformation rate and activated macrophage phagocytic function in relation to the presence of SMO and SMP in the diet. There were no significant differences in the Newcastle disease virus (NDV)-specific antibody titers between treatments at 7, 14, 28 and 42 days of age, between birds receiving different diets. The effect of enhanced immunity with dietary SMO and SMP declined with age. It was shown that SMO and SMP can stimulate both the systemic cellular and caecal local humoral immune response, and promote the development of the lymphocyte germinal center in caecal lamina propria. These diets did not have an effect on the systemic humoral immune response in the first two weeks post hatching. Therefore, both SMO and SMP, but especially SMO, could be used as a substrate for antibiotic substitution in the diet of young broiler chickens.

Keywords: T lymphocyte transformation rate, macrophage phagocytic function, immunoglobulin positive plasma cells, soybean meal oligo- and water soluble polysaccharides (SMO & SMP), broilers.

INTRODUCTION

Antibiotics added to feed in sub-therapeutic doses have until recently, been used worldwide as growth-promoting additives for broilers. However, their use seems increasingly to be leading to problems, such as the build-up of resistance to pathogens and the presence of antibiotic residues in meat, which may be harmful for humans. These problems are the main reasons why there is now a search for alternatives to growth promoting antibiotics. The substitution of antibiotics by other components which may promote broiler immunity is under investigation.

The intestine is continuously exposed to antigens from the external environment. It is also the first line of defense against the invasion of pathogenic bacteria. Gut-associated lymphoid tissue (GALT) is distributed along the intestine of broilers. It plays an important role in making specific antibodies for broilers (Bryant *et al.,* 1973; Befus *et al.,* 1980). Furthermore, microorganisms which reside in the distal intestinal segments of broilers, may trigger the gut mucosal defense of the host (Blum *et al.,* 1999.) Recently, results of several separate experiments have demonstrated that soybean meal oligo- and water-soluble polysaccharides (SMO and SMP) can be fermented by the caecal digesta microflora and can significantly increase the short chain fatty acid (SCFA) concentrations in vitro (Chapter 2). When SMO and SMP were used in diets of birds in an early stage of post-hatch, the birds had a decreased faecal oocyst output and an increase in the number of immunoglobulin A, M and G positive plasma cells in the caecal lamina propria of the broilers which were infected with *Eimeria tenella* (Chapter 7). Meanwhile, dietary SMO and SMP also promoted the development of lactic acid bacteria (LAB) population in the cecal contents of healthy broilers. This may thus support the survival of populations of LAB in *E. tenella* challenged broilers (Chapters 4 and 6).

The aim of the present investigation was to investigate the effect of dietary SMO and SMP on the humoral and cellular immune response of broilers, when SMO and SMP were added to the diets during 1-14 days post-hatch. It also aimed to study these responses in birds after withdrawal of these dietary components.

MATERIALS AND METHODS

Extraction of SMO and SMP

The extraction of SMO and SMP from soybean meal has bean described previously as follows: 1 kg soybean meal was refluxed twice in 10 l alcohol (80% (v/v)) at 75°C for 1 h. This supernatant was then used for SMO isolation and the residue for SMP isolation. The supernatant was condensed by rotavapor (Büchi RE 120) and protein was subsequently removed with 5 % trichloroacetic acid (TCA). The liquid was freeze-dried after passing a column filled with Amberlite 200 and Amberlite IRA-900 for removing TCA and amino acids from the supernatant. The residue was hydrolyzed in a 0.2 M NaOH solution at 80 °C for 2 h and filtrated using cheesecloth. The filtrate was neutralised with 30% acetic acid and freeze-dried. The freeze-dried sample was dissolved in 400 ml water. Protein was removed with 5 % TCA. After 1 h, the supernatant was centrifuged at 13000 g for 30 min. SMP was precipitated by adding alcohol to the supernatant until the alcohol concentration was 80 % (v/v) and kept at 4 °C over night. The polysaccharide precipitate was centrifuged at 2500 g for 10 min. The polysaccharide product was freeze-dried. Replicate products extracted at different times were mixed together and stored at – 20 °C .

Animal and diets

A total of 240 1-day-old male Arbor Acres broilers were randomly assigned to one of 4 groups of 60 animals. Every group of 60 birds was then randomly allocated to one of four dietary treatments. For each treatment, four cages with fifteen birds each, served as four replicates. The experiment lasted for 42 days. The diets were divided into a first (1-30 days) and second (31-42 days) phase; during phase 2 the diet was a commercially available diet and was the same for all groups. The formulation of the diets is shown in Table 1. There were four dietary treatments (CON, NSM, SMO, and SMP). Two basal diets were a commercial diet (positive control group, CON: diet II) and a non-soybean meal diet (negative control group, NSM: Diet I). Soybean meal oligo- (SMO) and water-soluble polysaccharides (SMP) were used in

the SMO and SMP groups, by diluting the basal soybean meal-free diets (diet I) by 1% SMO and 0.5 % SMP during 1-14 days of age respectively. These diets were replaced by the basal diet till 30 days of age as follows: ratio basal diet to experimental diet = 30 to 70 at the 15 th day; 60 to 40 at the 16 th day; 90 to 10 at the 17 thday and 100 to 0 at the 18 th day. Diet of all groups in this investigation changed to Diet III from 31 days of age onwards.

Immunization procedures

All broilers were vaccinated with attenuated vaccine H120 (ABV) for Avian infectious bronchitis. They were vaccinated by nose and eye drops on the first day post-hatch. Infectious bursa disease virus vaccine (IBDV) was given in the drinking water on Day 7 (primary) and 20 (secondary). Newcastle disease virus IV attenuated vaccine (NDV), was given via nose and eye drops, on Day 14 (primary) and 28 (secondary). All vaccines were obtained from Inner Mongolia Biomedicine Co., China.

Experimental methods

Immunohistochemistry: Four birds from each group were sacrificed at Day 15 of life by cervical dislocation. Mid-segments of both caeca were collected and washed in cold phosphate buffer solution (PBS) and then fixed in a 10 % formalin-buffered saline solution (pH 7) for 24 h. The fixed samples were rinsed to get rid of formalin and then embedded in paraffin, cut into 4-mm-thick slices, and attached to poly-L-lysine-coated slides. The slices were kept at 37 °C for 3 h and then stored at 4 °C prior to use. Prior to staining, the slides were allowed to dewax and to thenrehydrated. Endogenous peroxidase and non-specific binding were blocked by 3% H_2O_2 solution and 10 % normal goat serum at room temperature. The Avidin-Biotin Complex (ABC) technique was engaged for the immunohistochemistry procedure (Jingshan, 1992). Firstly, slides were incubated at 37 °C for 30 min. and then at 4 °C overnight in a humidity chamber with one of three kinds of monoclonal antibody (Mab), (mouse anti-chicken α-heavy chain specific IgA, γ-heavy chain specific IgG, and μ-heavy chain specific IgM, Southern Biotechnology Associates, Inc. USA). Secondly, the

slides were incubated with biotin-conjugated goat anti-mouse immunoglobulin G (IgG, Sigma, Zhong Shan Co.) at 37 °C for 30 min. Thirdly, they were incubated with peroxidase-conjugated streptavidin at 37 °C for 30 min., and then, the stained immunoglobulin-containing plasma cells were detected by 3, 3 Diamino Benzidine (DAB). There were three washes in 0.01 M PBS (pH7.4) between each reagent. Finally, all nucleoli were stained by haematoxylin. The negative control slices were stained in the same way as the experimental ones, but by substituting Mab with PBS.

T lymphocyte transformation rate (TLTR): A total of 48 birds were sacrificed at Day 14, 28 and 42 for analyzing T lymphocyte transfermation rate (TLTR), 16 birds (4 birds per treatment) at each of the three ages. TLTR was analyzed using a morphological method (Tianbang *et al.,* 1999). In brief, 10 ml blood sample was taken from each bird by cardiac puncture and heparinized (50 I.U./ml) (Hua Mei Biochemical products, China) immediately in a sterile cuvette and Kept at room temperature for 1h after gentle mixing. Lymphocytes were separated using lymphocyte delamination solution (Hua Mei Biochemical products, China). The separated lymphocytes were rinsed in Hank's solution (Hua Mei Biochemical products, China) three times. The concentration of lymphocyte cells was adjusted to 3×10^6/ml in RPMI-1640 medium (Hua Mei Biochemical products, China) in incubation bottles, that contained 16.67 μ l haemagglutinin (PHA) (Hua Mei Biochemical products, China) per ml incubation solution. The bottles were incubated at 37 °C for 72 h.

After incubation was terminated, the incubation solution was centrifuged at 100 g for 10 min. The supernatant was discarded and the lymphocyte cells were dispersed by air current. One to two drops of lymphocyte solution was placed on a cold slide, laid on, dried, fixed by formalin and rinsed by mol/15 (pH=6.8) PBS. The slides were stained with Giemsa (at least 3h). Lymphocytes were examined by light microscope. The transitional lymphocytes and lymphoblasts were identified according to Roitt *et al.,* (1989). The morphological picture is shown in Figure 1. TLTR was calculated according to the formula as follows:

$$TLTR = ((\text{transitional lymphocyte} + \text{lymphoblast})/\text{total lymphocyte}) \times 100.$$

Phagocyte carbon clearance test: A total of 32 birds was used for this test at 14 and 28 days of age. The carbon clearance test (Lijuan, 1981) was used to determine phagocyte activities. Of 16 birds (4 birds per treatment) at each of the two ages, live body weight was recorded, and then the birds were injected with 0.2 ml of East Indian ink (1:3 diluted solution) via the wing vein. At 2 min. and 10 min. post-injection a 0.2 ml blood sample was collected by cardiac puncture, and mixed with 20 ml 0.1 % Na_2CO_2 solution. OD values were examined at 600 nm wavelength by spectrophotometer (UD-7, Beckman Company, USA) with the Na_2CO_2 solution serving as a negative control. Livers and spleens were taken and weighed after the birds were sacrificed. Phagocytic function index (A) was calculated according the to the following:

$$K = (\lg OD_1 - \lg OD_2) / t_2 - t_1$$

$$A = (\text{body weight} / (\text{spleen weight} + \text{liver weight})) \times K^{1/3}$$

Where: K: carbon clearance rate.

A: phagocytic function index.

OD_1 and OD_2: light penetration rate of blood samples at 2 min. and 10 min., respectively

t_1 and t_2: time of the first and the second blood collection

NDV specific antibody titer determination: Of 32 birds' (8 birds at each of the 4 ages) blood was collected at Day 7, 14, 28 and 42 for the titres of NDV analysis. The birds were returned to their original cage after sampling. NDV antibody was checked by the hemadsorbent (HA) and the hemagglutination inhibition (HI) method (Tianbang *et al.,* 1999). In brief, 0.5-1.0 ml blood samples were obtained from the wing vein and allowed to clot for 4 h, after which serum was collected by centrifugation of the sample 3000 g for 10 min, 25 µ l NaCl solution (0.9 %) was added to each well of 96-well round-bottom microtitre plates. 25 µ l serum samples was added into the first well and mixed well, then 25 µ l solution was moved from the first well into the second one. etc. This procedure was repeated until the needed dilution had been reached. The grads concentration solution was incubated at 37 °C for 15-20 min.,

then 25 μ l NDV antigen (4 units, Ding Guo Biotechnique Co., Chian) was added to each well and incubated at 37 °C for 15-20 min. Twenty-five μ l chicken red blood cells (1 %, Ding Guo Biotechnique Co., Chian) were added in each well, and this was followed by 30 min incubation at 37 °C. There was a gentle vibration for 1-3 min. after serum, NDV antigen and red blood cells. The highest serum dilution that protected 50 % of the cells was then the NDV HI antibody titer.

Statistics

The number of caecal mucosa immunoglobulin A, M and Y positive plasma cells (IgA^+, IgM^+ and IgY^+) in the caecal lamina propria were expressed per villus. In total, 20 integrated villi were used in one group, with 5 from one bird of the same experimental group. The data of NDV specific titer, T lymphocyte transformation rates and microphage phagocytic function index, each treatment was considered as an experimental unit. The results were compared as means using a one-way ANOVA model, the significant difference between groups were analyzed by LSD t test, using SPSS 8.0 (Statistical Package for the Social Science, 1997) statistical software.

RESULTS

The NDV specific antibody titer changes in serum of broilers

The titres of Newcastle disease virus (NDV)-specific hemagglutination inhibition (HI) antibody in serum were analyzed for treatment effects at Days 7, 14, 28 and 42. These results are shown in Table 2. The data indicated that there were no significant differences in the NDV-specific antibody titres between treatments at the same investigation time. The titres were higher at Day 28 and 42 compared with Day 7 and 14 for all groups.

Table 1. Composition of the diets and diet fitted in different treatments and feeding phases [1, 2, 3] (% as fed)

Ingredient	Diet I	Diet II	Diet III
Ground corn	47.90	60.25	66.18
Soybean meal		11.00	26.95
Corn germ meal	15.00		
Corn gluten meal	24.00	17.00	
Fish meal	7.00	7.00	1.00
$CaHPO_4$	0.70	1.25	1.15
Limestone	1.60	1.20	1.35
Salt	0.30	0.30	0.30
Sunflower oil	2.50	1.00	2.00
Vitamin-mineral mixture*	1.00	1.00	1.00
L-Lysine HCI			0.07
Crude protein	20.21	12.62	20.22
Metabolism energy (MJ / Kg)	12.58	17.83	12.51
Ca	1.00	1.01	0.95
P	0.64	0.64	0.57
SMO		0.96	2.34
SMP		0.42	0.78
Experimental design			
Treatments	Diet I	Diet II	Diet III
SMO	1-30 days [4]		31-42 days
SMP	1-30 days [4]		31-42 days
NSM	1-30 days		31-42 days
CON		1-30 days [5]	31-42 days [6]

[1] Provide vitamins and minerals as previously described (Lee and Leeson, 2001); [2] Commercial diet control group (CON), soybean meal-free starter diet control group (NSM) and soybean meal oligo- and water soluble polysaccharides (SMO & SMP)groups were treatments in this experiment; [3] Two feeding phases: the first phase started from 1 day until 30 days of age, and the second started from 31 days to 42 days of age; [4] diluting the basal diets which were free of soybean meal (Diet I) by 1 % SMO and 0.5 % SMP during 1-14 day old respectively, then the diets were replaced by the basal diet till 30 day of age as follows: ratio of Diet I to experimental diet = 30 to 70 at Day 15; 60 to 40 at Day 16; 90 to 10 at Day 17 and 100 to 0 at Day 18; [5] Maduramicin (5 ppm) and aureomycin (0.22 %); [6] Aureomycin (0.22 %).

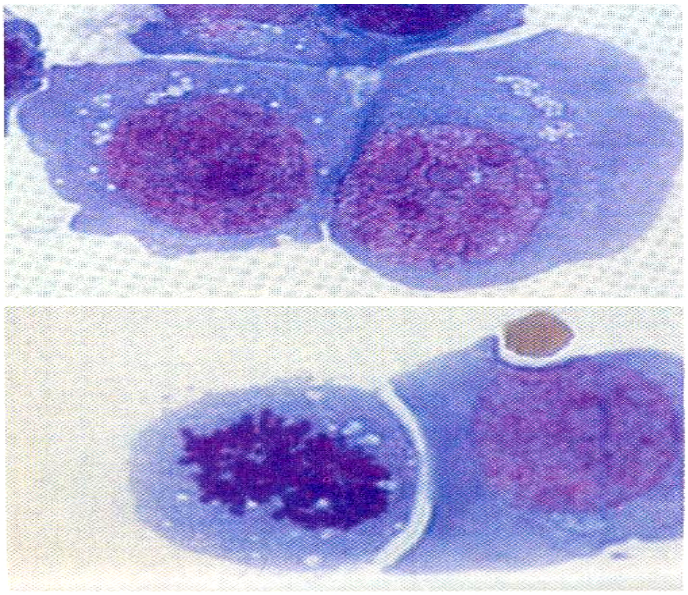

Figure 1 Phytohaemagglutinin (PHA)-induced lymphocyte blastogenesis (Roitt *et al.,* 1989) The human lymphocytes shown here have been stimulated by PHA. There is increased basophilia in the cytoplasm and an increase in the cell size (about 10-20μm) (upper picture). The chromosomes condense and can be clearly seen during metaphase (lower picture). 1-3 nucleolus and some vacuole can be seen in the cytoplasm. Giemsa stain × 2000. The virgin lymphocytes have smaller cell size (about 6-12 μm). There is no nucleolus and seldom a cavity in the cell (picture is not available here). The cells of which morphological characteristics are between virgin and lymphoblast are identified as transitional lymphocytes.

Table 2. The titres of NDV-specific antibody (log2) in serum of broilers as affected by soybean meal oligo-and polysaccharides (SMO and SMP) [1, 2]

Diet/Age	Day 7	Day 14	Day 28	Day 42
SMO [3]	6.25	5.50	8.00	7.60
SMP [3]	6.13	5.00	8.13	7.67
NSM [3]	5.75	5.12	8.25	8.50
CON [3]	6.00	5.38	8.00	6.60
SEM	0.19	0.16	0.22	0.29

[1] Newcastle disease virus IV attenuated vaccine (NDV) was vaccinated at Day 14 (primary) and 28 (secondary). Titres of NDV-specific antibody in serum was detected at Day 7, 14, 28 and 42; [2] Each value is the mean of 8 values and expressed as log2. [3] SMO, SMP, NSM and CON represent 1 % SMO, 0.5 % SMP treatments groups (SMO, SMP), soybean meal-free and commercial diets control groups (NSM and CON)

T lymphocyte transformation test

The results of T lymphocyte transformation rates (TLTR) are shown in Table 3. The non-soybean meal and commercial diet control groups (NSM and CON) had quite similar TLTRs at 14, 28 and 42 days of age. Compared with NSM-diet fed birds, the animals which were fed SMO and SMP had a numerically increased TLTR with 13. 3% and 26.7% at Day 14, 7.4 % and 11.0 % at Day 28 and 3.9 % and 6.9 % at Day 42, respectively, given the large variation between animals, but the differences was not significant ($p>0.05$).

Table 3. T lymphocytes transfer rates in broilers receiving different diets and checked at different ages (%)[1]

Diet/Age	Day 14	Day 28	Day 42
SMO [2]	13.23	15.50	24.14
SMP [2]	14.80	16.02	24.85
NSM [2]	11.68	14.43	23.24
CON [2]	11.90	13.37	23.75
SEM	0.67	0.58	0.58

[1] Each value is the mean of 4 replicates; [2] SMO, SMP, NSM and CON represent 1 % SMO, 0.5 % SMP treatments groups (SMO, SMP), soybean meal-free and commercial diet control groups (NSM and CON).

Phagocyte carbon clearance test

The macrophage phagocytic activity index (A) was examined for animals at Days 14 and 28. These results are shown in Table 4. Results indicated that dietary SMO and SMP promoted A at 14 days of age. Dietary SMO increased A values by 29.4 % and 23 %, compared to NSM and CON respectively. SMP increased A by 26.9 % and 20.9 % on Day 14, as compared to NSM and CON diets. These differences were not statistically significant ($P>0.05$). "A" values were similar for animals fed all diets on Day 28.

Table 4. Macrophage Phagocytic function index (A) in broilers receiving different diets for Days 14 and 28 [1].

Diet/Age	Day 14	Day 28
SMO [2]	8.13	10.88
SMP [2]	7.98	9.78
NSM [2]	6.29	10.02
CON [2]	6.60	10.40
SEM	0.34	0.29

[1] Each value is the mean of 4 replicates; [2] SMO, SMP, NSM and CON represent the 1 % SMO, 0.5 % SMP treatment groups (SMO, SMP), and the soybean meal-free and commercial control groups (NSM and CON).

Caecal lamina propria immunoglobulin A, Y and M positive plasma B cells (IgA$^+$, IgY$^+$ and IgM$^+$)

The numbers of caecal lamina propria immunoglobulin A, M and Y positive plasma cells (IgA$^+$, IgY$^+$ and IgM$^+$) were examined on Day 15 of age by MAbs and ABC immunohistochemistry techniques. They were counted per villi. A total of 20 integrated villi were used per group, with 5 villi from each bird of the same experimental group. The results are shown in Table 5. The distribution of IgA$^+$, IgY$^+$ and IgM$^+$ plasma cells is shown in Figure 2.

Compared with the NSM and CON diet groups, dietary SMO and SMP significantly increased the amount of IgA$^+$ and IgM$^+$ plasma B cells in the caecal lamina propria ($P<0.05$), but not for IgY$^+$. SMO had remarkably higher IgA$^+$ and IgM$^+$ plasma cells compared with the other three groups ($P<0.05$). Dietary SMO and SMP did not lead to a remarkable change in IgY$^+$ B cell numbers. There was no significant difference between NSM and CON groups for any of the three immunoglobulins.

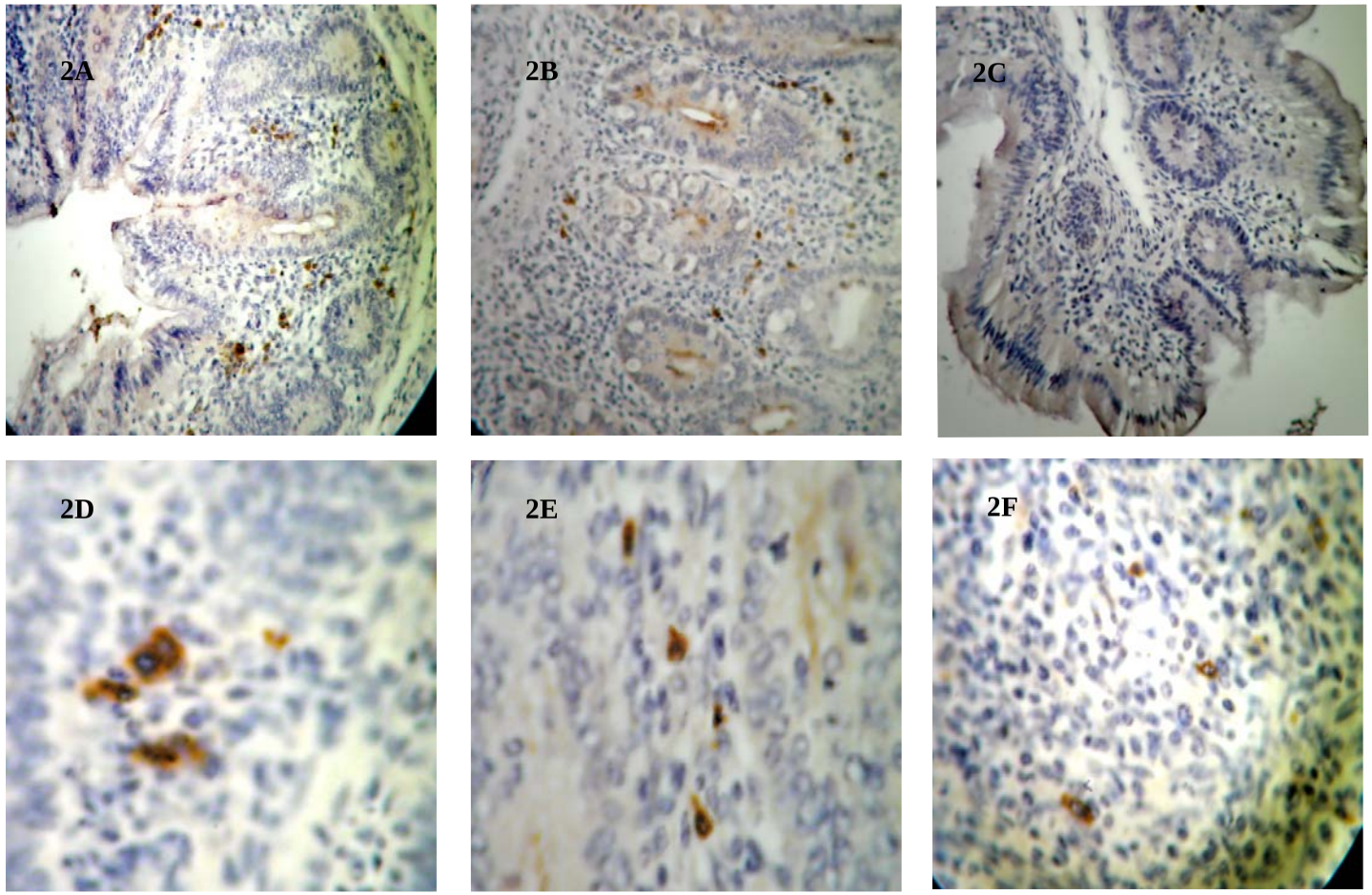

Figure 2. Distribution of IgA $^{+}$, IgM $^{+}$ and IgY $^{+}$ plasma cells under light microscope in caecal lamina propria at Day 15. 2A: IgA $^{+}$ in caecal villus crypt (x 400), 2B: IgA $^{+}$ in caecal villus body (x 400), 2C: caecal villus negative stained slice, substitute Mabs with PBS (x 400), 2D: IgA $^{+}$ (x 1000), 2E: IgY^{+}(x 1000), 2F: IgM^{+} (x 1000). MAbs and ABC immunohistochemistry technique is engaged in experiment. Cell nuclear was stained by haematoxylin. The cells with yellow plasma are positive cells.

Table 5. The number of IgA^+, IgM^+, and IgY^+ plasma cells in cecal lamina propria in broilers receiving different diets on Day 15 (/villus) [1, 2]

	IgA^+	IgM^+	IgY^+
SMO [3]	16.70 [a]	10.10 [a]	1.55
SMP [3]	10.50 [b]	6.95 [b]	1.55
NSM [3]	7.45 [c]	4.40 [c]	1.85
CON [3]	6.55 [c]	3.65 [c]	1.70
SEM	0.62	0.40	0.17

[1] Each value is the mean of 20 replicates; [2] Difference is significant between data marked different letter in the same column ($p<0.05$); [3] SMO, SMP, NSM and CON represent 1% SMO, 0.5% SMP treatments groups (SMO, SMP), soybean meal-free and commercial control groups (NSM and CON).

DISCUSSION

In this experiment, SMO and SMP were only added to the diet from Days 1-14 post-hatch, after which the SMO and SMP diets were gradually substituted by a soybean-meal-free basal diet within 3 days. Thereafter, the SMP and SMO-free diets were fed continuously until 30 days of age. From Day 31 a diet which contained soybean meal was given until the end of the experiment.

The results show clear changes in immune responses as an effect of feeding the SMO and SMP containing diets fed for Days 1-14. Furthermore, in this experiment, the lymphocyte germinal center of the caecal lamina propria were presented in only 50 % of the birds of the CON and NSM groups, also in 50 % of the birds of the SMP group and in 75 % of the birds of the SMO group at Day 15 (Figure is not shown here). This could have been an effect of the application of the diet during this part of life or as a result of the diet. The age component is likely to be important because at the beginning of that period, the broiler immune system is still undeveloped. The possible immunostimulatory mechanisms which may take place are as follows.

Firstly, the microorganisms which inhabit the lumen of the cecum of broilers are likely to have a significant stimulatory effect. In previous work, it has been shown that SMO and SMP stimulated the growth of the caecal microbial population and lactic acid bacteria (LAB) of the genera *Lactobacillus, Pediococcus, Weissella* and *Leuconostoc* as detected by scanning electron-microscopy, real-time PCR and fluorimetry technique (Chapter 4). Kazuhito *et al.* (1993) reported that the results of the immuno-histochemical analysis of tissue distribution of B and T cells in studies with germ-free and conventional chickens suggested that the development of gut-associated lymphoid tissues was affected by the intestinal flora. Also, LAB are gram-positive bacteria with cell wall components such as peptidoglycans, polysaccharide and teichoic acid, all of which have been shown to have immunostimulatory properties (Takahashi *et al.,* 1993). Peptidoglycan and muramyl dipeptide (MDP), which make up 30-70 % of the LAB cell wall (Rook *et al.,* 1989), can be released by lysozyme (Peeters *et al.,* 1975). Peptidoglycans are known to have adjuvant effects on the immune response (Stewart-Tull DES, 1980), especially on the intestinal mucosal surface (Link-Amster *et al.,* 1994). Binding sites for peptidoglycans were identified on lymphocytes and macrophages (Dziarski, 1991).

