EVALUATION OF PURIFIED LIGNIN AND MANNANOLIGOSACCHARIDES AS ALTERNATIVES TO ANTIBIOTIC GROWTH PROMOTERS IN POULTRY PRODUCTION

By

BUSHANSINGH BAURHOO

Department of Animal Science

McGill University, Montreal, Canada

January, 2007

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of

Master of Science

Your file Votre référence
ISBN: 978-0-494-32659-6
Our file Notre référence
ISBN: 978-0-494-32659-6

Suggested short title:

LIGNIN AND MANNANOLIGOSACCHARIDES IN POULTRY PRODUCTION

CONTRIBUTION OF AUTHORS

The original idea to initiate this study was provided by Dr. Ciro Ruiz-Feria and Dr. Leroy Phillip. The farm trial, experimental design, and statistical analysis were carried out by Bushansingh Baurhoo. The laboratory analyzes were performed in the Microbiology Laboratory of McGill University (Chapters 2 and 3) and Microbiology Laboratory of the Veterinary Department of the University of Montreal (Chapter 3) by Bushansingh Baurhoo. Dr. Ann Letellier from the Veterinary Department of the University of Montreal assisted and provided the pathogenic strains of *E. coli* used in the challenge study (Chapter 3). The manuscripts were written by Bushansingh Baurhoo and edited by Dr. Ciro Ruiz-Feria and Dr. Leroy Phillip.

ABSTRACT

Master of Science Animal Science

Bushansingh Baurhoo

EVALUATION OF PURIFIED LIGNIN AND MANNANOLIGOSACCHARIDES AS ALTERNATIVES TO ANTIBIOTIC GROWTH PROMOTERS IN POULTRY PRODUCTION

The potential of lignin and mannanoligosaccharides (MOS), as alternatives to antibiotic growth promoters was evaluated in broilers. Dietary treatments included: 1) negative control (CTL-, antibiotic free); 2) positive control (CTL+, 11 mg / kg virginiamycin); 3) MOS (diet 1 + Bio-Mos: 0.2 % to 21 d and 0.1 % thereafter); 4) LL (diet 1 + 1.25 % Alcell lignin); 5) HL (diet 1 + 2.5 % Alcell lignin). Bodyweight and feed conversion were not different when broilers were fed the CTL+, MOS, LL or HL diet. Birds fed MOS or LL had increased jejunum villi height ($P < 0.05$) and greater goblet cell number per villus ($P < 0.05$) when compared to those fed the CTL+ diet. MOS and LL increased ($P < 0.05$) the cecal populations of *Lactobacilli* and *Bifidobacteria* when compared to CTL+ fed birds. However, *Lactobacilli* and *Bifidobacteria* loads were lowest ($P < 0.05$) in birds fed the CTL+ or HL diet. Litter *E. coli* load was reduced ($P < 0.05$) when birds were fed MOS than when fed the CTL+ diet, but comparable to LL or HL fed birds. In birds challenged with pathogenic strains of *E. coli* (O2 and O88 serotypes) and fed the MOS or HL diet, the cecal population of total *E. coli* was lower ($P < 0.05$) than those fed the CTL+ diet; LL fed birds tended to have lower *E. coli* load than CTL+ fed birds. In summary, birds fed the MOS or LL diet had comparative advantage over CTL+ fed birds as evidenced by increased cecal populations of *Lactobacilli* and *Bifidobacteria*, increased villi height and greater goblet cell number in the jejunum, lower *E. coli* load in the litter, and lower cecal population of *E. coli* after an *in vivo* challenge with pathogenic strains of *E. coli*. Therefore, MOS and lignin could be regarded as natural alternatives to antibiotic growth promoters in poultry production.

(Key words: Antibiotics, mannanoligosaccharides, lignin, gut health, broilers)

RESUME

M.Sc. Zootechnie

Bushansingh Baurhoo

L'UTILISATION DE LA LIGNINE PURIFIEE ET D'OLIGOSACCHARIDES DE MANNANE COMME ALTERNATIVES À L'UTILISATION D'ANTIBIOTIQUES DE CROISSANCE DANS LA PRODUCTION AVICOLE

La possibilité d'utiliser de la lignine purifiée et des oligosaccharides de mannane (MOS) comme alternatives aux antibiotiques de croissance a été évalué sur des poulets à griller. Cinq différents traitements alimentaires ont été testés: 1) Contrôle-Négatif (CTL-, sans antibiotique); 2) Contrôle-Positif (CTL+, 11 mg / kg Virginiamycine); 3) MOS (Diète 1 + Bio-Mos: 0.2 % de 1 à 21 jours et 0.1 % par la suite); 4) LL (Diète 1 + 1.25 % Alcell lignine); 5) HL (Diète 1 + 2.5 % Alcell lignine). Les poulets à griller recevant les diètes CTL+, MOS, LL ou HL ont obtenu des résultats comparables en terme de gain de poids et de conversion alimentaire. La longueur des villosités du jéjunum et le nombre de cellules en gobelet par villosité des oiseaux consommant les diètes MOS ou LL étaient supérieurs ($P < 0.05$) lorsque comparés à ceux recevant la diète CTL+. Les concentrations au niveau du caecum en *Lactobacilli* et *Bifidobacteria* étaient plus élevées ($P < 0.05$) chez les poulets consommant les diètes MOS ou LL comparativement a ceux recevant la diète CTL+. Mais les oiseaux recevant les diètes CTL+ ou HL avaient les plus basses ($P < 0.05$) concentrations en *Lactobacilli* et *Bifidobacteria*. La population en *E. coli* dans la litière était réduite ($P < 0.05$) lorsque les oiseaux consommaient la diète MOS en comparaison avec ceux recevant la diète CTL+. Toutefois des niveaux comparables ont été observés lorsque les poulets recevaient les diètes MOS, LL ou HL. Les oiseaux ayant subit des tests de provocation avec les sérotypes pathogènes d' *E. coli* (O2 et O88), ceux recevant les diètes MOS ou HL avaient une concentration réduite ($P < 0.05$) en *E. coli* au niveau du caecum comparativement a ceux recevant la diète CTL+ ; les oiseaux recevant la diète LL avaient une tendance d'avoir une concentration inférieure en *E. coli* comparativement a ceux recevant la diète CTL+. En conclusion, des avantages tels: l'accroissement de la population en *Lactobacilli* et *Bifidobacteria* au niveau du caecum, l'allongement des villosités et l'augmentation du nombre de cellules en gobelet au niveau du jéjunum, une réduction dans la population en *E. coli* dans la litière et une réduction

dans la population d'*E. coli* chez les poulets ayant subit des tests de provocation avec les souches pathogènes d'*E. coli* ont été observés chez les poulets consommant les diètes MOS ou LL par rapport à ceux recevant la diète CTL+. Alors MOS et la lignine purifiée pourraient être utilisée comme des remplaçants des antibiotiques de croissance dans la production d'avicole.

(Mots clés : Antibiotiques, oligosaccharides de mannane, lignine purifiée, santé intestinale, poulets à griller)

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Ciro Ruiz-Feria and Dr. Leroy Phillip, my supervisors, for their invaluable guidance, stimulating suggestions, interest and enthusiasm in my work. I acknowledge to have benefited immensely in my professional development.

My deepest appreciation is also extended to Dr. Arif Mustafa, member of my graduate committee, for his useful advice and allowing me to use his laboratory facilities. A special thanks and gratitude to Dr. Ann Letellier for her scientific collaboration, guidance and assistance during my M.Sc. study.

I am thankful to Mr. David Meek for granting me permission to use the laboratory facilities of the microbiology department. My sincere thanks for any help and encouragement of fellow students and friends of the Animal Science department. I would also like to thank the poultry unit technicians B. Mitchell and R. McEwen for their help during the farm trials.

I am very grateful to my family for their loving support and care at all times. Last but not least, heart felt thanks to my fiancée for her moral support, encouragement and love.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure No. **Page**

LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Percentage
Cal	Calorie
AGP	Antibiotic Growth Promoter
CTL-	Negative control
CTL+	Positive control
MOS	Mannanoligosaccharides
LL	Low lignin
HL	High lignin
GCV	Goblet cell per villus
EU	European Union

CHAPTER 1: GENERAL INTRODUCTION

1.0 INTRODUCTION

For the past 40 years, poultry farmers have relied on the use of sub-therapeutic antibiotics in poultry feeds to improve growth performance, feed efficiency and to maintain flock health, and hence the term Antibiotic Growth Promoters (**AGP**). The addition of AGP in poultry diets is also a means of controlling post-slaughter shedding of pathogenic bacteria onto poultry products, thereby ensuring food safety. However, the use of sub-therapeutic levels of antibiotics in animal production is under severe scrutiny because livestock production practices have contributed to the development of antibiotic-resistant bacteria within the human population (Phillips, 1999; Smith et al., 2003). In 1986, with the objective of preventing transfer of antibiotic resistant genes between bacteria of animal and human interest, Sweden imposed a ban on all AGP in livestock production. The European Union initiated a ban on avoparcin in 1997 and all the other AGP were banned on the 1st of January, 2006 (Burch, 2006). Although a ban on AGP has not yet been initiated in other countries, such a scenario is most likely to occur in the near future due to international pressure and public health concerns. The poultry industry is hence compelled to develop natural alternatives to AGP to sustain or improve efficiency of production and safety of poultry meat and egg products.

Alternative approaches to improve poultry productivity and maintain flock health include the use of prebiotics, probiotics, enzymes, organic acids and plant secondary compounds (Ferket, 2004). However, studies conducted with broilers revealed some limitations in the use of probiotics, enzymes and acidifiers as alternatives to AGP. Limitations into the use of probiotics include the limited knowledge and difficulty in culturing the beneficial bacteria (Patterson and Burkholder, 2003; Rastall et al., 2005), inconsistency in the quantitative and qualitative microbial compositions of probiotic

products (Weese, 2002; Huyghebaert, 2003), and the highly variable results in animal species (Huyghebaert, 2003). The addition of exogenous enzymes in broilers diets improved performance (Odetallah et al., 2003), reduced the intestinal population of pathogenic bacteria (Hogberg et al., 2004), and increased the population of beneficial bacteria, such as *Lactobacilli* (Hogberg et al., 2004). However, the use of enzymes as alternatives to AGP in the European Union countries has necessitated major changes in feed formulation, management practices, disease control and significant modifications in the broiler housing facilities with air filtering systems for controlled ventilation and reduced stocking densities, as means to reduce the pathogen challenge conditions on the farm, and therefore represents a costly means of production (Page, 2003). The use of organic acids in poultry production is limited because these are easily neutralized in the duodenum (Ferket, 2004), performance benefits are highly variable (Langhout, 2000), and efficacy in the control of enteric diseases is low (Ferket, 2004).

There is increasing interest in the role of mannanoligosaccharides and lignin which seem to produce effects similar to those of prebiotics. But, very little research on the role of prebiotics has been carried out with poultry. Bio-Mos, a commercially available mannanoligosaccharide (**MOS**) is one of the few prebiotics that has been more commonly studied in chickens. In the absence of AGP, the addition of MOS to broiler diets showed health benefits by suppressing intestinal colonization of enteric pathogens (Spring et al., 2000), stimulating the immune response (Savage and Zakrzewska, 1996), and improving gut integrity (Iji et al., 2001; Ferket et al., 2002). However, studies conducted with MOS revealed inconsistent results on broiler performance and the intestinal population of the beneficial bacteria.

Other natural additives that have potential usage as alternatives to antibiotics include the phenolic substance, lignin. Lignin is a natural component of plant cell walls, and in its intact form lignin represents a barrier to digestion of feedstuffs. Interest in lignin derives from previous research at McGill University showing that a purified type of lignin, Alcell lignin, reduced the population of *E. coli in vitro* and increased growth rate and feed efficiency of veal calves (Phillip et al., 2000). Studies conducted with mice revealed that the addition of Alcell lignin to the diet significantly reduced the aerobic bacterial population in the cecum and their translocation to the mesenteric lymph nodes and the liver following burn injury, and lignin also reduced bacterial growth *in vitro* (Nelson et al., 1994). Research reports involving the use of lignin in poultry is limited. Ricke et al. (1982) reported that, when added to the diet, a purified lignin product (Indulin) improved weight gain and feed efficiency of broiler chickens and reduced the concentration of VFA in the ceca and large intestine.

The literature, therefore, suggests that prebiotics, such as MOS as well as lignin, could alter the microbial ecology of the gut in poultry; therefore, these natural feed additives should be viewed as alternatives to antibiotics in reducing the pathogen load in the gastrointestinal tract and reducing shedding of *E. coli* and *Salmonella* into the litter and poultry products.

1.1 Overall research objective

To determine the effects of MOS and a purified lignin on growth performance, gut integrity, gut microbiology and litter microbiology, and to determine whether these dietary additives could be regarded as alternatives to antibiotic growth promoters in broiler production.

1.2 Specific research objectives

To evaluate and compare the effects of an AGP-free diet and the AGP-free diet containing lignin, MOS, or the AGP, virginiamycin, on:

- growth performance, feed intake and feed efficiency
- gut integrity as measured by villi height, number of goblet cells within villus, crypt depth, muscularis thickness and thickness of muscularis mucosa of the jejunum
- microbial ecology of the ceca with special reference to *Lactobacilli* and *Bifidobacteria* as the beneficial bacteria, and *E. coli* and *Salmonella* as the pathogenic bacteria
- populations of *E. coli* and *Salmonella* in the broiler litter
- cecal populations of *E. coli* after *in vivo* challenge with known pathogenic strains of *E. coli*

CHAPTER 2: LITERATURE REVIEW

LITERATURE REVIEW

2.1 Introduction

The poultry industry has undergone remarkable improvements over the last 30 years, with marked scientific progress and modernization involving the use of genetically high yielding breeds, significant improvement in animal husbandry, disease control and nutrition. Poultry production is expected to expand in the coming years to meet higher demands for low-cost, healthy and convenient products. The global demand for poultry meat is projected to increase by more than 60 percent of the current consumption by the year 2020 (CAST, 1999). Technologies that could lower the quantity of feed consumed per unit of meat produced will benefit the producer and the consumer because feed is a major component, representing as much as 70 percent, of total production cost (Albright et al., 1994).

However, selection for improved growth has resulted in changes in the gastrointestinal development of chickens; the commercial poultry species are, therefore, more susceptible to enteric health problems (Tottori et al., 1997). Intestinal health and development of chickens has a major influence on performance as these affect feed digestion, nutrient absorption and utilization (Ferket, 2000). The gastrointestinal tract is the organ that supplies all the necessary nutrients to support growth and maintenance requirements of the body and is hence considered as a 'rate-limiting' step in the conversion of feedstuff nutrients into high-value food protein for humans (Ferket, 2000). Colonization and proliferation of one or more of the enteric pathogens, including parasites, bacteria and viruses, may result in enteric diseases leading to productivity losses and increased mortality, and increased contamination of poultry products by

pathogenic bacteria. Poor intestinal health, therefore, negatively impacts the poultry industry by reducing productivity, animal welfare and safety of poultry products is mostly likely to be compromised.

Since the discovery and development of the first antibiotics prior to the Second World War, these drugs had important applications in farm animals. Antibiotics are either therapeutically used to treat sick animals or added at sub-therapeutic levels to the feed to improve growth performance, feed efficiency and improve intestinal health, hence termed Antibiotic Growth Promoters (AGP). Therapeutic antibiotics, such as erythromycin, lincomycin, oxytetracycline, enrofloxacin and penicillin, are used for the treatment of enteric or systemic disease because these are readily absorbed in the gut and distributed throughout the body. These antibiotics are either added to the feed or drinking water as recommended by a veterinarian. The most common AGP in poultry production, such as virginiamycin, bacitracin and bambercycin, are not absorbed by the gut and these elicit their effects by altering the gut microflora to improve digestion, metabolism and nutrient absorption and the control of a number of enteric diseases for improved production of high quality, safe and efficient poultry products (Ferket, 2000; Page, 2003). However, the isolation of antibiotic-resistant bacteria in human patients has been associated with prolonged usage of AGP in livestock production (Phillips, 1999; Smith et al., 2003). For this reason, the use of AGP in poultry production for improved performance benefits is being severely scrutinized. Several natural alternatives are available for enhancing poultry performance in the absence of AGP. These are discussed in this section.

2.2 Antibiotic Growth Promoters (AGP) in poultry production

2.2.1 AGP usage in poultry production

Antibiotics are compounds that can either be naturally produced by specific micro-organisms or synthetic in nature, which when used at low concentrations, inhibit the growth of or destroy bacteria and other micro-organisms (NRC, 1980). The era of antibiotic growth promotion began when Moore et al. (1946) reported that sub-therapeutic usage of the antibiotic streptomycin in chickens resulted in substantial improvement in growth performance. At a time when livestock production management was rapidly changing from low-performance, high morbidity, free-range farming to a more controlled and intensive husbandry, and when post-war demands on increased global food supply were high, the discovery of an unexpected way to accelerate growth was received with enormous interest and enthusiasm by scientists and the public (Page, 2003). Since the 1950's, following several studies, the sub-therapeutic usage of antibiotics which had no application in human health, have been used in the poultry industry to promote growth and improve feed efficiency, thus improving economics of poultry production (Waldroup et al., 2003a).