Secondly, the physical and chemical characteristics of caecal contents can also change as a result of microbial fermentation end-products of SMO and SMP (Chapter 2). These characteristics and the SCFA may also be of influence on the intestinal lamina propria and on epithelial immune cells. The lower chyme pH is almost certainly influenced by the increased concentrations of fermentation end products such as acetic, propionic, butyric and lactic acids. It has been reported that changes of intracellular pH often parallel changes of extra cellular pH and may trigger cell division. The lower lumen pH has been associated with an increase in the number of cells that are actively synthesizing new DNA in the cecal wall of rats (Jacobs and Lupton, 1984; Lupton and Jacobs, 1987).

The SMP used in this experiment was mainly composed of galactose (12 %) and rhamnose (5 %) (Chapter 2). Dietary SMP is a water-soluble material containing large molecules. It has been reported that polysaccharides could play an

immunostimulating role by both the antigenic properties of polysaccharides themselves and as an adjuvant. Some polysaccharides could stimulate the secretion of cytokines and result in an enhancement of the cellular immune response function (Patchen *et al.,* 1981; Womble *et al.,* 1988; Tizard *et al.,* 1989; Linna *et al.,* 1996, Bergquist *et al.,* 1997 and Sonoda *et al.,* 1998).

In conclusion, SMO and SMP can stimulate both the systemic cellular and caecal local humoral immune response, promote the development of lymphocyte germinal center in caecal lamina propria. These diets did not have an effect on the systemic humoral immune response in the first two weeks after hatching. This work has shown that dietary soybean meal oligosaccharides (SMO) and water soluble polysaccharides (SMP) added to the diet of chickens from Day 1 to 14 after hatching, can affect some immunological characteristics of broilers especially in relation to systemic cellular and mucosal cellular immune responses after vaccination. The experimental results indicate that SMO and SMP can significantly promote the broilers' immune response with regard to local immunity. Furthermore, these diets also tend to stimulate cellular immunity. However, the effects of immunity enhancement decreased with age and also after withdrawal of SMO and SMP from the diet. Therefore, SMO and SMP, especially the former one, could be used as antibiotic substitution in the diet of young broiler chickens.

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REFERENCES

Befus, A. D., N. Johnston, G. A. Leslin and J. Bienenstock, 1980. Gut-associated lymphoid tissue in the chicken. I. Morphology, ontogeny, and some functional characteristics of Peyer's patches. The Journal of Immunology. 125(6):2626-2632.

Bergquist, C., T. Lagergård and J. Holmgeren, 1997. Anticarrier immunity suppresses antibody response to polysaccharide antigens after intranasal immunization with polysaccharide-protein conjugate. Infection and Immunity. May 1579-1583.

Blum, S., Y. Delneste, S. Alvarez, D. Haller, P. F. Perez, C. H. Bode, W. P. Hammes, A. M. A. Pfeifer and E. J. Schiffrin, 1999. Interactions between commensal bacteria and mucosal immunocompetent cell. International Dairy Journal. 9:63-68.

Bryant, B. J., H. E. Adler, D. R. Cordy, M. Shifrine and A. J. DaMassa, 1973. The avian bursa-independent humoral immune system: serological studies. European Journal of Immunology. 3:9.

Dziarski, R., 1991. Demonstration of peptigolycan-binding sites on lymphocytes and macrophages by photoaffinity cross-linking. Journal of Biology Chemistry. 266:4713-4718.

Jacobs, L. R., and J. R. Lupton, 1984. Effect of dietary fibers on rat large bowel mucosal growth and cell proliferation. American Journal of Physiology. 264:378-385.

Jingshan, Y., 1992. Cytochemical and Cytobiological Techniques. Published by BeiJing Medical University Publisher House. P105-107.

Kazuhito, H., H. Takehiro, I. Kikuji, T. Eiji and H. Yoshikazu, 1993. Immunohistochemical analysis of tissue distribution of B and T cells in germfree and conventional chickens. The Journal of Veterinary Medical Science. 55:1031-1034.

Lee, K. H. and S. Leeson, 2001. Performance of broilers fed limited quantities of feed or nutrients during seven to fourteen days of age. Poultry Science. 80:446-454.

Lijuan, C., 1981. Effect of astragalus polysaccharides on macrophage phagocytize function of rats. Pharmacology Journal of China. 2(3): 200-204.

Link-amstr, H., F. Rochat, K. Y. Saudan, O. Mignot and J. M. Aeschlimann, 1994. Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intakes. FEMS Immunology and Medical Microbiology. 10:55-64.

Linna, Z. and I. R. Tizard, 1996. Activation of a mouse macrophage cell line by acemannan: the major carbohydrate fraction from *Aloe vera* gel. Immunopharmacology.35:119-128.

Lupton, J. R. and L. R. Jacobs, 1987. Fiber supplementation results in expanded proliferative zones in rat gastric mucosa. The American Journal of Clinical Nutrition. 46:980-984.

Patchen, M. L. and E. Lotzova, 1981. The role of microphages and T-lymphocytes in glucan-

mediated alteration of murine hemopoiesis. Biomedicine.34:71.

Peeters, T., G. Vantrappen, 1975. The paneth cell: a source of intestinal lysozyme. Gut. 16: 553-558.

Roitt, I., J. Brostoff, and D. Male, 1989. Immunology (Second edition). Churchill Livingstone, Edinburgh London and Melbourne. Gower Medical Publishing. London. New York. 2.2-2.10.

Rook, G., 1989. Immunity to bacteria. In: Roitt I, Brostoff J, Male D. Eds. Immunology. St Louis: Mosby.

Sonoda, Y., T. Kasahara, N. Mukaida, N. Shimizu, M. Tomoda and T. Takeda, 1998. Stimulation of inteleukin-8 production by acidic polysaccharides from the root of *Panax ginseng.* Immunopharmacology.38:287-294.

SPSS, Inc. 1997. SPSS for windows graduate pack advanced version, Version 8.0. Chicago, IL: SPSS Inc.

Stewart-Tull, D. E. S., 1980. The immunological activities of bacterial peptidoglycans. Annual Review of Microbiology. 34:311-340.

Takahashi, T., T. Oka, H. Iwana, T. Kuwata and Y. Yamamoto, 1993. Immune response of mice to orally administered lactic acid bacteria. Bioscience, Biotechnology, and Biochemistry. 57:1557-1560.

Tianbang, Y. and L. Xingyou, 1999. Poultry Immunology. Published by Chinese Agricultural Science and Technology Publisher house. P277-326.

Tizard, I. R., R. H. Carpenter, B.H. McAnalley and M.C. Kemp, 1989. The biological activities of mannans and related complex carbohydrates. Molecular Biotherapy. 1:290.

Womble, D. and J. H. Helderman, 1988. Enhancement of allo-responsiveness of uman lymphocytes by acemannan (Carisyn™). International munopharmacology. 0:967.

Chapter 6

REAL-TIME BASED DETECTION OF LAB IN CAECAL CONTENTS OF *E. TENELLA* INFECTED BROILERS FED SOYBEAN OLIGOSACCHARIDES AND WATER-SOLUBLE POLYSACCHARIDES

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To be submitted

Real-Time Based Detection of LAB in Caecal Contents of *E. Tenella* Infected Broilers Fed Soybean Oligosaccharides and Water-Soluble Polysaccharides

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Abstract: Two-hundred and 40 1-day-old male Arbor Acres broilers were assigned to six different treatment groups with 40 birds each per treatment. The basal diet was a soybean meal-free diet, consisting of corn, corn gluten, corn germ meal, fish-meal and mineral and vitamin additives, containing 12.6 MJ of metabolizable energy (ME) per kg and 20.2 % of crude protein (CP). The six experimental treatments were as follows: two groups were fed diets containing either soybean meal oligosaccharides (SMO) or water-soluble polysaccharides (SMP). The chickens in these SMO and SMP groups were fed the basal diet diluted by 1 % SMO and 0.5 % SMP during 1-14 days of age, respectively. A third treatment was the vaccinated group (VAC). The birds in this group were vaccinated with 100 oocytes of *Eimeria tenella* when 3 days old. A fourth group was an anti-coccidial medicated group (ANT), in which Maduramasin (5 ppm) was included in the diet on the five days before infection at 15 days of age and until 7 days after infection. Finally, positive (COR) and negative (COW) control groups were included as treatments. The COR animals were infected at 15 d of age. Chickens of all treatments except COW were orally infected with 1000 sporulated oocysts of *E. tenella* on Day 15. COW animals received only the basal diet. Fecal oocyst shedding was monitored per treatment group between Day 5 and 13 after infection. Lactic acid bacteria (LAB) in caecal contents were evaluated on Day 7 after infection by a real-time PCR technique. The results showed: SMO and SMP groups had a lower number of oocysts per gram of faeces (OPG) during the monitoring period, compared with the COR group. SYBR Green I labeled stranded DNA was detected at the amplification cycles 12.97, 13.99, 14.85, 14.92, 22.207 and 27.68 for VAC, SMO, ANT, SMP, COW and COR groups respectively, and specific PCR products were confirmed by the result of melting curve analysis and agarose gel electrophoresis. The results suggest that these LAB communities were promoted by SMO and SMP and have a competitive exclusion function when the

broiler chickens are infected by *E. tenella*. It was therefore concluded that SMO and SMP had prebiotic properties for these broilers.

Keywords: lactic acid bacteria (LAB), real-time PCR, melting curve analysis, caecal contents, broilers

INTRODUCTION

Broiler chickens have been selected for a high rate of gain within the intensive poultry industry. They are therefore usually slaughtered at around six weeks of age. There is sufficient evidence to show that they are more susceptible to ascites, and the sudden death malabsorption syndrome, compared with conventional birds. Therefore, they are more likely to have metabolic disorders than animals which have a lower rate of gain. In addition, they are assumed to have a poor immunocompetence. This means that these chickens are considered more susceptible to various types of infections (Havenstein *et al.,* 1994). Coccidiosis, an infection by protoza, is a worldwide problem, especially in broiler industry. In fact, coccidiosis or coccidiasis infection is one of the major contributors to production loss, especially in young chickens.

Generally, a complex community of microorganisms colonizes the broiler's distal intestine, and has a very important role during the broiler life span. Lactic acid bacteria (LAB), as part of a normal bacterial flora, may reduce the risk of an infection with enteropathogens by several mechanisms: (1) decreasing the number of pathogens and their virulence from the production of antagonistic compounds, (2) occupation at all the mucosal surface thus preventing adhesion by potencial pathogens, (3) stimulation of the host's immune response (Bernet *et al.,* 1994; Link-Amster *et al*., 1994; Schiffrin *et al.,* 1995). These mechanisms all contribute to the concept known as competitive exclusion (CE).

Competitive exclusion was first described by Nurmi and Rantala (1973), and has the potential to control pathogens in poultry. By pre-feeding non-pathogenic microorganisms, such as normal intestinal bacteria from adult fowl to young birds, the ecological niche may become occupied by some of these bacterial species and further prevent the invasion of pathogens (Kaldhusdal *et al.,* 2001). The role of competitive exclusion by LAB on some pathogenic bacteria has been well documented (Van der Wielen *et al.*2002*,* Hofacre *et al.,* 2002). Yet, there is not as much information available about this phenomenon with regard to coccidiosis infection. Young broiler

chickens are susceptible to coccidial infections. Consequently, vaccination before infection and dosing with antibiotics are regularly used in poultry production to prevent coccidial infection.

The intestinal LAB community, as beneficial commensal bacteria, can be modulated and stimulated by the presence of prebiotics (Jenkins, *et al.,* 1999; Buddington, *et al.,* 1999; Rao, 1999; Gibson, 1999; Reddy, 1999; Menne *et al.,* 2000; Brady *et al.,* 2000). The result of previous research (Yu Lan et al., unpublished, Chapter 4) have shown that the caecal LAB communities of broiler chickens were stimulated by dietary water-soluble soybean oligosaccharides (SMO) and polysaccharides (SMP), especially when added to the diet for two weeks post hatching. It was hypothesized that LAB communities stimulated by SMO and SMP could exert a competitive exclusion function for broiler chickens infected by *E. tenella.* This work describes an experiment designed to test this hypothesis.

MATERIALS AND METHODS

Isolation of oligo- and water-soluble polysaccharides from soybean meal

Soybean meal oligosaccharides (SMO) and water-soluble polysaccharides (SMP), were extracted from soybean meal has bean described previously as follows: 1 kg soybean meal was refluxed twice in 10 l alcohol (80 % (v/v)) at 75 °C for 1 h. This supernatant was then used for SMO isolation and the residue for SMP isolation. The supernatant was condensed by rotavapor (Büchi RE 120) and protein was subsequently removed with 5 % trichloroacetic acid (TCA). The liquid was freeze-dried after passing a column filled with Amberlite 200 and Amberlite IRA-900 for removing TCA and amino acids from the supernatant. The residue was hydrolyzed in a 0.2 M NaOH solution at 80°C for 2 h and filtrated using cheesecloth. The filtrate was neutralised with 30 % acetic acid and freeze-dried. The freeze-dried sample was dissolved in 400 ml water. Protein was removed with 5 % TCA. After 1 h, the supernatant was centrifuged at 13000 g for 30 min. SMP was precipitated by adding alcohol to the supernatant until the alcohol concentration was 80 % (v/v) and kept

at 4 °C over night. The polysaccharide precipitate was centrifuged at 2500 g for 10 min. The polysaccharide product was freeze-dried. Replicate products extracted at different times were mixed together and stored at – 20 °C.

Animal and diets

Two-hundred and forty 1-day-old male Arbor Acres broilers were obtained from a commercial producer. They were divided into six groups with similar mean weight ($p>0.05$) and housed per group in cages (100 x 100 x 60 cm) until 30 days of age. The basal diet free of soybean meal, consisted of corn, corn gluten, corn germ meal, fish meal and a mineral and vitamin additive, and contained about 12.62 MJ/kg of metabolizable energy (ME) and 20.2 % of crude protein (CP). The basal diet was heated to 80 ºC for 1h for killing the possible coccidia, then mixed with SMO, SMO or Maduramasin respectively after which the diet was cooled to room temperature. The diets were kept at – 25 ºC until the day before use, and then allowed the temperature of 31 ºC.

Six experimental treatments were as follows: a negative (COW) (1) and positive control (COR) (2): these were only fed the basal diet. Negative controls (COW) received the basal diet only while positive controls (COR) received the same diet and, in addition, were infected with *E. tenella* at 15 d of age. The treatment diets contained soybean meal oligosaccharides (SMO) (3) and water-soluble polysaccharides (SMP) (4). These diets were formulated by diluting the basal diets by 1 % SMO and 0.5 % SMP respectively. The birds received this diet from 1-11 days of age. Thereafter, the experimental diets were substituted by a 30% basal diet at Day 12; 60 % at Day 13; 90 % at Day 14 and 100 % by Day 15. Further treatments were: a vaccination group (VAC) (5), in which the birds were vaccinated with 100 oocytes of *E. tenella* at 3 days of age; and finally, an anti-coccidial medicated group (ANT) (6), in which Maduramasin (5 ppm) was added to the diet on Day 5 before infection up to and including day 7 after infection.

Chickens had access to feed and water *ad libitum*. Room temperature was

around 31 ºC for the first 5 days and was then gradually reduced to around 27 ºC and kept at this temperature until the end of the experiment. Light was provided for 24 hours per day throughout the study.

Parasite and experimental infections

The strain of *E.tenella* used was developed from a single oocyst, this was multiplied to ensure that coccidia used for infection were all *E.tenella*. Oocysts originally came from the *E. tenella* Houghpon strain and was maintained at the Laboratory of Parasitology and Parasitological Disease, College of Veterinary Medicine, Chian Agricultural University, China. Chickens, excluding the birds in the COW group, were orally infected with 1000 sporulated oocysts of *E. tenella* at 20:00 on Day 15.

Oocyst counting

Fecal oocyst shedding was monitored following infection per treatment group between the 5 th and 13 th days after infection by the McMaster's method (Hodgson, 1970). Fecal samples were collected per 24 h according to group. The sample was homogenized in a mixer for 15 min. Two g of sample was suspended in 10 ml water and then diluted in 50 ml of a saturated salt solution. The oocysts were counted microscopically in a McMaster chamber. Oocyst numbers per gram of faeces (OPG) was calculated by number of oocysts counted/chamber x 100 (Xun *et al.,* 1998).

Sampling and DNA extraction

A total of 48 birds, eight from each of the six treatments, were sacrificed by cervical dislocation on Day 7 after infection. Caecal contents were examined for the presence of lactic acid bacteria (LAB). To diminish the effect of individual differences on the experimental result, the caecal contents of the birds from each treatment group was mixed in a sterilized bottle immediately after death. The samples were kept at –80 ºC pending further analysis. Caecal contents was purified and total DNA was extracted using the method based on that of Lewington *et al.*(1987) and Wang

et al.(1996).

Primers and Real-time quantitative PCR

PCR primers specific for LABs of the genera *Lactobacillus, Pediococcus, Weissella* and *Leuconostoc* were used for this work (Walter *et al.,* 2001). The primers included forward primer Lac1 (5'-AGC AGT AGG GAA TCT TCC A-3') and reverse primer Lac2GC (5'-GC-Clamp-ATT YCA CCG CTA CAC ATG-3'). PCR with primers Lac1 and Lac2GC amplified a 340-bp fragment of the V3 region of the 16S rRNA gene.

Real-time PCR was performed using a Light Cycler rapid thermal cycler instrument (BIORAD icycler, USA). Reactions were performed in a 20 μ l volume with 2.5 nM each Lac1 and Lac2GC primers, 2.5 nM Mg^{++}, 20 μM each dNTP, 0.2 μ l Taq DNA polymerase, 1μ l SYBR Green I and 1 μ l extracted DNA. The amplification program included the initial denaturation step at 94 °C for 3 min followed by 35 cycles at 94°C for 30 s, at 60 °C for 30 s, at 72 °C for 30 s; and finally at 72 °C for 2 min. Fluorescence was measured through the slow heating phase. The specificity of the amplified product was then determined by melt curve analysis according to Komurian-Pradel *et al.* (2001). Melting curve determinations were done immediately after PCR was completed, by heating at 95°C for 2 s, cooling to 65 °C for 40 s and heating slowly at 0.2 °Cs until 95 °C with continuous fluorescence recording. Melting curves were recorded by plotting fluorescence signal intensity versus temperature, and the PCR amplified product melting temperature (Tm) were determined by calculating the derivatives of the curve with the Light Cycler software and visualized by plotting the negative derivative against the temperature (Komurian-Pradel *et al.,* 2001). Each sample had three repeats in a real-time PCR run. Subsequently, the PCR products were further confirmed using agarose gel electrophoresis.

Data analysis

The results were compared as means using a one-way ANOVA model, the significant difference between groups were analyzed by LSD t test, performed using SPSS 8.0

(Statistical Package for the Social Science, 1997) statistical software.

RESULTS

Fecal oocyst shedding in broilers.

Fecal oocyst shedding was monitored per group between the 5 th and 13 th day following infection. Figure 1 shows the plot of oocyst numbers per gram of faeces (OPG) versus days after infection. There were no oocysts found in feces for the negative control group (COW) throughout the study period.

Compared with the positive control group (COR), the coccidiostat treatment group (ANT) had negligible OPG; the vaccination group (VAC) and soybean meal oligosaccharide or polysaccharides diet groups (SMO or SMP) had lower OPG from the 7th to the 13 th days after infection ($p<0.05$). The VAC group had higher OPG than the others at Days 5 and 6 after infection ($p<0.05$), and the curve of the VAC group shows that OPG declined throughout the study following infection. The SMP group did not show a significant peak.

Melting curve analysis.

To demonstrate that the amplification was specific for the target lactic acid bacteria (LAB) gene, a melting curve analysis was performed immediately after the real-time PCR procedures. Melting curve analysis showed VAC, SMO, ANT, SMP and COW with melting peaks (Tm) at 90.9 °C, 91.3 °C, 91.3 °C, 91.2 °C and 91.2 °C respectively (Figure 2a), and the predominant peak was unique for each group. There was no significant differences between Tms ($p>0.05$) and the standard error of the means was 0.08. Meanwhile, the agarose gel electrophoresis analysis (Figure 2b) demonstrated that this peak corresponded to a single band of the predicted size of 340 bp. In the case of the COR group, the melting curve did not show a clear peak around 91 °C, and the electrophoresis result showed only a dim band.

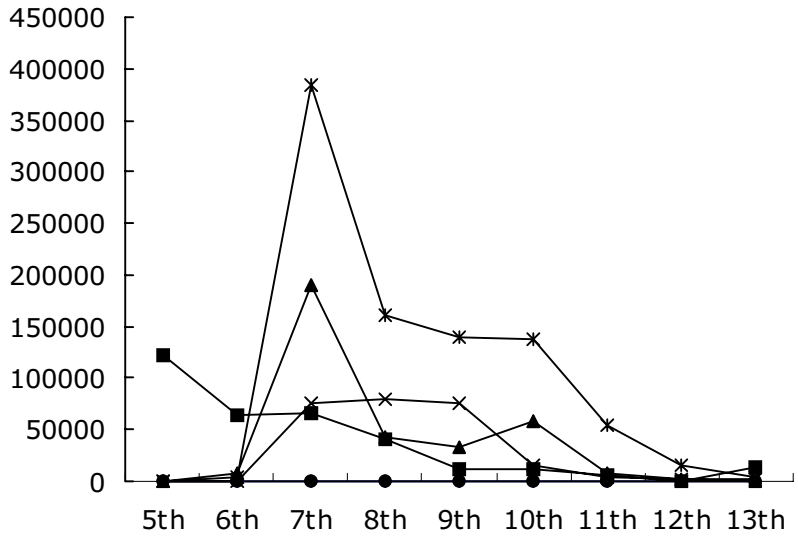

Figure 1. Oocysts number per gram of feces (OPG) (y axis) against days after infection (x axis), showed fecal oocysts shedding of broilers infected with 10^3 sporulated oocysts of *E.tenella* crop at 15 days of age. Mean came from three counts for each sample. Error bars represent standard deviation between mean. -∗- line is the representation of COR group; -▲- line is SMO; -× - line is SMP; -■- one is VAC; -●- one is COW and -◆- one is ANT.

Real-time PCR amplification

Real-time PCR amplification of LAB V3 region gene of the genera *Lactobacillus, Pediococcus, Weissella* and *Leuconostoc* was carried out by a real-time PCR technique performed on a Light Cycler instrument on Day 7 after *E. tenella* infection of the birds. SYBR Green I is nucleic acid dye specially binds with standard DNA (Weinbauer *et al.,* 1998). During real-time PCR, amplification products synthesis was monitored by SYBR Green I binding to stranded DNA. Figure 3 showed a plot of fluorescence versus cycle number driven from six groups using Lac1 and Lac2GC group specific primers of LAB. SYBR Green I was detected at the amplification cycle 12.97, 13.99, 14.85, 14.92, 22.207 and 27.68 for VAC, SMO, ANT, SMP, COW and COR groups with (SEM was 1.31) respectively. The cycle number of VAC, SMO, ANT and SMP groups was significantly lower than that of COW and COR groups ($p<0.05$), and cycle number in COW was lower than that in COR ($p<0.05$). In other words, a comparison of the LAB population in caecal contents in term of diets was: VAC > SMO > ANT > SMP > COW > COR.

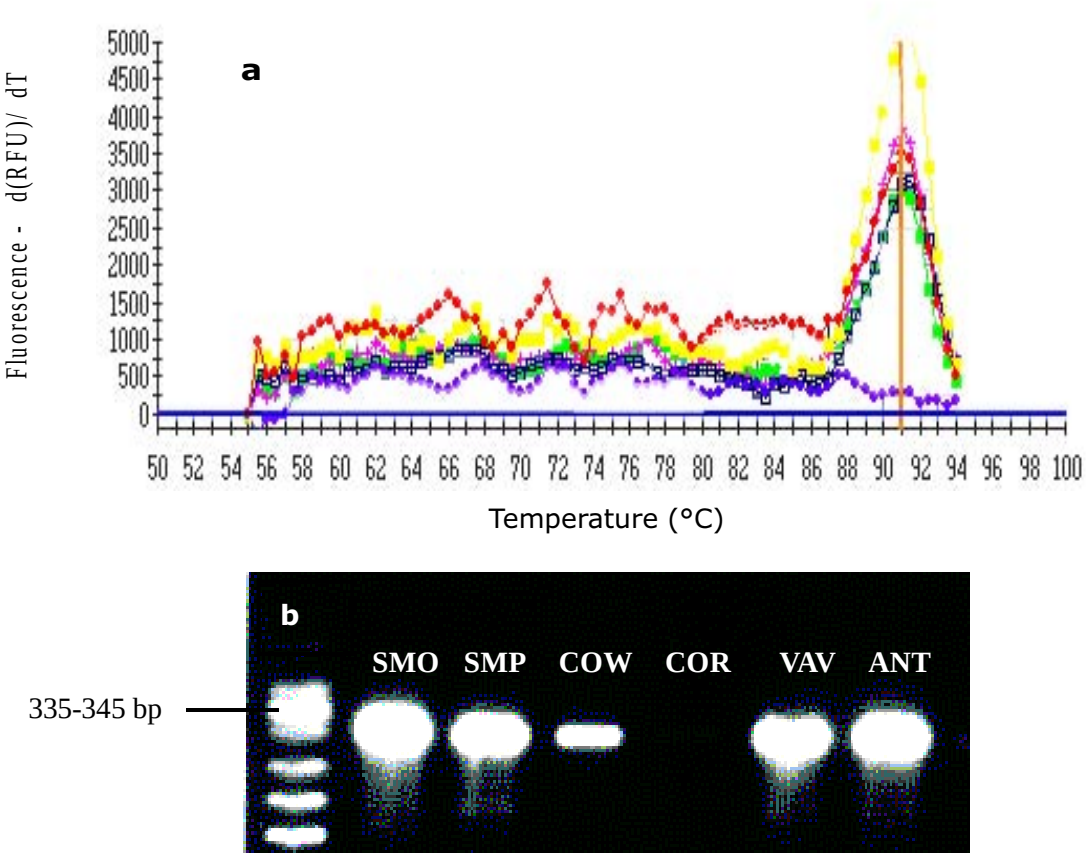

Figure 2. Plot of the negative derivative (-d*F*/d *T*) of the melting curves versus temperature (a). Except for the COR group, others with similar Tms around 91°C. Agarose gel electrophoresis analysis (b) demonstrated that these peaks correspond to a single band of the size predicted 340 bp.

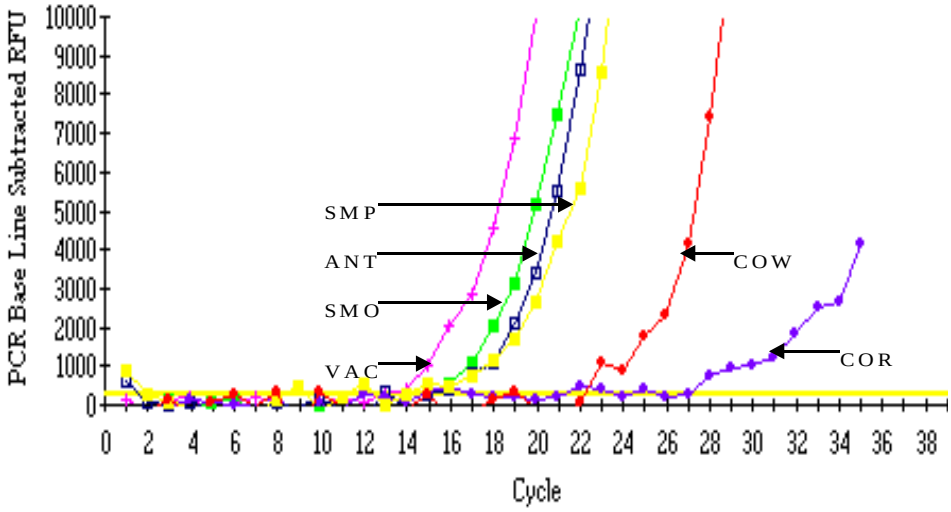

Figure 3. A plot of fluorescence (y) against cycle number (x) for each treatment: VAC = advanced vaccination group; SMO = soybean meal oligosaccharides adding group; ANT = anti coccidial medicine adding group; SMP = soybean meal water-soluble polysaccharides adding group; COW = non-infection control group; and COR = infection control group.