Data on the use of AGP in livestock production are not publicly available. The Union of Concerned Scientists (2001) estimated that about 70 % of all the antibiotics used in the United States are meant for non-therapeutic purposes. The study revealed that the yearly usage of AGP in livestock production has increased from 16.1 million pounds in 1980 to reach about 24.6 million pounds in 2001. In 2001, about 10.5 million pounds were used in poultry production, 10.3 million pounds in swine production and 3.7 million pounds in cattle production. In Europe, in 1999, around 52 % of the total antibiotics were used for therapeutic purposes in humans whereas the remaining 48 % had important

applications in agriculture (Pfizer Animal health, 1999; Hughes and Heritage, 2004). From the part involved in agriculture, about 15 % were used as AGP and 33 % therapeutically to treat sick animals.

2.2.2 Benefits and constraints of AGP usage in poultry production

In studies conducted with broilers and turkeys, the addition of different types of AGP to the diets significantly improved growth performance and feed efficiency. Belay and Teeter (1996) observed that the addition of virginiamycin (15 g / ton and 20 g / ton) to broiler diets significantly improved growth performance, feed efficiency, carcass weight and breast meat yield. In a study conducted with broilers, an antibiotic shuttle program (55 mg / kg of bacitracin to 42 d followed by 16.5 mg / kg virginiamycin to 56 d), significantly improved growth performance and feed conversion at 21 days of age (Waldroup et al., 2003a). The addition of virginiamycin (15 g / ton in starter and 10 g / ton in grower) and bacitracin (50 g / ton in starter and 25 g / ton in grower) to broiler diets significantly improved body weight at all times over a 7 week experimental period (Miles et al., 2006). At 7 weeks of age, body weight averaged to 2.48 ± 0.024 kg for birds fed the control diet, 2.54 ± 0.022 kg for those fed virginiamycin and 2.53 ± 0.023 kg for those fed bacitracin. As compared to the control diet (1.99 ± 0.022), feed conversion was improved in birds fed virginiamycin (1.95 ± 0.015), but was not different in birds fed bacitracin (1.97 ± 0.020). Turkeys fed diets containing bacitracin (55 mg / kg to 6 weeks and 27.5 mg / kg thereafter) had improved body weight and feed conversion at 15 weeks of age (Sims et al., 2004). The addition of virginiamycin (22 mg / kg) to turkey diets, significantly improved body weight and feed conversion over a 14 week experimental period (Parks et al., 2005). Body weight of turkeys fed virginiamycin averaged to 7.54 ±

0.06 kg whereas those fed the control diet was 7.21 ± 0.06 kg on average. Feed conversion was 2.26 ± 0.05 and 2.37 ± 0.05 for turkeys fed the virginiamycin and control diets respectively.

The most important health benefit of AGP in poultry production is the control of necrotic enteritis, a major cause of economical losses. Necrotic enteritis is an intestinal infection caused by *Clostridium perfringens.* The disease severely affects feed digestibility, thus lowering feed efficiency and growth performance, and is characterized by sudden increases in mortality reaching up to 20 % (Page, 2003). Infection mostly occurs at the age of 2 to 6 weeks old. Engberg et al. (2000) reported that the addition of 20 mg / kg of zinc bacitacin to broiler diets significantly reduced the populations of *C. perfringens* in the ileum and ceca. Elwinger et al. (1998) reported that the cecal load of *C. perfringens* in broilers was significantly reduced when diets contained either avoparcin or avilamycin. In several experiments, Stutz and Lawton (1984) concluded that bacitracin, penicillin, chlortetracycline, oxytetracycline, erythromycin, tylosin, virginiamycin, lincomycin, bambermycins, and carbadox at 55 mg / kg of the diet significantly reduced the load of *C. perfringens* in the ileum of broilers. These studies demonstrated that a decrease in the intestinal population of *C. perfringens* was accompanied by an improvement in growth and feed efficiency in broilers. Lowering the intestinal concentration of *C. perfringens* in broilers improves animal welfare and improves food safety due to a decrease in carcass contamination at the slaughter house (Page, 2003).

The addition of AGP to broiler diets decreased flock variability (Miles and Harms, 1984), improved meat quality and pigmentation (Ferket, 2004), improved litter characteristics (Ferket, 2004) and increased environmental benefits due to lower excretion of nitrogen, phosphorus and other nutrients as a result of improved nutrient utilization

(Cromwell, 1999). However, the most important attribute of AGP in poultry production is the reduction of production costs associated with improved growth performance and feed efficiency (Page, 2003). Feed accounts to as much as 70 % of the total poultry production cost (Albright et al., 1994; Fernandez and Woodward, 1999). The magnitude of benefits depends on the type of animal management, sanitary conditions on the farm, age of the farm buildings, and feed quality (Ferket, 2004). It is reported that AGP are more effective under large scale commercial farms conditions, whereby the pathogenic bacterial challenge is considered to be high (SOU, 1997; Sims et al., 2004). Under stress conditions, the intestinal population of enteropathogen, such as *Staphylococcus*, *Streptococcus*, *Corynebacterium*, *Enterobacteriasease*, *Peptococcaceae*, *Bacteriodacae*, and *Clostridium*, increases whereas the beneficial bacterial population, such as *Lactobacilli*, decreases (Tazume et al., 1993).

The economic benefits associated with improved growth and feed efficiency, as a result of AGP usage, were substantial at the time of their introduction, 30 years ago, when livestock management was mostly based on low performance, high morbidity and free-range farming (JETACAR, 1999). From a review of published data in the literature, Page (2003) reported that in a study conducted with more than 250, 000 broilers in 1979, growth performance and feed efficiency were significantly improved by 5 % and 3.5 % respectively. Similarly, from a comprehensive review of published data from 1980 to 1989, CEAS (1991) reported that the addition of AGP to broiler diets resulted in an improvement of 4 % in growth performance and 5 % in feed efficiency. Rosen (1995) reported, from published data in the literature, that the addition of AGP to broiler diets significantly improved growth performance and feed efficiency by 2 % and 3 % respectively. In a comprehensive review of literature, growth performance and feed

efficiency were significantly improved by 3 to 10 % and 3 to 5 % respectively (SOU, 1997) and 3.9 % and 2.9 % respectively (Thomke and Elwinger, 1998), when AGP was added to broiler diets.

However, during recent years, with genetically superior animal breeds, significant advances in animal husbandry, disease control and nutrition, the magnitude of benefits of AGP usage has become marginal. To assess the effect of a ban on AGP on broiler productivity, Emborg et al. (2001) conducted a large scale study in Denmark with over 200 million broiler chickens from the year 1995 to 1999. Results indicate that growth performance and morality rates were not affected by the discontinued use of AGP. The only benefit of AGP usage was a marginal increase of 1.6 % in feed efficiency. In a study conducted with over 7 million broilers in the US, Engster et al. (2002) observed that feed efficiency was only improved by 0.9 % and that withdrawal of AGP from the diets resulted in only 0.5 % losses in body weight and 0.2 % increase in mortality rates. It can, therefore, be concluded, that under the modern system of production, the addition of AGP to broiler diets may not be needful to maximize production responses in broilers.

2.2.3 AGP ban in poultry production

The discovery of new types of antibiotics in the near future is not promising; there is, therefore, need to preserve the existing ones for human health benefits (Soil Association, 2001). Moreover, it is most unlikely that new types of antibiotics can be developed that would be effective against multi-resistant bacteria (Aarestrup et al., 2001). Hence, interventions that could reduce the reservoir of antibiotic-resistant genes among livestock animal, would prolong the lifetime of the antibiotics used in human medicine. The most attractive area to reduce the risk of selecting antibiotic-resistant bacteria is to

ban the use of antibiotics as growth promoters in food animals (Hughes and Heritage, 2004). AGP usage differs dramatically in different countries across the world. Sweden makes no use of AGP for growth promotion purposes whereas the US still uses a wide range of antibiotics, including some considered as medically important in human medicine (JETACAR, 1999). A ban on AGP has also not been initiated in Canada. However, due to international pressures and public scrutiny, a complete ban on AGP usage in poultry production is most likely to occur in the near future.

In 1986, Sweden imposed a complete ban on AGP usage in livestock production, not to compromise human health. Denmark initiated a ban on AGP usage in 1995, starting with the glycopeptide antibiotic, avoparcin. The European Union (EU) banned avoparcin in 1997 as a 'precautionary principle' because of avoparcin cross-resistance with the vancomycin antibiotic. The streptogamin class of antibiotic, virginiamycin, was banned in Denmark in 1998 because of the possibility of cross-resistance with the human streptogamins, such as quinupristin. In 1999, the EU banned virginiamycin, tylosin, spiramycin and zinc bacitracin as AGP usage in livestock production, and the remaining two AGP, namely bambermycin and avilamycin, were banned on the 1st of January, 2006 (Burch, 2006).

However, in 1986, the discontinued use of AGP in the Swedish broiler production system had led to serious necrotic enteritis problems (Ferket, 2004). In 1987, the antibiotic virginiamycin was used as prophylactic treatment to all chickens and a 2-day treatment with phenoxy methyl penicillin was administered in drinking water during outbreak of the disease. In 1988, following joint research carried out by the feed industry and broiler producers to find alternative ways to prevent incidence of necrotic enteritis, Sweden brought significant changes in broiler feed formulations, reducing the protein

contents, increasing contents of fibre and coarse grain particles and adding exogenous enzymes. Because high standard of hygienic conditions and managements are termed 'crucial' to the success of an AGP-free production system, significant changes were brought in the management practices, biosecurity measures, disease control and to the housing system to reduce stocking density and spread of diseases (JETACAR, 1999; Doyle, 2001; Wireup, 2001). Today, Sweden is a model for successful AGP-free poultry production. However, the Swedish poultry products are more expensive and less competitive on the market, and the costs of the venture into the AGP-free production system are high (Hughes and Heritage, 2004). Because most of the world's populations reside in developing countries and that global meat demand is projected to increase more than 60 % of current consumption by the year 2020 (CAST, 1999), the Swedish model might not be economically feasible as a worldwide poultry production system.

In order to protect their position on the European market, the Danish government instituted a voluntary ban on the use of AGP in poultry production along with a penalty tax for any usage in 1998. However, in 2000, a complete ban on AGP usage in Denmark resulted in increased enteric diseases and mortality rates in broilers. Therapeutic antibiotics, that have important implications in human medicine, such as ampicillin, erythromycin, streptomycin and tetracycline, were intensively used for treatment purposes. The use of tetracycline increased from 12,100 kg in 1998 to 27,000 kg in 2001. Casewell et al. (2003) reported that a simple ban on AGP usage may not solve the antibiotic resistance problem, but may lead to greater risks to human and animal health. There is, therefore, need to develop alternative feed additives that could mitigate productivity losses and improve safety of poultry products after a ban of AGP usage in poultry production.

2.2.4 Modes of action of AGP

Table 2.1 represents the commonly used AGP in poultry feed and their antibacterial mode of action (Gaskins et al., 2002). Most of the AGP function either at the cellular or the ribosomal level of the bacteria, thereby resulting in impaired growth or death. The AGP bacitracin interferes with the building and maintenance of bacterial cell wall, while streptogamins, such as virginiamycin, interrupt proper protein translation at the ribosomal level (Gaskins et al., 2002). By limiting the growth and proliferation of certain bacteria, AGP limit the production of bacterial metabolites and restrict the detrimental effects of these bacteria on the host. The host, therefore, grows and performs better than if grown under challenged conditions.

Although, the exact mechanism by which AGP promote growth of poultry is not clearly understood, several modes of action have been proposed. Most of the AGP used in poultry production have a broad spectrum of activity. However, gram-negative bacteria are resistant to most of the AGP because of their complex cell wall structure (Ferket, 2000; Page, 2003). It is reported that AGP act mainly on gram-positive bacteria, such as *Clostridium perfringens* which are pathogenic to poultry (Truscott and Al-Sheikhly, 1977; Engberg et al., 2000), and the beneficial bacteria, such as *Lactobacilli*, *Bifidobacteria* and *Streptococcus* (Page, 2003). By lowering the bacterial load in the gut, AGP reduce the competition for vital nutrients between the bird and the microbial flora and reduce stimulation of the immune system (Ferket, 2004). A stimulated immune system diverts nutritional resources away from growth and production, hence lowering animal performance (Klasing, 1988; Cook, 2000). Dietary inclusion of AGP reduced intestinal tract length (Stutz et al., 1983; Miles et al., 2006) and

TABLE 2.1: Major Antibiotic Growth Promoters used in poultry feed and their mode of action[1]

Class	Generic Name	Spectrum	Mechanism of action
Streptogamin	Virginiamycin	Gram +	Protein synthesis inhibition
Phosphoglycolipid	Bambermycin	Gram +	Cell wall synthesis inhibition
Peptides	Bacitracin	Gram +	Cell wall synthesis inhibition
Oligosaccahrides	Avilamycin	Gram +	Protein synthesis inhibition
Macrolide	Tylosin	Gram +	Protein synthesis inhibition
Glycopeptide	Avoparcin	Gram +	Cell wall synthesis inhibition
Lincosaminides	Lincomycin	Gram +	Protein synthesis inhibition

[1] Gaskins et al. (2002)

small intestinal weight (Stutz and Lawton, 1984; Dafwang et al., 1985; Henry et al., 1986), thus enhancing nutrient absorption and reducing the nutrient demand of the gut (Ferket, 2004). The reduction in gut mass due to dietary addition of AGP is similar to those observed in germ-free birds (Murakami et al., 1994).

2.3 Antibiotic resistance in poultry and human bacteria

2.3.1 Development of antibiotic resistance

Antibiotic resistance is defined as a microbiological phenomenon, which may or may not have clinical implications depending on pharmacokinetic and pharmacodynamic parameters as they apply to specific antibiotics (Phillips et al., 2004). Although resistance to antibiotics has existed since 1950 (Gustafson and Bowen, 1997), concerns were only

expressed in 1980, when antibiotic-resistant pathogenic bacteria were identified in hospital patients (Revington, 2002). The threat that these pathogenic bacteria may pose to human health has driven much scientific consideration and investigations. The more an antibiotic is used, the more likely are resistant populations to develop among pathogens and commensal bacteria in the livestock species (Phillips et al., 2004). However, there is great diversity in the development of antibiotic resistance as some bacteria very rapidly develop resistance in the individual treated, while others remain susceptible.

In response to selection pressure in the presence of antibiotics, the strong in-built survival mechanisms of bacteria enable single or multiple genetic mutations to develop resistance to the antibiotics (Revington, 2002). Studies indicate that *E. coli* developed resistance to different types of antibiotics as a result of mutation at the gene level (McDonald et al., 2001). In the presence of an antibiotic, most of the bacteria may be killed, but the resistant survivors may eventually re-establish themselves and transfer their antibiotic-resistant genes to future generations or to different bacterial species (Doyle, 2001). The transfer of antibiotic-resistant genes between gram-positive and gram-negative pathogenic bacteria enables bacteria to acquire multi-drug resistance (Witte, 1999). Resistance to antibiotics may be acquired or transferred through horizontal flow of genetic material via conjugative plasmids between different genera of bacteria (Van den Bogaard et al., 2001) or via integrons (distinct families of DNA elements) between completely different species of micro-organisms (Revington, 2002; Vo et al., 2006). Although resistance to antibiotics may be transmitted directly through antibiotic residues in meat, it is insignificant as compared to indirect transmission via different strains of bacteria (Hughes and Heritage, 2004). The rapid rate of bacterial cell division allows the

establishment of a predominant antibiotic-resistant bacterial population within host animal and animal-human populations.

The method of antibiotic usage has a major impact on the development of antibiotic resistance. In contrast to therapeutic dosage of penicillin over short periods of time, the administration of low doses of penicillin over a relatively long period of time significantly increased penicillin resistance in *Streptococcus pneumoniae* in human patients (Guillemot et al., 1998). These findings are, therefore, in agreement to reports of Phillips (1999) and Smith et al. (2003), that the prolonged usage of sub-therapeutic levels of antibiotics favoured bacterial resistance to the antibiotics. In addition to dosage of antibiotics used, the number of individuals receiving the antibiotics and the confinement of the area under treatment are important factors in the development and transfer of antibiotic resistance (Levy, 2001). These are characteristics of an intensive system of poultry production that favours the development of antibiotic-resistant bacteria.

2.3.2 Correlation between AGP usage in poultry production and antibiotic resistance in humans

The elucidation of these antibiotic resistance transfer mechanisms has important implications in agriculture. The transfer of antibiotic resistance is twofold (Lees and Aliabadi, 2002). The major concern is the transfer of antibiotic-resistant genes from antibiotic-resistant bacteria in livestock animals to the human microflora. Secondly, antibiotic-resistant bacteria in animal products cause serious human health problems, either when consumed or by direct contact mostly at the slaughter house. Antibiotic resistant strains of *Campylobacter*, *Salmonella* and *E. coli* have been isolated from poultry and retail meat products in many countries (Sackey et al., 2001; Zhao et al., 2001;

Mayrhofer et al., 2004). The livestock population is a potential reservoir of antibiotic-resistant bacteria or antibiotic-resistant genes which are transferred to the human population via the food chain causing infections (Phillips, 1999; Aarestrup et al., 2001; Revington, 2002). Human infections with antibiotic-resistant pathogenic bacteria are difficult to treat and therefore increase hospitalization costs (Lees and Aliabadi, 2002).