DISCUSSION

Competitive exclusion of LAB

E. tenella experience schizogony and gametogony in the caecal wall of broilers, and finally, the oocyst which is shed in the faeces is the mature form of the infective coccidia. Merozoites of the first generation schizonts invade the caecal lamina propria via the crypt epithelial cells. The second generation schizonts develop in the lamina propria within these crypt cells, and the new merozoites invade other caecal cells either directly through the connective tissue or via the caecal epithelial cells (Fernando *et al.,* 1983). Generally, the amount of shed oocysts is dependent on the number of sporozoites and merozoites that penetrate the enterocytes for a certain inoculum dose, and represents the infection-resistant ability of broilers. Caecal LAB have been shown to play an important role in competitive exclusion of pathogenic bacteria by several mechanisms during the bacterial infection (van der Wielen *et al.*, 2002, Hikkinen *et al.* and Fukata *et al.,* 1999) and these results suggest that the same may also be true for coccidia, eg. *E. tenella.*

The results indicate (Figure 1) that the number of oocysts per gram of faeces (OPGs) of all experimental groups were much lower during the monitoring period of 7 to 13 d post-infection when compared with the COR group. The inhibitory mechanism may have been different for the different groups. In the case of the SMO and SMP groups, there are four possible mechanisms involved. Firstly, SMO and SMP stimulated the development of caecal LAB when these two non-digestible carbohydrates were used in the diets of the 1-13 day-old broilers (Chapter 4). Other gut bacteria may also have been stimulated by SMO and SMP concurrently with LAB(Chapters 3 and 4). Indigenous bacteria occupy gut mucosal surfaces, and the coccidial parasite could be unable to attach to the mucosa therefore unable to penetrate the surface epithelial layer. This is one of the mechanisms of the so-called competitive exclusion. Secondly, the relatively short intestine and the fact that birds can eat as much as their gut capacity allows, means that a large amount of undigested carbohydrates and other substrates can potentially reach the hindgut. VFA represent a major-end

product when microorganisms ferment these materials, and can lead to a lower pH of caecal contents. Such a low pH may also have negative effects on *E. tenella* growth. Thirdly, some kind of LAB can produce bacteriocins which are antimicrobial proteins or oligopeptides, and display a spectrum of activity against strains taxonomically related to the producer organism (Klaenhammer 1988). During this experiment, the caecal supernatant was collected from 15 day-old broilers of SMO, SMP and COW groups, and its inhibitory activity was investigated in vitro by the agar diffusion method (Tianbang *et al.,* 1999). Both the SMO and SMP groups did not show any specific anti- salmonella activity (Figure not shown here). These results suggest that bacteriocin secretion may have only been of trivial importance when the pathogen did not incubate together with caecal microflora. However, the situation would be different under in vivo conditions, which need further study. Finally, some strains of LAB, especially the *Lactobacilli* can either augment the non-specific defense mechanism of the host in rat (Bloksma *et al.,* 1979) or modulate the specific immune response in a different fashion in human (Vidal *et al.,* 2002). It is apparent that the lowest OPG of the ANT group greatly contribute to the inhibition activity of the coccidiostat medicine. However, in the case of the VAC group, *E. tenella* specific antibody are made after vaccination with *E. tenella* at Day 3, and then some memory immune cells may still have existed in the birds' body. The quick immune response and large amount of *E. tenella* specific antibody secretion play a key role on *E. tenella* exclusion, when the birds are infected on Day 15 of age. This work will be reported separately (Chapter 7).

Diet, LAB and E. Tenella infection

In this experiment, a soybean meal-free basal diet was used, and apart from the SMO and SMP groups, this diet was not changed throughout the study. SMO and SMP were used in the diet during 1-11 days of age. In order to eliminate the direct effect of SMO and SMP on coccidia infection, the experimental diets were totally substituted by the basal diet 24 hours before infection, because it has been reported that polysaccharides can block the receptor-mediated invasion of some pathogens (Dalton *et al.,* 1991).

The defense against coccidial infection would have declined after withdrawal of the SMO and SMP from diets. This effect will most likely occur, because LAB and other native bacterial populations would decrease after withdrawal of the fermentation substrates leading to a relatively lower concentration of acids and potentially more gut wall exposure to *E. tenella*.

Real-time PCR and melting curve analysis.

We investigated the effect of dietary soybean meal oligosaccharide (SMO) and water-soluble polysaccharide (SMP) on caecal contents lactic acid bacteria (LAB) population of broilers challenged by *E. Tenella* on Day 15 post-hatch. Six experimental treatments were applied in this study, including a negative and a positive control group (COW and COR); an coccidiostat group (ANT); a vaccinaed group (VAC), vaccinated prior to infection, and SMO and SMP diet groups (SMO or SMP). Lactic acid bacteria (LAB) of caecal contents were determined 7 days after infection. The assay was based on fluorescence melting curve analysis of PCR products generated from the LAB of the genera *Lactobacillus, Pediococcus, Weissella* and *Leuconostoc* DNA.

SYBR Green I dye is a nonspecific intercalating dye, and the reaction is made specific by using "hot-start" PCR and empirically determined annealing and signal acquisition temperatures for each gene-specific primer (De Preter, *et. al.,* 2002). It implies that the analysis of the melting curve provides a check that specific amplification has occurred, It also distinguishes products from nonspecific primer-dimers and highlights any contamination (Ririe *et al.,* 1997; Pietilä *et al* 2000). In this study, the melting curve demonstrated that primers were specific for LAB of the genera *Lactobacillus, Pediococcus, Weissella* and *Leuconostoc,* because they amplified one major specific product with a distinct melting peak. For the melting curve of the COR group, no peak was present around 91 °C, This result indicated that no specific PCR product was melted at that temperature. However, a stranded PCR product was detected at amplification cycle 27.68 (Figure 3), which may have originated from non-specific PCR products with a melting temperature below 86 °C (Figure 2a).

The shape and position of a DNA melting curve is characterized by its GC/AT ratio, sequence and length (Ririe *et.al.,*1997). During PCR amplification, non-specific products, primer-dimers, or even the fold of Lac2GC itself, can form double stranded DNA that can be bound by SYBR Green I. In this melting curve, there were some trivial non-specific peaks before the predominant peaks. The non-specific peaks were apparently distinguishable from the specific amplification PCR product. However, no primer-dimer products were visible in the gel electrophoresis analysis of the same samples. It was therefore assumed that these peaks were associated with the DNA samples isolated directly from caecal contents and consisted of a complex DNA mixture of various bacteria. Meanwhile, the Lac2GC reverse primer, which was originally designed for DGGE analysis and relatively longer (68bp) because of the 40bp GC clamp (Wu *et al.,* 1998), may fold during the real-time PCR procedure. There were only small differences in the dominant amplification Tms among groups, and that may have resulted from different components of LAB DNA that were covered by the group-specific primers used in this experiment.

Real-time PCR allows continuous detection of PCR products by monitoring SYER Green I from the initial synthesis procedure. The sensitivity of SYBR Green I analysis is more than adequate for the majority of research applications, and its quantitative nature is demonstrated by the linear relationship between the log of the template concentration and the cycle number at which fluorescence rises above the baseline (Simpson *et al.,* 2000, Bates *et al.,* 2001). The relative DNA expression levels are further quantified by constructing a standard curve using DNA dilution of high expressed gene.

Comparing the cycle numbers at which standard DNA bound SYBR Green I was detected, VAC, SMO, ANT and SMP groups had a lower cycle number than the COW and COR groups, especially the COR group. Combined with the melting curve analysis results of the COR group, it may be concluded that there was hardly any DNA of LAB in the caecal contents seven days post- infection. The results suggest that SMO and SMP can stimulate LAB growth in the cecum of broilers during the early stage post-hatching (Chapter 4), and the LAB may play a competitive exclusion role in coccidia

infection. As a matter of fact, there was more survival of LAB in SMP and SMO animals after the infection with coccidia. The birds of the VAC and ANT group had more surviving LAB than that in COW, an interesting finding, which needs to be studied further.

In this case, it was impossible to quantify the DNA in samples because as mentioned above, the amplified DNA consists of several genera of LAB and the proportion of each genus is difficult to estimate. Consequently, the amplified DNA did not have the corresponding standard curve. Whether the standard cure can be done by single species of LAB or not needs further research.

In conclusion, it has been shown that soybean oligosaccharide and water-soluble polysaccharide (SMO and SMP) can promote growth of the lactic acid bacteria population, which can lead to competitive exclusion in the caecal contents of the *E. tenella* challenged broiler. It is clear that when SMO and SMP are used in the diet in the early stage post-hatch there will be an anti-coccidial effect. The result suggests that under the conditions described here, SMO and SMP can have prebiotic properties in broiler diets.

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REFERENCES

Bates, J. A., E. J. A. Taylor, D. M. Kenton and J. E. Thomas, 2001. The amplication of real-time PCR to the identification, detection and quantification of *Pyrenophora* species in barley seed. Molecular Plant Pathology. 2(1):49-57.

Bernet, M.F., D. Brassart, J.R. Neeser, A.L. Servin, 1994. *Lactobacillus acidophilus* LA1 binds to intestinal cell lines and inhabits cell attachment and cell invasion by enterovirulent bacteria. Gut 35, 483-489.

Bloksma, N., E. de Heer, M. van Duk and M. Willers, 1979. Adjuvanticity of lactobacilli. I. Differential effects of viable and killed bacteria, Clinical and experimental Immunology, 37: 367-375.

Brady, L., J. Daniel, D. Gallaher and Frank F. Busta, 2000. The role of probiotic cultures in the prevention of colon cancer. Journal of Nutrition 130:410S-414S.

Buddington, R. K., and E. Weiher, 1999. The application of ecological principles and fermentable fibers to manage the gastrointestinal tract ecosystem. Journal of Nutrition 129:1446S-1450S.

Dalton, J. P., D. Hudson, J. H. Adams and L. H. Miller, 1991. Blocking of the receptor-mediated invasion of erythrocytes by Plasmodium knowlesi malaria with sulfated polysaccharides and glycosaminoglycans. European Journal of Biochemistry. 14;195(3):789-794.

De Preter, K., F. Speleman, V. Combaret, J. Lunec, G. Laureys, B. H. Eussen, N. Francotte and J. Board, 2002. Quantification of MYCN, DDX1, and NAG gene copy number in neuroblastoma using a real-time quantitative PCR assay. : Modern Pathology. 2002 15(2):159-166.

Fernando, M. A., A. M. Lawn, M. E. Rose and M. A. Al-Attar, 1983. Invasion of chicken caecal and intestinal lamina propria by crypt epithelial cells infected with coccidia. Parasitology. 86 (Pt 3):391-398.

Fukata, T., K. Sasai, T. Miyamoto and E. Baba, 1999. Inhibitory effect of competitive exclusion and fructooligosaccharide, singly and in combination, Salmonella colonization of chicks. Journal Food Protection. 62(3): 229-33.

Gibson, G. R., 1999. Dietary modulation of the human gut microflora using prebiotics oligofructose and lnulin Journal of Nutrition 129:1438S-1441S.

Hakkinen, M. and C. Schneitz, 1999. Efficacy of a commercial competitive exclusion product against Campylobacter jejuni. British Poultry Science. 40(5):619-21.

Havenstein, G. B., P. R. Ferkel, S. E. Scheideler and B. T. Larson, 1994. Growth, livability, and feed conversion of 1957 vs 1991 broilers when fed "typical" 1957 and 1991 broiler diets. Poultry Science 73: 1785-1794.

Hodgson, J.N., 1970. Coccidiosis: oocyst-counting technique for coccidiostat evaluation. Experimental Parasitology. 28:99-102.

Hofacre, C. L., A. C. Johnson, B. J. Kelly and R. Froyman, 2002. Effect of a commercial competitive exclusion culture on reduction of colonization of an antibiotic-resistant pathogenic Escherichia coli in day-old broiler chickens. Avian Disease. 46(1):198-202.

Jenkins, D. J. A., C. W. C. Kendall and V. Vuksan, 1999. Inulin, oligofructose and intestinal function Journal of Nutrition 129:1431S-1433S.

Kaldhusdal, M., C. Schneitz, M. Hofshagen and E. Skjerve, 2001. Reduced incidence of Clostridium perfringens-associated lesions and improved performance in broiler chickens treated with normal intestinal bacteria from adult fowl. Avian Disease. 45 (1):149-56.

Klaenhammer, T. R., 1988. Bacteriocins of lactic acid bacteria. Biochimie. 87:337-349.

Komurian-Pradel, F., G. Paranhos-Baccalà, M. Sodoyer, P. Chevallier, B. Mandrand, V. Lotteau and P. André, 2001. Quantitation of HCV RNA using real-time PCR and fluorimetry. Journal of Virology Methods. 95:111-119.

Lewington, J., S. D. Greenaway and B. J. Spilline, 1987. Rapid small scale preparation of bacterial genomic DNA, suitable for cloning and hybridization analysis. Letters in Applied Microbiology. 5:51-53.

Link-Amster, H., F. Rochat, K. Y. Saudan, O. Mignot, J. M. Aeschlimann, 1994. Modulation of specific humoral immune response and changes in intestinal flora mediated though fermented milk intake. FEMS immunology and medical microbiology. 10, 55-64.

Menne, E. and N. Guggenbuhl, 2000. Fn-type chicory inulin hydrolysate has a prebiotic effect in humans. Journal of Nutrition 130:1197-1199.

Nurmi, E. and M. Rantala, 1973. New aspects of Salmonella infection in broiler production. Nature. 19;241(5386):210-211.

Pietilä, J., Q. He, J. Oksi and M.K. Viljanen, 2000. Rapid differentiation of *borrelia garinii* from *Borrelia afzelii* and *Borrelia burgdorferi*sensu stricto by Lightcycler fluorescence melting curve analysis of PCR product of the *recA* gene. Journal of Clinical Microbiology. 38(7): 2756-2759.

Rao, A. V., 1999. Dose-response effects of lnulin and oligofructose on intestinal bifidogenesis effects. Journal of Nutrition 129:1442S-1445S.

Reddy, B. S., 1999. Possible mechanisms by which pro- and prebiotics influence colon carcinogenesis and tumor growth. Journal of Nutrition 129:1478S1482S.

Ririe, K. M., R. P. Rasmussen,and C. T. Wittwer, 1997. Product differentiation by analysis of DNA melt curves during the polymerase chain reaction. Analytical. Biochemistry. 245:154-160.

Schiffrin, E. J., F. Rochat, H. Link-Amster, J. M. Aeschimann, A. Donnet-Hughes, 1995. Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. Journal of Dairy Science, 78, 491-497.

Simpson, D. A. C., S. Feeney, C. Boyly and A. W. Stitt, 2000. Retinal VEGF mRNA measured by SYBR Green I fluorescence: A versatile approach to quantitative PCR. Molecular Vision.

6:178-183.

SPSS, Inc. 1997. SPSS for windows graduate pack advanced version, Version 8.0. Chicago, IL: SPSS Inc.

Tianbang, Y. and L. Xingyou, 1999. Poultry Immunology, Published by China Agriculture Technology Publishing House, p 260-263.

van der Wielen, P. W., L. J. Lipman, F. van Knapen and S. Biesterveld, 2002. Competitive exclusion of Salmonella enterica serovar Enteritidis by Lactobacillus crsepatus and Clostridium lactatifermentans in a sequencing fed-batch culture. Applied and Environmental Mirobiology. 68(2):555-559.

Vidal, K., A. Donnet-Hughes, and D. Granato, 2002. Lipoteichoic Acids from *Lactobacillus johnsonii* Strain La1 and *Lactobacillus acidophilus* Strain La10 Antagonize the Responsiveness of Human Intestinal Epithelial HT29 Cells to Lipopolysaccharide and Gram-Negative Bacteria. Infection and Immunity. 70: 2057-2064.

Walter, J., C. Hertel, G. W. Tannock, C. M. Lis, K. Munro and W. P. Hammes 2001. Detection of *Lactobacillus, Pediococcus, Weissella* and *Leuconostoc* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Applied and Environmental Mirobiology. June, 2578-2585.

Wang, R.-f., W.-W. Cao and C. E. Cerniglia, 1996. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Applied and Environmental Microbiology. 62;1242-1247.

Weinbauer, M. G., C. Beckmann and M. Höfle, 1998. Utility of green fluorescent nucleic acid dyes and aluminum oxide membrane filters for rapid epifluorescence enumeration of soil and sediment bacteria. Applied and Environmental Microbiology. Dec. 5000-5003.

Wu, Y., V. M. Hayes, J. Osinga, I. M. Mulder, M. W. G. Looman, C. H. C. M. Buys, and R. M. W. Hofstra, 1998. Improvement of fragment and primer selection for mutation detection by denaturing gradient gel electrophoresis. Nucleic Acids Research. 54:32-40.

Xun, S. and Q. Guo, 1998. Coccidia and Coccidiosis of Domestic Fowl. Pressed by China Agricultural University publisher, ISBN 7-81002-966-5. p 254-255.

Chapter 7

DIETARY SOYBEAN MEAL OLIGO- AND POLYSACCHARIDES AFFECT IMMUNOGLOBULIN ON CAECAL LAMINA PROPRIA OF *EIMERIA TENELLA* INFECTED BROILERS

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Dietary Soybean Meal Oligo- and PolysaccharidesAffect Immunoglobulin on Caecal Lamina Propria of *Eimeria Tenella* Infected broilers

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Abstract: An experiment was conducted to investigate the effect of soybean meal water-soluble oligosaccharides (SMO) and polysaccharides (SMP) on the caecal lamina propria immunoglobulins A, M and Y positive plasma cells (IgA^+, IgM^+ and IgY^+) after broilers were challenged by *Eimeria tenella.* The experiment hypothesized that SMO and SMP can promote the maturation of B lymphocytes in the cecal lamina propria of coccidia infected broilers. Two hundred and forty 1-day-old male Arbor Acres broilers were assigned to six treatment groups with forty birds for each experimental group. The basal (control) diet (COW) was a soybean meal-free diet, consisting of corn, corn gluten, corn germ meal, fish meal and a mineral and vitamin additive, containing 12.5 MJ/Kg of metabolizable energy (ME) and 20.21% crude protein (CP). The six treatments were as follows: water soluble soybean meal oligosaccharides and polysaccharides groups (SMO and SMP). Chickens in SMO and SMP groups were fed the basal diet (COW) diluted by 1 % SMO and 0.5 % SMP during 1-14 day-old, respectively. The advanced vaccination group (VAC): the birds in the VAC group were vaccinated with 100 oocytes of *E. tenella* on Day 3 post-hatch. Anti-coccidial medication group (ANT) which received Maduramasin (5 ppm) in the diet on Day 5 before infection until Day 7 after infection. Uninfected and infection control groups (COW and COR) were only fed the basal diet. All birds, except those in the COW group, were orally infected with 1000 sporulated oocysts of *E. tenella* on Day 15. Caecal lamina propria plasma B cells, IgA^+, IgM^+ and IgY^+ were monitored at 12, 24, 48, 72, 96, 120, 144 and 168 hours post-infection by using monoclonal antibody and immunohistochemistry techniques. The results showed that animals in both the SMO and the SMP treatment had a significant increase in the number of caecal lamina propria IgA^+ and IgM^+ cells compared with the control group (COR). The IgA^+ cells composed the majority of the three immune response B cells. Birds fed with dietary SMO had a significantly increased number of IgA^+ and IgM^+ at 12 and 168 hours

post-infection (PI) ($p<0.05$), and IgY^+ cells at 24, 48 and 168 hours PI. As for SMP, IgA^+, IgM^+ and IgY^+ numbers were increased dramatically from the 120th hour PI onwards. IgA^+ and IgY^+ were significantly higher than COR at 120, 144 and 168 hours PI ($p<0.05$). There was an insignificant difference in IgM^+ between SMP and COR ($p>0.05$). The predominant amount of IgY^+ appeared in the VAC group. In conclusion, the presence of both SMO and SMP in the diets, were associated with a significant increase in the number of B plasma cells (IgA^+ and IgM^+) of *E. Tenella* infected broilers. The IgA^+ forms the majority of the three immune response B cells.

Keywords: soybean meal oligo- and polysaccharides (SMO & SMP), immunoglobulins, *Eimeria tenella,* broilers.

INTRODUCTION

Eimeria tenella is an important protozoal parasite in the alimentary tract of broilers. It is one of the major causes of reduced productivity during the early stage of post-hatch. *E. tenella* infection usually leads to a significant increase of immunoglobulin A, M and G in the caecal lamina propria. These antibodies are produced locally in the intestine after an *E.* infection and may play a role in the protection against coccidiosis in chickens (Girard *et al.,* 1997).

IgA is the major immunoglobulin of mucosal secretions, and can function in multiple ways as a first line of immune defense at the boundaries with the pathogens (Lamm, 1997). The origin and homing of intestinal IgA antibody-secreting cells are both well documented (Michael *et al.,* 2002). IgA B plasma cells in the intestine were switched from IgM in lymphoid tissue of the germinal centers, showed further proliferation and maturation in the secondary lymphatic organs and finally homed back into the mucosa via the blood. Meanwhile, IgA B cells can be transported to the intestinal lumen by bile (Gebhardt**,** 1983).

Resistance against adhesion and penetration of pathogens is achieved by immune mechanisms of the host and it is likely that IgA in the gut prevents the transfer of antigens through the gut-wall (Kaetzel *et al.,* 1991 and Mazanec *et al.,* 1992). IgA-rich post–infection caecal contents have been shown to inhibit sporozoites invasion and these sporozoites can lose their ability to differentiate, even if they can still invade the enterocytes (Davis *et al.,* 1979). Meanwhile, specific IgA can bind to the sporozoites membrane (Tress *et al.,* 1989). Furthermore, specific anti- *E. tenella* IgM and IgG are also involved in the local immune defence against the infection (Girard *et al.,* 1997) though their anti-parasitic mechanisms are not as specific as for IgA.

IgY is the predominant isotype immunoglobulin in serum (Michael *et al.,* 2002). In the circulation it can activate complement by classical pathways and it can participate in antibody-dependent, cell-mediated cytotoxicity (ADDC), Therefore it can be

expected to produce a greater inflammatory response at the mucosal surface than IgA (Gruskay *et al.,* 1955). IgM is the major secretory immunoglobulin in the intestinal tract of children, young animals (Volkheimer *et al.,* 1968 and 1969) and during the early stage of a local immune response (Hemming *et al.,* 1977).

Numerous species of commensal microorganisms are attached to the intestinal mucosa. Their activity and relative numbers can be modulated by dietary non-digestible carbohydrates. These bacteria play an important role in the modulation of the host gut mucosal defence (Blum *et al.,* 1999). Polysaccharides are generally poor immunogens and they are used as adjuvant to conjugate with a carrier protein (Bergquist *et al.,* 1997). Some polysaccharides however, have biological activity for stimulation humoral and cell-mediated immune response of animal (Womble *et al.,* 1988 and Tizard *et al.,* 1989).

In this investigation the hypothesis was that IgA^+, IgM^+, IgY^+ in the caecal mucosa of *E. tenella* challenged broilers, would stimulated by dietary SMO and SMP used during the early stage of post-hatch and that these components would promote the maturation of B-cells.

MATERIALS AND METHODS

Extraction of oligo- and water-soluble polysaccharides from soybean meal

The extraction of SMO and SMP from soybean meal has bean described previously as follows: 1 kg soybean meal was refluxed twice in 10 l alcohol (80% (v/v)) at 75°C for 1 h. This supernatant was then used for SMO isolation and the residue for SMP isolation. The supernatant was condensed by rotavapor (Büchi RE 120) and protein was subsequently removed with 5 % trichloroacetic acid (TCA). The liquid was freeze-dried after passing a column filled with Amberlite 200 and Amberlite IRA-900 for removing TCA and amino acids from the supernatant. The residue was hydrolyzed in a 0.2 M NaOH solution at 80 °C for 2 h and filtrated using cheesecloth. The filtrate was neutralised with 30 % acetic acid and freeze-dried. The freeze-dried

sample was dissolved in 400 ml water. Protein was removed with 5 % TCA. After 1h, the supernatant was centrifuged at 13000 g for 30 min. SMP was precipitated by adding alcohol to the supernatant until the alcohol concentration was 80 % (v/v) and kept at 4 °C over night. The polysaccharide precipitate was centrifuged at 2500 g for 10 min. The polysaccharide product was freeze-dried. Replicate products extracted at different times were mixed together and stored at – 20 °C .

Animal and diets

Two-hundred and forty 1-day-old male Arbor Acres broilers were obtained from a commercial producer. They were divided into six groups of similar mean weight and housed per group in cages (100 x 100 x 60 cm) until 30 days of age. The basal diet was free of soybean meal, and consisted of corn, corn gluten, corn germ meal, fish meal and a mineral and vitamin additive, and contained about 12.62 MJ/kg of metabolizable energy (ME) and 20.2 % of crude protein (CP). The diet was heated to 80 ºC for 1 h, then mixed with SMO, SMP or Maduramasin respectively after the diet had cooled to room temperature. The diets were kept at – 25 ºC until the day before use, and then allowed to reach a temperature of 31 ºC.

There were six experimental treatments as follows: a negative (COW) (1) and positive control (COR) (2): these were only fed the basal diet. Negative controls (COW) received the basal diet only, while positive controls (COR) received the same diet and, in addition, were infected orally with 1000 sporulated oocysts at Day 15 post-hatch. Experimental treatment diets contained soybean meal oligosaccharides (SMO) (3) and water-soluble polysaccharides (SMP) (4). These diets were formulated by diluting the basal diets by 1 % SMO and 0.5 % SMP respectively. The birds received this diet from 1-11 days of age. Thereafter, the experimental diets were substituted by a 30 % basal diet at Day 12, 60% at Day 13, 90% at Day 14 and 100% at Day 15. Further treatments were: a vaccination group (VAC) (5), in which the birds were vaccinated with 100 oocytes of *E. tenella* at 3 days of age; and finally an anti-coccidial medicated group (ANT) (6), in which Maduramasin (5 ppm) was added to the diet at Day 5 before infection up to and including Day 7 after infection.

Chickens had access to feed and water *ad libitum*. Room temperature was around 31 ºC for the first 5 days and was then gradually reduced to around 27 ºC and they were kept at this temperature until the end of this experiment. Light was provided for 24 hours per day throughout the study.

Table 1. Diet, soybean meal oligo- and water soluble polysaccharides (SMO and SMP), antibiotic and vaccine fitted to experimental treatments

Treatments [1]	COW	COR	ANT	VAC	SMO	SMP
Basal diet [2]	Yes	Yes	Yes	Yes	Yes	Yes
SMO	No	No	No	No	Yes	No
SMP	No	No	No	No	No	Yes
Cococcidiostat	No	No	Yes [3]	No	No	No
Vaccination	No	No	No	Yes [4]	No	No

[1] The six treatments in this experiment was uninfection and infection control groups (COW and COR), dietary SMO and SMP groups (SMO and SMP), antibiotic used group (ANT) and advanced vaccination group (VAC)

[2] Did not have antibiotics.

[3] Maduramicin (5 ppm) was used on the 5th day before infection and until the 7th day after infection.

[4] The birds in this group were vaccinated with 100 oocytes of *E.tenella* at 3 day of age.

Parasite and experimental infections

The strain of *E. tenella* used was developed from a single oocyst, which was multiplied to ensure that coccidia used for infection were all *E. tenella*. The single oocyst originally came from the *E. tenella* Houghpon strain and was maintained at the Laboratory of Parasitology and Parasitological Disease, College of Veterinary Medicine, Chian Agricultural University, P. R. China. Chickens, excluding the birds in the COW group, were orally infected with 1000 sporulated oocysts of *E. tenella* at 20:00 at Day 15.