Page (2003) reported that, in the past, antibiotics that had no direct implications in human medicine were considered safe to be used as AGP in livestock production for improved performance benefits. However, recent studies indicate that antibiotics are chemically related in terms of their functional groups and modes of action, and that resistance to one class of antibiotics may facilitate the acquisition of resistance to other classes (Revington, 2002). The classes of antibiotics, namely β-lactams (penicillin and cephalosporins), sulphonamides, teraycyclines, macrolides, lincosamides, streptogamins and quinolones, that are used as antibiotic-growth promoters are closely related to antibiotics used in human medicine (Phillips et al., 2004).

The most glaring example of the relationship between agriculture and antibiotic-resistant bacteria in humans is the prevalence of vancomycin-resistant enterococci (VRE). Since the year 1986, human infections with VRE, commonly identified in hospital patients, were not treatable by the vancomycin antibiotic and necessitated the use of the streptogamin antibiotics, namely quinnupristin / dalfopristin (QD). However, the prolonged usage of sub-therapeutic levels of virginiamycin in poultry feeds for growth enhancement has established a reservoir of streptogamin-resistant genes in *E. faecium* in chickens (Smith et al., 2003). The cross-resistance between virginiamycin-resistant *E. faecium* in poultry and human *E. faecium* has led to the emergence of QD-resistant *E. faecium* in human health (Smith et al., 2003). In a study conducted with poultry, pigs and

humans, Van den Bogaard et al. (1997) concluded that the vanX gene that encodes for vancomycin resistance in *E. faecium*, was transferred from the animal sources to humans. In another study, Van den Bogaard et al. (2001) concluded that plasmids were responsible for the transfer of antibiotic-resistance in pathogenic bacteria from broilers and turkeys to the farmers and slaughters.

However, in a scientific evaluation of the threat that sub-therapeutic doses of virginiamycin for growth promotion in livestock production may pose to human health, the European Scientific Committee for Animal Nutrition (SCAN, 1998) concluded that there was no evidence of virginiamycin resistance transfer to humans and that the efficacy of the QD antibiotics was not compromised in the US and France. Phillips et al. (2004) reported that, in a critical review of published data, the hazard of antibiotic resistance to human health from AGP usage in livestock is extremely small, and concluded that a ban on AGP usage in livestock production is a non-scientific approach. The authors stressed that, other than farm animals, other potential sources may have important contributions in the development of antibiotic-resistance among bacteria in the food chain. The abuse of therapeutic antibiotics, such as erythromycin, clindamycin, tetracycline and aminoglycosides, in human medicine is a major cause of antibiotic-resistance in human bacteria (Hughes and Heritage, 2004). Antibiotic-resistant bacteria have also been isolated in wild rodents (Mallon et al., 2002) and crop plants (Zinniel et al., 2002). However, in a more recent study, Collingnon (2004) opposed the report of Phillips et al. (2004), and concluded that these findings were based on lack of scientific evidence and affirmed that the usage of AGP in livestock production represents a major threat in the development of antibiotic resistance in pathogenic bacteria of human concerns.

2.3.3 Effects of AGP withdrawal and antibiotic resistance in humans

The sub-therapeutic usage of antibiotics favours the development of antibiotic resistance in both the commensal and pathogenic intestinal microflora in poultry (Phillips et al., 2004). However, a reduction or discontinued use of these antibiotics generally reduces antibiotic resistance in intestinal bacteria because these resistant bacteria are replaced by susceptible strains (Phillips et al., 2004). Following a complete ban on AGP usage in the Danish broiler production system, Aarestrup et al. (2001) tested the susceptibility of 856 isolates of *E. faecium* to four of the most commonly used AGP. A ban on avoparcin in 1995 significantly decreased glycopeptide-resistant *E. faecium* from 72.7 % in 1995 to 5.8 % in 2000. Increasing the use of virginiamycin from 1995 to 1997 consequently increased virginiamycin-resistant *E. faecium* from 27.3 % in 1995 to 66.2 % in 1997. However, a ban on virginiamycin in 1998 significantly decreased virginiamycin resistance to 33.9 % in 2000. An increase in the use of avilamycin from 1995 to 1996, resulted in increased avilamycin resistance from 63.6 % in 1995 to 77.4 % in 1996. Following a decrease in the use of avilamycin in 1996, resistance to avilamycin decreased to 4.8 % in 2000. However, studies demonstrate that once resistance to antibiotics is acquired, bacteria do not become sensitive to the antibiotic over generations. Although the use of streptomycin was discontinued for more than 10 years in human medicine, strains of *E. coli* were still resistant to the antibiotic; the streptomycin-resistant gene, rpsL, undergoes mutations over generations to maintain resistance to the antibiotic (Morell, 1997).

2.4 Alternatives to AGP in poultry production

2.4.1 Prebiotics

Prebiotics are 'non-digestible feed ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of intestinal microflora, such as *Lactobacilli* and *Bifidobacteria*' (Gibson and Roberfroid, 1995). Prebiotics are non-digestible oligosaccharides, especially those that contain fructose, xylose, galactose, glucose and mannose (Gibson and Roberfroid, 1995; Gibson, 1998); the most common and successful ones are those based on fructose and mannose (Gibson, 1998; Ferket, 2004). Although prebiotics have a similar mode of action to probiotics, these do not contain live microbes, but stimulate the growth of commensal or beneficial bacteria in the gut of the host (Vijaya Kumar et al., 2005). The establishment of a population of commensal bacterial exerts gut health benefits by competitively excluding pathogenic bacteria for binding sites and competing for nutrients (Rolfe, 2000), modulating the immune functions (Rastall et al., 2005), and by producing toxic compounds that inhibit pathogens (*Lactobacilli* secrete bacteriocins and *Bifidobacteria* secrete organic acids and other bactericidal substances) (Gibson and Wang, 1994; Kawai et al., 1994; Jin et al., 1996a,b).

2.4.1.1 Fructo-oligosaccharides (FOS)

FOS, a polymer of fructose and glucose, exerts health benefits by 'feeding' the beneficial bacteria in the gut, which competitively excludes the colonization of pathogens (Ferket, 2004). Dietary FOS is not digested by the host digestive enzymes because of β-linkages between fructose monomers and is hence available for fermentation by the gastrointestinal microflora (Xu et al., 2003). In studies conducted with humans, FOS

selectively enhanced the growth of *Lactobacilli* (Mitsuoka et al., 1987) and *Bifidobacteria* in the gut (Hidaka and Hirayama, 1991). Broilers fed FOS enriched diets significantly increased the cecal populations of *Lactobacilli* and *Bifidobacteria* by 7-folds and 24-folds respectively (Patterson et al., 1997), thereby reducing enteric diseases (Patterson and Burkholder, 2003). FOS is well utilized by *Bifidobacteria* strains (*B. longum, brevis* and *infantis*) with the exception of *B. bifidum* whereas *E. coli* and *Clostridium perfringens*, pathogenic bacteria in poultry, failed to utilize FOS as a fermentative carbohydrate source (Hidaka and Hirayama, 1991). The addition of FOS to broiler diets reduced the cecal pH (Chambers et al., 1997) and the population of *Salmonella* in the ceca (Chambers et al., 1997; Fukata et al., 1999). At dietary inclusion levels of 0.25 %, 0.4 % or 0.5 %, FOS significantly improved growth performance and feed efficiency in broilers (Ammerman et al., 1988; Xu et al., 2003) and, at the 0.5 % inclusion level, significantly reduced mortality rates (Ammerman et al., 1988).

2.4.1.2 Mannanoligosaccharides (MOS)

MOS is a polymer of the hexose monosaccharides, glucose and mannose. Bio-Mos (Alltech Inc., Kentucky, US) is the most commonly available MOS type and consists of a complex mixture of mannoproteins, mannose and glucose (Dawson, 2006; pers. comm.). Bio-Mos, derived from cell wall fragments of the yeast *Saccharomyces cerevisiae*, is neither digested by endogenous digestive enzymes nor absorbed by the host (Zimmerman et al., 2001). Live yeast is used as a probiotic, but yeast cell-wall is a prebiotic. Although MOS is classified as a prebiotic, it is however not used as a substrate in microbial fermentation to selectively enrich the intestinal beneficial bacteria (Ferket, 2004). Instead, MOS functions to selectively adsorb and remove gram-negative

pathogenic bacteria from the intestine, stimulate the immune system and enhance gut integrity of chickens. These are described below.

Published reports show highly variable production performance responses in broilers fed MOS enriched diets. The addition of 5 g / kg MOS to broiler diets resulted in minor improvements in body weight, but no improvement in feed conversion (Iji et al., 2001). Body weight and feed conversion of broilers were significantly improved when 3 g / kg MOS were included in the finisher diets (Kumprecht and Zobac, 1997). The addition of 1 g / kg MOS to broiler diets significantly improved body weight gain and feed conversion (Eren et al., 1999). However, broiler diets containing 3 g / kg MOS did not significantly alter body weight gain, feed utilization and nutrient utilization (Shafey et al., 2001). Moreover, in a study conducted by Waldroup et al. (2003b), broilers fed diets containing 1 g / kg MOS from 0 to 42 days followed by 0.75 g / kg to 63 days, did no show any improvement in growth performance and feed efficiency as compared to broilers fed AGP-containing diets (55 mg / kg bacitracin methylene disalicyclate from 0 to 42 d followed by 16.5 mg / kg virginiamycin to 63 d). In another study conducted by Waldroup et al. (2003a), broilers fed diets containing AGP (55 mg / kg of bacitracin methylene disalicyclate to 42 d of age followed by 16.5 mg / kg virginiamycin to 56 d of age), MOS (1 g / kg from 0 to 42 d and 0.75 g / kg to 56 d) or a combination of AGP and MOS, showed comparable growth performance.

The addition of MOS to poultry diets inhibits the colonization of pathogenic bacteria in the gastrointestinal tract of the birds. In contrast to the mode of action of AGP, MOS acts as high affinity ligands, offering competitive binding sites for gram-negative bacteria (Ofek et al., 1977). Gram-negative bacteria, such as *E. coli* and *Salmonella*, with the mannose-specific Type-1 fimbriae adsorb to MOS instead of attaching to the intestinal

epithelium and are then excreted from the gut without colonization (Newman, 1994). Adherence of the pathogenic bacteria to the gut mucosa is a prerequisite for the onset of infection and subsequent enteric diseases (Spring et al., 2000). In an effort to confirm the inhibitory effects of MOS on pathogen colonization, Spring et al. (2000) screened different bacterial strains for their ability to agglutinate MOS in yeast cell preparations *in vitro*. Results indicated that 5 of 7 *E. coli* strains and 7 of 10 strains of *Salmonella typhimurium* and *S. enteritidis*, pathogenic bacteria in poultry, agglutinated MOS. In challenge studies conducted by Spring et al. (2000), when 3-d old broiler chicks fed a MOS enriched diet (0.4 % of DM), were orally challenged with 10^4 CFU of *S. typhimurium*, the cecal population of *S. typhimurium* was significantly reduced from 5.40 to 4.01 log CFU / g at d 10; the cecal population of *S. dublin* was significantly reduced from 90 % to 56 % when the chicks were challenged with *S. dublin*. Fernandez et al. (2002) reported that broilers fed MOS and challenged with *Salmonella enteritidis* (PT4) had reduced population of the specific strain of *Salmonella* in the ceca. Denev et al. (2005) observed that MOS fed broilers had significantly lower cecal coliform concentrations. However, in turkeys orally challenged with pathogenic strains of *E. coli* (O2, O19, O88 and O159 serotypes), the intestinal concentrations of coliforms did not differ whether the turkeys were fed AGP-free diets or diets containing MOS or AGP (Fairchild et al., 2001). The intestinal coliforms and *E. coli* concentrations of MOS fed turkeys did not differ to those fed AGP or AGP-free diets (Sims et al., 2004).

Moreover, MOS stimulates the immune functions of birds and has important positive effects on the humoral immunity and immunoglobulin status. Humphrey et al. (2002) reported that a good humoral immune response is nutritionally a more efficient mean to resist disease than an active inflammatory response. Savage and Zakrzewska

(1996) reported that turkeys fed diets containing 0.11 % MOS had increased plasma IgG and bile IgA and concluded that the increase in antibody response to MOS was due to the ability of the immune system to react to foreign antigenic material of microbial origin. Portions of the cell wall structure of the yeast *Saccharomyces* contained in MOS elicit powerful antigenic properties (Ballou, 1970). Because MOS enhances IgA secretion into the gut mucosal layer, pathogenic bacteria become more labile to the phagocytic action of gut-associated lymphocytes (Ferket, 2004).

In comparison to the well documented effects of AGP on gut integrity in poultry, little is known about the effects of MOS. However, in a study conducted with turkeys, diet containing MOS (0.1 % of DM) significantly increased the villi height:crypt ratio than those fed AGP containing diets (Ferket et al., 2002). An increased villi height had also been reported in birds fed a prebiotic-based diet (Solis de los Santos et al., 2005). It is well known that long villi are typically correlated with an improved gut health status. Ferket et al. (2002) also reported that turkeys fed MOS (0.1 % of DM) exhibited thinner muscularis layer and increased number of goblet cells per mm of villus height as compared to AGP-fed birds. The mucus gel layer coating the surface of the intestinal epithelium is the first major barrier to enteric infection and is a function of the number of goblet cells present in the villi (Ferket et al., 2002). The production of mucus is increased, as a consequence of increased number of goblet cells, due to stimulation of the immune system (Edens et al., 1997). The glycoproteins of gut mucins specifically bind pathogens and reduce their colonization by serving as alternative binding sites to receptors on host enterocytes (Blomberg et al., 1993).

2.4.2 Purified lignin

Lignin, a natural component of plant cell wall, is a useful pellet binder in feed manufacture, as lignosulphonate, but represents a barrier to nutrient digestibility. Fragmentation of the lignin macromolecule by alkalis or acids is the basic step of all chemical procedures of wood processing (Zemek et al., 1979). The Alcell process of wood for paper manufacturing involves aqueous ethanol as the cooking liquor at temperatures between 185 °C to 195 °C (Pye, 1996). During the pulping process the chemical integrity of native lignin is disrupted, yielding Alcell lignin (Alcell Technologies Inc., QC, Canada) having low molecular weight fragments, that possess biological effects not characteristics of the native lignin (Lora et al., 1983). Zemek et al. (1979) reported that lignin contains 11 different phenolic fragments, namely eugenol, isoeugenol, syringaldehyde, coniferylalcohol, ferulic acid, coumaryl, dehydrodiferulic acid, di-O-acetylpinoresinol, vanillyl, 4-hydroxy-3-methoxy-β-hydroxy-propiophenone and 2-(4-hydroxy-3-methoxy-β-hydroxy-propiophenone)-2-propanone.

Very little research has been conducted with lignin in livestock production. However, from the limited published data, lignin has been reported to possess antimicrobial properties and to improve growth performance and microbial ecology of the hindgut of several livestock species. Jung and Fahey (1983) provided several examples of the microbiological effects of lignin, and Zemek et al. (1979) reported that the phenolic components of lignin inhibited the growth of *E. coli*, *Saccharomyces cerevisiae*, *Bacillus licheniformis* and *Aspergillus niger* in studies conducted *in vitro*. Although the exact mechanism of lignin action remains unclear, there is evidence that the poly-phenolic compounds of lignin cause cell membrane damage and lysis of bacteria, with subsequent release of cell contents (Jung and Fahey, 1983). Alcell lignin when incorporated in the

diet altered the volatile fatty acid (VFA) production pattern in the cecum of rats, reduced the population of *E. coli* K 12 *in vitro*, and improved calf growth rate and feed efficiency at a dietary inclusion level of 1.25 % (Phillip et al., 2000). Using a mouse model, Nelson et al. (1994) observed that dietary addition of Alcell lignin (10 % of DM) significantly reduced the population of aerobic bacteria in the cecum and their translocation into the mesenteric lymph nodes and the liver following burn injury, and also reduced the *in vitro* growth of *E. coli*, *Staphylococcus aureus* and *Pseudomonas*. In studies with pigs, dietary addition of Alcell lignin (1.25 % of DM) had no effect on growth performance and feed efficiency (Valencia and Chavez, 1997). However, Ligmed-A, a lignin product derived from sugar cane, when orally administered at a dose of 2 g / kg for 4 days, was successful in treating diarrhoea in piglets (Mitjans et al., 2001). Research into the application of lignin in poultry production is limited. Ricke et al. (1982) reported that, when added to the diet (4 and 8 % of DM), a purified lignin product (Indulin) improved weight gain and feed efficiency of broiler chickens and reduced the VFA concentration in the ceca and large intestine.

2.4.3 Probiotics

Probiotics are defined as 'live microbial feed supplements that beneficially affect the host animal by improving its intestinal balance' (Fuller, 1989). *Lactobacilli* and *Bifidobacteria* are extensively used in humans, whereas species of *Enterococcus*, *Bacillus* and *Sacharomyces* are most frequently used in livestock production (Salminen et al., 1998). Probiotic-based diets when fed to broilers significantly improved growth performance and feed efficiency (Jin et al., 1996a; Safalaoh, 2006). However, the addition of FeedFree, a commercial probiotic consisting of 2 *Lactobacilli* strains, to

broiler diets did not significantly affect growth performance nor the cecal population of *E. coli* (Murry et al., 2004). Although diets containing the commercial probiotic, Protexin, had no effect on growth performance and feed efficiency in broilers, the cecal population of gram-negative bacteria was significantly reduced (Gunal et al., 2006). In a study conducted with broilers, Pelicano et al. (2005) reported that the effects of probiotics on intestinal integrity were inconsistent and concluded that the response was dependent on the type of micro-organisms contained in the products.