Immunohistochemistry

Three birds from each group were sacrificed at 12, 24, 48, 72, 96, 120, 144 and 168 hours post-infection. The tissue of both middle segments of the caecae were collected and washed in cold phosphate buffer solution (PBS) and then fixed in a 10 % formalin-buffered saline solution (pH7) for 24 h. The fixed samples were embedded in paraffin and sectioned as 4-mm-thick slices, attached to poly-L-lysine-coated slides. Prior to staining, the slides were allowed to dewax and were rehydrated. Endogenous peroxydase and non-specific binding were blocked by 3 % H_2O_2 solution and 10 % normal goat serum at room temperature, respectively. The Avidin-Biotin Complex (ABC) technique was engaged in the immunohistochemistry procedure (Jingshan, 1992). Firstly, slides were incubated at 37 °C for 30 min. Subsequently they were stored at 4 °C overnight in a humidity chamber with one of three kinds of monoclonal antibody (Mab) (mouse anti-chicken α-heavy chain specific IgA, γ-heavy chain specific IgG and μ-heavy chain specific IgM, Southern Biotechnology Associates, Inc. USA). Secondly, the slides were incubated with biotin-conjugated goat anti-mouse immunoglobulin G (IgG; Sigma, Zhong Shan Co.) for 30 min at 37 °C. Thirdly, they were incubated with peroxidase-conjugated streptavidin at 37 °C for 30 min. Finally, the stained immunoglobulins positive plasma cells were detected by 3,3 Diamino Benzidine (DAB). There were three washes in 0.01 M PBS (pH7.4) between each reagent. Finally, all nucleoli were stained by haematoxylin. The negative control slices were stained the same as the experimental ones, but substituting Mabs with PBS.

Statistics

Caecal lamina propria immunoglobulin A, M and Y positive plasma cell (IgA^+, IgM^+, IgY^+) in the caecal lamina propria were calculated per villi. A total of 15 integrated villi were used in each group five villi came from each of three birds of the same experimental group. The results were compared as means by a one-way ANOVA model, the significant difference within groups were analyzed by Turkey test at $p<0.05$ level, and performed by SPSS (8.0) (Statistical Package for the Social Science,

1997) statistical software.

RESULTS

The immunohistochemistry-stained sections were examined by light microscope, and the results are shown in Figure.1, and Tables 2, 3 and 4. Immunoglobulin A, M and Y positive plasma cells (IgA^+, IgM^+ and IgY^+) were distributed among the dispersal lymphocytes in the cecal lamina propria (Figure 2). Both SMO and SMP diets brought about a significant increase in the number of caecal lamina propria IgA^+ and IgM^+. was the majority of the three isotypes, and IgM^+ was next to the IgA^+. The IgA^+, IgM^+ and IgY^+ were detected in all groups throughout the study period, and except for COW and ANT groups, their numbers increased with time.

Compared with animals in the COR group, which received a soybean meal free diet, the animals in the other three treatment groups (SMO, VAC and SMP) had much higher numbers of IgA^+ and IgM^+ cells at 12 h post-infection (PI), and this increased with time. Significantly higher numbers of IgA^+ and IgM^+ plasma cells were detected at 168 h PI for animals receiving diets SMO and SMP ($p<0.05$) (Tables.2 and 4). Animals of the VAC group had a much higher number of IgY^+ plasma cells throughout the experimental period than those of other treatments (Table.3). Birds in the SMO and SMP groups had lower initial IgY^+ numbers, however there were significantly higher numbers at 168 h PI. as compared with COR ($p<0.05$) (Table 3). The rapid increases in IgM^+ and IgY^+ appeared earlier than IgA^+ in birds of the SMO group, starting from 144 h, 24 h and 168 h PI respectively. However the quantity of IgA^+ was higher throughout the experimental period as compared to IgY^+ and IgM^+. For animals in the SMP group, the number of IgA^+, IgM^+ and IgY^+ cells increased dramatically from 120 h, 96 h and 96 h PI respectively.

Compared with the VAC group, the SMO group had significantly higher IgA^+ plasma B cells at 24 h and 168 h PI., and higher numbers of IgM^+ cells at 144 and 168 h PI. ($p>0.05$) (Tables 2 and 4). There was a noticeably lower number of IgY^+ cells in these animals at hours before 120 h PI ($p<0.05$). In the birds of the SMP group,

there were lower initial numbers of IgA^+ and IgM^+ cells at 12 h PI. The significant increase started from 120 h and144 h until 168 h PI. respectively, compared with the VAC group ($p<0.05$) (Tables 2 and 4). The SMP group had relatively lower numbers of IgY^+ cells than the VAC group during the whole investigation, and it was significant in animals from 12 h until 72 h PI ($p<0.05$) (Table 3).

The difference in the number of IgA^+ and IgM^+ cells per villi between birds of the SMO and SMP groups, indicated that these immunoglobulins increased faster in SMP than that in SMO birds. While birds of SMO had more IgA^+ and IgM^+ cells at the beginning than that of SMP ($p<0.05$), there was no significant difference at 168 h PI. (Tables 2 and 4).

Compared with SMO, SMP, COR and VAC treatment animals, both ANT and COW had relatively lower amounts of IgA^+, IgM^+ and IgY^+, which did not increase dramatically afterwards. However, the VAC group had a significantly higher number of initial IgA^+ and IgM^+ compared with other groups except for SMO, and IgY^+ compared with all other groups.

Table 2. Numbers of cecal mucosa immunoglobulin A positive plasma cell (IgA^+) per villus of E. Tenella infected broilers in different treatments

Time(h)	COW	COR	ANT	VAC	SMO	SMP	SEM
12	1.73 ^{b}	1.27 ^{b}	0.73 ^{b}	11.40 ^{a}	11.67 ^{a}	5.23 ^{b}	0.67
24	1.87 ^{c}	12.67 ^{ab}	1.33 ^{c}	11.67 ^{b}	16.87 ^{a}	12.07 ^{ab}	0.79
48	3.80 ^{b}	23.07 ^{a}	2.00 ^{b}	19.20 ^{a}	21.13 ^{a}	14.60 ^{a}	1.27
72	3.87 ^{c}	36.20 ^{a}	2.80 ^{c}	33.40 ^{ab}	28.40 ^{ab}	22.27 ^{b}	1.91
96	4.27 ^{b}	40.20 ^{a}	4.33 ^{b}	34.33 ^{a}	33.13 ^{a}	32.07 ^{a}	1.93
120	2.53 ^{c}	45.13 ^{b}	4.60 ^{c}	31.73 ^{b}	31.20 ^{b}	85.87 ^{a}	3.32
144	4.47 ^{d}	50.13 ^{b}	3.47 ^{d}	32.80 ^{c}	39.33 ^{bc}	89.27 ^{a}	3.46
168	3.07 ^{c}	47.20 ^{b}	3.13 ^{c}	41.27 ^{b}	83.80 ^{a}	95.20 ^{a}	4.14

$^{abc.}$ Figures with different letters in same row values was significantly different ($p<0.05$), comparisons was made within treatments.

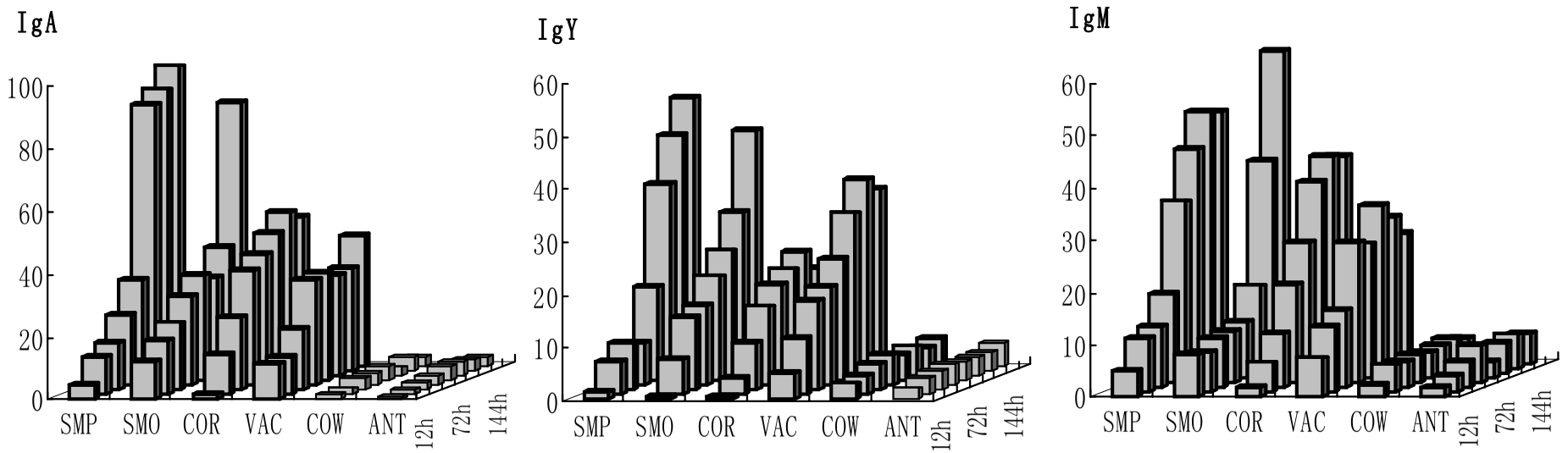

Figure 1. Treatments (x axis) versus the number (y axis) of cecal mucosa immunoglobulin A, M and Y positive cells (IgA^+, IgM^+ and IgY^+) of per cecal villus at 12h, 24h, 48h, 72h, 96h, 120h, 144h and 168h (z axis) post oral infection with 1000 *E. tenella* oocysts on Day 15. Means come from 3 male AA broilers per observation. The six treatments in this experiment were uninfected and infected control groups (COW and COR), dietary SMO and SMP groups (SMO and SMP), antibiotic group (ANT) and advanced vaccination group (VAC).

Table 3. Numbers of cecal mucosa immunoglobulin G positive plasma cell (IgY^+) per villus of E. Tenella infected broilers in different treatments

Time(h)	COW	COR	ANT	VAC	SMO	SMP	SEM
12	3.00 ^{ab}	0.60 ^{c}	2.13 ^{b}	4.87 ^{a}	0.60 ^{c}	1.40 ^{b}	0.26
24	3.67 ^{bc}	3.27 ^{c}	3.33 ^{bc}	10.67 ^{a}	6.73 ^{b}	6.47 ^{b}	0.42
48	5.00 ^{cd}	9.13 ^{bc}	3.60 ^{d}	16.93 ^{a}	13.67 ^{ab}	8.87 ^{b}	0.71
72	6.00 ^{b}	14.93 ^{a}	4.27 ^{b}	18.67 ^{a}	15.00 ^{a}	7.93 ^{b}	0.82
96	4.93 ^{b}	18.27 ^{a}	3.73 ^{b}	23.07 ^{a}	19.80 ^{a}	17.67 ^{a}	1.31
120	5.60 ^{b}	20.27 ^{a}	3.67 ^{b}	30.87 ^{a}	23.80 ^{a}	26.87 ^{a}	1.54
144	4.47 ^{c}	22.60 ^{b}	3.53 ^{c}	36.53 ^{a}	28.67 ^{ab}	27.07 ^{ab}	1.68
168	5.33 ^{c}	18.60 ^{b}	4.53 ^{c}	33.73 ^{a}	30.13 ^{a}	28.40 ^{a}	1.43

$^{abc.}$ Figures with different letters in same row values was significantly different ($p<0.05$), comparisons was made within treatments.

Table 4. Numbers of cecal mucosa immunoglobulin M positive plasma cell (IgM^+) per villus of E. Tenella infected broilers in different treatments[1, 2]

Time(h)	COW	COR	ANT	VAC	SMO	SMP	SEM
12	2.20 ^{c}	1.67 ^{c}	1.67 ^{c}	7.33 ^{ab}	8.13 ^{a}	4.80 ^{bc}	0.42
24	5.13 ^{cd}	5.33 ^{cd}	3.00 ^{d}	12.33 ^{a}	7.53 ^{bc}	10.33 ^{ab}	0.52
48	4.93 ^{b}	10.13 ^{ab}	5.00 ^{b}	14.60 ^{a}	9.40 ^{b}	11.40 ^{a}	0.62
72	5.20 ^{d}	18.53 ^{ab}	6.93 ^{cd}	26.60 ^{a}	9.73 ^{b}	16.80 ^{abc}	1.32
96	4.93 ^{b}	25.73 ^{a}	3.31 ^{b}	25.53 ^{a}	10.73 ^{b}	33.60 ^{a}	1.70
120	5.20 ^{b}	36.53 ^{a}	5.87 ^{b}	31.87 ^{a}	16.67 ^{b}	42.53 ^{a}	2.02
144	5.93 ^{c}	40.47 ^{ab}	6.40 ^{c}	29.00 ^{b}	39.40 ^{ab}	48.80 ^{a}	2.16
168	5.00 ^{d}	39.67 ^{bc}	5.80 ^{d}	24.80 ^{c}	59.40 ^{a}	47.93 ^{ab}	2.72

[1.] The six treatments in this experiment were uninfected and infected control groups (COW and COR), dietary SMO and SMP groups (SMO and SMP), antibiotic used group (ANT) and advanced vaccination group (VAC); [2.] The number IgM of per cecal villus at 12h, 24h, 48h, 72h, 96h, 120h, 144h and 168h post oral infection with 1000 *Eimeria tenella* oocysts on Day 15. n=3 male AA broilers per observation; $^{abc.}$ Figures with different letters in same row values was significantly different ($p<0.05$), comparisons was made within treatments.

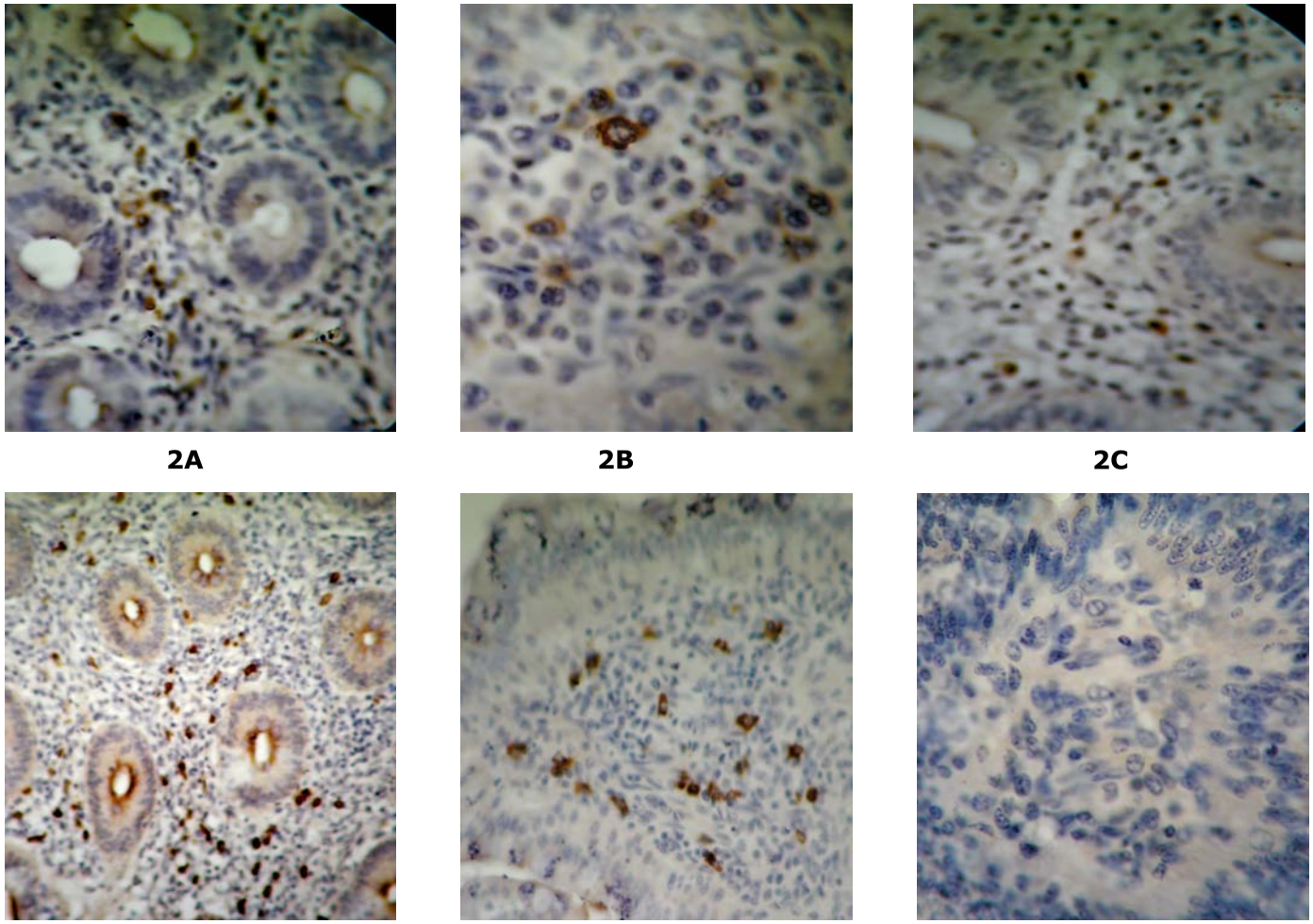

Figure 2. Pictures of light microscope of the caecal mucosal immunoglobulin A, M and Y positive plasma B cells (IgA^+, IgM^+ and IgY^+) in caecal lamina propria. Avidin-Biotin Complex (ABC) technique was engaged in immunohistochemistry procedure. Caecal villus negative stained slice, substitute Mabs with PBS. Cell nuclear was stained by haematoxylin. The cells with yellow plasma are positive cells. In Figure 2 2A: IgA^+ (× 1000); 2B: IgM^+ (× 1000); 2C: IgY^+ (× 1000); 2D: IgA^+ (× 400); 2E: IgM^+ (× 400) and 2F: negative stained slice (× 1000).

DISCUSSION

This experiment was conducted to investigate the differential effect of soybean meal oligosaccharides (SMO) and water soluble polysaccharides (SMP) on the caecal lamina propria immunoglobulin A, M and Y positive plasma B cells (IgA^+, IgM^+ and IgY^+) after broilers had been challenged by *E. tenella.*

The results showed that both dietary SMO and SMP stimulated the development of IgA^+ and IgM^+ in the caecal lamina propria of *E. tenella* challenged broilers. Interestingly, the birds in the SMO group had significantly higher IgA^+ at 12 h PI compared with COR, and the number of IgA^+ was similar to that of the VAC group at this time. This is supported by the results of another separate experiment (Chapter 5). In this other experiment, a study was made into the effect of dietary SMO and SMP on immunology characteristics of broilers with regard to humoral, systemic cellular and mucosal cellular immune responses when these were used in the diet during the first two weeks post-hatch. The numbers of IgA^+, IgM^+ and IgY^+ plasma cells in the caecal lamina propria were examined on Day 15 post-hatch. Noticeably greater numbers of IgA^+ and IgM^+ were observed in SMO and SMP fed group compared to the other treatments. The result suggests that SMO and SMP have promoted the number of cells containing these immunoglobulins, even before the birds were infected with *E. tenella.* That means there would be natural antibody stimulated by dietary SMO and SMP, and this need study further.

SMO comprises mainly the α-galactoside family of oligisaccarides, including raffinose, stachyose etc. SMO cannot be hydrolyzed by the monogastric digestive enzymes due to the lack of α-1,6 galactosidase activity in the intestinal mucosa (Gitzelmann *et al.,* 1965). However it may be fermented by microorganisms in the GI tract. Soybean non-starch polysaccharides (NSP) mainly consist of arabinans, arabinogalactans and acidic polysaccharides (pectin-type compounds). About one-third of NSP in soybeans are water-soluble (SMP) (Bach-Knudsen, 1997). According to monosaccharide sugar analysis, the SMP used in this experiment mainly comprised galactose (12%) and rhamnose (5%) (Chapter 2). SMO had a lower molecular

weight than SMP. Both of them, however, can be fermented by intestinal commensal microorganisms. That means both SMO and SMP can affect the activity of microbial community which either in caecal contents or attached on caecal mucosal surface. These microorganisms would have some effect on gut mucosal immune responses and need further study.

The chicken gut associated lymphoid tissues (GALT) are critical for the development of B-lymphocytes as this is the main development occurs primarily in GALT (Michael *et al.,* 2002). It has previously been shown that the development of GALT is affected by the intestinal bacteria normally present in the chicken (Kazuhito *et al.,* 1993). Therefore the possible mode of action, which is supported by the findings in the experiment described here, is that the stimulated caecal microorganisms trigger both the mucosal non-specific and *E. tenella* specific B plasma cells. The immunostimulatory effect of the intestinal lactic acid bacteria (LAB) has been confirmed in many investigations in human and animals (De Simone *et al.,* 1989, 1993; Takahashi *et al.,* 1993). It is believed that the LAB' cell wall materials are composed mainly of peptidoglycan (30-70 % of the total cell wall), polysaccharides and teichoic acid (Adams, *et al.,* 1981) and that these are responsible for the immunostimulating role (Stewart-Tull, 1980; De Ambrosina *et al.,* 1998). The real-time PCR analysis result showed that SMO and SMP can stimulate LAB of the genera *Lactobacillus, Pediococcus, Weissella* and *Leuconostoc* to grow in the caecal content during the early stage of post-hatch, particularly the SMO (Chapter 4).

The increased number of IgA^+, IgM^+ and IgY^+ cells is a phenomenon, that is a consequence of complex processes which are regulated by T lymphocytes and various cytokines and which lead to plasma cell differentiation (James *et al.,* 1983). The process of the mucosal immune response has been well documented (Shanahan, 2000) in the human. When an antigen invades the intestinal lumen, microfold cells (M cells) covering lymphoid tissue in cecal tonsils (Suzan *et al.,* 1999) transport particulate antigens to dendritic and other antigen presenting cells (macrophages). Dendritic cells process and present the antigen in the context of the major histocompatibility complex (HMC) and in association with costimulatory molecules to

T cells. Under normal circumstances, for innocuous antigens, the usual outcome is the generation of interleukin (IL)-10 and transforming growth factor-β(TGF-β), These drive the differentiation of T helper type 2 (Th2) and regulatory T cells (Th3 and Tr1), thereby promoting IgA responses. Therefore, the initial higher numbers of immunoglobulins positive plasma cells represent a highly activated immune response system in the cecal lamina propria. It appears that as a result, the birds receiving dietary SMO or SMP will have a considerable protection against *E. tenella* infection just as vaccination.

SMP significantly enhanced the development of IgA^+, IgM^+ and IgY^+ plasma cells in this experiment. Apart from this happening as a consequence of the stimulated gut bacterial population, there may also be another explanation. It may be that there is a immunostimulating role of polysaccharides such as SMP itself. It has been reported that some polysaccharides, acting as an adjuvant or soluble antigen, have biological activity with regard to the humoral and cell-mediated immune response by stimulation of cells from the histiocytic lineage, resulting in increased secretion of cytokines and an enhanced cellular function (Patchen *et al.,* 1981; Womble *et al.,* 1988; Tizard *et al.,* 1989; Linna *et al.,* 1996, Bergquist *et al.,* 1997 and Sonoda *et al.,* 1998).

IgA^+, IgM^+ and IgY^+ plasma cells were also detected in both the ANT and COW group, although compared with other groups they had a relatively lower number of Ig positive cells which did not increase noticeably throughout the experimental period. The presence of these Ig positive cells in the ANT and COW groups, may have been a result of intestinal microorganisms and some antigens present in the diet and environment.

In conclusion, both SMO and SMP in broiler diets, were associated with significant increases in the number of IgA^+ and IgM^+ plasma B cells of *E. Tenella* infected broilers. The counts for IgA^+ was the greatest of the three immune response B cells. SMO or SMP will have a considerable protection against *E. tenella* infection just as vaccination.

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REFERENCE

Adams, A., J. F. Petit, P. Lefrancier and E. Lederer, 1981. Muramyl peptides. Molecular and Cellular Biochemistry. 41:27-47.

Bach-Kundsen, K. E., 1997. Carbohydrate and lignin contents of plant material used in animal feeding. Animal Feed Science Technology. 67:319-338.

Bergquist, C., T. Lagergård and J. Holmgeren, 1997. Anticarrier immunity suppresses antibody response to polysaccharide antigens after intranasal immunization with polysaccharide-protein conjugate. Infection and Immunity. May 1579-1583.

Blum, S., Y. Delneste, S. Alvarez, D. Haller, P. F. Perez, C. H. Bode, W. P. Hammes, A. M. A. Pfeifer and E. J. Schiffrin, 1999. Interactions between commensal bacteria and mucosal immunocompetent cell. International Dairy Journal. 9:63-68.

Davis, P. J. and P. Porter, 1979. A mechanism for secretary IgA-mediated inhibition of the cell penetration and intracellular development of Emiera tenella. Immunology 36:471-477.

De Ambrosina V. M., S. Gonzalez, G. Perdigon, A. P. De Rruiz Holgado and G. Oliver, 1998. Immunostimulating activity of cell walls from lactic acid bacteria and related species. Food and Agricultural Immunology. 10:183-191.

De Simone, C., B. Bianchi Salvadri, E. Jirillo, L. Baldinelli, F. Bitonti and R. Vesel, 1989. Modulation of immune activities in human and animals by dietary lactic acid bacteria. In: Chandan RC. Ed. Yogurt: nutrition and health properties. McLean, VA: Kirby Lithographics. 201-214.

De Simone, C., R. Vesel, B. Bianchi Salvadri and E. Jirillo, 1993. The role of probiotics in modulation of the immune system in men and animals. International Journal of Immunotherapy. 9:23-28.

Girard, F., G. Fort, P. Yvoré and P. Quéré, 1997. Kinetics of specific immunoglobulin A, M and G production in the duodenal and cecal mucosa of chickens infected with Eimeria acervulina or Eimeria tenella. International Journal of Parasitology. 27:7 803-809.

Gitzelmann, R. and S. Auricchio, 1965. The handling of soy alpha-galactosidase by a normal and galactosemic child. Pediatrics. 36:231.

Gruskay, F. L. and R. E. Cooke, 1955. The gastrointestinal absorption of unaltered protein in normal infants and in infants recovering from diarrhea. Pediatrics. 16:763.

Hemmings, W. A. and E. W. Williams, 1977. Quantitative and visualization studies of the transport of rat and bovine IgG and ferritin across the segments of the small intestine of the sucking rat. Proceedings of the Royal Society of London. Series B. Biological sciences. 197:425.

James, S. P., M. Zenz, G. Mullin and L. Braun-Elwert, 1983. Role of lymphokinesis in functions of gastrointestinal mucosal T cells. Immunphysiology of the Gut. Vol 11(Walker A., Harmatz P. and Werskil B., Eds), Academic Press, New York, NY, USA, pp. 129-143.

Jingshan, Y., 1992. Cytochemical and Cytobiological Techniques. Published by BeiJing Medical University Publisher House. P105-107.

Kaetzel, C. S., J. K. Robinson, K. R. Chintalacharuvu, J. P. Vaerman, M. E. Lamm, 1991. The polymeric immunoglobulin receptor (secretary component) mediates transport of immune complexes across epithelial cell: A local defence function of IgA. Proceedings of the National Academy of Sciences of the United States of America. 88:8796-8800.

Kazuhito, H., H. Takehiro, I. Kikuji, T. Eiji and H. Yoshikazu, 1993. Immunohistochemical analysis of tissue distribution of B and T cells in germfree and conventional chickens. The Journal of Veterinary Medical Science. 55(6):1031-1034.

Lamm, M. E., 1997. Interaction of antigens and antibodies at mucosal surfaces. Annual Review of Microbiology. 51:311-340.

Linna, Z. and I. R. Tizard, 1996. Activation of a mouse macrophage cell line by acemannan: the major carbohydrate fraction from Aloe vera gel. Immunopharmacology.35:119-128.

Mazanec, M. B., C. S. Kaetzl, M. E. Lamm, D. Fletcher, J. G. Nedrud, 1992. Intercellular neutralization of virus by immunoglobulin A antibodies. Proceedings of the National Academy of Sciences of the United States of America. 89:6901-6905.

Michael, J. H. Ratcliffe, 2002. B cell development in gut associated lymphoid tissues. Veterinary Immunology and Immunopathology.87:337-340.

Patchen, M. L. and E. Lotzova, 1981. The role of microphages and T-lymphocytes in glucan-mediated alteration of murine hemopoiesis. Biomedicine.34:71.

Shanahan, F., 2000. Nutrient tasting and signaling mechanisms in the gut. V. Mechanisms of immunologic sensation of intestinal contents. American journal of physiology.

Gastrointestinal and liver physiology. 278:G191-G196.

Sonoda, Y., T. Kasahara, N. Mukaida, N. Shimizu, M. Tomoda and T. Takeda, 1998. Stimulation of inteleukin-8 production by acidic polysaccharides from the root of Panax ginseng. Immunopharmacology.38:287-294.

SPSS, Inc. 1997. SPSS for windows graduate pack advanced version, Version 8.0. Chicago, IL: SPSS Inc.

Stewart-Tull, D. E. S., 1980. Immunological activities of bacterial peptidoglycan. Annual Review of Microbiology. 34:311-340.

Suzan, H. M. Jeurisen, Frans Wagenaar and E. Marga Janse, 1999. Further characterization of M cells in gut-associated lymphoid tissues of the chicken. Poultry Science. 78:965-972.

Takahashi, T., T. Oka, H. Iwana, T. Kuwata and Y. Yamamoto, 1993. Immune response of mice to orally administered lactic acid bacteria. Bioscience, biotechnology, and biochemistry. 57:1557-1560.

Tizard, I. R., R. H. Carpenter, B. H. McAnalley and M. C. Kemp, 1989. The biological activities of mannans and related complex carbohydrates. Molecular biotherapy. 1:290.

Tress, A. J., M. J. Karim, S. B. McKerlar and S. D. Carter, 1989. Emiera tenella: local antibodies and interactions with the sporozoite surface. Journal of protozology 36:326-333.

Volkheimer, G. and F. H. Schulz, 1968. The phenomenon of persorption, Digestion. 1:213.

Volkheimer, G., F. H. Schulz, A. L. Lindenau and C. Wood, 1969. Persorption of metallic iron particles, Gut. 10:32.

Womble D. and J. H. Helderman, 1988. Enhancement of allo-responsiveness of human lymphocytes by acemannan (Carisyn™). International immunopharmacology. 10:967.

Gebhardt, R., 1983. Primary cultures of rat hepatocytes as a model system of canalicular development, biliary secretion, and intrahepatic cholestasis. Gastroenterology. 84:1462-1470.

Chapter 8

PRODUCTION EFFECTS OF OLIGO- AND WATER-SOLUBLE POLYSACCHARIDES (SMO & SMP) IN EARLY STAGE OF POSTHATCH DIETS FOR BROILERS

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Production Effects of Soybean Meal Oligo- and Water-Soluble Polysaccharides (SMO & SMP) in Early Stage of Post-hatch Diets for Broilers

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Abstract: Three trials were conducted to investigate the effect on growth of diets with water-soluble soybean oligosaccharides (SMO, 1 % of diet) and polysaccharides (SMP, 0.5 % of diet) during the first two weeks of life. Subsequent compensatory growth was also measured. In **Trial 1.** Three hundred and sixty 1-day-old male Arbor Acres broilers were used. The experimental design was as follows: 11 % soybean meal offered 0.96 % SMO and 0.42 % SMP in the basal diet of the first phase (CON), During Days 1-14 the CON diet was diluted by an additional 1 % SMO and 0.5 % SMP respectively. This resulted in diets SMO-1 and SMP-1. The second phase diet (Diet C, started from Day 22) was the same for all three groups, consisting of 26.95 % soybean meal which offers 2.34 % SMO and 0.78 % SMP. Results showed a growth and feed intake suppressive effect of supplemental SMO or SMP and subsequent complete compensatory growth of the broilers. The lowest body weight occurred on Days 14 and 28 for the SMO group, and on Day 14 for the SMP group, compared with the birds fed the antibiotic-containing diet. The SMO and SMP diets resulted in lower feed intake and lower feed utilization during the period of Days 1-14. Birds of the SMO-1 and SMP-1 groups had a lower feed efficiency during the period of Days 1-14, but a higher one later, when compared with the CON group. In **Trial 2**, two hundred and forty 1-day-old male Arbor Acres broilers were divided into four groups. In Phase I which lasted 30 days, one diet contained soybean meal (CON) and the other three diets were free of soybean meal (NSM, SMO-2 and SMP-2). SMO and SMP were used in the diet in the same way as described for Trial 1. The second phase diet started from Day 31 post-hatch, and had the same design as for Trial 1. Results indicated that the body weight of birds in the CON group, which received soybean meal and antibiotics throughout the experiment, was significantly higher than NSM, SMO-2 and SMP-2 groups at the Days 14, 28 and 42 ($p<0.05$). Birds in the SMO-2 group had a noticeably lower body weight compared with the birds receiving the NSM diet when measured on Days 14 and 28

post-hatch ($p<0.05$). This difference in weight had disappeared by Day 42. The use of SMP resulted in similar body weight throughout the experiment compared with NSM animals. In **Trial 3**, two-hundred and forty 1-day-old male Arbor Acres broilers were assigned into six groups as follows: soybean meal oligosaccharides and water soluble polysaccharides groups (SMO-3 and SMP-3). Chickens receiving SMO-3 and SMP-3 diets were given the same basal diet NSM but diluted by 1 % SMO and 0.5 % SMP for the period of Days 1-14. The VAC group of birds were vaccinated with 100 oocytes of *Eimeria tenella* on Day 3 post-hatch. The anti-coccidial medicine birds (ANT), received Maduramasin (5 ppm) in the diet from Day 5 before infection until Day 7 after infection (Day 11 to Day 22 post-hatch). Uninfected (COW) and infected (COR) control groups were only given NSM. Chickens, in the SMO-3, SMP-3, VAC, ANT and COR groups, were orally infected with 1000 sporulated oocysts of *E. tenella* on Day 15 post-hatch. All birds in Trial 3 had a large part of soybean meal-free basal diet (NSM) during Day 1-30 of age, and D¢ò started from Day 31. Results showed that the SMO-3 group had a lower body weight by Day 14 compared with the other groups ($p<0.05$). Birds of the SMP-3 group had a significantly higher body weight compared with those of VAC group by Day 42 ($p<0.05$). In **conclusion**, soybean meal oligosaccharides (SMO) and water-soluble polysaccharides (SMP) had a growth depressing effect when they were used in broiler diets both with and without soybean meal during the early stage of post-hatch, which was resulted from the feed intake restrict role of dietary SMO and SMP. Compensatory growth took place after SMO and SMP were withdrawn from the diet of broiler chickens after 14 days of age. The results suggest that SMO and SMP can be used as safety feed restriction substitute in diet of broiler chickens during the early stage of post-hatch.

Keywords: soybean meal oligo- and polysaccharides (SMO and SMP), productivity, compensatory growth, broilers.

INTRODUCTION

Soybean meal oligosaccharides (SMO) are composed of sugars mainly from the α-galactoside family including raffinose, stachyose etc. SMO cannot be hydrolyzed by the chicken's digestive enzymes due to the lack of α-1,6 galactosidase activity in the intestinal mucosa (Gitzelmann et al., 1965). Furthermore, oligosaccharides cannot pass through the intestinal wall. Therefore, either they remain undigested, or they may be fermented by gastrointestinal bacteria. Consequently, they are assumed, at least partly, to be the cause of the lower metabolizable energy (TME) content of soy protein concentrate (Leske et al., 1993). Soybean nonstarch polysaccharides (NSP) consist mainly of arabinans, arabinogalactans and acidic polysaccharides (pectic-type compounds). About one-third of NSP in soybeans are water-soluble (SMP) (Bach-Kundsen, 1997). These SMP contribute to a high chyme viscosity (Thompson et al., 1987) and may therefore reduce nutrient digestibility for chickens (Almirall et al., 1995; Bedford et al.,1992; Choct *et al*., 1996).

SMO and SMP are present at 88 g/kg and 40 g/kg soybean meal respectively (Yulan et al., unpublished data-Chapter 2). SMO and SMP are responsible for the presence of hygroscopic properties of the feces. Such droppings will be sticky and this will cause wet litter (Bedford, 1995). The wet litters promote the growth of bacteria, which make the birds on such litters more prone to infection and productivity will then be reduced. However, reports concern about the effect of soy soluble carbohydrates on poultry productivity has somewhat contradiction. Graham et al. (2002) reported that when soybean meal is treated with enzymes to hydrolyze raffinose and stachyose and then used in birds' diet for 14 days from 7 to 21 days post-hatch, chick growth performance was not significantly improved by enzyme treatment.

On the other hand, information about how SMO and SMP in the diet affect productivity of broilers is far from complete. The effects of consumption of SMO and SMP during a short period are not well known. It is also not known whether there are carry-over effects such as compensatory gain after the application has stopped. The

primary aim of this study was to examine whether in healthy and in *E. tenella* challenged broilers, growth is affected by SMO and SMP in post-hatch diets and whether there are carryover effects during the four weeks thereafter.

MATERIALS AND METHODS

Extraction of oligo- and water-soluble polysaccharides from soybean meal

The extraction of SMO and SMP from soybean meal has bean described previously as follows: 1 kg soybean meal was refluxed twice in 10 l alcohol (80 % (v/v)) at 75°C for 1h. This supernatant was then used for SMO isolation and the residue for SMP isolation. The supernatant was condensed by rotavapor (Büchi RE 120) and protein was subsequently removed with 5 % trichloroacetic acid (TCA). The liquid was freeze-dried after passing a column filled with Amberlite 200 and Amberlite IRA-900 for removing TCA and amino acids from the supernatant. The residue was hydrolyzed in a 0.2 M NaOH solution at 80 °C for 2 h and filtrated using cheesecloth. The filtrate was neutralised with 30% acetic acid and freeze-dried. The freeze-dried sample was dissolved in 400 ml water. Protein was removed with 5 % TCA. After 1h, the supernatant was centrifuged at 13000 g for 30 min. SMP was precipitated by adding alcohol to the supernatant until the alcohol concentration was 80 % (v/v) and kept at 4 °C over night. The polysaccharide precipitate was centrifuged at 2500g for 10 min. The polysaccharide product was freeze-dried. Replicate products extracted at different times were mixed together and stored at – 20 °C .

Animals and housing

In Trial 1. Three hundred and sixty 1-day-old male Arbor Acres broilers were randomly assigned to three groups of 120 birds each as an experimental treatment group. Each group of 120 birds was assigned randomly to a diet (Table 1), and the trial was performed from 1 to 42 days of age. From each group, subgroups of 100 birds and 20 birds were located on 20 m^2 and 4 m^2 floor with rice hulls as bedding material. Birds had free access to diets and water. Light management was as follows: birds

were exposed to 23-24 h of light during Days 1-3; 12 h during Days 4-15; 16 h during Days 16-22 and 18-23 h from Day 23 until the end of experiment. The temperature was around 31 °C for the first 5 days and then reduced to around 27 °C over 2 days for the remainder of the study.

In Trial 2. Two hundred and forty 1-day-old male Arbor Acres broilers were randomly divided into four groups of 60 animals. Each group of sixty birds was randomly allocated to one of four diets. For each diet group, four cages with 15 birds per cage served as replicates. Light and temperature management were as for Trial 1. Birds had free access to both diets and water during the entire 42 days experimental period.

***In Trial 3*.** Two hundred and forty 1-day-old male Arbor Acres broilers were randomly divided into six groups of 40 animals each as an experimental group. Animals were housed in cages (100 x 100 x 60cm) in a coccidia-free room. Chickens had access to feed and water ad libitum. Temperature management was the same as in Trial 1 and constant light was provided throughout the study period of 42 days.

Diets and experiment design

***General treatment*.** The dietary treatments were divided into a first and a second phase. The first phase diet was given for 21days in Trial 1 and for 30 days in Trials 2 and 3. The diets were a soybean meal containing and a soybean free meal (Diet II and Diet I). The second phase diet was fed from 22-42 days of age (Trial 1) and 31-42 days of age (Trials 2 and 3). All birds received the same Diet III in this phase (Table 1). In all Trials, the basal diets of SMO or SMP groups were diluted by 1% SMO or 0.5 % SMP. The experimental design is shown in Table 2.

Trial 1. There were three treatment groups (SMO, SMP and CON) in Trial 1. Additional SMO and SMP were used in the SMO-1 and SMP-1 groups. The CON group received the basal diet (Diet II) shown in Table 1. This basal diet consisted of corn, soybean meal, corn gluten, fishmeal, mineral and vitamin additives. The soybean meal containing

basal diet (Diet II) was diluted by 1 % SMO and 0.5 % SMP respectively during the first phase. After Day 14, the diets were gradually replaced by the basal diet (Diet II) within three days (ratio of basal diet to experimental diet = 30 to 70 at Day 15; 60 to 40 at Day 16; 90 to 10 at Day 17, and 100 to 0 at Day 18). Birds of SMO-1 and SMP-1 groups received the basal Diet II until 21 days of age. All groups (CON, SMO-1 and SMP-1) received the same diet (Diet III in Table 1) in the second phase of the experiment within two days starting from Day 22 until Day 42.

Trial 2. In Trial 2, there were four dietary treatments (SMO, SMP, NSM and CON). A commercial diet control group (CON) (Diet II in Table 1) and a non-soybean meal diet control group (NSM) (Diet I in Table 1.) SMO and SMP were used as additives in SMO-2 and SMP-2 groups, diluting the basal diets, which were free of soybean meal (Diet I) by 1% SMO and 0.5 % SMP and fed from Day 1-14post-hatch respectively. From Day 15, the diets were replaced by the basal diet (Diet I in Table 1) until Day 30 in the same way as in Trial 1. Diets of all groups in the experiment were changed to Diet III from Day 31.

Trial 3. In Trial 3, six treatments were applied (SMO-3, SMP-3, VAC, ANT, COW and COR). SMO and SMP were used as additives in SMO-3 and SMP-3 groups, diluting the basal diet (Diet I) by 1 % SMO and 0.5 % SMP respectively, from Days 1-11 post-hatch. The experimental diet was gradually replaced by 30 % of Diet I on Day 12, 60 % on Day 13, 90 % on Day 14 and 100 % on Day 15. All other groups received Diet I in Table 1. On Day 3 post-hatch, the VAC group of birds were vaccinated as follows: 100 oocytes of *E. tenella* were administered via the crop (vaccine supplied by Parasites Research Group of China Agricultural University). Another group (ANT) received the anti-coccidial drug Maduramasin (5 ppm) in their diet started from Day 5 before infection, until Day 7 after infection (Day 11 to Day 22 post-hatch). Birds in the SMO-3, SMP-3, VAC, ANT, and COR groups, were orally infected with 1000 sporulated oocysts of *Eimeria tenella* via the crop at 20:00 p.m. on Day 15 post-hatch. Birds in the COR and COW groups served as infected and uninfected control groups.

Table 1. Ingredient composition of the diets fed in Trials 1, 2 and 3

Ingredient	Diet I (% as fed)	Diet II (% as fed)	Diet III (% as fed)
Ground corn	47.90	60.25	66.18
Soybean meal		11.00	26.95
Corn germ meal	15.00		
Corn gluten meal	24.00	17.00	
Fish meal	7.00	7.00	1.00
$CaHPO_4$	0.70	1.25	1.15
Limestone	1.60	1.20	1.35
Salt	0.30	0.30	0.30
Sunflower oil	2.50	1.00	2.00
Vitamin-mineral mixture*	1.00	1.00	1.00
L-Lysine HCI			0.07
Crude protein	20.21	12.62	20.22
Metabolism energy (MJ / Kg)	12.58	17.83	12.51
Ca	1.00	1.01	0.95
P	0.64	0.64	0.57
SMO		0.96	2.34
SMP		0.42	0.78

* Provided vitamins and minerals as previously described (Lee et al., 2001).

Health treatments of birds

Birds in Trials 1 and 2 were immunized as follows: avian attenuated infectious bronchitis vaccine H120 (ABV) on Day 1 post-hatch ,via nose and eye drops; infectious bursal disease virus vaccine (IBDV)- primary and secondary vaccination on Days 7 and 20 respectively, in the drinking water; Newcastle disease virus IV attenuated vaccine (NDV)- vaccinated primary and secondary on Days 14 and 28 via nose and eye drops. The birds in Trial 3 did not receive ABV, IBDV and NDV vaccines.

Table 2. Experimental design according to the diets fed in different periods, in different trial and treatments

Trail	Group	1-21 day-old		22-42 day-old
		Diet I	Diet II	Diet III
Trail 1	SMO-1		Yes [1]	Yes [1]
	SMP-1		Yes [1]	Yes [1]
	CON		Yes [2]	Yes [3]
		1-30 day-old		31-42 day-old
		Diet I	Diet II	Diet III
Trail 2	SMO-2	Yes [1]	Yes [1]	
	SMP-2	Yes [1]	Yes [1]	
	NSM	Yes [1]	Yes [1]	
	CON		Yes [2]	Yes [3]
Trial 3	COW	Yes [1]		Yes [1]
	COR	Yes [1]		Yes [1]
	ANT	Yes [4]		Yes [1]
	VAC	Yes [1]		Yes [1]
	SMO-3	Yes [1]		Yes [1]
	SMP-3	Yes [1]		Yes [1]

[1.] Did not have antibiotics;

[2] with Maduramicin (5 ppm) and Aureomycin (0.22 %);

[3.] Aureomycin (0.22 %);

[4.]Maduramicin (5 ppm) is used on Day 5 before infection and until Day 7 after infection.

Non-soybean meal basal diet (Diet I), soybean meal containing basal diet (Diet II and Diet III)

Body weight and feed consumption

Body weight was measured on Days 1, 14, 28 and 42 post-hatch in all trials. Birds did not receive feed and water for 12 hours before weighing. In Trial 1, 20 birds were kept in a sub-group of the three experimental groups throughout the experiment. In this way, any dead birds in each treatment group were replaced by

birds which had been raised separately, but had the same diet and initial body weight as the 100 birds chosen initially. Accumulating feed consumption was recorded per group at 14, 28 and 42 days of age in Trial 1.

Statistics

The results are analyzed means by One-Way ANOVA per trial with each treatment as a different factor, The significant difference within groups were analyzed by LSD t test, performed by SPSS (8.0) (Statistical Package for the Social Science, 1997) statistical software.

RESULTS

The body weights on Days 1, 14, 28 and 42 post-hatch for Trials 1, 2 and 3 are shown in Table 3. The results of body weight gain, feed consumed and feed utilization efficiency are given in Table 4. Initial body weight on Day 1 post-hatch was similar among groups within the same trial ($P>0.05$).

Performance data for Trial 1, in which the effects of SMO and SMP were studied in terms of productivity of broilers during the early stage of post-hatch, showed a remarkably lower body weight on Days 14 and 28 for birds fed the SMO diet. Moreover, on Day 14, the SMP animals also had a lower body weight compared to the CON animals (Table 3). However, there were no significant differences in body weight between the treatment groups by 42 days of age ($P>0.05$). Feed intake by SMO-1 and SMP-1 birds during Days 1-14 were 8.2 % and 8.4 % of total feed consumption. This was less than in the CON animals (8.9 %). This situation persisted for another two weeks (Table 4). Most of the feed consumption in SMO-1 and SMP-1 birds (63. 7 and 64.5 % respectively), took place during Days 29-42. This was higher than in the CON animals (61.9 %) over the same period (Table 4). Body weight gain of birds in SMO-1 and SMP-1 was somewhat less during the first two weeks compared to the birds in the CON group. This coincides with the SMO and SMP addition period. After the extra SMO and SMP were withdrawn from the diet, the birds of the SMO-1 and

SMP-1 groups appeared to have a faster body weight gain when compared to birds of the CON group (Table 4). SMO-1 and SMP-1 groups had lower feed utilization efficiency during Days 1-14. However, later, it was higher compared with the CON group (Table 4).

Table 3. Body weight at Day 1, 14, 28 and 42 in Trials 1, 2 and 3 (g)

	Day 1	Day 14	Day 28	Day 42
Trial 1				
CON	42.8	342.1 ^{a}	973.2 ^{a}	2045.8
SMO-1	42.4	275.9 ^{c}	899.3 ^{b}	1991.8
SMP-1	42.3	297.6 ^{b}	939.0 ^{ab}	2005.9
SEM	0.2	2.9	10.0	19.9
Trail 2				
CON	42.3	272.9 ^{a}	700.6 ^{a}	1437.0 ^{a}
NSM	42.6	182.2 ^{b}	449.3 ^{b}	981.7 ^{b}
SMO-2	42.2	161.2 ^{c}	384.6 ^{c}	1016.2 ^{b}
SMP-2	42.2	169.1 ^{bc}	408.6 ^{bc}	1076.6 ^{b}
SEM	0.3	4.3	14.1	24.1
Trial 3				
COW	41.5	147.2 ^{a}	378.5	925.7 ^{ab}
COR	41.9	148.6 ^{a}	398.0	895.0 ^{ab}
ANT	43.0	148.3 ^{a}	375.4	924.3 ^{ab}
VAC	41.6	151.1 ^{a}	338.8	868.6 ^{b}
SMO-3	42.2	131.2 ^{b}	323.4	941.7 ^{ab}
SMP-3	42.5	156.0 ^{a}	403.6	1174.9 ^{a}
SEM	0.2	1.5	13.2	27.4

There was significant difference between the data marked different letters in the same trial and column ($p<0.05$). in Trial 1 each mean comes from 100 birds. In Trial 2, body weight data at 1st mean comes from 61, 60, 61, and 61 birds, at 14th comes from 40, 37, 39 and 40 birds; at 28th comes from 31, 29, 30 and 32 birds; at 42nd comes from 23, 21, 22 and 24 birds for CON, NSM, SMO-2 and SMP-2 group respectively. In trail, body weight data at 1st mean comes from 40, 41, 41, 40, 40, and 40 birds, at 14th comes from 40, 39, 39, 39, 38 and 39 birds; at 28th and 42nd comes from 7, 6, 7, 7, 6, and 6 birds for COW, COR, ANT, VAV, SMO-3 and SMP-3 group respectively.

Table 4. Feed consumption (g/day/bird), body weight gain (g/day) and feed to gain ratio in basal diet control group (CON) and the basal diet diluted by SMO or SMP experimental groups (SMO-1 or SMP-1)

	1-14 day	15-28 day	29-42 day	total
Group feed consumption (g/day/bird)				
CON	25.6	84.1	178.5	96.1
SMO-1	21.7	74.9	169.3	88.6
SMP-1	22.8	73.4	174.8	90.3
Mean body weight gain (g/day)				
CON	21.4	45.1	76.6	47.7
SMO-1	16.7	44.5	78.0	46.4
SMP-1	18.2	45.8	76.2	46.8
Feed to gain ratio (feed/body weight gain)				
CON	1.20	1.87	2.33	2.01
SMO-1	1.30	1.68	2.17	1.91
SMP-1	1.25	1.60	2.29	1.93

Trials 2 and 3 were conducted to investigate the effect of SMO and SMP on body weight in healthy (Trial 2) and in *E. tenella* challenged (Trial 3) broilers. The birds were all given a soybean meal-free diet and the diet was diluted by 1 % SMO and 0.5 % SMP respectively during Days 1-14. The results are given in Table 4. The body weight of the birds in the CON group, which were fed diets containing soybean meal and antibiotics throughout the experiment, was significantly higher than in the animals which were fed the soybean meal-free basal diets (NSM), supplemented with SMO and SMP (SMO-2 and SMP-2). On Days 14, 28 and 42 post-hatch, these differences were significant ($p<0.05$). SMO-2 birds had a significantly lower body weight when compared with NSM at Days 14 and 28 ($p<0.05$). However, this difference had

disappeared by Day 42. The use of the SMP diet did not cause any effect on body weight throughout the experiment, compared with animals receiving the NSM diet. In Trial 3, the SMO-3 group had lower body weights at Day 14 compared with other groups ($p<0.05$). The birds of the SMP-3 group had significantly higher body weight compared with those of the VAC group on Day 42($p<0.05$).

DISCUSSION

Three growth trials were conducted to investigate the effect of soybean meal oligosaccharide (SMO) and soybean meal water-soluble polysaccharides (SMP) on the productivity of broiler chickens when the SMO and SMP were added in diet of the first two weeks post-hatch. In trial 1, SMO and SMP were used in corn-soybean based diet; in Trial 2, SMO and SMP were added in soybean meal-free basal diet of healthy birds; and in Trial 3, SMO and SMP were added in soybean meal-free basal diet of *E. tenella* infection birds.

SMO and SMP had a negative effect on productivity of broiler chickens in the early stage after hatching in all three Trials. Apparently some growth depression a lower feed utilization resulted from SMO and SMP supplementation in Trial 1. This result is in disagreement with results of the research of Irish et al., (1995). Who studied the influence of stachyose and raffinose (alpha-galactosides of sucrose) present in soybean meal (SBM) on the nutritional value of the meal using adult broilers. In our work, the feed to gain ratio was higher when SMO or SMP was used in diet (Table 1). Therefore, this growth depression is attributable to the reduced feed intake. In addition, it can also be assumed that this was the cause of a lower digestibility of nutrients and thus a lower nitrogen-corrected true metabolizable energy (TMEn). It is most likely that oligosaccharides, raffinose and stachyose are the major reasons for the low ME of a soybean meal (Coon et al., 1990). During experiments described here, an appetite suppressant effect of SMO and SMP observed in SMO- and SMP-fed birds, therefore the growth depression can be partly attributed to the appetite suppressive effect of SMO and SMP. Furthermore, earlier research (Refstie et al., 1999) showed that the digestibility of dry matter had a clear negative relationship with the amount of

indigestible soybean NSP because of the high chyme viscosity. It is known that the indigestible SMO and SMP can produce diarrhea (Wiggins, 1984), which is probably related to an increased digesta passage rate. As a result, this can decrease digestion and absorption of dietary nutrients. The experimental results showed that the growth depression lasted somewhat longer in the SMO-consuming birds than of the SMP-consuming birds. This difference may have been caused by the differences in the dose of SMO (1 %) and SMP (0.5 %) as used in the diets, rather than to the chemical properties of the two carbohydrate fractions.

Compensatory growth was observed in SMO and SMP consuming birds and a higher feed efficiency was noted in both the SMO and SMP group after SMO and SMP had been withdrawn from the basal diet. This compensatory growth is attributable to the removal of the appetite suppressant and anti-nutritional effect of both SMO and SMP. Secondly, the fermentation characteristics of SMO and SMP may also be a factor. This can trigger off the compensatory growth. SMO and SMP which passes undigested into the lower gut of chicken will be at least partly fermented by microbes (Calloway et al., 1966; Wagner et al., 1976). This process results in gas production and a high concentration of volatile fatty acids (VFAs). Both these effects were found in previous research (Yulan et al., unpublished data which was described in Chapter 2). Furthermore, SMO and SMP promoted the development of lactic acid bacteria (LABs) in caecal contents when they were used in broilers' diet during the early stage of posthatch (Yulan et al., unpublished data, which was described in Chapter 4). These results showed the birds fed SMO and SMP had higher lactic acid and VFA in the caecal digesta, produced by the microflora, resulting in a lower lumen chyme pH in the hindgut when compared with the control group. It has been reported that changes in intracellular pH often parallel changes in extracellular pH which may trigger cell division. A low luminal pH has been shown to be associated with an increase in the numbers of cells that actively synthesize new DNA in the cecum of rats (Jacobs and Lupton, 1984; Lupton and Jacobs, 1987). The effects of SMO and SMP on intestinal growth were investigated by using the birds from Trial 2. The results indicated that SMO and SMP had no significant effect on the relative growth of the intestines on Day 15 post-hatch, when compared with NSM birds. SMO and SMP birds had a smaller

villus compared with those of the NSM group. Furthermore, pictures of the ileal and cecal surface at 8000 times magnification by scanning electronic microscopy showed that the density of micro-villi was higher and that they were relatively less developed in SMO and SMP birds compared to NSM birds. Compensatory growth of duodenal and ileal growth was noticed in SMO- and SMP- consuming birds after SMO and SMP were withdrawn from their diet (Yulan et al., unpublished data-Chapter 3.). According to these results, we may contribute the compensatory growth to the compensatory growth of intestines. Because it is possible that the surface of intestine mucosal villi and micro-villi increased after SMO and SMP were withdrawn from diet, and those increased mucosal cells are positively related to total digestive enzyme activity (Ija *et al.,* 2001). Thirdly, the nutritional restriction in SMO and SMP birds during the early stage of post-hatch could cause a very fast adaptive response with an assumed increase of growth hormone secretion during the period of compensatory growth (Gonzales et al., 1998; Buyse et al., 1997).