Probiotics have limited practical applications in poultry production, partly because results are highly variable and that the quantitative and qualitative microbial compositions of probiotic products are inconsistent (Weese, 2002; Huyghebaert, 2003). The limited knowledge and difficulty of culturing beneficial micro-organisms to achieve suitable high viable counts limit the success of developing successful probiotic products (Rastall et al., 2005). Furthermore, the culture of different species of important commensal bacteria under laboratory conditions is very limited. Recent molecular techniques indicate that only 20 to 50 % of the commensal bacterial have successfully been cultured under laboratory conditions (Patterson and Burkholder, 2003). The use of probiotics as alternatives to AGP in poultry production is, therefore, not promising.

2.4.4 Exogenous enzymes

The common exogenous enzymes used in poultry diets include xylanases, phytases, cellulose, arabixoxylanase and β-glucanase. The addition of a blend of enzymes to the diet yield greater beneficial effects than single-activity enzymes (Odetallah et al., 2002). When added to cereal-based diets of broilers, enzymes significantly improved growth performance and feed efficiency (Odetallah et al., 2003), significantly reduced the

intestinal population of pathogenic bacteria, such as coliforms and *Clostridium perfringens* (Engberg et al., 2004; Hogberg et al., 2004), decreased the susceptibility to enteric diseases, such as necrotic enteritis (Kaldhusdal and Skjerve, 1996), and increased the intestinal beneficial bacterial population, such as *Lactobacilli* (Hogberg et al., 2004).

In a comprehensive review of the literature, Rosen (2001) concluded that enzymes had near equivalent effects to AGP on growth performance and feed efficiency in broilers and can potentially limit performance losses following a ban on AGP. Following a complete ban on AGP, the Swedish broiler production system makes use of enzyme blends. However, this production system has necessitated major changes in feed formulation, management practices, disease control and significant modifications in the broiler housing facilities with air filtering systems for controlled ventilation and reduced stocking densities, as means to reduce the pathogen challenge conditions on the farm (Page, 2003). The Swedish broiler production system, therefore, represents a costly method of production which is economically not sustainable for the developing countries

2.4.5 Organic acids

Organic acids, such as fumaric acid, formic acid, butyric acid and malic acid, have not gained much interest in poultry production because these are easily neutralized in the duodenum of birds (Ferket, 2004), due to limited responses in weight gain and feed conversion (Langhout, 2000), and low effectiveness in the control of enteric diseases (Ferket, 2004). The effects of dietary organic acid mixtures on growth performance and feed efficiency in broilers are highly inconsistent. In studies conducted with broilers, organic acids did not affect growth performance and feed efficiency (Moharrery and Mahzonieh, 2005; Gunal et al., 2006). However, Skinner et al. (1991) observed

significant improvements in growth performance and feed efficiency when broiler diets contained mixtures of organic acids. The anti-bacterial properties of organic acids significantly lowered the intestinal load of *E. coli* (Moharrery and Mahzonieh, 2005) in broilers and significantly reduced *Salmonella* invasion in cecal epithelial cells *in vitro* (Van Immerseel et al., 2004). However, lactic acid bacteria, among other beneficial gram-positive bacteria, are able to survive at low intestinal pH because of their high intracellular potassium concentration which counteracts acid anions (Russell and Diez-Gonzalez, 1998). Recent technology has developed a protected form of organic acids that is coated with a fat layer to bypass the neutralizing effect into the duodenum of chickens (Gauthier, 2002). Organic acids are released in the lower part of the gut after action of digestive enzymes on the fat layer. Scientists have, therefore, recently renewed their interest in organic acids. If applied correctly, after the removal of AGP, organic acids may not only be effective as a growth promoter but also a meaningful dietary additive for the prevention of necrotic enteritis, caused by *Clostridium perfringens*, that cause serious economic losses in poultry production (Gauthier, 2002).

2.4.6 Herbs, spices and essential oils

Essential oils have long been recognized for their anti-microbial activity (Lee et al., 2004). Lee and Ahn (1998) reported that the essential oil cinnamaldehyde, derived from cinnamon, strongly inhibits *Clostridium perfringens* and *Bacteroides fragilis in vitro*, and moderately inhibits *Bifidobacterium longum* and *Lactobacillus acidophilus* isolated from humans. Oregano contains phenolic compounds, such as carvacrol, that possess antimicrobial properties against fungi and multi-resistant strains of *E. coli* and *Pseudomonas* (Bozin et al., 2006), whereas allicin, derived from garlic, inhibits the

growth of *E. coli*, *Salmonella*, *Campylobacter*, *Staphylococcus aureus* and *Clostridium perfringens* (Amagase et al., 2001). However, the exact anti-microbial mechanism of essential oils is poorly understood. Helander et al. (1998) reported that carvacrol and thymol cause membrane disintegration of the bacteria leading to the release of cell contents, whereas cinnamaldehyde penetrates the membrane, due to its lipophilicity, to reach the inner part of the bacterial cell and impair bacterial enzyme system. Oussalah et al. (2006) reported that oregano oils mostly induced depletion of the intracellular ATP concentration of the bacteria whereas cinnamaldehyde significantly reduced the intracellular pH of the bacteria. The addition of commercial types of essential oils, such as Liv-52 and Crina to broiler diets significantly improved growth performance and feed efficiency (Williams and Losa, 2001).

2.5 Summary and conclusion of literature review

A healthy gut is a key factor to achieve optimum performance in modern poultry production. Intestinal health is important to prevent or reduce pathogens of poultry flocks, a major source of contamination of poultry carcasses and products at the slaughter house that cause food-borne disease outbreaks in humans. Dietary addition of sub-therapeutic levels of antibiotics to poultry diets has been a common practice to improve growth performance, feed efficiency and maintain intestinal health of chickens. However, the use of AGP in poultry production has been associated with the development of antibiotic-resistance in human pathogenic bacteria, and therefore poses a threat to human health. A global ban on the use of AGP in poultry production, therefore, seems to be inevitable in the near future. The poultry industry is bound to develop 'biologically safer' alternatives to mitigate losses or improve productivity, food safety and economic benefits. Several new products have been shown to partially improve poultry productivity in the absence of AGP but so far, no product can be regarded as a total replacement. Therefore, this study provides an evaluation of the prebiotics, mannanoligosaccharides (Bio-Mos) and purified lignin (Alcell lignin), as natural alternatives to AGP in broiler production.

Preface to Chapter 3

Chapter 3 is comprised of a manuscript, co-authored by B. Baurhoo, L. Phillip, and C. A. Ruiz-Feria, which has been submitted for publication in the journal, *Poultry Science*. All literature cited in this chapter is listed in the "References" section at the end of the thesis. The tables and figures contained in the manuscript are presented at the end of this chapter, and tables not reported in the manuscript are presented in the Appendix section of the thesis.

Chapter 3 describes Experiment 1 carried out to evaluate the effects of the prebiotics, mannanoligosaccharides (Bio-Mos) and purified lignin (Alcell lignin), on broiler performance, gut integrity, gut microbiology and litter microbiology; the effects of these dietary additives were then compared with those of the antibiotic, virginiamycin.

CHAPTER 3

Effects of Purified Lignin and Mannanoligosaccharides on Intestinal Integrity and Microbial Populations in the Ceca and Litter of Broiler Chickens [1]

Baurhoo Bushansingh, Leroy Phillip, and Ciro A. Ruiz-Feria

McGill University

Department of Animal Science,

Ste. Anne de Bellevue, QC, H9X 3V9, Canada.

[1] Submitted for publication in *Poultry Science*: September, 2006

Effects of Purified Lignin and Mannanoligosaccharides on Intestinal Integrity and Microbial Populations in the Ceca and Litter of Broiler Chickens

3.1 ABSTRACT

A study was conducted to evaluate lignin and mannanoligosaccharides as potential alternatives to antibiotic growth promoters in broilers. Dietary treatments included an antibiotic free diet (CTL-), a positive control (CTL+, 11 mg/kg virginiamycin), and an antibiotic free diet containing Bio-Mos (MOS, 0.2 % to 21 d and 0.1 % thereafter), or Alcell lignin at 1.25 % (LL) or 2.5 % (HL) of the diet. Each treatment was randomly assigned to 4 floor pen replicates (40 birds each). Body weight and feed conversion were recorded weekly throughout 42 d. Jejunum histology was analyzed at d 14, 28 and 42. At d 28 and 42, cecal contents were assayed for *E. coli*, *Salmonella*, *Lactobacilli* and *Bifidobacteria*, and the litter was analyzed for *E. coli* and *Salmonella*. Birds fed the CTL- diet were heavier ($P < 0.05$) than those fed the other dietary treatments, but feed conversion was not affected by dietary treatments. Birds fed MOS and LL had increased jejunum villi height and a higher number of goblet cells per villus ($P < 0.05$) when compared to those fed the CTL+ diet. At d 42, birds fed MOS, LL or HL had greater *Lactobacilli* numbers than those fed the CTL+ diet. Compared to the CTL+ diet, the MOS diet increased the populations of *Bifidobacteria* ($P < 0.05$) in the ceca. Litter *E. coli* load was lower in birds fed MOS ($P < 0.05$) than in birds fed the CTL+ diet, but comparable to that of birds fed the LL or HL diet. Broiler performance was similar in birds fed antibiotics or antibiotic-free diets containing either MOS or lignin. However, birds fed MOS and LL had a comparative advantage over birds fed antibiotics as evidenced by an

increased population of beneficial bacteria in the ceca, increased villi height and number of goblet cells in the jejunum, and lower population of *E. coli* in the litter.

(**Key words**: Antibiotics, mannanoligosaccharide, lignin, gut health, broilers)

3.2 INTRODUCTION

Since the early 1950's, antibiotics have been widely used in poultry feeds; at first to control disease but subsequently, sub-therapeutic levels of antibiotics have been used to increase growth rate and improve feed efficiency. The introduction of antibiotic growth promoters (**AGP**) in livestock production did lead to substantial economic benefits (JETACAR, 1999) but with advances in animal genetics, nutrition and vaccination programs the magnitude of benefits from AGP has lessened. Based on a comprehensive review of studies published between 1980 and 1989, the Centre for European Agricultural Studies (CEAS, 1991) reported that the use of AGP in broiler diets improved growth performance by 4 % and feed efficiency by 5 %. Research in Denmark involving over 200 million broilers revealed that the use of AGP improved feed efficiency by only 1 %; no losses in body weight gain were recorded when AGP were withdrawn from broiler feeds (Emborg et al., 2001). In a more recent study with over 7 million broilers in the US, Engster et al. (2002) reported that feed efficiency was improved by only 0.9 %; the withdrawal of AGP resulted in only 0.5 % reduction in body weight gain.

Currently, the sub-therapeutic usage of antibiotics in livestock production is under severe scientific and public scrutiny because AGP have been linked to the development of antibiotic–resistant pathogenic bacteria, which pose a threat to human health (Smith et al.,

2003). As result of such concerns, the European Union initiated, in 1997, a ban on sub-therapeutic usage of the antibiotic avoparcin, in animal production; and all AGP were banned on January 1, 2006 (Burch, 2006). Although a complete ban on AGP has not been implemented in many countries, international pressure and public health concerns are likely to lead to such a scenario. Consequently, the poultry industry must develop alternatives to AGP to address public health concerns without compromising the efficiency of poultry production.

Prebiotics are non-digestible feed ingredients which beneficially affect the host by selectively stimulating the growth or metabolic activity of a limited number of intestinal micro organisms (Gibson and Roberfroid, 1995). Fructo-oligosaccharides and mannanoligosaccharides are among the classes of prebiotics that beneficially affect gut health, but they do so by different modes of action (Ferket, 2004). Research comparing Bio-Mos, a commercial mannanoligosaccharide, to AGP shows that it can effectively suppress enteric pathogens, enhance the immune response, and improve the integrity of the intestinal mucosa in broilers (Spring et al., 2000; Iji et al., 2001). However, the effects of mannanoligosaccharides on the beneficial micro-organisms in the chicken gut are not very consistent (Spring et al., 2000; Fairchild et al., 2001; Fernandez et al. 2002; Denev et al., 2005).

Lignin has been investigated for its effects on the hindgut microflora and animal performance, and its ability to inhibit the growth of pathogenic enteric bacteria. Lignin is a natural component of plant cell walls and in its intact form, it represents a barrier to digestion of feedstuffs. Alcell lignin is a co-product of paper manufacture, composed of low molecular weight polyphenolic fragments (Lora et al., 1993). Alcell lignin (1.25 % of DM) has been reported to improve growth performance of veal calves and to inhibit

the growth of *E. coli in vitro* (Phillip et al., 2000). In studies with chickens, the dietary inclusion of Indulin (4 and 8 %), a purified form of lignin, has been shown to improve weight gain and feed efficiency, and to reduce the concentrations of volatile fatty acids in the ceca and large intestine (Ricke et al., 1982). Nelson et al. (1994) reported that Alcell lignin reduced intestinal translocation of pathogenic bacteria following burn injury in rats, and inhibited *in vitro* growth of *E. coli, Staphylococcus aureus,* and *Pseudomonas*. It seems likely, therefore, that purified lignin has the potential to improve poultry performance by altering the microbial ecology of the hind gut.

The objectives of this study were to determine the effects of dietary addition of purified lignin (Alcell lignin) or a mannanoligosaccharide (Bio-Mos) to broiler diets free of antibiotics on growth performance, intestinal integrity, and microbial populations in the ceca and litter. The effects of the prebiotics were then compared to those of an antibiotic growth promoter supplemented diet.

3.3 MATERIALS AND METHODS

3.3.1 Bird management and experimental design

Eight hundred one-day-old male Cobb 500 broilers were obtained from a commercial local hatchery (Couvoir Simetin, QC, Canada) and grown over a 42 d experimental period. The chicks were raised in concrete-floor pens covered with 8 cm of clean pine wood shavings and each pen was equipped with one tube feeder and one automatic waterer. Throughout the study, the birds were brooded following standard temperature regimes, which gradually decreased from 32° to 24 °C, and under a 20:4 light:dark cycle. Procedures for bird management and care were approved by the Animal Care Committee of McGill University.

Birds were randomly assigned to 5 treatments (4 pen replicates; 40 birds per pen). The five experimental diets included: 1) negative control diet (**CTL-**), antibiotic free; 2) positive control diet (**CTL+**), containing 11mg / kg virginiamycin; 3) CTL- with the addition of Bio-Mos (Alltech Inc., Kentucky, US, **MOS**, 0.2 % of the starter diet and 0.1 % of the grower diet); 4) CTL- with the addtion of Alcell lignin (Alcell Technologies Inc., QC, Canada) at 1.25 % of the diet (**LL**); 5) CTL- with the addtion of Alcell lignin at 2.5 % of the diet (**HL**). The diets were formulated to be iso-nitrogenous, iso-energetic and to meet or exceed NRC (1994) requirements for macro and micronutrients. The ingredient composition and nutrient content of the diets are shown in Table 3.1. A two-phase feeding program was used with a starter diet from d 1 to 21 and a grower diet from d 22 to 42. Feed consumption and body weight (by pen) were recorded at weekly intervals.

3.3.2 Gut parameters and histology

At weekly intervals, one bird from each pen (4 birds / treatment) was euthanized by electrical stunning and bleeding of the carotid artery; the gizzard and pancreas were removed and the weight of the remainder of the gastrointestinal tract was recorded. The contents of the ceca and small intestine (duodenum, jejunum and ileum) were carefully hand-stripped and the weight of the empty gut segments was individually recorded. A 1-cm segment of the jejunum (2 cm from the end of duodenum) was excised, washed in physiological saline solution, and fixed in 10 % buffered formalin. The tissue samples were later embedded in paraffin, and a 2-μm section of each sample was placed on a glass slide and stained with haematoxylin and eosin. Histological sections were examined with a Nikon phase contrast microscope coupled with a MicroComp integrated digital imaging

analysis system (Nikon Eclipse 80i, Nikon Corporation, Japan). The variables measured were villus height, crypt depth, goblet cell number into villi membrane, thickness of the muscularis mucosae and muscularis layer. Villus height was measured from the top of the villus to the top of the lamina propria and the crypt depth was measured from the base upwards to the region of transition between the crypt and villus (Aptekmann et al., 2001). Ten measurements were taken per bird for each variable; for purposes of statistical analysis the average of these values was used. Digesta from the duodenum, jejunum, ileum and ceca was diluted with distilled water (1:10) and the pH was measured using a glass electrode pH meter (Denver Instrument, Mansfield, TX).

3.3.3 Microbial populations of cecal digesta and litter

At 28 and 42 d of age, the cecal contents from each bird were aseptically emptied into sterile plastic bags and stored at -20 °C for later microbiological analysis. Samples of the cecal contents were serially diluted in 0.85 % sterile saline solution and used to assay *Lactobacilli*, *Bifidobacteria*, *E. coli* and *Salmonella*. All microbiological analyzes were performed in duplicates and the average value of these were used for statistical analyses. *Lactobacilli* was anaerobically assayed using Lactobacilli MRS Agar (Fischer Scientific, ON, Canada) and incubated at 37 °C for 48 h. Enumeration of *Bifidobacteria* was performed using Wilkins-Chalgren agar (Oxoid, ON, Canada) supplemented with glacial acetic acid (1 ml/L) and mupirocin (100 mg/L) extracted from antimicrobial discs (Oxoid). Seventy-five discs were placed into 15 ml of Wilkins-Chalgren broth (Oxoid) and shaken for 30 minutes. Thereafter, 10 ml of this broth was added to 90 ml of agar medium (Rada et al., 1999). The petri dishes were placed in anaerobic jars, using Anaeropacks (Oxoid), and incubated at 37 °C for 5 days. *E. coli* was assayed using Rapid

E. coli 2 agar (Bio-Rad laboratories, ON, Canada) modified using E. coli supplement (Bio-Rad) to be selective for *E. coli*. Populations of *Salmonella* were assayed using Salmonella Shigella Agar (Fischer Scientific).

Litter sampling was performed using a modification of the method described by (Rybolt et al., 2005). Litter samples, from each pen, were taken at 5 different locations per sample, in the middle and equidistant from each other at each end both longitudinally and vertically using examination gloves. The 5 sub-samples were thoroughly mixed by hand and sealed into sterile Whirlpak microbiological bags and sealed. All samples were then immediately kept at -20 °C for later microbiological analysis. A 10 g sample was serially diluted in sterile saline solution and *E. coli* and *Salmonella* were enumerated as previously described. The 100 mm petri dishes were then incubated at 37 °C overnight and colonies counted.

3.3.4 Statistical analysis

Data were analyzed as a one-way ANOVA, using the General Linear Models (GLM) procedure of SAS (SAS Institute, 2003) with pen serving as the experimental unit for performance parameters and bird as the experimental unit for histology and microbiology parameters. Treatment means were separated using the Bonferroni's Multiple Comparison test. Statistical significance was declared at a probability of $P < 0.05$. All microbiological concentrations were subject to $\log_{10}$ transformation prior to analysis.

Statistical model used:

$$Y_{ij} = \mu + TRT_i + e_{ij}$$

where: TRT_i : i = 1, 2, 3, 4, or 5 (i.e 5 dietary treatments).

e_{ij} : j = 1, 2, 3, or 4 (i.e 4 pens or bird / treatment); $e_{ij} \sim N(0, \sigma^2_e)$.

Fixed effect parameters of the model:

(a) μ is the overall mean of microbial populations (log CFU / g)

(b) TRT_i is the fixed effect of i^{th} treatment (diet) on microbial populations

Random effect parameters of the model:

σ^2_e is the random residual variation of the model

3.4 RESULTS

3.4.1 Growth performance

The effects of dietary addition of virginiamycin, MOS and lignin on broiler performance are shown in Table 3.2. Beyond the first week of the experiment, birds fed the CTL- diet were consistently heavier than those fed the diet containing AGP. At 28, 35 and 42 d of age, birds fed the CTL- diet were also heavier than those fed diets containing lignin (LL and HL); only at d 42 birds fed the CTL- diet were heavier than those fed the MOS diet. Throughout the entire 42 d, birds fed the diets containing virginiamycin, MOS, or lignin (LL or HL) exhibited similar growth performance. Prior to d 28 of the experiment, feed intake was not different among treatment groups (Table 3.2). However, during the last two weeks of the study, birds fed the CTL- diet consumed more feed than did those in the other treatment groups. Feed conversion did not differ among dietary treatments at any point during the 6 wk experimental period.

3.4.2 Gut parameters

The villi height in the jejunum was similar for all treatments at d 14 of the study (Table 3.3). However, at d 28, MOS-fed birds had longer villi than birds in any treatment group, except the LL group. At d 42, birds fed the CTL+ or HL diet exhibited the smallest villi height. At d 42, there were no differences in villi height among birds fed the CTL- diet or diets containing MOS or LL. The number of goblet cells per villus (**GCV**) was not different among treatment groups at d 14 (Table 3.3). At d 28, however, MOS fed birds had significantly greater number of GCV than birds in any other treatment group, except the LL diet. At this age, birds fed the LL diet had greater number of GCV than those fed virginiamycin; the number of GCV obtained with the LL diet was not different from those obtained with the HL diet or the CTL- diet. By d 42, birds fed MOS had a greater number of GCV than birds in the other treatment groups. Estimates of crypt depth, muscularis layer thickness, and thickness of the muscularis mucosae were not affected by dietary treatment (Table 3.3). The whole gut weight, and weights of empty duodenum, jejunum, ileum and ceca, and the pH of the intestinal and cecal digesta were not affected by dietary treatments at any time (Appendix 1 and 2 respectively).

3.4.3 Microbial populations of cecal digesta and litter

The populations of *Lactobacilli* in the cecal digesta are shown in Figure 3.1. At both d 28 and 42, birds fed the CTL+ diet had the lowest population of *Lactobacilli*. At d 28, the population of *Lactobacilli* in MOS-fed birds exceeded only that of birds fed the CTL+ diet; however, at d 42, birds fed the MOS diet had the largest population of *Lactobacilli* among all dietary treatments. At d 42, birds fed both levels of lignin also had greater populations of *Lactobacilli* in the ceca than those fed the CTL+ diet. However,

there were no differences in the cecal population of *Lactobacilli* between the two levels of lignin, or between lignin and the CTL- diet, whether the measurements were made at d 28 or d 42.

At d 28, *Bifidobacteria* was detected only in birds fed the CTL- and the MOS diets; at d 42, *Bifidobacteria* was detected in birds from all treatment groups, except when birds were fed the HL diet (Figure 3.2). At d 28, the population of *Bifidobacteria* was not different in birds fed MOS or CTL-. At d 42, birds fed the MOS diet had a higher population of *Bifidobacteria* than those fed the CTL+ or the HL diet, but not different from birds fed the CTL- diet nor the LL diet. Populations of *E. coli* and *Salmonella* could not be enumerated because it appears that the concentrations in the cecal digesta were too low to be enumerated.

The litter was found to be free of *Salmonella* but, at both d 28 and 42, birds fed the MOS diet had lower populations of *E. coli* in the litter than those fed either the CTL- or CTL+ diet (Figure 3.3). At d 28, birds fed LL or HL had lower populations of *E. coli* loads in the litter than birds fed the CTL- or CTL+ diet but the effects were not significant. Birds fed the HL diet had a lower population of *E. coli* in the litter than those fed the CTL- but only at d 42. At both d 28 and d 42, the effects of MOS and lignin (LL and HL) were similar.

3.5 DISCUSSION

We found that growth performance was better with birds fed the AGP-free diet compared with a diet containing an AGP (virginiamycin); moreover, growth performance with the AGP-free diet exceeded that of the diets containing MOS or lignin. This was an unexpected finding but can be partially attributed to the effects of feed intake because during the last two weeks of the study, the presence of additives decreased feed consumption compared to the AGP-free diet. There are no reports of a suppression in feed consumption when either antibiotics or MOS has been added to broiler feeds (Waldroup et al., 2003; Hooge, 2004). In studies with lignin fed to pigs (Valencia and Chavez, 1997) and to calves (Phillip et al., 2000) there were no effects on feed consumption.

Feed efficiency was not affected by any of the additives. In studies with broilers fed MOS, AGP, a combination of MOS and AGP or an AGP free diet, Waldroup et al. (2003) reported no improvement in growth performance and feed efficiency. However, based on a meta-analysis of 44 research trials with broilers, Hooge (2004) concluded that birds fed MOS showed improved growth performance and feed efficiency compared with those fed AGP-free diets; performance was similar between MOS and AGP. In a study with broiler chickens, Ricke et al. (1982) reported that dietary addition of indulin improved weight gain and feed efficiency. However, indulin is a different source of lignin than the one used in this study, which may explain the different results.

It is reported that AGP (Sims et al., 2004) and most beneficial additives (Hooge, 2004) are most effective under disease and stress conditions, such as extremes of ambient temperature, crowding and poor management, which are invariably present in commercial broiler production. The present study was conducted under good hygienic conditions (new experimental facility, strict biosecurity measures, clean litter, good ventilation and

low stocking density), thus implying minimum bacterial challenge. Under such conditions, the birds may not have required any feed additive for maximum productive response.

Dietary addition of MOS caused a major increase in the height of the villi in the jejunum when compared to AGP and AGP-free diets. The effect of feeding the low level of lignin was similar to that of MOS. An increase in villi height in the duodenum has been previously reported in broilers fed a prebiotic-based diet compared to an antibiotic free diet (Solis de los Santos et al., 2005), and has been explained by indigenous microbes that stimulate vascularization and development of the intestinal villi, thus enhancing the efficiency of digestion and absorption (Stappenbeck et al., 2002). Thus our findings suggest that *Lactobacilli and Bifidobacteria*, among other types of beneficial bacteria favoured by the dietary addition of MOS or LL, have important contributions to villi height. Since long villi are correlated with improved gut health, MOS and LL diets offer a comparative advantage over the CTL+ diet in improving the gut health status of the birds. However, at d 42, birds fed the CTL+ or HL diet had shorter villi than those fed the CTL- diet. Miles et al. (2006) also reported that virginiamycin fed broilers had shorter villi in the ileum and duodenum than when fed an AGP-free diet. Both diets (CTL+ and HL) had lower cecal populations of beneficial bacteria and this could explain the shorter villi.

The results revealed that the addition of MOS also increased the number of GCV when compared to all other dietary treatments. Compared to the CTL+ diet, the LL diet also had a positive effect on the number of GCV but only at d 28. In studies with turkeys, Ferket et al. (2002) reported that, when compared to an AGP-free diet, MOS significantly increased the goblet cell numbers. Goblet cells are responsible for the production of mucins which bind pathogenic microorganisms and reduce their colonization of the gut

mucosa (Blomberg et al., 1993). The mechanism by which MOS increases mucin production is through stimulation of the immune system (Janeway, 1993). Since LL also increased goblet cell numbers, it may be possible that low levels of lignin act via a mechanism similar to MOS.

Gut weight and other measures of gut integrity (crypt depth, muscularis thickness and muscularis mucosae thickness) were not influenced by either lignin, MOS or AGP at any stage of the experiment. Ferket et al. (2002) reported that intestinal weight and crypt depth were similar when turkeys were fed MOS or an AGP-free diet; however, muscularis thickness was significantly reduced. Broilers fed diets containing the AGP virginiamycin or bacitracin had reduced length and weight of the intestinal tract (Stutz et al., 1983; Dafwang et al., 1985; Miles et al., 2006). Increases in gut mass are associated with inflammation following bacterial infection (Walton, 1988) and this notion is supported by the observation that germ-free birds have thinner muscularis mucosae than conventional birds (Gordon and Bruckber-Kardoss, 1961). The reason for the lack of an effect of the feed additives on gut parameters may be that under the conditions of this experiment the pathogen load in the gut was low.

The cecal population of *Lactobacilli,* at d 42 of the study, was highest in birds fed MOS. At this age, adding an AGP to the diet caused a major reduction in *Lactobacilli* population. The effects of MOS on the population of beneficial bacterial in the gut of broilers are inconsistent. Fernandez et al. (2002) and Denev et al. (2005) reported increases in *Lactobacilli* and *Bifidobacteria* populations in the ceca of broilers fed MOS compared to an AGP-free diet. Sims et al. (2004) observed increased cecal population of *Bifidobacteria* in turkeys fed MOS compared to an AGP-free diet but there were no differences in cecal load of *Lactobacilli.* Spring et al. (2000) also reported no effect of

MOS on *Lactobacilli* populations in the ceca of broilers. In studies with turkeys, Fairchild et al. (2001) reported that intestinal populations of *Lactobacilli* and *Bifidobacteria* did not differ among an AGP-free diet or those containing MOS or flavomycin. Factors contributing to variability in the effects of MOS on population of beneficial bacteria in the gut may include differences in experimental conditions, diet formulation, seasonal effects, and health status of the flock. Published data on the effects of lignin on the populations of *Lactobacilli* and *Bifidobacteria* in the gut of chickens are not available. It was obvious that lignin had no effect on cecal populations of *Lactobacilli* and *Bifidobacteria* when compared to the AGP-free diet; however, when compared to the AGP diet, lignin had a positive effect on the population of *Lactobacilli* in the ceca, and this represents a novel finding. We observed that HL inhibited the growth of *Bifidobacteria* in the ceca, which implies that lignin was beneficial only at the low level.

Then MOS, and to a less extent LL, increased villi height, goblet cell numbers and the population of beneficial bacteria (*Lactobacilli* and *Bifidobacteria)* in the ceca of broiler chickens. These events may be linked. For instance, there is evidence that *Lactobacilli* and *Bifidobacteria* can increase the synthesis and secretion of mucin in the gut (Smirnov et al., 2005) as a result of an increase in goblet cell number (Ferket et al., 2002). Hence, the greater populations of *Lactobacilli* and *Bifidobacteria* in the ceca of birds fed MOS or the low level of lignin could explain the increase number of GCV associated with these treatments. There is also evidence that *Lactobacilli* and *Bifidobacteria* promote gut health by competing against potential pathogens for nutrients and binding sites, and by producing bacteriocins which act as antimicrobial compounds to control pathogens in the gut (Gibson and Wang, 1994; Kawai et al., 2004). Therefore, the

use of MOS and low levels of lignin in the diet may be an effective strategy to maintain the integrity and health of the gut in chickens.

We could not enumerate *E. coli and Salmonella* in the cecal digesta and this was probably due to undetectably low concentrations of these pathogenic bacteria under the conditions of this experiment. The use of an enrichment medium, before the samples were plated, may have allowed for detection of low numbers of *E. coli* and *Salmonella.* However, this approach was not adopted.

Chicken litter is a potential reservoir and transmission vehicle for pathogens and potential pathogens and a major source of *E. coli* (Garrido et al., 2004; Schrader et al., 2004). Our results reveal that the litter from MOS fed birds showed reduced population of *E. coli* when compared to birds fed the CTL+ or CTL- diet. According to Newman (1994), *E. coli* and *Salmonella* adsorb to MOS in the chicken gut and less is excreted in feces. This explains the reduced population of *E. coli* in the litter of MOS fed birds. The effect of MOS in reducing *E. coli* load in the litter is consistent with the results of Stanley et al. (2000). It is possible that *E. coli* remains bound to MOS, thereby limiting *E. coli* proliferation in the litter. Compared to CTL-, adding AGP to the diet did not influence the *E. coli* load in litter. Gram-negative pathogenic bacteria, such as *E. coli*, are resistant to most of the AGP used in poultry production (Page, 2003), and therefore our finding is expected.

There was a tendency for lignin to reduce *E. coli* load in the litter. Although not statistically different from the control diets, the effects were comparable to that of MOS. Research conducted *in vitro* with the lignin product used in this study demonstrated that it has inhibitory effects on growth of *E. coli, Staphylococcus aureus,* and *Pseudomonas* (Nelson et al., 1994; Phillip et al., 2000). Nelson et al. (1994) reported that addition of

lignin to the diet had a tendency to inhibit growth of aerobic bacteria in the cecum of rats and reduced the translocation of these bacteria in lymph nodes and the liver. Although the exact mechanism of lignin action remains unclear, it has been suggested that the phenolic compounds in lignin cause cell membrane damage and lysis of bacteria (Jung and Fahey, 1983). Lignin could, therefore, be a dietary strategy to reduce *E. coli* load in the gut and litter of chickens.

E. coli is the principal pathogenic organism implicated in cellulitis, the major cause of carcass condemnation at the processing plants in Canada (Kumor et al., 1998). Cellulitis is characterized by subcutaneous inflammatory reaction resulting from an infection by *E. coli* associated with litter (Schrader et al., 2004). The findings from this study indicate that MOS, and to a less extent lignin, can be used to reduce *E. coli* proliferation in poultry litter. This would offer an opportunity for dietary control of the cellulitis problem.

In conclusion, adding MOS to broiler diets improved gut integrity, as measured by changes in villi height, goblet cell number and populations of the beneficial bacteria, *Lactobacilli* and *Bifidobacteria*, in the ceca; MOS also resulted in a major reduction in *E. coli* load in the litter, and this might have implications for the control of cellulitis in chickens. The effect of lignin in reducing *E. coli* load in litter was similar to that of MOS. Under the conditions of this study, AGP failed to improve growth performance and feed efficiency when compared to an antibiotic free diet or one containing MOS or lignin. The addition of MOS and perhaps low levels of lignin, to the diet could be an alternative to the use of antibiotics as growth promoters in poultry production.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Saskatchewan Chicken Industry Development Fund (SCIFD). The technical support of Mr. David Meek, Mr. Francois Ouellette, Mrs. Elisabeth Nourtier, Mr. Keyvan Amini, and Ms. Marie Claude Viau is greatly appreciated.

TABLE 3.1. Composition of basal diets and calculated analysis of the finalized dietary treatments

Ingredients	Starter[1] (1 to 21 d)	Grower[1] (22 to 42 d)
	Composition, g / kg	
Corn	530.3	564.4
Soybean meal[2]	385.1	340
Soybean Oil	13.7	30
Lyrco Starter Premix[3]	40	0
Lyrco Grower Premix[4]	0	38.5
Biolys65[5]	3.2	0
DL Methionine	2.5	2.1
Threonine	0.2	0
	Calculated analysis	
Energy (Kcal/kg)	3100	3200
Crude Protein (%)	22.0	20.0
Arginine (%)	1.48	1.35
Lysine (%)	1.37	1.09
Methionine (%)	0.59	0.52
Calcium (%)	1.00	0.90
Av. Phosphorus (%)	0.50	0.47

[1]Basal diets were completed to 1000 by adding the feed additives or an inert filler or both, for a total of 25 g / kg

[2]Partially defatted extruded soybean meal, composition per kg: Energy 3100 Kcal; Crude Protein 475 g; Lysine 27.8 g; Methionine 6.3 g; Calcium 1.9 g; Av. Phosphorus 7 g.