Data in Table 3 indicated that the birds in the CON group from Trial 1 had a much higher body weight than that in Trial 2 at 14, 28 and 42 days of age even though the two groups had received the same diet. That may have been at least partly due to the stress caused by sampling in Trial 2. The birds which consumed SMO or SMP and which had been infected with *E. Tenella* in Trial 3, had a lower body weight than their soybean meal-free diet counterparts in Trial 2 (Table 3), not only because gut mucosa could have been damaged by parasites, but also because of the corresponding immune responses. For the same reason, the vaccinated animals (VAC) in Trial 3 had a low body weight at 42 days of age (Table 3).

In conclusion, the addition of soybean meal oligosaccharides (SMO) and water-soluble polysaccharides (SMP) to young broiler diets resulted in slower growth. However, compensatory growth took place after SMO and SMP were withdrawn from the diet of broiler chickens. So the results suggest that SMO and SMP can be used as safety feed restriction substitute in diet of broiler chickens during the early stage of post-hatch.

REFERENCES

Almirall, M., M. Francesch, A. M. Perez-Vendrell, J. Brufau and E. Esteve-Garcia, 1995. The differences in intestinal viscosity produced by barley and ¦Â-glucanase alter digesta enzyme activities and ileal nutrients digestibilities more in broiler chickens than cocks. Journal of Nutrition. 125:947-955.

Bach-Kundsen, K. E., 1997. Carbohydrate and lignin contents of plant material used in animal feeding. Animal Feed Science and Technology. 67:319-338.

Bedford, M. R. and H. L. Classen, 1992. Reduction of intestinal viscosity through manipulation of dietary rye and pentosanase concentrations is effected through changes in carbohydrate composition of the intestinal aqueous phase and results in improved growth and food conversion efficiency of broiler chickens. Journal of Nutrition. 122:560-569.

Bedford, M. R., 1995. Mechanism of action and potential environmental benefits from the use of feed enzymes. Animal Feed Science and Technology. 53:145-155.

Buyse, J., E. Decuypere and J. D. Veldhuis, 1997. Compensatory growth of broiler chickens is associated with an enhanced pulsatile growth hormone (GH) secretion: preferential amplification of GH secretory burst mass. British Poultry Science. July; 38(3):291-296.

Calloway, D. H., D. J. Colasito and R. D. Matthews, 1966. Gases produced by human microflora. Nature. 212:1238.

Choct, M., R. J. Hughes, J. Wang, M.R. Bedford, A. J. Morgan and G. Annison, 1996. Increased small intestinal fermentation is partly responsible for the anti-nutritive activity of non-starch polysaccharides in chickens. British Poultry Science. 37:609-621.

Coon, C. N., K. L. Leske, O. Akavanichan and T. K. Cheng, 1990. Effect of oligosaccharide-free soybean meal on the true metabolizable energy and fiber digestion in adult roosters. Poultry Science. 69:787-793.

Gitzelmann, R. and S. Auricchio, 1965. The handling of soy alpha-galactosidase by a normal and galactosemic child. Pediatrics. 36:231.

Gonzales, E., J. Buyse, M. M. Loddi, T. S. Takita, N. Buys and E. Decuypere, 1998. Performance, incidence of metabolic disturbances and endocrine variables of food-restricted male broiler chickens. British Poultry Science. ; 39(5):671-678.

Graham, K. K., M. S. Kerley, J. D. Firman and G. L. Allee, 2002. The effect of enzyme treatment of soybean meal on oligosaccharide disappearance and chick growth performance. Poultry Science. 81:1014-1019.

Ija, P. A., A. Saki, and D. R. Tivey, 2001. Body and intestinal growth of broiler chicks on a commercial starter diet. 1. Intestinal weight and mucosal development. British Poultry

Science. 42(4):505-513.

Irish, C. G., G. W. Barbour, H. L. Classen, R. T. Tyler and M. R. Bedford, 1995. Removal of the alpha-galactosides of sucrose from soybean meal using either extraction or exogenous alpha-galactosides and broiler performance. Poultry Science. 74:1484-1494.

Jacobs, L. R., and J. R. Lupton, 1984. Effect of dietary fibers on rat large bowel mucosal growth and cell proliferation. American Journal of Physiology. 264:378-385.

Leske, K. L., C. J. Jevne and C. N. Coon, 1993. Effect of oligosaccharide additions on nitrogen-corrected true metabolizable energy of soy protein concentrate. Poultry Science. 72:664-668.

Lee, K. H. and S. Leeson, 2001. Performance of broilers fed limited quantities of feed or nutrients during seven to fourteen days of age. Poultry Science. 80:446-454.

Lupton, J. R. and L. R. Jacobs, 1987. Fiber supplementation results in expanded proliferative zones in rat gastric mucosa. American Journal of Clinical Nutrition. 46:980-984.

SPSS, Inc. 1997. SPSS for windows graduate pack advanced version, Version 8.0. Chicago, IL: SPSS Inc.

Refstie, S., B. Svihus, K. D. Shearer and T. Storebakken, 1999. Nutrient digestibility in Atlantic salmon and broiler chickens related to viscosity and non-starch polysaccharide content in different soybean products. Animal Feed Science and Technology. 79:331-345.

Thompson, D. B. and J. W. Erdman, 1982. Phytic acid determination in soybeans. Journal of Food Science. 47:513-517.

Wagner, J. R., R. Becker, M. R. Gumbmann and A. C. Olson, 1976. Hydrogen production in the rat following ingestion of raffinose, stachyose, and oligosaccharide-free bean residue. Journal of Nutrition. 106:466-470.

Wiggins, H. S., 1984. Nutritional value of sugars and related compounds undigested in the small gut. Proc. Nutr. Soc.43:69-75.

GENERAL DISCUSSION

INTRODUCTION

Soybean meal is used worldwide as an ingredient of poultry production diets both for the quantity and quality of its protein. Word-wildly large amounts of soybean meal are being used annually as poultry feed. However, apart from its high protein content, soybean meal also contains relatively large amounts of water-soluble non-digestible carbohydrates, namely both oligosaccharides (SMO) and water-soluble polysaccharides (SMP). The amount of galacto-oligosaccharides in soy solubles is about 20.59 %, of which 3.96 % from raffinose, 15.94 % from stachyose and 0.69% from verbascose (Smiricky-Tjardes *et al.,* 2003). SMO and SMP are usually considered to be anti-nutritional factors in broiler diets, because they are thought to reduce the available dietary energy. Consequently, enzymes have been added (Graham *et al.,* 2002). To eliminate these non-digestible carbohydrate from diet of broiler chickens, SMO are removed from soybean meal by alcohol extraction (Coon *et al.,* 1990).

However, poultry raised for meat production, have been selected based on their growth rate, which means that these birds have a much higher feed intake than lower growth rate birds. As a result, broilers are susceptible to metabolism disorders and sudden death malabsorption syndrome. The birds generally also have poor immune competence, and are therefore susceptible to infection by pathogens. Sub-therapeutic doses of antibiotics, and coccidiostats are therefore usually added to diets to promote growth and keep the birds healthy. However given growing concerns about the effect which these additives could have on human health, many investigations in recent years have focussed on finding safe feed additives, such as prebiotics, as an alternatives. Prebiotics may promote health and enhance the immune responsiveness of the animals themselves, by stimulating appropriate the commensal bacteria which live naturally in the gut. Given that SMO and SMP are often already present in broiler diets, the costs associated with their inclusion would be minimal, if it could be shown that they have desirable effects on gut health.

As fermentable carbohydrates, SMO and SMP could affect the activity of hindgut

microflora. If so, may therefore have the potential to be used as prebiotics in the diet of broiler chickens. To test this hypothesis, SMO and SMP were isolated from soybean meal, and then added to a soybean meal-free basal diet. The amount of SMO or SMP were matched the normal content of corn-soybean meal basal diet for broilers of the same age. Meanwhile, SMO or SMP were also added to a corn-soybean meal basal diet of broiler chickens. This thesis describes several experiments related to the *in vitro* fermentation mechanisms of SMO and SMP, and their prebiotic characteristics on young broiler chickens *in vivo*.

This study can be divided into four separate, but linked themes:

- *In vitro* evaluation of the fermentation characteristics of SMO and SMP with broilers' caecal microbial community as inoculum;
- The effect of SMO and SMP on intestinal physical development;
- The prebiotic role of SMO and SMP in terms of bacterial colonization in the gut;
- The effect of SMO and SMP on productivity of broilers when used in the broiler diet for two weeks post-hatch.

One *in vitro* experiment and three separate animal experiments were conducted in this study. The diets and animals are described in Tables 1 and 2. In this Chapter, the results of these experiments are summarized and some interesting phenomena in the experiments are discussed. The results are compared with other similar studies. Further study and future research suggestions are given at the end of this Chapter.

Table 1. Diet in different trials and experimental periods

Feeding trial				
Group/Period	Days 1-14	Days 15-17	Days 18-30	Days 31-42
CON	Diet II	Diet II	Diet II	Diet III
NSM	Diet I	Diet I	Diet I	Diet III
SMO	Diet I diluted by 1 % SMO	Transitional diet	Diet I	Diet III
SMP	Diet I diluted by 0.5 % SMP	Transitional diet	Diet I	Diet III
E. tenella infection trial				
Group/Period	Days 1-11	Days 12-14	Days 15-30	Days 31-42
COW	Diet I	Diet I	Diet I	Diet III
COR	Diet I	Diet I	Diet I	Diet III
ANT	Diet I	Diet I	Diet I	Diet III
VAC	Diet I	Diet I	Diet I	Diet III
SMO	Diet I diluted by 1 % SMO	Transitional diet	Diet I	Diet III
SMP	Diet I diluted by 0.5 % SMP	Transitional diet	Diet I	Diet III
Field trial				
Group/Period	Days 1-14	Days 15-17	Days 18-21	Days 22-42
CON	Diet II	Diet II	Diet II	Diet III
SMO	Diet II diluted by 1 % SMO	Transitional diet	Diet II	Diet III
SMP	Diet II diluted by 0.5 % SMP	Transitional diet	Diet II	Diet III

Diet I : The basal Diet I free of soybean meal, consisted of corn, corn gluten, corn germ meal, fish meal and a mineral and vitamin additive, and contained about 12.6 MJ/kg of metabolizable energy (ME) and 20.2 % of crude protein (CP).

Diet II : Commercial control diet, consisted of soybean meal, corn, corn gluten, fish meal and a mineral and vitamin additive, and contained about 12.6 MJ/kg of metabolizable energy (ME) and 22.2 % of crude protein (CP).

Diet III: Diet consisted of soybean meal, corn, corn gluten, fish meal and a mineral and vitamin additive, and contained about 12.5 MJ/kg of metabolizable energy (ME) and 17.8 % of crude protein (CP).

Transitional diet: the ratio of basal diet to experimental diet = 30 to70 at the first day; 60 to 40 at the second day; 90 to10 at the third day.

Table 2. Initial number of animals and numbers of animals sacrificed in different time and trials (head)

Feeding trial [*]										
Groups	Total animal	Day 1[1]	Day 3[1]	Day 6[1]	Day 9[1]	Day 12[1]	Day 14[2,3]	Day 15[1,4,5,6]	Day 28[1, 2, 3]	Day 42[2]
CON	61+20	4	4	4	4	4	8 [***]	8	8	4
NSM	60+20	4	4	4	4	4	8 [***]	8	8	4
SMO	61+20	4	4	4	4	4	8 [***]	8	8	4
SMP	61+20	4	4	4	4	4	8 [***]	8	8	4

E. tenella infection trial									
Groups	Total animal	Day 15[4]	Day16[4]	Day17[4]	Day 18[4]	Day19[4]	Day 20[4]	Day 21[4, 5]	Day 22[4]
COW	40	3	3	3	3	3	3	11	3
COR	40	3	3	3	3	3	3	11	3
ANT	40	3	3	3	3	3	3	11	3
VAC	40	3	3	3	3	3	3	11	3
SMO	40	3	3	3	3	3	3	11	3
SMP	40	3	3	3	3	3	3	11	3

Field trial [**]	
Groups	Total animal
CON	100+20
SMO	100+20
SMP	100+20

[1.] For intestinal length and weight measurement; [2.] For T lymphocyte transfer rate analysis; [3.] For macrophages phagocytic function assay; [4.] For immunohistochemistry analysis of IgA, IgM and IgY containing B cells in caecal lamina propria; [5.] For caecal LAB analysis; [6.] For intestinal morphology analysis by scanning Electro-microscope.

[*.] Blood samples collected from 8 birds of each group and at days 7, 14, 28 and 42, respectively. The birds were not sacrificed.

[**.] Birds were not sacrificed in field trial.

[***]Birds came from the extra 20 birds, which received same diet with the treatment group but in a separate cage.

IN VITRO EVALUATION OF FERMENTATION MECHANISMS OF SOME SOY CARBOHYDRATE FRACTIONS USING THE BROILER CAECAL MICROBIAL COMMUNITY AS INOCULUM

Soybean meal is one of the major protein ingredient resources in poultry diets. Alfalfa meal is sometimes also used as an ingredient of commercial diets, because it can enhance the color of egg yolk and of broiler skin. As a legume, soybean contains a considerable amount of water-soluble carbohydrates, known to comprise both oligosaccharides and polysaccharides. These carbohydrates cannot be digested by digestive enzymes of broiler chickens, but they can be fermented by the intestinal microflora. In this study (Chapter 2), an investigated was made of the fermentation characteristics of four extracted non-digestible carbohydrates (ENC): soybean meal oligosaccharides (SMO), soybean meal water-soluble polysaccharides (SMP), alfalfa meal oligosaccharides (AMO) and alfalfa meal water-soluble polysaccharides (AMP); and two pure sugars, raffinose (RAFF) and stachyose (STA). The caecal contents of 81-day old broilers was used as the microbial inoculum.

Results indicated that all test substrates are utilized by caecal bacteria. The amount of gas and VFA of the fermentation fluid were positively related to the sugar content of the substrates. The high butyric acid production and low ammonia nitrogen concentration at the end of fermentation of SMO deserves more attention. Butyric acid is considered to be beneficial for animal health as it is not only the energy source of mammalian colonocytes, but also can inhabit the promotion of colon carcinogenesis (Potter, 1999).

Ammonia nitrogen.

Ammonia (including the ammonium ion) produced from amino acid degradation in the body is converted to uric acid in the liver of the chicken. A significant amount of this uric acid later appears in the gastrointestinal tract (GIT) by bill excreting, and is hydrolyzed into ammonia by microbial urease (Wrong, 1981). Most ureolytic activity takes place in the caeca of chickens (Lee, 1977). This ammonia together with that

produced from other nitrogenous substrates, may be used for microbial protein synthesis in the presence of sufficient carbohydrate as an energy source, or may enter the blood stream in the case of excessive protein fermentation.

Ammonia is known to be harmful to animal health (Visek, 1978). Some species of urease-producing bacteria are known to have a negtive effect on growth of chickens (Lev *et al.*,1957). Therefore, increased microbial protein synthesis ability or a decrease in urease activity can reduce ammonia nitrogen concentration both in the GIT and environment. In this experiment, the caecal microflora fermented non-digestible carbohydrates in an *in vitro* gas production system. Uric acid was not added to the medium, though there were added peptides, amino acids, and some ammonia, to supply bacterial needs. There was supposed to be a low concentration of uric acid in the fermentation solution. Any uric acid present in the fermentation solution may therefore have originated from the inoculum. Since the inoculum was only a small proportion of the total solution (1.25 ml of 87 ml total solution), the uric acid concentration must have been low, though this was not measured. The low ammonia concentration found relating in vitro fermentation of SMO was probably related to microbial utilization. However, the situation would be different in the case of an *in vivo* environment, given that the amounts of nitrogen in the GIT are less easily controlled.

When SMO was used in the diet of early post-hatch, there was an increase in the population of some lactic acid bacteria (LAB) in the caecal contents (Chapter 4). It is known that LAB can decrease urease activity in the small intestinal contents of young chicks (Yeo and Kim, 1997). So the dietary SMO would result in a lower ammonia concentration in their living environment. It is also known that the gut bacteria can convert ammonia nitrogen to microbial protein (Karasawa, 1999). On the other hand, LAB stimulated by dietary SMO might partly inhibit the growth of urease-producing bacteria by several mechanisms (Barrow, 1992). Therefore, if it considers both of these mechanisms, dietary SMO could reduce ammonia nitrogen concentration from uric acid in droppings of broiler chickens by stimulating the production of more microbial biomass. However, the current experimental results could not provide

conclusive proof on the selective stimulation role of SMO on LAB (Chapter 4), so this point needs further investigation.

Microbial community

The gas production experimental results indicated that the non-digestible carbohydrates tested in this experiment, could influence the composition and/or activity of the caecal microbial community of broiler chickens. The caecal bacterial DNA fingerprint after fermentation of the different carbohydrate fractions showed that there was some shift in the caecal microbial community (Figure 1), even though the caecal contents came from 81-day old broiler chickens. The gut microbial ecosystem is considered to be quite stable in adult birds. When the intestinal microflora was supplied with SMO and SMP from an early stage of post-hatch, the lactic acid bacterial population was significantly increased in the caecal contents of SMO consuming birds (Chapter 4). Therefore, SMO appeared to modulate the gut microbial community in a beneficial direction, especially when SMO used in the diet of early stage post-hatch.

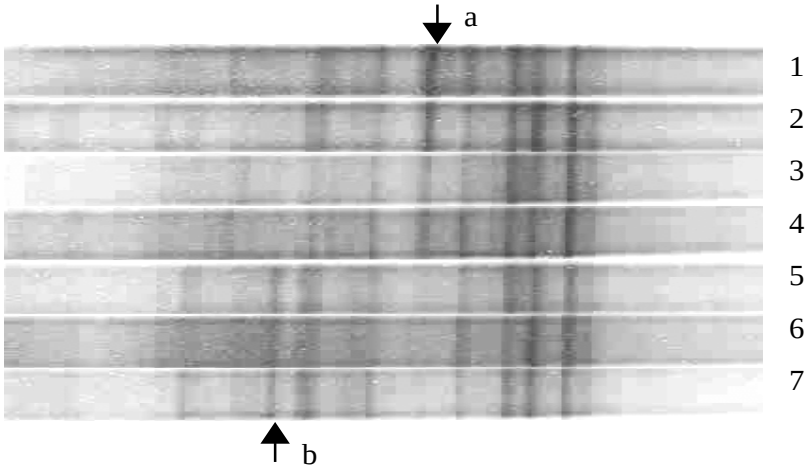

Figure 1 DGGE band pattern of bacteria community profiles of sample containing the extracted non-digestible carbohydrates from soybean meal and alfalfa meal. DGGE profile lanes: 1: alfalfa meal oligosaccharides (AMO); 2: soybean meal oligosaccharides (SMO); 3: alfalfa meal water-soluble polysaccharides (AMP); 4 soybean meal water-soluble polysaccharides (SMP); 5: blank before incubation; 6: blank after incubation; 7: inoculum. Solid arrowheads indicate the position where the band become predominant (a) or disappear (b) as compared with inoculum and blank.

Comparison of between natural and synthetic oligosaccharides in terms of fermentation kinetics and products

Smiricky-Tjardes *et al.* (2003) compared the fermentation characteristics of soy fermentable carbohydrates with some synthetic oligosaccharides which had been shown to have prebiotic properties, by *in vitro* experiments using pig caecal contents. Soy solubles were fermented faster and produced more gas than the synthetic oligosaccharides, fructo-oligoaccharides (FOS, short, middle and long chain) and mannan-oligosaccharides (Table 3). This may have been partly due to the high sucrose concentration (24.45 %) in soy solubles. In terms of VFA produced from SMO, the proportion of butyric acid production in total VFA production was 14 % when fermented by pig gut bacteria, and this proportion is lower than the result of our experiment (24 %)(Chapter 2). The fermentation kinetics of natural fermentable carbohydrates extracted from soybean meal and alfalfa meal was compared with synthetic oligosaccharides raffinose and stachyose (Chapter 2). The results showed that those natural carbohydrates had lower total gas production than that of synthetic oligosaccharides.

THE EFFECT OF SMO AND SMP ON INTESTINE PHYSICAL DEVELOPMENT OF BROILER CHICKENS WHEN SMO AND SMP WERE USED IN THE DIET FOR TWO WEEKS POST-HATCH

SMO and SMP used in broiler diets for two weeks post-hatch had no effect on the intestinal weight. This was especially the case, when expressed either as a percentage of the digestive tract-free body weight or as intestinal weight in length (g / cm), and compared with animals fed a soybean meal-free diet up to 15 days of age. However, dietary SMO and SMP did significantly affect the morphological development of the gut mucosa. Birds in the SMO and SMP groups had smaller villi than the birds in the soybean meal-free control group (NSM) at Day 15. Meanwhile, a higher density and less developed micro-villi were observed in the duodenum and ileum of the birds of the SMO and SMP groups, compared with the birds of the NSM group (Chapter 3). Compensatory intestinal growth took place at 14 to 28 days of age after SMO and

SMP had been withdrawn from the diet. The results suggest that the main effect of dietary SMO and SMP on intestinal development occurs at the microvilli. It is also possible that dietary SMO and SMP increase the microvillus number per unit area of intestinal mucosa surface. This may occur via stimulation of the microflora and these in turn may stimulate gut cell proliferation by their metabolic end-products, such as VFA. It has been reported that the microvillus length increases from the day of hatching to Week 12. The microvillus diameter exhibits only few changes during development (Mitjans *et al.,* 1997). So it is also possible that the intestinal compensatory growth occurs as an increase of microvillus length after withdrawal SMO and SMP from diet.

Brush border enzyme activity is expressed over a large proportion of the villus. Enzyme activity per unit villus surface area is similar between ages (Ija *et al.*, 2001). The digestive capacity and feed intake of birds in the SMO and SMP groups may have increased due to an increase in the total enzyme activity brought about by the increased surface area. As a result, some compensatory growth was noticed in the birds of SMO and SMP groups after SMO and SMP were withdrawn from diet (Chapter 8).

THE PREBIOTIC ROLE OF SMO AND SMP USED IN DIETS DURING TWO WEEKS POST-HATCH

Competitive exclusion (CE)

"Competitive exclusion" is defined as any action by the gastrointestinal tract commensal microorganisms which acts to resist colonization by invading pathogens, such as occupation of attachment sites on the intestinal mucosal surface, competition for nutrients, or the production of bacteriocins (Chapter 1). Some feed ingredients can promote the CE ability of chickens. For example, the inclusion of fructooligosaccharide (FOS) in the diet of chickens may lead to a shift in the intestinal gut microflora, and under some circumstances may result in reduced susceptibility to Salmonella and Campylobacter colonization (Bailey *et al.*, 1991; Schoeni and

Wong,1994). Feeding FOS, tannic acid or mannose in the diet of chicks together with the administration of defined competitive exclusion bacteria has been also shown to had to a greatly reduced susceptibility to Salmonella colonization (Schoeni and Wong, 1994; Fukata *et al.*, 1999; Kubena *et al.*, 2001). In order to test whether SMO and SMP could promote the resistance of broiler chickens to coccidiosis, SMO and SMP were used in diet for two weeks post-hatch. Caecal wall attached microflora and the population of a group of lactic acid bacteria (LAB) in caecal contents were determined on Day 15 post-hatch, using scanning electric microscopy and real-time PCR technique (Chapters 3 and 4). Furthermore, the LAB population in the caecal contents was detected by real-time PCR technique at Day 7 after the birds had been infected with *E. tenella* (Chapter 6).

Experimental results indicated that SMO and SMP, which were used in bird diets from Days 1-15 post-hatch, led to an increase in the bacterial population attached to the caecal wall, and increased the LAB population in caecal contents when compared to the birds of soybean meal-free control group, and the commercial diet control group containing antibiotics (Chapter 3 and 4). The LAB population in caecal the contents of post-infection birds was significantly higher in the SMO and SMP groups than in the infected control group (Chapter 6). Furthermore, the number of oocysts shed per gram droppings was remarkably lower in the SMO and SMP groups compared with the control group (Chapter 6). Based on these results, it was concluded that SMO and SMP can promote the CE ability against *E. tenella* infection, by stimulation of the GIT microbial community.

Several studies have reported that feeding prebiotics, such as fructo-oligosaccharides (FOS), in the diet of chickens may reduce the susceptibility of animals to Salmonella and Campylobacter colonization (Bailey *et al.*, 1991; Schoeni and Wong, 1994; Fukata *et al.*, 1999; Kubena *et al.*, 2001). However, few studies have focussed on the CE promotion effect of prebiotics in poultry diet against coccidial infection. The results of this experiment is the first report about the role of soybean meal fermentable carbohydrates in promoting CE ability against coccidial infections in broiler chickens.

Table 3. Comparison fermentation characteristics of soybean meal natural carbohydrates (soy solubles) with some synthetic carbohydrates which used as prebiotic (fructo- and Mannan- oligosaccharides) in animal diet, when swine fecal microflora as an inoculum [1]

	Gas production (mL/g of OM)	Time to attain maximal rate of gas production, h	PH	Acetic acid (mmol/g OM)	Propionic (mmol/g OM)	Butyric acid (mmol/g OM)	Total SCFA (mmol/g OM)
Short-chain FOS	230.1	5.0	5.1	2.2	2.3	1.2	5.7
Medium-chain FOS	243.0	6.1	5.1	2.3	2.6	1.2	6.1
Long-chain FOS	231.5	6.0	5.1	2.3	2.7	1.0	6.0
Raffinose	1,227.9	—	6.4	3.6	0.8	0.7	3.5
Stachyose	45.5	3.0	6.4	3.1	2.5	1.1	6.7
Soy solubles [3]	510.3	3.2	5.8	2.4	1.9	0.7	5.0
Mannan oligosaccharides	155.3	8.3	6.1	1.6	1.4	0.4	3.4

[1] Smiricky-Tjardes *et al.* (2003).

[2] Soy solubles contain OM: 90.2 %, N: 2.37 %, total amino acid: 11.93 %, sucrose: 24.45 %, raffinose: 3.96 %, stachyrose: 15.94 %, verbascose: 0.68 %

Immune competence

An *in vivo* experiment was conducted to investigate the effects of SMO and SMP on the immune response of broilers during 42 days of life. (Table 1) (Chapter 5). Animals were vaccinated against avian infectious bronchitis (ABV) on Day 1, against infectious bursa (IBDV) on Days 7 and 20, and against Newcastle disease virus (NDV) on Days 14 and 28. Several aspects of humoral, cellular and mucosal immunity were measured on Days 7, 14, 28 and 42 post-hatch. The experimental results indicated that the SMO and SMP diets did not have any effect on the systemic humoral immune response in the first two weeks after hatching. However, SMO and SMP did stimulate both the systemic cellular and caecal local humoral immune response and could promote the development of the lymphocyte germinal center in the caecal lamina propria. Therefore, both SMO and SMP, but particularly SMO, could possibly be used as anti-pathogen substitution in the diet of young broilers. Furthermore, an *E. tenella* infection animal trial was also conducted to investigate the effect of SMO and SMP on the caecal lamina propria immunoglobulins A, M and Y positive plasma cells, of broilers challenged with *E. tenella* (Table 1). The six treatments were as follows: 1) water-soluble soybean meal oligosaccharides (SMO), and 2) polysaccharides groups (SMP). 3) Advanced vaccination group (VAC), birds vaccinated with 100 oocytes of *E. tenella* on Day 3 post-hatch, 4) anti-coccidial medication group (ANT), containing Maduramasin (5 ppm) in diet from five days before infection to seven days after infection. 5) Uninfected, non-medicated (COW), and 6) infection control groups (COR) were only given basal diet. Chickens, except for the birds in COW group, were orally infected with 1000 sporulated oocysts of *E. tenella* on Day 15 post-hatch. The caecal lamina propria IgA-, IgM- and IgY- containing B plasma cells were monitored at 12, 24, 48, 72, 96, 120, 144 and 168 hours post-infection by used monoclonal antibodies and immunohistochemistry techniques. The results showed that animals in both the SMO and the SMP treatments had a significant increase in the number of caecal mucosal IgA-and IgM-containing B cells, compared with the infection control group (COR), and was comparable with VAC group. The higher number of immunoglobulin containing B cells detected at 12 h post-infection in birds of the SMO group, might have be on associated the with natural antibody present.

Natural antibodies (NAb) are defined as antigen-binding antibodies present in non-immunized individuals (Parmentier *et al.,* 2004). Natural antibodies are often dismissed from immunological analysis as "background," but they may play an important role in conferring immunity against infections (Ochsenbein *et al.,*1999). In mammals, NAb are preferentially derived from $CD5^+$ (B1) B cells (Casali and Notkins, 1989) located in the peritoneal cavity (Ochsenbein, *et al.,* 1999) and along the intestinal tract (Quan *et al.,* 1997). NAb are also present in chickens (Longenecker and Mosmann, 1980; Jalkanen *et al.,* 1983; Neu *et al.,* 1984; Barua and Yoshimura, 2001; Parmentier *et al.,* 2004). In this experiment, dietary SMO significantly increased the number of IgA and IgM containing B plasma cells at 12 h post-infection. It is possible that the natural antibody in broilers, especially the intestinal lamina propria had been stimulated either by the dietary SMO itself or because of the increased the LAB population and decreased lumen pH. The exact mechanisms need further study.

FEED RESTRICTION WELFARE ROLE OF SMO AND SMP ON BROILER CHICKENS WHEN USED IN DIET FOR TWO WEEKS POST-HATCH

In modern broiler strains, fast growth ocurs in the first four weeks (Marks, 1979) post-hatch. This fast growth of broiler chickens is accompanied by a number of problems, namely, increased body fat deposition, a high incidence of metabolic diseases, high mortality, a high incidence of skeletal diseases (Leeson and Summers, 1988), and poor immune competence (Tottori *et al.*, 1997). The problems of using sub-therapeutic doses of antibiotics in the diet have been increasingly highlighted in recent years (General Introduction). Therefore, some feed restriction during the early stages post-hatch would probably be helpful to improve the health condition of broiler chickens. Feed restricted birds have fewer bone, joint, and foot problems than *ad libitum* fed birds (Hocking and Duff, 1989). Feed restricted birds also show an improved antibody response and disease resistance, as well as significantly decreased mortality, as compared to *ad libitum* fed birds (Attia *et al.,*1993). Feed restriction has important welfare benefits, and is standard practice in broiler breeding farms. The two most commonly used commercial restriction programmes are

skip-a-day, in which amounts of feed calculated to achieve desired body weight are fed on alternative days; and limited every other day, on which only half of the usual amount is fed (Mench, 2002). In addition, some alternative methods have been used, such as adding chemical components to the diet, or diluting the diet with non-digestible fiber, but none of them have proven completely satisfactory (Savory *et al.,* 1996). There is also evidence indicating that current feed restriction practices also have negative welfare effects, such as chronic hunger (Savory *et al.,* 1993).

The dilemma of current feed restriction practices, and the ban on the use of anti-bacterial feed additives are increasing the urgency pressure to finding alternatives to optimize bird performance and health.

Animal experiments were performed to investigate the effects of SMO and SMP on the productivity of broilers (Chapter 8). SMO and SMP were used in diets (either containing soybean meal or not, Table 1) during the first two weeks post-hatch, diluting the basal diet with 1 % and 0.5 % SMO and SMP, respectively. All birds had free access to feed and water. Results showed an appetite suppressive effect of supplemental SMO or SMP when applied during the first two weeks of life. Significantly lower feed intake and body weight were noticed in SMO and SMP consuming birds. Compensatory growth took place after SMO and SMP were withdrawn from the diet of broiler chickens after 14 days of age, and complete compensatory body weight was reached by Day 42. Furthermore, SOM and SMP showed prebiotic properties, both in terms of increasing the LABs population in caecal contents, and in promoting the immune response both in healthy and *E. tenella* infected birds, as well as improving the CE ability against coccidial infection, when used in diet in the early stage of post-hatch (Chapters 4, 5, 6, and 7). Therefore, based on our experimental results, it should be possible to use SMO and SMP as a feed restriction additive in broiler diets of early post-hatch.

CONCLUSION

To sum up, our experimental results have led to the following conclusions:

When non-digestible carbohydrates were fermented by the caecal contents microbial community of broiler chickens *in vitro*:

- SMO, SMP, AMO and AMP RAFF and STA can stimulated the bacterial activity of caecal bacteria. After fermentation, SMO produced the lowest ammonia nitrogen and the highest butyric acid of these tested products.
- SMO, SMP, AMO and AMP RAFF and STA could lead to a shift in the caecal microbial community in some extent.

When the diet of the first two weeks post-hatch was diluted by 1 % SMO and 0.5 % SMP, respectively.

- Dietary SMO and SMP had no effect on the development of intestinal length and relative weight. However, dietary SMO and SMP decreased the villus surface size and tended to increase the microvilli density on the villus surface.
- Dietary SMO could significantly increase the LAB population in caecal contents both in healthy birds and in the birds infected with *E*. *tenella.*
- Dietary SMO and SMP enhanced cellular and had no effect on the humoral immune response following vaccination.
- SMO and SMP led to an increase in immunoglobulin A, M and Y positive plasma cells (IgA^+, IgM^+ and IgY^+) within the caecal mucosa, both in healthy birds and the birds infected by *E. tenella.*
- SOM and SMP led to feed restriction during its used as a dietary additive, and complete compensatory growth had taken place after SMO and SMP were withdrawn from the diet.

Based on the results of this thesis, it is concluded that soybean meal oligosaccharides (SMO) and water-soluble polysaccharides (SMP) can be used as harmless feed restriction additives in young broiler chickens. In addition, both products would have beneficial effects on certain microbiological and immunological promotion

within the GIT, might therefore be suitable additives for the promotion of GIT health.

SUGGESTION FOR FURTHER STUDIES

- To investigate whether soybean meal fermentable carbohydrate (SMFC) can selectively stimulate specific gut beneficial bacteria or not; and its effects on the urease-producing bacterial population in the ileum and cecae, when SMFC are used in broiler diets at early post-hatch.
- To study the relationship between SMFC and urease activity in caecal contents, and the effect on ammonia nitrogen concentration in their raising facilities (i.e. environmental effect).
- To study the effect of SMFC on intestinal microvillus development and brush border digestive enzyme activity in young broiler chickens.
- To study the relationship between SMFC dosage used in diet of young broiler chickens and feed restriction target.
- To investigate the effect of SMFC on natural antibody and CE ability against other gut pathogens, when SMFC are used in broilers diets at early post-hatch.
- To further identify the ingredients of SMFC, given the isolation method for SMO and SMP, which was used in this work, it cannot be excluded that the effect was also associated with other substrates.

REFFERENCES

Attia, Y. A., K. A. Yamani and W. H. Burke, 1993. Daily energy allotment and reproductive performance of broiler breeder males. Poultry Science. 72:42-45.

Bailey, J. S., L. C. Blankenship, N. A. Cox, 1991. Effect of fructooligosaccharide on Salmonella colonization of the chicken intestine. Poultry Science. 70(12):2433-2438.

Barrow, P. A., 1992. Prebiotics for chickens. Pages 225-257 in Probiotics. R.Fuller, ed. Chapman and Hall, London, UK.

Barua, A. and Y. Yoshimura, 2001. Ovarian autoimmunity in relation to egg production in laying hens. Reproduction 121:117–122.

Casali, P. and A. L. Notkins, 1989. $CD5^+$ B lymphocytes, polyreactive antibodies and the human B-cell repertoire. Immunology Today. 10:364–368.

Coon, C. N., K. L. Leske, O. Akavanichan and T. K. Cheng, 1990. Effect of oligosaccharide-free soybean meal on the true metabolizable energy and fiber digestion in adult roosters. Poultry Science. 69:787-793.

Fukata, T., K. Sasai, T. Miyamoto, and E. Baba, 1999. Inhibitory effects of competitive exclusion and fructooligosaccharide, singly and in combination, on Salmonella colonization of chicks. Journal of Food Protection. 62(3):229-233.

Graham, K. K., M. S. Kerley, J. D. Firman and G. L. Allee, 2002. The effect of enzyme treatment of soybean meal on oligosaccharide disappearance and chick growth performance. Poultry Science. 81:1014-1019.

Hocking, P. M. and S. R. I. Duff, 1989. Musculo-skeletal lesions in adult male broiler breeder fowls and their relationship with body weight and fertility at 60 weeks of age. British Poultry Science. 30:777-784.

Ija, P. A., A. Saki, and D. R. Tivey, 2001. Body and intestinal growth of broiler chicks on a commercial starter diet. 1. Intestinal weight and mucosal development. British Poultry Science. 42(4):505-513.

Jalkanen, S., K. Granfors, M. Jalkanen and P. Toivanen, 1983. Immune capacity of the chicken bursectomized at 60 hours of incubation: failure to produce immune, natural, and autoantibodies in spite of immunoglobulin production. Cell Immunology. 80:363–363.

Karasawa, Y., 1999. Significant role of the nitrogen recycling system through the ceca occurs in protein-depleted chickens. Journal of Experimental Zoology. 283:418-425.

Kubena, L. F., J. A. Byrd, C. R. Young, and D. E. Corrier, 2001. Effects of tannic acid on cecal volatile fatty acids and susceptibility to Salmonella typhimurium colonization in broiler chicks. Poultry Science. 80(9):1293-1298.

Lee D. J. W., 1977. Urease activity in the digestive tract of the chick and its role in the utilisation of urea as a source of non-amino nitrogen. British Poultry Science. 18:449-458.

Leeson, S. and J. D. Summers, 1988. Some nutritional implications of leg problems with poultry. British Veterinary Journal. 144:81-92.

Lev, M., C. A. E. Briggs, and M. E. Coates, 1957. The gut flora of chick. 3. Differences in cecal flora between 'infected', uninfected and penicillin-fed chicks. Journal of Animal Science. 16:346-372.

Longenecker, B. M. and T. R. Mosmann, 1980. Natural antibodies to chicken MHC antigens are present in mice, rats, humans, alligators and allogeneic chickens. Immunogenetics 11:293–302.

Marks, H. L., 1979. Growth rate and feed intake of selected and nonselected broilers. Growth. 43:80-90.

Mench, J. A., 2002. Broiler breeders: feed restriction and welfare. World's Poultry Science Journal. 58:23-29.

Mitjans, M., G. Barniol and R. Ferrer, 1997. Mucosal surface area in chicken small intestine during development. Cell Tissue Research. 290:71-78.

Neu, N., K. Hala and G. Wick, 1984, Natural chicken antibodies to red blood cells are mainly directed against the B-G antigen, and their occurrence is independent of spontaneous autoimmune thyroiditis. Immunogenetics 19:269–277.

Ochsenbein, A. F., T. Fehr, C. Lutz, M. Suter, F. Brombacher, H. Hengartner and R.M. Zinkernagel, 1999. Control of early viral and bacterial distribution and disease by natural antibodies. Science. 286:2156–2159.

Parmentier H. K., A. Lammers, J. J. Hoekman, Gde V. Reilingh, I. T. Zaanen, and H. F. Savelkoul, 2004. Different levels of natural antibodies in chickens divergently selected for specific antibody responses. Developmental & Comparative Immunolog. 28(1):39-49.

Potter, J. D., 1999. Fiber and colorectal cancer. New England Journal of Medicine. 340:223-224.

Quan, C. P., A. Berneman, R. Pires, S. Avrameas and J-P. Bouvet, 1997. Natural polyreactive secretory immunoglobulin A autoantibodies as a possible barrier to infection in humans. Infection Immunology. 10:3997–4004.

Savory, C. J., K. Maros and S. M. Rutter, 1993. Assessment of hunger in growing broiler breeders in relation to a commercial restricted feeding programme. Animal Welfare. 2:231-152.

Savory, C. J., P. M. Hocking, J. S. Mann and M. H. Maxwell, 1996. Is broiler breeder welfare improved by using qualitative rather than quantitative food restriction to limit growth rate? Animal Welfare. 5:105-127.

Schoeni, J. L. and A. C. Wong, 1994. Inhibition of Campylobacter jejuni colonization in chicks by defined competitive exclusion bacteria. Applied Environmental Microbiology. 60(4):1191-1197.

Smiricky-Tjardes, M. R., E. A. Flickinger, C. M. Grieshop, L. L. Bauer, M. R. Murphy, and G. C. Fahey Jr., 2003. In vitro fermentation characteristics of selected oligosaccharides by swine fecal microflora. Journal of Animal Science. 81(10):2505-2514.

Tottori, J., R. Yamaguchi, Y. Murakawa, M. Sato, K. Uchida, and S. Tateyama, 1997. The use of feed restriction for mortality control of chickens in broiler farms. Avian Disease. 41(2):433-437.

Visek, W. J., 1978. The mode of growth promotion by antibiotics. Journal Animal Science. 46: 1447-1469.

Wrong, O. M., 1981. Nitrogen compounds. Pages 133-211 in: The Large Intestine: Its Role in Mammalian Nutrition and Homeostasis. O. M. Wrong, C. J. Edmonds, and V. S. Chadwick, ed. John Wiley and Sons, New York, NY.

Yeo, J. and K. Kim, 1997. Effect of feeding diets containing an antibiotic, a probiotic, or Yucca extration on growth and intestinal urease activity in broiler chicks. Poultry Science. 76: 381-385.

SUMMARY

Summary

Soybean meal is world wide used as an ingredient of poultry diet, due to both the high quality and quantity of its protein content. Meanwhile soybean meal contains relatively high levels of soluble carbohydrates, known as oligosaccharides (SMO) and polysaccharides (SMP). SMO and SMP can not be digested by digestive enzymes and tend to reduce the available energy in the diet, therefore behaving as an anti-nutritional factor. However, SMO and SMP can be utilised by the intestinal microflora. They may affect gut microbial community population consistent, and promote the proliferation of gut mucosal cells including natural antibodies, especially, when SMO and SMP are used in broilers' diet during the early stage of post-hatch. As a result, SMO and SMP may have an effect on the development of the gastrointestinal tract and furthermore on productivity; stimulate the growth of gut lactic acid-producing bacteria (LAB), and promote immune competence ability. To test the prebiotic and welfare role of SMO and SMP on broiler chickens, and to investigate the possibility of using SMO and SMP as an antibiotic substitution, one in vitro fermentation evaluation experiment (in vitro experiment) and three animal trials (Trials 1, 2 and 3) were described in this thesis.

SMO and SMP were isolated from soybean meal. SMO consisted of mainly stachyose and raffinose, and galactose and raffinose were the main ingredients of SMP in this study. These two components were added in small amounts to diets of young chickens. The levels of adding was approximately equated to the amount of soybean meal SMO and SMP, which usually used in diet of broiler chickens of the same age. We choose to add them to diet of birds from Days 1-14, because we hypothesised that these fermentable carbohydrates will affect intestinal development and gastrointestinal microbial community ecosystem establishment, as well as the immune responsibility. Further to affect the healthy condition and productivity of broiler chickens. The diet and treatments of the three animal trials were as follows:

Trial 1. Three hundred and 60 1-day-old male Arbor Acres broilers were used. The experimental design was as follows: 11 % soybean meal offered 0.96 % SMO and

0.42 % SMP in the basal diet of the first phase (CON). During Day 1-14, the CON diet was diluted by 1 % SMO and 0.5 % SMP respectively. This resulted in diets SMO-1 and SMP-1. The second phase diet started from Day 22 and lasted until Day 42. This was the same in all three groups, consisting of 26.95 % soybean meal which offers 2.34 % SMO and 0.78 % SMP. The results of this experiment are presented in **Chapter 8.**

Trial 2. Two hundred and 40 1-day-old male Arbor Acres broilers were divided into four groups. In phase I which lasted 30 days, one diet contained soybean meal (CON) and other three diets were free of soybean meal (NSM, SMO and SMP). SMO and SMP were used as a supplement in the diet in the same way as described in trial 1. The second phase diet started from 31 days of age and had the same design as in trial 1. The results are presented in **Chapter**s **3, 4, 5,** except production figures which are presented in **Chapter 8.** .

Trial 3. Two hundred and 40 1-day-old male Arbor Acres broilers were assigned into six groups as follows:

- **SMO** and **SMP** groups**:** soybean meal oligosaccharides and water soluble polysaccharides groups (SMO and SMP). Chickens receiving SMO and SMP diets were given the same basal diet NSM but diluted by a supplement of 1 % SMO and 0.5 % SMP during 1-14 day of age, respectively.
- **VAC** group: the birds were vaccinated with 100 oocytes of Eimeria tenella at three days of age.
- **ANT** group: The anti-coccidial medicine birds (ANT) received Maduramasin (5 ppm) in their diet from the 5th day before infection until Day 7 after infection.
- **COW** and **COR** groups: Uninfected (COW) and infected (COR) control groups were only given NSM.

Chickens, that in SMO, SMP, VAC, ANT and COR groups, were orally infected with 1000 sporulated oocysts of *Eimeria tenella* at Day 15 of age. All birds in trial 3 had a large part of a soybean meal-free basal diet (NSM) during 1-30d of age, and the second phase diet (as same in **Trial 1**) started from 31 d of age. The results of this

experiment were included in **Chapter**s **6 and 7**, except production figures which were included in **Chapter 8.**

Chapter 1. In this **Chapter**, a literature review is presented. It focused on the intestinal physical and microbial ecosystem development in broiler chickens, and aimed to elucidate the relationship between commensal microflora and digestive system development. It focused on competitive exclusion and the immune responses of chickens, and also evaluated the evidence to suggest that modulation of the gut microbial community is possible by addition of prebiotics to the diet.

Chapter 2. An *in vitro* experiment and its results is described in this Chapter. The experiment was conducted to investigate the fermentation kinetics of some non-digestible carbohydrates (NDC) by the caecal microbial community of broiler chickens by an assessment of the cumulative gas production during fermentation of each substrate. It also aimed to study changes in the microbial community, following fermentation of these non-digestible carbohydrates by use of polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Four water-soluble non-digestible carbohydrates (ENC): soybean meal oligosaccharides (SMO), soybean meal water-soluble polysaccharides (SMP), alfalfa meal oligosaccharides (AMO) and alfalfa meal water-soluble polysaccharides (AMP) were studied. Two pure sugars, raffinose (RAFF) and stachyose (STA) were also included.

To assess the fermentability of the substrates, cumulative gas pressure was monitored continuously, for 72 hours, and at the end of the fermentation, pH, and volatile fatty acid (VFA) and ammonia (NH_3) concentrations were measured in the fermentation solution. The inoculum for the *in vitro* gas production was obtained from a mixture of caecal contents of 40 81 day-old broiler birds. The PCR-DGGE technique was applied to compare microbial DNA fingerprints between substrates at the end of fermentation.

Results showed that SMO had the highest total sugar (720 g/kg in DM) content and SMP had the highest protein concentration (504.6 g/kg in DM) among the ENC.

Experimental results suggested that the amount of gas and VFA produced as well as the decrease in pH of the fermentation fluid were positively related to the sugar content in ENC. SMO led to significantly more butyric acid production ($P<0.05$) after fermentation compared with other ENC. The production of butyric acid was 155.2 mg/g DM, 100.3 mg/g DM, 84.5 mg/g DM and 71.8 mg/g DM for SMO, SMP, AMP and AMO, respectively. SMO had the lowest pH (5.5) and ammonia nitrogen concentration (199.3 mg/L) after fermentation. This was significantly different from the other ENCs and the pure sugars. DGGE analysis revealed that the fingerprint of caecal bacterial communities showed some variation (C, value >60%-80%) between some of the substrates.

It was concluded from this chapter, that mixed oligosaccharides and water-soluble polysaccharides extracted from soybean meal (SMO and SMP) and alfalfa meal (AMO and AMP), as well as the pure oligosaccharides raffinose (RAFF) and stachyose (STA) were significantly different both in terms of their fermentation kinetics and end-products using caecal contents from adult broilers. The extent to which these non-digestible, but fermentable, carbohydrates could change the microbial community of the broiler caecum, either in terms of the species detected, or its activity, needs to be investigated further, and then related to its effect on gut health in the animal itself.

Chapter 3. In this **Chapter**, the effect of dietary SMO and SMP on intestinal growth and mucosal morphology development in broiler chickens was studied in **Trial 1**. Intestinal weight and length were recorded on four segments (duodenum, jejunum, ileum and caecum) at 1, 3, 6, 9, 12, 15 and 28 days of age. The digestive tract-free body weight was recorded at 15 and 28 days of age. Intestinal morphology of duodenum, jejunum, ileum and caecum of broilers were investigated by scanning electron microscopy at 15 days of age.

The results on intestinal weight, expressed as a percentage of the digestive tract-free body weight (IW/DTFBW x 100) showed that the birds fed the soybean meal-free diet had a higher IW/DTFBW for duodenum, jejunum and ileum than that of the CON group at 15 and 28 days of age. Birds in the SMO and SMP groups had a higher

relative duodenum weight and a thinner caecal wall than the NSM group at day 28 d of age ($p<0.05$). Compensatory duodenal and ileal growth was noticed in the SMO and SMP birds after SMO and SMP were withdrawn from the diet. Birds in the SMO and SMP group had a significantly higher IW/DTFBW for duodenum than those in the CON and the NSM group ($P<0.05$) and a higher value for ileal relative weight than CON animals ($P<0.05$) at 28 days of age. Birds in the SMP and SMO groups had a smaller size of villi in duodenum, jejunum and ileum than in the NSM animals. A higher density and undeveloped micro-villi were observed in duodenum and ileum of the birds of the SMO and SMP groups compared with the birds of the NSM group. Attached microorganisms were observed on the caecal mucosal surface for birds receiving the SMO diet.

It was concluded that water-soluble soybean meal oligo- and polysaccharides administered in the early stage of post-hatch do not affect the development of the intestines relative weight and length when compared to the soybean meal-free fed animals at 15 days of age. However, SMO and SMP diets did significantly affect the morphological development of the gut mucosa. The SMO diet promoted the attached population of microorganisms on the caecal wall. Compensatory intestinal growth took place during 14 to 28 days of age after SMO and SMP were withdrawn from the diet.

Chapters 4 & 6. In these two Chapters, studies were performed to determine whether SMO and SMP can affect the growth of caecal lactic acid bacteria (LAB) in the early stage of post-hatch. This was done in chickens fed a soybean meal-free basal diet in **Trial 2(Chapter 4)**. Furthermore it was aimed to detect LAB population after the birds had been infected with E. tenella in **Trial 3 (Chapter 6)**. LAB of the genera Lactobacillus, Pediococcus, Weissella and Leuconostoc in cecal contents of broilers were detected with Light Cycler real-time PCR and fluorescence (SYBR Green I), followed by melting curve analysis of specific primer PCR products,

In **Chapter 4**, the caecal contents LAB population was detected at Day 15. The results of melting curve analysis showed that amplification melting peaks (Tm)

appeared at 91.1 °C for SMO, 90.85 °C for SMP, 91.05 °C for NSM, and 90.50 °C for CON respectively. A single predominant peak also appeared for each group. Real-time PCR result showed that SYBR Green I labeling stranded DNA was detected at the amplification cycles 13.36, 15.24, 16.11 and 18.00 for the four groups respectively. The cycle number is an indication of target DNA concentration in the PCR reaction solution, and the two are inversely proportional. These results suggested that SMO and SMP stimulated the LAB growth in the caecal contents during the early stage of post-hatch of broiler chickens.

In **Chapter 6**, fecal oocyst shedding was monitored per treatment group between the 5th and 13th day after infection. This was between 20 d and 28 d of age. Lactic acid bacteria (LAB) in caecal contents were evaluated on the 7th day after infection. The results showed that SMO and SMP groups had a lower number of oocysts per gram of faeces (OPG) during the monitoring period, compared with the COR group. SYBR Green I labeled stranded DNA was detected at the amplification cycles 12.97, 13.99, 14.85, 14.92, 22.21 and 27.68 for VAC, SMO, ANT, SMP, COW and COR groups respectively, and specific PCR products were confirmed by the result of melting curve analysis and agarose gel electrophoresis. The results suggest that these LAB communities were promoted by SMO and SMP and have a competitive exclusion function when the broiler chickens are infected by *E. tenella*. It was therefore concluded that SMO and SMP had prebiotic properties for these broilers.

The results of **Chapter**s **4 & 6** suggested that SMO can significantly increase the population of a group of lactic acid bacteria (genera *Lactobacillus, Pediococcus, Weissella* and *Leuconostoc*) both in healthy birds at day 15 and in infected birds at Day 7 pi. Dietary SMO and SMP lead to competitive exclusion in caecal contents of the *E. tenella* challenged broiler. The selective stimulation role of SMO on LAB could not be certified by current experimental results.

Chapters 5 & 7. In these two **Chapters**, the effects are reported of SMO and SMP on the immune responses of broilers during 42 days of life followed by vaccination in **Trial 2** (**Chapter 5**), and the cecal lamina propria immunoglobulin A, M and Y

positive plasma cells populations of broilers challenged with *E. tenella* in **Trial 3**. (**Chapter 7**).

In **Chapter 5**, animals were vaccinated against avian infectious bronchitis (ABV) on day 1, against infectious bursa (IBDV) on days 7 and 20, and against Newcastle disease virus (NDV) on days 14 and 28 respectively. Several aspects of humoral, cellular and mucosal immunity were measured on 7, 14, 28 and 42 days of age. The experimental results indicated that SMO and SMP led to an increase in immunoglobulin A, M and Y positive plasma cells (IgA^+, IgM^+ and IgY^+) within the caecal mucosa. There was also a tendency for increased T lymphocyte transformation rate and activate macrophages phagocytic function in relation to the presence of SMO and SMP. There were no significant differences in the Newcastle disease virus (NDV)-specific antibody titers among treatments at 7, 14, 28 and 42 days of age, between birds receiving different diets. The effect of enhanced immunity with SMO and SMP declined with age.

Chapter 7 the results of the study of the effect of SMO and SMP on the caecal lamina propria immunoglobulin A, M and Y positive plasma cells of broilers challenged with *E.tenella have been described.* This experiment hypothesised that SMO and SMP promote maturation of B lymphocytes in the caecal lamina propria of coccidia infected broilers. Caecal lamina propria IgA^+, IgM^+ and IgY^+ were monitored at 12, 24, 48, 72, 96, 120, 144 and 168 hours post infection applying immunohistochemistry with monoclonal antibody. The results showed that animals in both the SMO and the SMP treatment had a significant increase in the number of caecal mucosal IgA^+ and IgM^+ cells compared with the infection control group (COR). IgA^+ cells composed the majority of the B cells, and the number of IgM^+ cells was next to the IgA^+. Dietary SMO significantly increased the number of IgA^+ and IgM^+ at the 12^{th} and 168^{th} hour post-infection (pi) ($p<0.05$), and IgY^+ cells at the 24^{th} 48^{th} and 168^{th} hour pi. As for SMP, IgA^+, IgM^+ and IgY^+ numbers were increased dramatically from the 120^{th} hour pi. IgA^+ and IgY^+ were significant higher than COR at the 120^{th}, 144^{th} and 168^{th} hours pi ($p<0.05$). There was a non significant difference in IgM^+ between SMP and COR ($p>0.05$). IgY^+ predominantly appeared in the VAC group.