[3]Provided per kg of feed: Protein 1.63 g; Ca 8.37 g; P 3.39 g; Na 1.54 g; Mg 0.21 g; K 0.04 g; Co 0.39 mg; Cu 19.56 mg; Zn 48.7 mg; I 0.97 mg; Mn 79.88 mg; Fe 148.13 mg; Se 0.29 mg; Vitamin A 10,000 IU; Vitamin D 2,500 IU; Vitamin E 40 IU.

[4]Provided per kg of feed: Protein 1.2 g; Ca 8.2 g; P 3.16 g; Na 1.6 g; Mg 0.2 g; K 0.04 g; Co 0.4 mg; Cu 20 mg; Zn 50 mg; I 1 mg; Mn 82 mg; Fe 146 mg; Se 0.3 mg; Vitamin A 10,000 IU; Vitamin D 2,500 IU; Vitamin E 40 IU.

[5]Provided per kg of feed: 1.6 g Lysine.

TABLE 3.2. Effects of antibiotics, mannanoligosaccharide, and lignin on body weight (g), feed intake (g) and feed conversion of broiler chickens[1]

	Treatments[2]					
Age	CTL -	CTL +	MOS	LL	HL	SEM
	Body weight					
Day 1	47.15	46.84	46.88	46.80	46.89	0.38
Day 7	190.94^{a}	179.28^{b}	188.43^{ab}	186.69^{ab}	184.07^{ab}	2.02
Day 14	486.96^{a}	457.55^{b}	475.78^{ab}	474.56^{ab}	477.76^{ab}	5.83
Day 21	976.10^{a}	919.56^{b}	956.64^{ab}	936.20^{ab}	951.53^{ab}	10.91
Day 28	1688.12^{a}	1619.18^{b}	1657.77^{ab}	1630.65^{b}	1626.77^{b}	12.39
Day 35	2318.45^{a}	2164.75^{b}	2229.98^{ab}	2114.65^{b}	2154.90^{b}	22.05
Day 42	3047.45^{a}	2764.20^{b}	2826.18^{b}	2822.05^{b}	2745.98^{b}	46.57
	Feed Intake					
Day 1 - 7	167.19	144.16	161.44	152.81	150.47	8.58
Day 1 - 14	559.18	530.48	538.27	552.75	548.35	6.94
Day 1 - 21	1266.40	1212.58	1212.13	1215.55	1236.23	18.15
Day 1 - 28	2435.90	2398.04	2392.09	2419.02	2433.32	28.64
Day 1 - 35	3733.21^{a}	3397.44^{b}	3461.99^{b}	3466.62^{b}	3445.29^{b}	57.53
Day 1 - 42	5107.12^{a}	4577.64^{b}	4657.10^{b}	4595.21^{b}	4575.78^{b}	102.87
	Feed Conversion					
Day 1 - 7	0.88	0.80	0.86	0.82	0.82	0.05
Day 1 - 14	1.14	1.16	1.13	1.17	1.15	0.02
Day 1 - 21	1.30	1.32	1.27	1.30	1.30	0.01
Day 1 - 28	1.44	1.48	1.44	1.48	1.50	0.01
Day 1 - 35	1.61	1.57	1.55	1.57	1.60	0.01
Day 1 - 42	1.68	1.66	1.65	1.63	1.67	0.02

[1]Mean of four replicates

[2]CTL^{-}: antibiotic free diet; CTL+: commercial type diet with 11 mg / kg virginiamycin; MOS: antibiotic free diet supplemented with 0.2 % and 0.1 % Bio-Mos in the starter (1 to 21 d) and in the grower feed (22 to 42 d), respectively; LL and HL: antibiotic free diet supplemented with 1.25 % or 2.5 % Alcell lignin, respectively

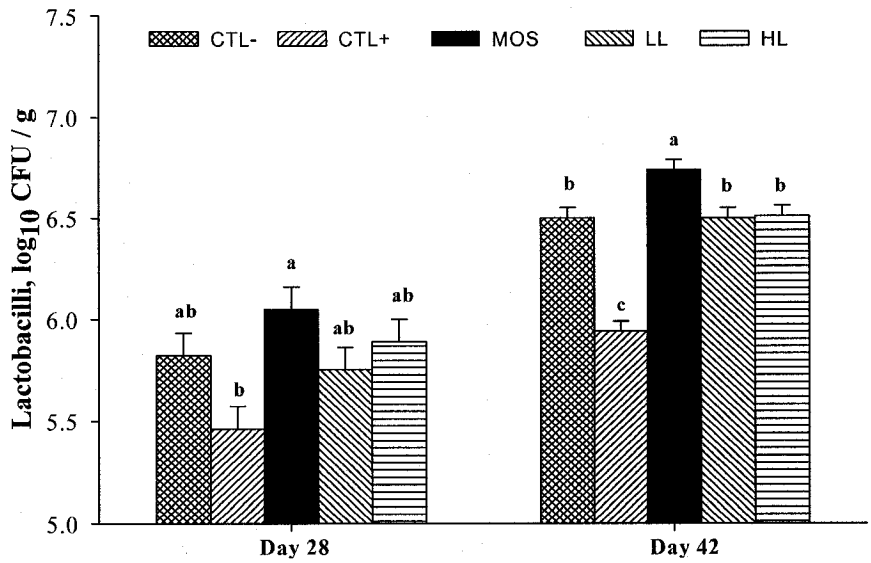
[a,b] Values with different superscript within the same row are different (Bonferroni t-test, $P < 0.05$)

TABLE 3.3. Effects of antibiotics, mannanoligosaccharide, and lignin on villi height, number of goblet cells per villus, crypt depth, muscularis layer thickness and muscularis mucosae thickness of broiler chickens[1]

	Treatments[2]					
Age	CTL -	CTL +	MOS	LL	HL	SEM
	Villi height (μm)					
Day 14	1962.21	1799.16	1868.47	1955.14	1839.95	68.13
Day 28	2114.57^{b}	2126.74^{b}	2627.46^{a}	2290.76^{ab}	2194.70^{b}	77.73
Day 42	2299.66^{a}	1748.41^{b}	2465.86^{a}	2338.29^{a}	1757.75^{b}	104.88
	Number of goblet cells/villus					
Day 14	68.68	52.81	69.20	78.00	51.75	8.53
Day 28	53.54^{bc}	35.17^{c}	95.79^{a}	77.99^{ab}	55.08^{bc}	8.77
Day 42	60.95^{b}	35.31^{b}	118.03^{a}	54.92^{b}	37.51^{b}	8.33
	Crypt depth (μm)					
Day 14	148.86	139.52	156.54	126.81	142.55	9.94
Day 28	117.53	126.65	148.62	125.43	150.77	10.18
Day 42	137.06	153.47	154.08	159.10	157.40	7.41
	Muscularis thickness (μm)					
Day 14	96.25	101.83	123.08	92.86	114.87	9.43
Day 28	151.40	114.55	128.53	137.59	145.02	14.72
Day 42	184.62	142.74	170.59	174.86	210.68	15.85
	Muscularis mucosae thickness (μm)					
Day 14	13.98	13.44	16.84	13.71	13.86	1.06
Day 28	19.41	16.13	21.13	24.75	20.94	2.18
Day 42	25.78	23.90	22.96	23.91	26.22	1.82

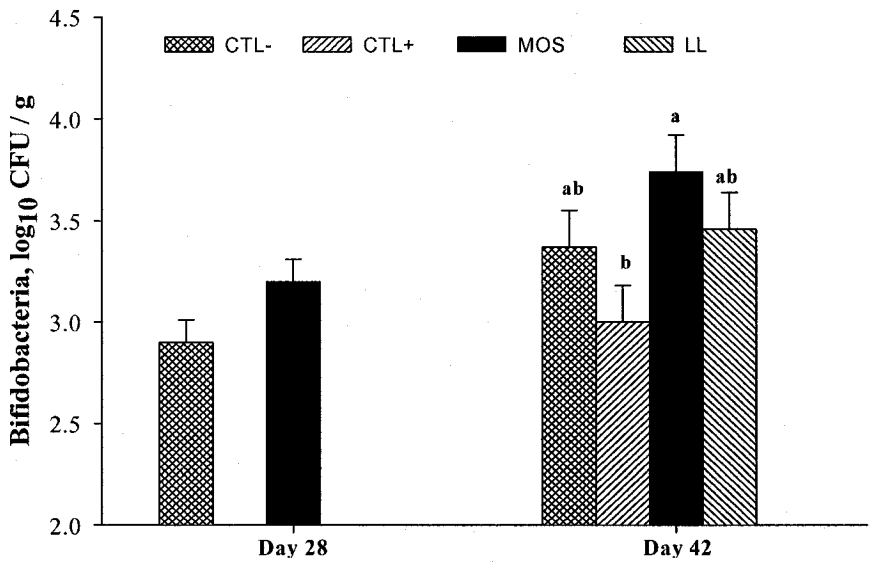
[1]Mean of 10 individual measurements per bird (40 replicates)
[2]CTL^{-}: antibiotic free diet; CTL+: commercial type diet with 11 mg / kg virginiamycin; MOS: antibiotic free diet supplemented with 0.2 % and 0.1 % Bio-Mos in the starter (1 to 21 d) and in the grower feed (22 to 42 d), respectively; LL and HL: antibiotic free diet supplemented with 1.25 % or 2.5 % Alcell lignin, respectively
[a,b] Values with different superscript within same row are different (Bonferroni t-test, $P < 0.05$)

FIGURE 3.1. Effects of antibiotic free diets (CTL-); antibiotic supplemented diets (CTL+, 11 mg / kg virginiamycin); antibiotic free diets supplemented with Bio-Mos (MOS) at 0.2 % and 0.1 % in the starter (1 to 21 d) and in the grower feed (22 to 42 d), respectively; and antibiotic free diet supplemented with low (LL, 1.25 %) or high (HL, 2.5 %) Alcell lignin, on the populations of *Lactobacilli* in the cecal digesta of broiler chickens



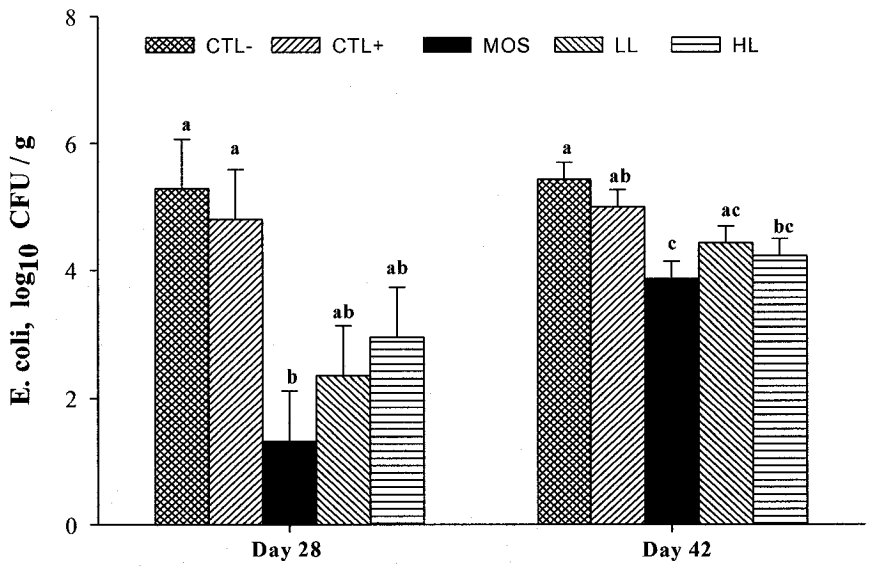
[a,b,c] Values with different superscript within a group are different (Bonferroni t-test, $P < 0.05$)

FIGURE 3.2. Effects of antibiotic free diets (CTL-); antibiotic supplemented diets (CTL+, 11 mg / kg virginiamycin); antibiotic free diets supplemented with Bio-Mos (MOS) at 0.2 % and 0.1 % in the starter (1 to 21 d) and in the grower feed (22 to 42 d), respectively; and antibiotic free diet supplemented with low (LL, 1.25 %) or high (HL, 2.5 %) Alcell lignin, on the populations of *Bifidobacteria* in the cecal digesta of broiler chickens



[a,b] Values with different superscript within a group are different (Bonferroni t-test, $P < 0.05$)

FIGURE 3.3. Effects of antibiotic free diets (CTL$^-$); antibiotic supplemented diets (CTL+, 11 mg / kg virginiamycin); antibiotic free diets supplemented with Bio-Mos (MOS) at 0.2 % and 0.1 % in the starter (1 to 21 d) and in the grower feed (22 to 42 d), respectively; and antibiotic free diet supplemented with low (LL, 1.25 %) or high (HL, 2.5 %) Alcell lignin, on the populations of *E. coli* in the litter of broiler chickens



[a,b,c] Values with different superscript within a group are different (Bonferroni t-test, $P < 0.05$)

Preface to Chapter 4

Chapter 4 comprises a manuscript, co-authored by B. Baurhoo, L. Phillip, A. Letellier, and C. A. Ruiz-Feria, soon to be submitted for publication in the journal, *Poultry Science*. The format has been modified to be consistent within this thesis, according to the guidelines set by the Faculty of Graduate Studies and Research. All literature cited in this chapter is listed in the "References" section at the end of the thesis. All tables and figures are presented at the end of this chapter.

Results from Experiment 1, presented in Chapter 3, demonstrate that the addition of mannanoligosaccharides (MOS) and low levels of lignin to broiler diets significantly increased villi height and goblet cell number in the jejunum, and increased the populations of *Lactobacilli* and *Bifidobacteria* in the ceca. Moreover, MOS and to a lesser extent a low level of lignin, significantly reduced *E. coli* loads in broiler litter. In that experiment, whether or not birds were fed MOS or lignin, *E. coli*, pathogenic bacteria that cause food-borne illnesses in humans, was not detected in the ceca, but *E. coli* was detected only in the litter. These findings created uncertainty as regarding the effects of reducing the intestinal population of pathogenic bacteria, and ultimately poultry products.

The aim of Experiment 2, described in Chapter 4, was to ascertain whether MOS and lignin would be effective in reducing the intestinal loads of pathogenic bacteria when challenged with known infectious strains of *E. coli*. Experimental and feeding conditions utilized in Experiment 1 were repeated in Experiment 2 to maintain consistency, and to confirm experimental findings reported in Chapter 3.

CHAPTER 4

Effects of Purified Lignin and Mannanoligosaccharides on Intestinal Microbial Populations after *E. coli* Challenge in Broiler Chickens [1]

B. Baurhoo[*], L. Phillip[*], A. Letellier[†], and C. A. Ruiz-Feria[*]

[*]McGill University, Department of Animal Science, Ste. Anne de Bellevue, H9X 3V9, QC, Canada ; [†]University of Montreal, Veterinary Department, St-Hyacinthe, J2S 7C6, QC, Canada.

[1] To be submitted for publication in *Poultry Science*

Effects of Purified Lignin and Mannanoligosaccharides on Intestinal Microbial Populations after *E. coli* Challenge in Broiler Chickens

4.1 ABSTRACT

Two experiments were conducted to evaluate lignin and mannanoligosaccharides (MOS) as alternatives to antibiotic growth promoters in broilers. Dietary treatments for the 2 studies were: 1) negative control (CTL-, antibiotic free); 2) positive control (CTL+, 11 mg / kg virginiamycin); 3) MOS (diet 1 + Bio-Mos: 0.2 % to 21 d and 0.1 % thereafter); 4) LL (diet 1 + 1.25 % Alcell lignin); 5) HL (diet 1 + 2.5 % Alcell lignin). In experiment 1, each treatment was assigned to 4 pen replicates (52 birds each). Body weight and feed intake were recorded weekly throughout 38 d. At 28 and 38 d, cecal contents were assayed for *Lactobacilli* and *Bifidobacteria*. Body weight and feed intake did not differ among dietary treatments. At d 38, the population of *Lactobacilli* was highest ($P < 0.05$) in birds fed MOS, whereas LL fed birds had higher ($P < 0.05$) *Lactobacilli* load than CTL+ fed birds. Birds fed MOS or LL had higher ($P < 0.05$) populations of *Bifidobacteria* than those fed the CTL+ diet at both d 28 and 38. However, at d 28 and 38, *Lactobacilli* and *Bifidobacteria* loads were lowest ($P < 0.05$) in birds fed the CTL+ or HL diet. In experiment 2, at d 21, birds from the initial flock were transferred to cages for oral *E. coli* (O2 and O88 serotypes) challenge (3 birds / cage; 12 birds / treatment). After 3, 6 and 9 d, the cecal populations of total *E. coli* were determined. At d 9, *E. coli* load was lower ($P < 0.05$) in birds fed MOS or HL than those fed the CTL+ diet; at this time interval, LL fed birds tended to have lower *E. coli* load than those fed the CTL+ diet. At d 3, HL fed birds also had lower ($P < 0.05$) *E. coli* load than CTL+ fed birds. In summary, birds fed MOS or LL had comparative advantage over

CTL+ fed birds as evidenced by increased cecal populations of *Lactobacilli* and *Bifidobacteria* and, in the *E. coli* challenge study, lower cecal *E. coli* load. Therefore, MOS and lignin could potentially replace antibiotic growth promoters in poultry production.

(**Key words**: antibiotic, mannanoligosaccharides, lignin, gut health, food safety)

4.2 INTRODUCTION

Food safety is a major public health concern worldwide (Rodriguez-Morales et al., 2005). For example, in the United States, food contamination with pathogenic bacteria is responsible for over 76 million cases of food-borne illnesses annually; 325,000 cases result in hospitalization and 5,000 cases result in death (Mead et al., 1999). The principal pathogenic bacteria causing food-borne illnesses are *Campylobacter*, *Salmonella* and *E. coli* (Mead et al., 1999). The critical point of bacterial contamination of poultry products occurs at the slaughter house when pathogens in the intestinal contents make contact with chicken carcasses (Heyndrickx et al., 2002). *E. coli* is important in poultry production because different antibiotic-resistant strains have been isolated from poultry and poultry meat products in several countries (Sackey et al., 2001; Zhao et al., 2001; Mayrhofer et al., 2004). Human infections with antibiotic-resistant pathogenic bacteria are difficult to treat and therefore increase hospitalization costs, estimated at $ 7 to 10 billion (US) (Mead et al., 1999; Lees and Aliabadi, 2002).

Global demands for safe poultry products have prompted the need for effective biological modulators of enteric microflora in the poultry industry, and in this context,

there is increased interest in the prebiotics, mannanoligosaccharides (MOS) and purified lignin. These dietary additives have the potential to eliminate or kill the intestinal pathogenic bacteria (Nelson et al., 1994; Newman, 1994; Phillip et al., 2000), but very little research on these additives has been carried out with poultry. Spring et al. (2000) and Fernandez et al. (2002) conducted studies with broilers which showed that when birds were fed diets containing MOS and challenged with pathogenic strains of *Salmonella*, the cecal populations of these specific strains of *Salmonella* were significantly reduced. However, in studies with turkeys challenged with pathogenic strains of *E. coli*, there were no effects of MOS on the total intestinal population of *E. coli* (Fairchild et al., 2001). There is clearly a lack of research in which broilers have been fed MOS and challenged with *E. coli*. Such data are useful to establish the efficacy of MOS in reducing the intestinal population of *E. coli* in broilers.

The poly-phenolic fragments of Alcell lignin have been shown to inhibit *in vitro* growth of *E. coli, Pseudomonas* and *Staphylococcus aureus* (Nelson et al., 1994; Phillip et al., 2000). Nelson et al. (1994) also observed that dietary addition of Alcell lignin (10 % of DM) significantly reduced the population of aerobic bacteria in the cecum of rats. There are no published studies of the effects of lignin on *in vivo* growth of *E. coli*. Nevertheless, research findings suggest that lignin could have a role in reducing the load of pathogenic bacteria in the gut of poultry, thereby improving the safety of poultry products. Given the need for biological additives as alternatives to antibiotics in poultry production, research into lignin and MOS would be quite valuable.

The objectives of this study were to: 1) evaluate the effects on cecal population of total *E. coli* in broilers fed Bio-Mos and Alcell lignin after an *in vivo* challenge with known pathogenic strains of *E. coli*; (2) to confirm findings of the previous experiment of

the effects of Bio-Mos and Alcell lignin on cecal populations of *Lactobacilli* and *Bifidobacteria* in broilers; 3) to compare the effects of the antibiotic growth promoter (**AGP**), virginiamycin, with those of Bio-Mos and Alcell lignin.

4.3 MATERIALS AND METHODS

4.3.1 Bird management

One thousand and forty one-day-old male Cobb 500 broilers were obtained from a commercial local hatchery (Couvoir Simetin, QC, Canada) and grown over a 38 d experimental period under good hygienic (strict biosecurity measures and clean litter) and management conditions in a newly constructed experimental facility with concrete floor pens. Birds were randomly assigned to 5 dietary treatments (4 pen replicates; 52 birds per pen). Each pen was covered with 8 cm of clean pine wood shavings and was equipped with one tube feeder and one automatic waterer. The birds were brooded following standard temperature regimes, which gradually decreased from 32° to 24° C, and under a 20:4 light:dark cycle throughout the studies. Bird management and care were conducted following the animal care protocol approved by the University of McGill Animal Care Committee. Birds were group weighed by pen and feed consumption determined at weekly intervals.

4.3.2 Experimental diets

The birds were fed a corn-soybean meal based diet. All the diets were formulated to be iso-nitrogenous, iso-energetic and to meet or exceed NRC (1994) requirements for macro and micronutrients. The ingredient composition and nutrient contents of the diets are shown in Table 4.1. A two-phase feeding program was used, with a starter diet from d

1 to 21 and a grower diet from d 22 to 38. The five dietary treatments included: 1) negative control diet (**CTL-**, AGP-free); 2) positive control diet (**CTL+**, commercial type feed containing 11mg / kg virginiamycin); 3) **MOS** (diet 1 + Bio-Mos (Alltech Inc., Kentucky, US): 0.2 % of starter diet and 0.1 % of grower diet); 4) **LL** (diet 1 + 1.25 % Alcell lignin, Alcell Technologies Inc., QC, Canada); 5) **HL** (diet 1 + 2.5 % Alcell lignin).

4.3.3 Enumeration of *Lactobacilli* and *Bifidobacteria*

At d 28 and 38, one bird from each pen replicate was euthanized by electrical stunning and bleeding of the carotid artery; the ceca were aseptically collected into sterile plastic bags. Samples of the fresh cecal contents were diluted 10-folds by weight in buffered peptone water (Fischer Scientific, ON, Canada) and mechanically massaged using a stomacher (Model 400 Lab Blender, Seward Medical, London, UK) for 30 seconds. The samples were then serially diluted in 0.85 % sterile saline solution for enumeration of *Lactobacilli* and *Bifidobacteria* as the beneficial bacteria of the chicken gut. All microbiological analyzes were performed in duplicates and the average value of these were used for statistical analysis. *Lactobacilli* were anaerobically assayed using Lactobacilli MRS Agar (Fischer Scientific) and incubated at 37 °C for 48 h. *Bifidobacteria* were anaerobically assayed using Wilkins-Chalgren agar (Oxoid, ON, Canada) supplemented with glacial acetic acid (1 ml/L) and mupirocin (100 mg/L) extracted from antimicrobial discs (Oxoid) (Rada et al., 1999). The petri dishes were incubated at 37 °C for 3 days. After the incubation periods, colonies of *Lactobacilli* and *Bifidobacteria* were then counted.

4.3.4 *E. coli* challenge study

Bird transfer. At d 21, 6 birds from each pen replicate of the initial flock of birds were randomly removed, separated into two groups and then transferred to individual cages equipped with individual feeders and nipple drinkers, respective of treatments, for *E. coli* challenge. For each group, birds in cages (3 birds / cage, n = 60, 12 birds / dietary treatment) were housed in two separate rooms with environmentally controlled conditions following the same temperature and light conditions as above.

E. coli challenge and enumeration. Two *E. coli* serotypes (O2 and O88) were used based on pathogenicity to poultry (Menao et al., 2002), and agglutination to MOS (Mirelman et al., 1980). The O2 and O88 serotypes were isolated from chicken carcasses and obtained from the Veterinary lab of University of Montreal (St Hyacinte, QC, Canada). A growth curve was constructed to determine the point at which the cultures reached and maintained the concentration of 10^7 CFU / ml corresponding to a stationary phase of growth, which was the gavage target dose desired in this study. The *E. coli* concentrations were verified by serial dilutions and plated on Sheep Blood Agar (Oxoid) at 37 °C for 24 hours and colonies counted corresponding to the desired concentration.

Prior to the challenge study, litter samples were screened for *E. coli* to confirm that birds were free from the administered O2 and O88 serotypes of *E. coli*. At d 29, caged birds in the first room were orally challenged with a mixed culture of *E. coli* (O2 and O88 serotypes) at a concentration of 1 x 10^7 CFU / ml of sterile PBS (pH = 7.2), whereas birds in the second room were orally gavaged with 1 ml of sterile PBS, serving as control.

At 3, 6 and 9 d post-inoculation, 4 birds from each dietary treatment (1 bird per cage) of the 2 groups were euthanized and the ceca of each bird were aseptically removed and collected for enumeration of total *E. coli*. Samples of the fresh cecal contents were serially diluted and plated on Rapid *E. coli* 2 Agar (Biorad Lab, ON, Canada), modified using *E. coli* supplement (Biorad Lab) to be selective for *E. coli* for identification and quantification of *E. coli* of all types. Microbiological analyzes of the cecal samples were performed in duplicates and the average value of these were used for statistical analysis. Samples of *E. coli* isolates were sub-cultured on Sheep Blood Agar and then O serotyped by the Polymerase Chain Reaction **(PCR)** techniques to verify that the serotypes recovered in the ceca matched those administered. Any isolate of the O2 or O88 serotypes of *E. coli* recovered in the ceca of PBS gavaged birds and samples of isolates of the same serotypes from *E. coli* challenged birds were genotyped using the Pulsed Field Gel Electrophoresis method **(PFGE)** to verify if identical to the serotypes used to challenge the birds.

4.3.5 Statistical analysis

Data were analyzed by one-way ANOVA using the General Linear Models (GLM) procedure of SAS (SAS Institute, 2003) with pen serving as the experimental unit for performance parameters and bird as the experimental unit for microbiological parameters. Treatment means were separated using the least square means option of SAS. Differences between treatment means were tested using Bonferroni's Multiple Comparison test and statistical significance was declared at a probability of $P < 0.05$. All microbiological concentrations were subject to $\log_{10}$ transformation prior to analysis.

Statistical model used:

$$Y_{ij} = \mu + TRT_i + e_{ij}$$

where:

TRT_i : i = 1, 2, 3, 4, or 5 (i.e 5 dietary treatments).

e_{ij} : j = 1, 2, 3, or 4 (i.e 4 pens or birds / treatment); $e_{ij} \sim N(0, \sigma^2_e)$.

Fixed effect parameters of the model:

(a) μ is the overall mean of microbial populations (log CFU / g)

(b) TRT_i is the fixed effect of i^{th} treatment (diet) on microbial populations

Random effect parameters of the model:

σ^2_e is the random residual variation of the model

4.4 RESULTS

4.4.1 Bird performance and enumeration of *Lactobacilli* and *Bifidobacteria*

Dietary treatments did not alter growth performance or feed intake (Table 4.2). Feed conversion ratio was not different among dietary treatments up to d 28. However, at d 35, feed conversion ratio was higher in birds fed the CTL+ diet than those fed the CTL- diet; there were no other treatment effects on feed conversion ratio.

At d 28, the cecal population of *Lactobacilli* was highest in birds fed MOS or CTL-, but there were no differences between these two treatments (Figure 4.1). At d 38, the *Lactobacilli* population was significantly increased with the MOS diet than with the CTL- or any other diet. Birds fed the CTL+ or HL diet had lower *Lactobacilli* loads than those fed the CTL- diet at both d 28 and 38. However, the magnitude of reduction with HL was much more pronounced at d 28 than at d 38. When fed to birds, the LL diet increased the cecal populations of *Lactobacilli* when compared to those fed the CTL+ or

HL diets at d 28 and 38. However, *Lactobacilli* load was not different between birds fed LL or CTL- at d 38, but *Lactobacilli* load was lower in LL fed birds at d 28.

Observations made at d 28, revealed that neither MOS nor LL altered the cecal population of *Bifidobacteria* when compared to the CTL- diet (Figure 4.2). However, at d 38, birds fed the LL diet had lower population of *Bifidobacteria* than those fed the MOS or CTL- diet. When compared to the CTL-, MOS or LL diet, the CTL+ and HL diets significantly reduced the populations of *Bifidobacteria* at d 28 and 38, but there were no differences between these two diets (CTL+ and HL).

4.4.2 *E. coli* challenge and enumeration

PBS gavage. Serotyping results of *E. coli* isolates indicated that the O88 serotype was absent in the ceca of the birds at all intervals (d 3, 6 and 9) after PBS gavage. However, among all the *E. coli* isolates, about 10 % of the O2 serotype ("O2-PBS") was identified in the ceca. This was an unexpected finding. Results from PFGE revealed that these *E. coli* isolates of the O2 serotype were genetically different from the O2 serotype used to challenge the birds.

At 3 and 6 d, the HL diet significantly reduced the cecal populations of *E. coli* in PBS gavaged birds as compared to those fed the CTL- or CTL+ diet; at d 9, *E. coli* population was lower in HL fed birds than those fed the CTL- diet but not in birds fed the CTL+ diet (Figure 4.3). Moreover, at d 3, birds fed the HL diet had reduced *E. coli* population than those fed the LL diet and, at d 6, *E. coli* load was also lower than MOS fed birds. Birds fed MOS had reduced *E. coli* load than those fed the CTL- diet at d 3. There were no differences in the populations of *E. coli* among birds fed the CTL-, CTL+ or LL diet at any intervals after the gavage.

E. coli challenge. Results from serotyping indicated that the two serotypes of *E. coli* (O2 and O88) used in the challenge were recovered in the cecal digesta of birds at all intervals (d 3, 6 and 9) after the challenge. However, irrespective of time intervals after the challenge and dietary treatments, there were 11 times more of the O2 than the O88 serotype. Results from PFGE revealed that the O2 serotype used to challenge the birds ("O2-challenge") was recovered in the cecal digesta. Moreover, the O2 serotype identified in the ceca of PBS gavaged birds ("O2-PBS") was recovered in the ceca of *E. coli* challenged birds. Several other serotypes of *E. coli* were also recovered in the ceca of the birds but these were not relevant to the study.

At all intervals after the *E. coli* challenge, the effects of CTL- and CTL+ on the populations of *E. coli* were similar (Figure 4.4). At d 3 and 9, the HL diet significantly reduced the cecal populations of total *E. coli* when compared to the CTL- or CTL+ diet but at d 6, *E. coli* load was lower only in birds fed the CTL- diet. At d 9, birds fed MOS had a lower population of *E. coli* than those fed the CTL- or CTL+ diet; at this time interval, LL fed birds had lower *E. coli* load when compared to those fed the CTL- diet. At all intervals after the *E. coli* challenge, the effects of MOS, LL and HL on the populations of *E. coli* were similar.

4.5 DISCUSSION

The results of this study show that the addition of the antibiotic, virginiamycin, to an antibiotic-free diet did not alter growth performance or feed intake in broilers. Feed conversion ratio, however, was increased with the addition of virginiamycin. The impact of virginiamycin on performance parameters in this experiment was different from that observed in Experiment 1, where feed efficiency was unaltered, and growth rate and feed intake decreased following virginiamycin addition. As indicated in the previous discussion, there are no reports of a suppression in growth performance or feed consumption due to antibiotics (Waldroup et al., 2003; Hooge, 2004), but such an anomaly was not repeated in the current experiment. The decrease in feed efficiency observed in this study following the addition of virginiamycin occurred despite the lack of any change in growth performance and feed intake. This is an unexpected finding but the observation has been made before between diets containing bacitracin and enramycin (Pedroso et al., 2006).

The observations that MOS and lignin failed to alter production responses are consistent with results of the previous experiment, and demonstrate that dietary additives were not needful to maximize broiler performance. In a study conducted with broilers fed AGP, MOS, a combination of AGP and MOS or an AGP-free diet, Waldroup et al. (2003a,b) observed no difference in growth performance and feed efficiency. Hooge (2004) reported that birds fed MOS showed improved growth performance and feed efficiency compared with those fed AGP-free diets; performance was similar between MOS and AGP. The addition of virginiamycin, to broiler diets improved body weight and feed conversion compared with an AGP-free diet (Miles et al., 2006). Based on the current study and published research, it would appear that MOS and AGP have variable

effects on broiler performance. These may be attributed to differences in experimental conditions, diet formulation, and health status of the birds. It is reported that AGP (Sims et al., 2004) and the most beneficial additives (Hooge, 2004) are most effective under stress and disease conditions.

This is the first report of the impact of Alcell lignin on poultry performance but the results revealed no beneficial effect of this natural additive. Phillip et al. (2000) reported that Alcell lignin (1.25 % of DM) improved growth rate and feed efficiency in veal calves. However, in studies with pigs a similar dietary level of Alcell lignin did not alter growth rate and feed efficiency (Valencia and Chavez, 1997). Previous studies using different forms of lignin have been conducted with poultry. For example, the addition of indulin, purified Kraft lignin from wood in paper manufacturing industry (Ross et al., 1986), has been shown to improve weight gain and feed efficiency in broilers (Ricke et al., 1982). Differences in the forms and sources of lignin, as well as in animal species may contribute to variability in performance responses.

When compared to CTL-, the impact of virginiamycin, MOS and LL on cecal populations of *Lactobacilli* at d 28 and 38were similar to that observed in Experiment 1. With regards to *Bifidobacteria*, the effects of MOS and CTL+ were consistent with those observed in the previous experiment. In the case of HL, however, cecal populations of *Lactobacilli* were significantly reduced at both d 28 and 38 in this experiment whereas, in Experiment 1, HL had no effect. In contrast to the previous experiment, *Bifidobacteria* was detected in all treatment groups at both d 28 and 38. Moreover, in the present study, the LL diet reduced the population of *Bifidobacteria* at d 38 when compared to birds fed the CTL- diet whereas in Experiment 1, the LL diet did not alter the population of

Bifidobacteria. There is no reasonable explanation for the differential effects of the lignin diets between the two experiments.

Our findings that, at d 28 and 38, birds fed the CTL+ diet had significantly lower cecal loads of *Lactobacilli* and *Bifidobacteria* than CTL- fed birds were expected because antibiotic growth promotants are known to inhibit the growth and colonization of these intestinal gram-positive bacteria (Engberg et al., 2000).