In conclusion, SMO and SMP can stimulate both the systemic cellular and caecal local humoral immune response, and promote the development of the lymphocyte germinal center in caecal lamina propria. These diets did not have an effect on the systemic humoral immune response in the first two weeks after hatching. Both SMO and SMP significantly increased in the number of B plasma cells (IgA + and IgM +) of *E. Tenella* infected broilers. Therefore, SMO and SMP, especially the former one, could be used as an antibiotic substitution in the diet of young broiler chickens.

Chapter 8. The effects of SMO and SMP on productivity of broiler chickens in three animal trials were reported in this Chapter. The results in **trial 1** showed an appetite suppressive effect of supplemental SMO or SMP and subsequent complete compensatory growth of broilers. The lower body weight was at Day 14 and 28 in the SMO used group, and Day 14 in the SMP used group compared with their antibiotic used counterpart group. SMO and SMP resulted in lower feed intake and lower feed utilization during 1-14 day. Birds of SMO-1 and SMP-1 groups had lower feed utilization efficiency during 1-14 day, but higher afterwards when compared with the CON group. The results in **trial 2** indicated that body weight of birds in the CON group, which used soybean meal and antibiotics throughout the experiment, was significantly higher than in the soybean meal-free control group and the SMO and SMP adding groups at Day 14, Day 28 and Day 42 ($p<0.05$). Birds that consumed SMO had a noticeable lower body weight compared to the birds receiving soybean meal-free control diet when measured at the 14th and 28th day of age ($p<0.05$). This difference in weight disappeared at the Day 42 of life. The use of SMP in the diet resulted in similar body weight throughout the experiment compared with the birds of the soybean meal-free diet control group. Results of **Trial 3** showed that the birds which consumed SMO had lower body weight at the Day 14 compared with others ($p<0.05$). Birds that consumed SMP had significantly higher body weight compared with those of the VAC group at Day 42 ($p<0.05$).

Based on the results of three animal trials it was concluded that soybean meal oligosaccharide (SMO) and water soluble polysaccharide (SMP) decreased feed intake and had a growth depressing effect when they were used in diet either containing

soybean meal or not during the early stage of post-hatch. Compensatory growth took place after SMO and SMP were withdrawn from the diet of broiler chickens after 14 days of age. There was no abnormal behaviour of birds throughout the trials. Therefore, SMO and SMP can be used as safe feed restriction additives in diet of broiler chickens.

CONCLUSION

In summary, experimental results of this thesis lead to the following conclusions:

Non-digestible carbohydrates can be fermented by caecal contents microbial community of broiler chickens *in vitro*:

- SMO, SMP, AMO, AMP, RAFF and STA can stimulate bacterial activity in caecal contents. SMO produced the lowest ammonia nitrogen and highest butyric acid in these fermentable carbohydrates after fermentation.
- SMO, SMP, AMO, AMP, RAFF and STA could lead to a shift of caecal contents microbial community to some extent.

When the diet of the first two weeks were diluted by 1% SMO and 0.5% SMP, respectively, this resulted in:

- Dietary SMO and SMP didn't affect the development of intestinal length and relative weight. However, dietary SMO and SMP decreased the villus size, and tended to increase microvilli density on villus surface.
- Dietary SMO significantly increased LAB population in caecal contents both in healthy birds and the birds infected with *E. tenella.*
- Dietary SMO and SMP enhanced cellular but did not affect humoral immune response followed by vaccination.
- SMO and SMP led to an increase in immunoglobulin A, M and Y positive plasma cells (IgA, IgM and IgY) within the caecal mucosa both in healthy birds and in birds infected by *E. tenella.*
- SMO and SMP led to feed restriction when it was used in the diet as an additive, during 1-14 days of age. Compensatory growth occurred after SMO and SMP were withdrawn from the diet.

SAMENVATTING

Samenvatting

Soyaschroot wordt wereldwijd intensief gebruikt als ingrediënt voor pluimveevoeders omdat het een hoog gehalte aan eiwit heeft. Dat eiwit heeft bovendien ook een hoge kwaliteit.

Soyaschroot bevat een relatief hoog niveau aan oplosbare koolhydraten. Dat zijn de oligosaccharides (SMO) en de oplosbare polysaccharides (SMP). Zowel SMP en SMO kunnen niet door enzymen van het dier zelf worden verteerd. Ze verlagen dan ook enigszins het gehalte aan omzetbare energie en gedragen zich als anti-nutritionele factor. SMO en SMP's kunnen echter wel worden gefermenteerd door microorganismen in het maagdarmkanaal. Deze koolhydraten hebben een duidelijke invloed op de microflora bij kuikens en ze kunnen ook de proliferatie van cellen met antilichamen in de darmwand stimuleren. Dit is speciaal zo bij kuikens die SMO en SMP in het voer ontvangen.

Dit betekent dat SMO en SMP de ontwikkeling van het maagdarmkanaal microflora en cellen met antilichamen beïnvloeden. Daarnaast kunnen ze de produktie van de dieren zelf beïnvloeden. Ze stimuleren de groei van melkzuur producerende bacteriën.

Om deze zogenaamde prebiotische rol van SMO en SMP bij broiler kuikens te bestuderen en ook de mogelijke rol als vervanger van groeibevorderende antibiotica werd een in vitro proef uitgevoerd. Daarnaast zijn drie in vivo experimenten beschreven in deze thesis.

SMO en SMP werden geïsoleerd uit soyaschroot. SMO bestaat vooral uit stachyose en raffinose, terwijl galactose en raffinose de hoofdbestanddelen van SMP waren. Beide componenten werden in kleine hoeveelheden aan het rantsoen van jonge kuikens toegevoegd.

Deze geïsoleerde hoeveelheden komen overeen met datgene wat aanwezig is in het sojaschroot aandeel van het rantsoen voor kuikens van 1 tot 14 dagen.

De algemene hypothese die in deze studie werd geformuleerd is dat de twee groepen fermenteerbare koolhydraten (SMO en SMP) invloed hebben op de ontwikkeling van het maagdarmkanaal en ook op het microbiële ecosysteem. Bovendien werd in de hypothese gesteld dat deze twee soorten koolhydraten de immuun responses beïnvloeden. Dit kan gevolgen hebben voor gezondheid en productiviteit van broiler kuikens.

De rantsoensamenstelling en behandelingen in de drie in vivo studies waren als volgt.

Proef 1: 360 ééndagskuikens (haantjes Arbor Acres broiler) werden gebruikt in 3 proefbehandelingen. De proefbehandelingen waren als volgt: 11% sojaschroot in het basis rantsoen levert ongeveerd 0.96% SMO en 0.42% SMP in het voer voor de eerste fase (dag 1-14). Dit rantsoen heette (CON) en bevatte dus een dubbele hoeveelheid SMO en SMP . Dus de SMO en SMP verdunde de diëten met ongeveer 10 en 5 g/kg respectievelijk (rantsoen SMO-1 en SMP-1). De rest van fase 1 (dag 15-21) werd geen extra SMO en SMP toegediend. De 2e fase startte op dag 22 en duurde tot de dieren 42 dagen oud waren. Fase 2 was voor alle dieren in deze proef hetzelfde. In deze fase kregen alle dieren 26.95% sojaschroot in het rantsoen, dit leverde 2.34% SMO en 0.78% SMP op. De resultaten zijn weergegeven in **hoofdstuk8.**

Proef 2 : hiervoor werden 240 ééndagskuikens (Arbor Acres haantjes) gebruikt. Zij werden in 4 groepen onderverdeeld en duurde van 1 – 30 dagen leeftijd. Het CON dieet bevatte sojaschroot en de andere 3 waren soyaschroot vrij (NSM, SMO en SMP). SMO en SMP werden als supplement in het rantsoen gebruikt op dezelfde manier als in **proef 1.**

De 2e fase in **proef 2** startte vanaf 31 dagen en was identiek aan die in **proef 1**. De resultaten over darmimmunologie en gezondheid zijn gepresenteerd in **hoofdstukken 3, 4 en 5** en de produktiecijfers in **hoofdstuk 8**.

Proef 3: 240 ééndagskuikens van dezelfde genetische origine als in **proef 1** en 2

werden als volgt aan de 6 behandelingen toegewezen:

- **SMO** en **SMP** groepen: deze kregen de wateroplosbare SMO en SMP. Deze werden toegevoegd aan het sojavrije basisdieet NSM met 1 en 0.5 % aan SMO en SMP respectievelijk. Zij kregen dit dieet van 1 – 14 dagen leeftijd.
- **VAC** groep: de dieren in deze groep werden op een leeftijd van 3 dagen gevaccineerd met 100 oocysten van *Eimeria Tenella.*
- **ANT** groep: deze dieren kregen Maduramasin (5 ppm) als coccidistaticum in het rantsoen vanaf 5 dagen voor de infectie met *E. Tenella* tot dag 7 na de infectie.
- Verder waren in deze **proef 2** controlegroepen die beide een NSM rantsoen ontvingen. Eén controlegroep **COR** werd geïnfecteerd en één controlegroep **COW** werd niet geïnfecteerd.

Kuikens die in de SMO, SMP, VAC, ANT en COR groep zaten werden alle oraal geïnfecteerd met 1000 gesporuleerde oocysten van *E. Tenella* op dag 15. Alle dieren in **proef 3** hadden dus een soyaschroot vrij dieet gedurende fase 1 (tot 30 dagen). In de 2e fase van deze proef die identiek was aan die van **proef 1** kregen deze dieren allemaal hetzelfde dieet, dus met sojaschroot. De resultaten van deze proef zijn in **hoofdstukken 6 en 7** weergegeven, behalve de produktiecijfers die in **hoofdstuk 8** staan vermeld.

Hoofdstuk 1 bevat het literatuuroverzicht. De focus in dit hoofdstuk is op de fysische ontwikkeling van het maagdarmkanaal en de ontwikkeling van het microbiële ecosysteem. In dit hoofdstuk wordt nagegaan hoe de relatie is tussen de commensale microflora en de immuun response van kuikens. De literatuur laat proeven zien die aangeven dat modulatie van de microflora in de darm mogelijk is door de toevoeging van prebiotica (SMO en SMP).

Hoofdstuk 2 beschrijft de in vitro proef van deze thesis. Het experiment was opgezet om de kinetiek van de fermentatie door het microbiële systeem van de ceca

te bestuderen. Met name ging het erom na te gaan hoe sommige niet door dierenzymen afbreekbare koolhydraten (NOC) door de cecale microflora worden

gefermenteerd. De fermentatie werd gemeten via de cumulatieve gasproductie techniek met elk substraat. In deze proef werden 6 substraten getest, vier daarvan bestonden uit met water geëxtraheerde niet verteerbare koolhydraten (ENC). Dat waren SMO, SMP, alfalfa oligoscacchariden (AMO) en alfalfa wateroplosbare polysacchariden AMP. Verder werden 2 zuivere suikers raffinose (RAFF) en stachyose (STA) meegenomen in de vergelijking van gasproduktie.

Om de fermenteerbaarheid van de substraten te meten werd de cumulatieve gasproduktie gemeten via de methode zoals die in het laboratorium van Diervoeding wordt toegepast. De cumulatieve gasproduktie werd continu over 72 uur gemonitored. Op het eind van deze 72 uur werden pH, VFA en NH_3 concentraties gemeten in de fermentatieoplossing. Het inoculum voor de in vitro gasproduktie werd verkregen via een mengsel van ceca inhoud van 40 broilers van 81 dagen leeftijd. De PCR-DGGE techniek werd toegepast om microbiële DNA fingerprinting na fermentatie met verschillende substraten te kunnen vergelijken op het eind van de fermentatie.

De resultaten toonden aan dat van de verschillende substraten de SMO het hoogste suikergehalte had (720 g/kg DM) had en SMP het hoogste eiwitgehalte (504.6 g/kg DM). Verder werd gevonden dat de hoeveelheid gas en VFA evenals pH daling in positieve zin gerelateerd zijn aan het suikergehalte in de ENC. SMO leverde na fermentatie meer boterzuur op dan andere ENC ($P < 0.05$). De produktie van boterzuur in mg/g DM in SMO, SMP, AMP en AMO was 155.2, 100.3, 84.5 en 71.8 respectievelijk. SMO liet de laagste pH zien (5.5) en ook het laagste ammonia N gehalte (199.3 mg/L) na fermentatie.

Dit was significant verschillend met alle andere ENC en met de ruwe suikers. DGGE analyse toonde aan dat de fingerprint van de cecal bacteriegemeenschap enige variatie tussen substraten te zien gaf (C, waarde > 60-80%). Uit de resultaten van de studie met in vitro fermentatie met ceca inoculum werd geconcludeerd dat gemengde oligosaccharides en wateroplosbare polysaccharides geëxtraheerd uit sojaschroot (SMO en SMP) en uit alfalfa meel (AMO en AMP) alsook de zuivere raffinose in stachyose (RAFF en STA) duidelijk verschillen in termen van kinetics. Ook

de eindprodukten waren verschillend na fermentatie van deze verschillende substraten.

Er dient evenwel verder te worden onderzocht in hoeverre deze niet door dier verteerbare koolhydraten de microbiële samenleving kunnen veranderen. Dit geldt zowel voor het aantal soorten die aanwezig zijn en gedetecteerd worden alsook voor de activiteit van deze soorten. Verder dient ook de relatie van deze verandering met darmgezondheid nader te worden vastgesteld.

In hoofdstuk 3 is de studie naar de invloed van SMO en SMP op de ontwikkeling van het darmkanaal weergegeven. De dieren ontvingen 4 rantsoenen: 1 met sojaschroot (CON) en 3 zonder schroot. Deze laatste drie kregen voer zonder sojaschroot, SMO, SMP of NSM van dag 1 – 14. Na dag 14 kregen ze allemaal een sojavrij dieet.

Het darmkanaal werd in vier segmenten verdeeld, nl.: duodenum, jejunum, ileum en caecum. Dit werd gedaan bij dieren op de leeftijd van 1, 3, 6, 9, 12, 15 en 28 dagen. Het lichaamsgewicht (zonder verteringskanaal) werd bepaald op 15 en 28 dagen leeftijd. De morfologie van duodenum, jejunum, ileum en caecum van broilers werd via scanning electron microscopy bepaald bij dieren op 15 dagen leeftijd.

De resultaten m.b.t. darmgewicht (IW), uitgedrukt als % van darmvrij lichaamsgewicht (IW/DTFBW x 100) toonden aan dat de dieren die geen soyaschroot in het rantsoen kregen een hogere waarde van IW/DTFBW hadden voor duodenum, jejunum en ileum dan de dieren van de controlegroep (CON) op 15 en 28 dagen leeftijd.

Vogels in de SMO en SMP groep hadden een relatief hoger duodenum gewicht en een dunnere caecum wand dan de NSM dieren op dag 28 ($P < 0.05$). Nadat SMO en SMP uit de rantsoenen verdwenen (na dag 15) trad compensatie voor duodenum enileum op want de dieren in de GMO en GMP groep hadden hogere IW/DTFBW voor het duodenum dan dieren in CON en NSM ($P < 0.05$). Ze hadden ook een hogere waarde voor het relatief ileumgewicht dan CON dieren op 28 dagen leeftijd. Vogels in

de SMP en SMO groep hadden kleinere villi in duodenum, jejunum en ileum dan dieren met een sojavrij dieet (NSM).

Een speciale waarneming was nog dat aangehechte microorganismen gevonden werden op mucosa oppervlak bij dieren op SMO rantsoen.

Er werd geconcludeerd dat wateroplosbaar SMP en SMO in rantsoenen van kuikens tot 15 dagen de ontwikkeling van maagdarmkanaal qua gewicht en lengte niet beïnvloeden in vergelijking met dieren zonder sojaprodukten. SMO en SMP beïnvloeden echter wel de morfologische ontwikkeling van de darm mucosa. SMO stimuleerde kennelijk wel de aanhechting van micro-organismen op de cecum wand. Als SMO en SMP dieren op dag 14 aan het rantsoen onttrokken werden trad bij deze groepen wel een speciale compensatoire groei op in de periode 15 – 28 dagen wanneer de dieren allemaal NSM kregen.

Hoofdstukken 4 en 6. In deze twee hoofdstukken werd nagegaan of SMO en SMP de ontwikkeling van melkzuur bacteriën (LAB) in de vroege fase van het leven kan beïnvloeden met het oog op infectie met *E. Tenella*. Dit werd gedaan bij kuikens die NSM kregen (zie voor specificatie **hoofdstuk 4**).

In dit onderzoek werden LAB van de genera Lactobacillus, Pediococcus, Weisella en Leuconostoc gevonden middels "Light Cycler real time PCR en fluorescentie (SYBR Green I)" en door melting curve analyse van primaire PCR produkten.

Hoofdstuk 4 laat zien dat in de ceaca inhoud op dag 15 een LAB populatie werd gevonden.

De melting curve analyse toonde aan dat deze amplification melting peaks (Tm) verschenen bij 91.1°C, 90.85 °C, 91.05 °C en 90.50 °C in SMO, SMP, NSM en CON dieren respectievelijk.

Bij elke groep dieren verscheen een enkele dominante peak. Resultaten van real

time PCR lieten zien dat SYBR Green I labeling stranded DNA gedetecteerd werd na amplificatie cyclus nr. 13.36, 15.24, 16.11 en 18.00 voor respectievelijk SMO, SMP, NSM en CON. Dit cyclus nummer is een indicatie voor de lage DNA concentratie in de PCR oplossing en beide zijn omgekeerd evenredig met elkaar. Deze resultaten suggereren dat SMO en SMP de groei van LAB in de eerste levensfase van broilers stimuleert.

In hoofdstuk 6 is onderzoek beschreven dat de oocysten uitscheiding van kuikens na *E. Tenella* infectie bestudeerde tussen dag 5 en 13 na infectie (dit was op leeftijd 20 – 28). De melkzuurbacterie (LAB) in de ceca inhoud werd op dag 7 na infectie bestudeerd. De resultaten lieten zien dat SMO en SMP groepen minder oocysten per gram faeces (OPG) opleverden dan bij de COR dieren. SYBR Green I labeled stranded DNA werd gevonden bij amplificatiecyclus no. 12.97, 13.99, 14.85, 14.92, 22.21 en 27.68 voor dieren van VAC, SMO, ANT, SMP, COW en COR behandeling respectievelijk. De specifieke PCR produkten werden beïnvloed door de resultaten van de smeltpunt analyse en agarose gel electrophorese. De resultaten laten zien dat microbiële LAB gemeenschappen gestimuleerd worden door SMP en SMO en ook dat ze een competitieve exclusie functie hebben wanneer de dieren met *E. Tenella* geïnfecteerd raken. Er werd daarna geconcludeerd dat de SMO en SMP prebiotische eigenschapen hebben voor broilers.

De resultaten in **hoofdstukken 4 en 6** suggereren duidelijk dat speciaal SMO de populatie van de groep van melkzuurbacteriën (genera Lactobacillus, Pediococcus, Weissella en Leuconostoc) stimuleren zowel in gezonde dieren op dag 15 alsook bij geïnfecteerde dieren 7 dagen na infectie. SMO en SMP in het rantsoen leidde tot competitieve exclusie van *E. Tenella* in dieren die daarna gechallenged zijn. De selectieve stimulerende rol van SMO en SMP op LAB kon niet worden bevestigd in deze proef.

In **hoofdstukken 5 en 7** wordt verslag gedaan van de proef met de invloed vanSMO en SMP op de immune response bij broilers na vaccinatie. Dit waren dieren van experiment 2 (zie ook **hoofdstuk 5**).

In **hoofdstuk 7** werd onderzocht hoe SMO en SMP de immunoglobuline A, M en Y bevattende plasmacellen populatie beïnvloedde nadat de dieren gechallenged waren met *E. Tenella*.

In **hoofdstuk 5** werden de dieren gevaccineerd tegen ABV op dag 1, tegen IBDV op dag 7 en 20 respectievelijk, en tegen NDV op een leeftijd van 14 en 28 dagen respectievelijk. Verschillende aspecten van de humorale, cellulaire en mucose immuniteit werden gemeten op een leeftijd van 7, 14, 28 en 42 dagen.

Proefresultaten lieten zien dat opname van SMO en SMP in het rantsoen leidde tot meer immunoglobuline A, M en Y positieve plasmacellen in de mucosa van de ceca. (IgA^+, IgM^+, IgY^+). Er was ook een tendens voor toename in T lymphocyten transformatie ratio en een toename in geactiveerde macrofage functie bij dieren die SMO en SMP in rantsoenen kregen. Er werden echter geen significante verschillen in NDV specifieke antilichamen gevonden tussen dieren op verschillende behandeling op een leeftijd van 7, 14, 28 en 42 dagen. Het effect van hogere immuniteit met SMO en SMP in het rantsoen verminderde met leeftijd.

In **hoofdstuk 7** is beschreven hoe deze twee componenten SMO en SMP in het dier de aantallen immunoglobuline A, M en Y positieve plasmacellen in de lamina propria beïnvloeden bij dieren die gechallenged waren met *E. Tenella*. De hypothese voor deze proef was dat SMO en SMP de maturatie van B lymphocyten in de cecale lamina propria van broilers die geïnfecteerd zijn met coccidia stimuleert.

IgA^+, IgM^+, IgY^+ in cecale lamina propria werd gemonitored op respectievelijk 12, 24, 48, 72, 96, 120, 144 en 168 uur na de infectie. Deze monitoring gebeurde met monoclonale antilichamen na immunohistochemistry.

Dieren die SMO en SMP in het rantsoen hadden beide duidelijk toename in IgA^+ en IgM^+ cellen in hun cecale mucosa vergeleken met de controle dieren die geïnfecteerd waren (zonder deze componenten in het voer, COR). IgA^+ cellen maakten verreweg het grootste deel van de B cellen met op de 2e plaats IgM^+.

SMO in het rantsoen van kuikens verhoogde duidelijk IgA^+ en IgM^+ cellen op 12 en 168 uur post infectie (pi) ($p < 0.05$) en IgY^+ op 24, 48 en 168 uur pi ($p < 0.05$). In SMP dieren werd de toename in aantal IgA^+, IgM^+ en IgY^+ cellen vooral gevonden na 120 uur pi.

IgA^+ en IgY^+ in de drie COR groepen was duidelijk hoger dan die in de COR dieren op 120, 144 en 168 uur na infectie ($P < 0.05$). Tussen SMP en COR bestond enige verschil in IgM^+ (niet significant) In de VAC groep werd in hoofdzaak IgY^+ gevonden.

Het onderzoek zoals in **hoofdstuk 7** beschreven leidde tot de conclusie dat zowel SMO en SMP rantsoenen beide de systemische cellulaire en de locale immuno response in de ceca stimuleren. Ze stimuleren de ontwikkeling van het "lymphocyte germinal center" in de lamina propria. Beide rantsoenen hebben geen invloed op de humoral immune response in de eerste 2 weken na uitkomen. Zowel SMO als SMP deden het aantal plasma B cellen toenemen (IgA^+ en IgM^+) bij dieren die geïnfecteerd waren met *E. Tenella*. Men kan op basis van deze effecten concluderen dat zowel SMO als SMP maar speciaal SMO gebruikt kan worden als alternatief voor antibiotica bij jonge kuikens.

In **hoofdstuk 8** zijn de resultaten van drie experimenten weergegeven met betrekking tot de studie over de invloed van SMP en SMP op de produktiviteit van broilers.

De resultaten van **proef 1** laten zien dat er een voeropname depressie is na opname van SMO en SMP. Na het weglaten van deze twee componenten uit het voer is er een volledig inhaal effect (binnen 14 dagen). Vergeleken met de dieren die antibiotica in het voer ontvingen, hadden SMO dieren op dag 14 en 28 een lager lichaamsgewicht en de SMP dieren op dag 14. Resultaten laten vooral in deperiode 1-14 een lagere voeropname zien. Vergeleken met de CON dieren was er een verhoogde voer/groei verhouding maar na 14 dagen was er een betere groei en lagere voederconversie dan bij CON dieren.

De resultaten van **proef 2** laten zien dat bij CON dieren (met sojaschroot en antibiotica) het lichaamsgewicht op dag 14, 28 en 42 hoger was dan bij dieren die SMO, SMP en sojaschroot in het rantsoen ontvingen ($P < 0.05$). Dieren met SMO in het rantsoen hadden op dag 14 en 28 een lager gewicht dan dieren zonder sojaschroot ($P < 0.05$). Op 42 dagen leeftijd was dit verschil echter niet meer aanwezig. Het gebruik van SMP in het rantsoen gaf een gelijk gewicht als dieren op sojavrij rantsoen. Ook in **hoofdstuk 3** hadden SMO dieren een lager gewicht op 14 dagen in vergelijking met andere behandelingen ($P < 0.05$).

Uit de resultaten van alle drie produktieproeven werd geconcludeerd dat tijdens opname van SMO en SMP in het voer de voeropname en groei verminderde.

Echter nadat deze twee produkten uit het voer zijn weggelaten treedt er compensatie op. Er werd geen abnormaal gedrag geconstateerd bij de 3 proeven. Daarom lijkt het erop dat SMO en SMP kan worden gebruikt als additief voor het bereiken van enige voerrestrictie bij broiler kuikens.

Conclusies

Niet verteerbare koolhydraten kunnen door de cecale microflora van kuikens worden gefermenteerd in vitro.

- SMO, SMP, AMO, AMP, RAFF en STA kan de microbiële activiteit in lumen van ceca stimuleren. SMO gaf het laagste ammoniak gehalte en hoogste boterzuurgehalte na fermentatie.
- SMO, SMP, AMO, AMP, RAFF en STA kan leiden tot verschuiving in microbiële flora.

Wanneer 1% SMO of 0.5% SMP wordt gebruikt in het rantsoen van de 1e 2 weken resulteert dit in:

- Geen effect op lengte en gewicht van de darmen. Wel verlaagde de SMO en SMP de villusgrootte en tendeerde naar een grotere microvilli dichtheid op villi.
- Toename in populatie aan LAB in ceca inhoud zowel bij gezonde dieren als bij

dieren geïnfecteerd met *E. Tenella*.

- Een verhoogde cellulaire immuniteit na vaccinatie maar geen verhoogde humorale immuun response.
- Een toename in IgA, IgM en IgY positieve plasma cellen in de cecale mucosa zowel in geïnfecteerde als in gezonde dieren.
- Restrictie in voeropname tijdens opname van SMO en SMP in het rantsoen maar daarna trad bij weglaten volledige compensatie op.

CURRICULUM VITAE

Yu Lan was born on 9th of August, 1962 in Inner Mongolia of China. She obtained her Bachelor degree in Animal Husbandry in July 1984 at Inner Mongolia Agricultural University. After graduation, she was employed by Animal Husbandry Research Institute, YikeZhao Meng of Inner Mongolia, China. She worked as a chemist in the field of feed chemical ingredients analysis. She obtained her Master's degree in Animal Nutrition in November 1988. After graduation, she was employed by Grassland Research Institute, Chinese Academy of Agricultural Sciences. She worked as project leader of "The prebiotic effect of soybean meal oligosaccharides on broiler chickens" (2001-2003); Project leader of "Investigation of active peptides from enzyme degradation products of alfalfa RUBISCO"(1999-2001); Co-project leader of "Alfalfa leaves protein and water-soluble polysaccharides isolation technique and their healthy promotion role on rat and broiler chickens "(1999-2001). She also participated several projects during 1988-2000. From July 2000, she started her Ph.D study in Animal Nutrition Group of Wageningen University, the Netherlands. During this period, she also was an employee of Grassland Research Institute, Chinese Academy of Agricultural Sciences.

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