The present study shows that MOS increased the cecal populations of *Lactobacilli* and *Bifidobacteria* and, in the challenge study, reduced the population of *E. coli*. In the previous experiment, *E. coli* was not detected in the cecal digesta and therefore an effect of MOS on pathogenic bacteria could not be established. In the present study, the use of an *E. coli* challenge provided an opportunity to assess the impact of MOS on these pathogens. The results clearly show that MOS was effective in suppressing the growth of *E. coli* in broilers. Reports indicate that competitive exclusion is a mechanism involving the establishment of an intestinal population of beneficial bacteria, such as *Lactobacilli*, that prevents the colonization of pathogenic bacteria (Van der Wielen et al., 2002). It is quite possible that the increase in both *Lactobacilli* and *Bifidobacteria* may be based on the same principle. MOS competitively excludes gram-negative pathogenic bacteria from the intestine. The mannose-specific Type-1 fimbriae of *E. coli* adsorb to MOS and are ultimately excreted without colonizing the chicken gut (Newman, 1994). Research to-date have revealed equivocal responses in intestinal populations of *Lactobacilli* and *Bifidobacteria* in both broilers (Spring et al., 2000; Fernandez et al., 2002; Denev et al., 2005) and turkeys (Fairchild et al. 2001; Sims et al., 2004). That, in both Experiment 1 and 2, MOS consistently increased the cecal populations of *Lactobacilli* and

Bifidobacteria gives clear evidence of the beneficial attribute of MOS on the beneficial bacteria in the intestine of broilers.

Both experiments consistently showed that the LL diet increased the cecal populations of *Lactobacilli* and *Bifidobacteria* when compared to the AGP diet. These effects of the low level of lignin are similar to those observed with MOS. But when the comparison was made with the AGP-free diet, lignin did not show any beneficial effects. Mannose oligosaccharides are classified as prebiotics (Ferket, 2004). Prebiotics have the effect of selectively stimulating the growth or metabolic activity of a limited number of intestinal micro organisms (Gibson and Roberfroid, 1995). Given the similarity in the effects of MOS and LL on *Lactobacilli* and *Bifidobacteria*, lignin, at low levels, has the potential to be classified as a prebiotic. Maintenance of a good symbiotic relationship between the host and its intestinal microflora is recognized as being critical for optimal performance and health of broilers (Ferket, 2000). The intestinal populations of *Lactobacilli* and *Bifidobacteria* compete against potential pathogens for nutrients and binding sites, thereby reducing the intestinal population of pathogens (Rolfe, 2000). Furthermore, *Lactobacilli* secrete bacteriocins (Jin et al., 1996a,b) and *Bifidobacteria* produce organic acids and other bactericidal substances (Gibson and Wang, 1994); all of these substances suppress the colonization of the intestines by pathogenic bacteria. Therefore, under the conditions of this study, diets containing MOS and LL offered a significant advantage over virginiamycin in improving the microbial ecology of the gut in broilers.

In contrast to the LL diet, HL inhibited the growth of *Lactobacilli* and *Bifidobacteria* in the present experiment, and inhibited the growth of *Bifidobacteria* in the previous experiment. These findings demonstrate that lignin, at high levels, possess

antibacterial effects against the intestinal beneficial bacteria and would, therefore, preclude the use of lignin at dietary levels that exceed the 1.25 %. Previous studies, both *in vivo* and *in vitro*, have demonstrated that Alcell lignin (10 %) inhibited bacterial growth (Nelson et al., 1994; Phillip et al., 2000).

Results indicate that, in birds subjected to the *E. coli* challenge, 11 times as many O2 serotype were recovered in the ceca as the O88 serotype. This suggests that the O2 serotype can better colonize the gut. According to Menao et al. (2002), the O2 and O88 serotypes of *E. coli* are pathogenic to poultry, but the O2 serotype is most commonly isolated on large scale broiler farms and dead chicken carcasses. The O2 serotype ("O2-PBS") was isolated from the ceca of PBS gavage and *E. coli* challenged birds; this finding indicates that this O2 serotype was present in the gut of the birds prior to the experiment. Prior to the initiation of the challenge study, the litter was screened for the presence of O2 and O88 serotypes but neither was detected. The "O2-PBS" serotype may, therefore, have been present at low concentration and not detectable at the time the litter was screened.

In birds gavaged with PBS or challenged with *E. coli*, the cecal populations of total *E. coli* were not different whether birds were fed the CTL+ or CTL- diet. It is reported that *E. coli* is resistant to most of the AGP used in poultry production because of the complex cell wall structure (Ferket, 2000), and therefore our findings are expected. Moreover, by inhibiting the intestinal growth of *Lactobacilli* and *Bifidobacteria*, AGP limit the opportunity for competitive exclusion of *E. coli* from the gut.

In birds challenged with *E. coli* and fed MOS, a significant reduction in the cecal population of total *E. coli* was observed, but only at d 9. It seems likely, therefore, that there was a time delay for MOS to act on the cecal population of *E. coli*. Fernandez et al. (2002) reported similar findings when MOS fed broilers were orally challenged with

Salmonella enteritidis (PT4). Results of this study also indicate that the effects of MOS in reducing the cecal concentration of total *E. coli* were more pronounced in *E. coli* challenged birds than in PBS gavaged birds. These findings agree with reports of Hooge (2004), that MOS is most effective under disease and stress conditions.

In broilers fed MOS and challenged with pathogenic strains of *Salmonella*, the cecal populations of the specific strains of *Salmonella* were significantly lowered as compared to those fed AGP-free diets (Spring et al., 2000; Fernandez et al., 2002). However, in an *E. coli* (O2, O19, O88 and O159 serotypes) challenged study conducted with turkeys, Fairchild et al. (2001) observed that the intestinal concentration of coliforms did not differ when turkeys were fed an AGP-free diet or one containing MOS or AGP. Sims et al. (2004) also reported that the concentrations of *E. coli* and coliform in the large intestine of MOS fed turkeys did not differ from those fed an AGP or AGP-free diet. It seems likely, therefore, that a positive response to MOS in reducing the intestinal population of pathogenic bacteria occurs mainly in broilers rather than turkeys.

The challenge study indicates that both LL and HL reduced the cecal population of total *E. coli* compared to the CTL- diet. However, the effects were more pronounced with the HL diet. It is likely, therefore, that the inhibition of *E. coli* by lignin may be dose related. In studies conducted *in vitro*, Phillip et al. (2000) reported a greater inhibition of *E. coli* growth in culture media containing 10 % (wt/vol) rather than 5 % (wt/vol) Alcell lignin. Although the exact mechanism of lignin action is not clear, Jung and Fahey (1983) proposed that the poly-phenolic compounds of lignin cause cell membrane damage and lysis of bacteria. Other phenolic compounds, such as carvacrol, thymol and cinnamaldehyde, have been shown to exert anti-microbial effects against *Lactobacilli*, *Bifidobacteria* and *E. coli* (Lee et al., 2004; Bozin et al., 2006). Carvacrol and thymol are

reported to cause cell membrane disintegration and release of the bacterial cell contents, whereas cinnamaldehyde penetrates the bacterial cell membrane to impair the enzyme system, reduce the intracellular pH and cause ATP depletion (Helander et al., 1998; Oussalah et al., 2006).

That MOS and LL significantly reduced the cecal population of total *E. coli* in birds challenged with pathogenic strains of *E. coli* is an important finding from this study. Intestinal *E. coli* contaminates poultry carcasses during processing at the slaughter house (Heyndrickx et al., 2002), and this represents an important cause of food-borne illnesses in humans (Mead et al., 1999). Therefore, the addition of MOS or a low level of lignin to poultry diets could be a useful dietary strategy to improve the safety of poultry products. The cecal population of total *E. coli* was also significantly reduced with the HL diet but this treatment also caused a significant reduction in the cecal population of *Lactobacilli* and *Bifidobacteria*. Such an outcome is not desirable and would militate against the use of high levels of lignin in poultry diets.

In conclusion, the dietary addition of MOS increased the cecal populations of *Lactobacilli* and *Bifidobacteria*; in *E. coli* challenged birds, MOS and LL reduced the cecal populations of total *E. coli*. A higher level of lignin (2.5 % of DM) caused a major reduction in the cecal population of *E. coli*, but *Lactobacilli* and *Bifidobacteria* loads were also reduced. Under the conditions of this study, virginiamycin failed to improve production performance when compared to an AGP-free diet or one containing MOS or lignin. It seems that MOS and a low level of Alcell lignin could replace antibiotic growth promoters in poultry production; these natural feed additives have the potential to improve the safety of poultry products without risking the spread of antibiotic-resistance in bacteria.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Saskatchewan Chicken Industry Development Fund (SCIFD). Bio-Mos was provided by Alltech Inc. Assistance in diet formulation was provided by Mrs. Elisabeth Nourtier (Lyrco Nutrition Inc.). Technical assistance in microbiological analyses was provided by Mr. David Meek and PCR analyses by Ms. Valerie Normand. The assistance of Mr. Keyvan Amini and Ms. Marie Claude Viau in bird handling is greatly appreciated.

TABLE 4.1. Composition and calculated nutrient contents of the dietary treatments of broiler chickens

Ingredients	**Starter (1 to 21 d)**		**Grower (22 to 38 d)**	
	CTL+[1]	Basal[2]	CTL+[1]	Basal[2]
	Composition (g / kg)			
Corn	552.3	501.5	619.6	568.8
Soybean Meal [3]	394	404	325	334
Soybean Oil	8	24	12	29
Lyrco Starter Premix[4]	44	44	0	0
Lyrco Grower Premix[5]	0	0	40	40
L-Lysine	1.5	1.3	2.8	2.6
DL-Methionine	0.2	0.2	0.6	0.6
	Calculated nutrients contents			
Energy (Kcal/kg)	3150		3200	
Crude Protein (%)	22.5		20.0	
Arginine (%)	1.52		1.33	
Lysine (%)	1.35		1.20	
Methionine (%)	0.55		0.52	
Calcium (%)	0.95		0.90	
Av. Phosphorus (%)	0.45		0.45	

[1]Contained virginiamycin (11 mg / kg).
[2]Basal diets were completed to 1000 by adding the feed additives or an inert filler, or both, for a total of 25 g / kg.
[3]Partially defatted extruded soybean meal, composition per kg: Energy 3100 Kcal; Crude Protein 475 g; Lysine 27.8 g; Methionine 6.3 g; Arginine 32.6 g; Calcium 1.9 g; Av. Phosphorus 7 g.
[4]Supplied per kg of feed: Protein 1.67 g; Calcium 8.6 g; Phosphorus 3.5 g; Sodium 1.6 g; Magnesium 0.2 g; Potassium 0.04 g; Sulphur 0.18 g; Cobalt 0.4 mg; Copper 20 mg; Zinc 50 mg; Iodine 1 mg; Manganese 82 mg; Iron 152 mg; Selenium 0.3 mg; Vitamin A 9,998 IU; Vitamin D 2,499; Vitamin E 40 IU.
[5]Supplied per kg of feed: Protein 1.2 g; Calcium 8.2 g; Phosphorus 3.2 g; Sodium 1.6 g; Magnesium 0.2 g; Potassium 0.04 g; Sulphur 0.2 g; Cobalt 0.4 mg; Copper 20 mg; Zinc 50 mg; Iodine 1 mg; Manganese 82 mg; Iron 146 mg; Selenium 0.3 mg; Vitamin A 10,000 IU; Vitamin D 2,500 IU; Vitamin E 40 IU.

TABLE 4.2. Effects of antibiotics, mannanoligosaccharide and lignin on body weight (g), feed intake (g) and feed conversion of broiler chickens[1]

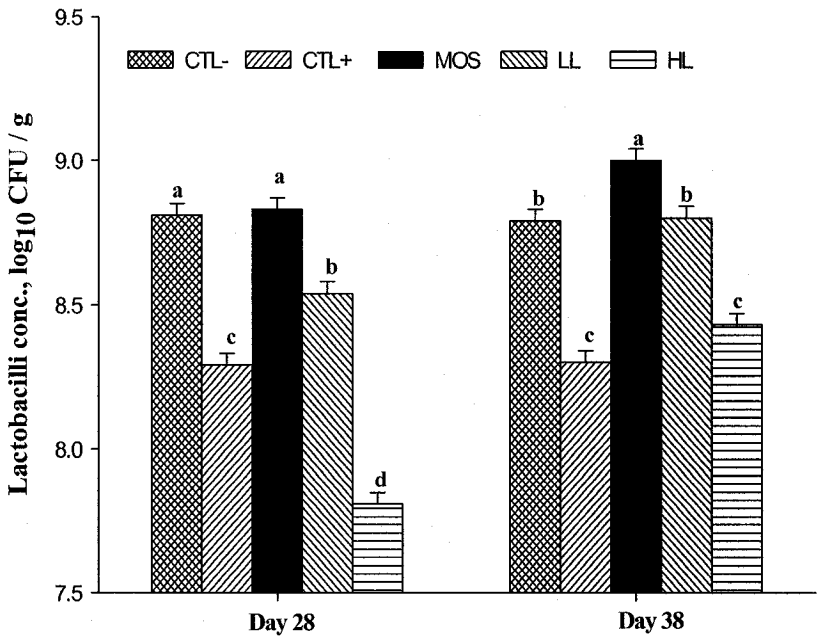
	Treatments[2]					
Age	CTL -	CTL +	MOS	LL	HL	SEM
	Liveweight performance					
Day 1	45.05	44.78	44.97	44.20	44.83	0.22
Day 7	145.00	145.87	142.16	139.16	138.42	2.13
Day 14	411.30	408.12	409.47	402.02	397.73	4.68
Day 21	856.43	839.90	849.54	829.72	831.15	6.77
Day 28	1430.76	1386.80	1417.21	1406.92	1376.07	14.71
Day 35	2135.64	2082.01	2144.57	2116.48	2070.01	18.27
	Feed Intake					
Day 1 - 7	124.33	113.85	115.64	114.01	109.83	4.21
Day 1 - 14	441.88	445.44	444.06	446.77	426.97	6.67
Day 1 - 21	966.26	1013.03	984.26	976.80	922.35	21.77
Day 1 - 28	2027.72	2111.83	2062.82	2050.34	2030.52	20.04
Day 1 - 35	3968.89	4088.27	4014.10	4041.57	3964.76	44.65
	Feed Conversion					
Day 1 - 7	0.86	0.78	0.82	0.82	0.79	0.03
Day 1 - 14	1.08	1.09	1.09	1.11	1.08	0.01
Day 1 - 21	1.13	1.21	1.16	1.18	1.11	0.03
Day 1 - 28	1.42	1.53	1.46	1.18	1.48	0.02
Day 1 - 35	1.86^{b}	1.97^{a}	1.87^{ab}	1.91^{ab}	1.92^{ab}	0.02

[1]Mean ± SE of four replicates.

[2]CTL-: antibiotic free diet; CTL+: commercial type diet containing 11 mg / kg virginiamycin; MOS: CTL- + 0.2 % and 0.1 % Bio-Mos in the starter (1 to 21 d) and grower feed (22 to 38 d), respectively; LL: CTL- + 1.25 % Alcell lignin; HL: CTL- + 2.5 % Alcell lignin.

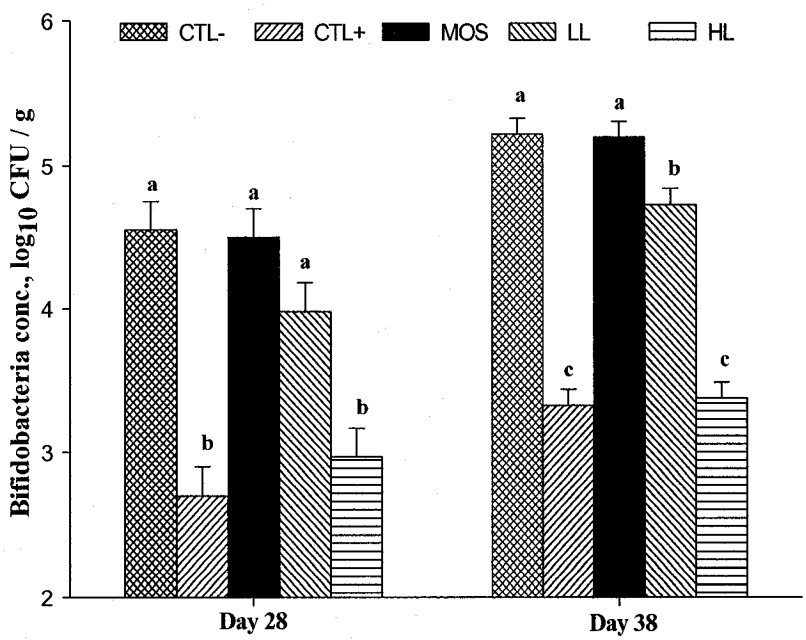
[a,b] Values with different superscript within same row are different (Bonferroni t-test, $P < 0.05$).

FIGURE 4.1. Concentrations (log_{10} CFU / g) of *Lactobacilli* in the ceca of broiler chickens fed CTL- (antibiotic free diet); CTL+ (commercial diet containing 11 mg / kg virginiamycin); MOS: (CTL- + 0.2 % and 0.1 % Bio-Mos in the starter (1 to 21 d) and grower feed (22 to 38 d), respectively); LL: (CTL- + 1.25 % Alcell lignin); HL: (CTL- + 2.5 % Alcell lignin) diets.



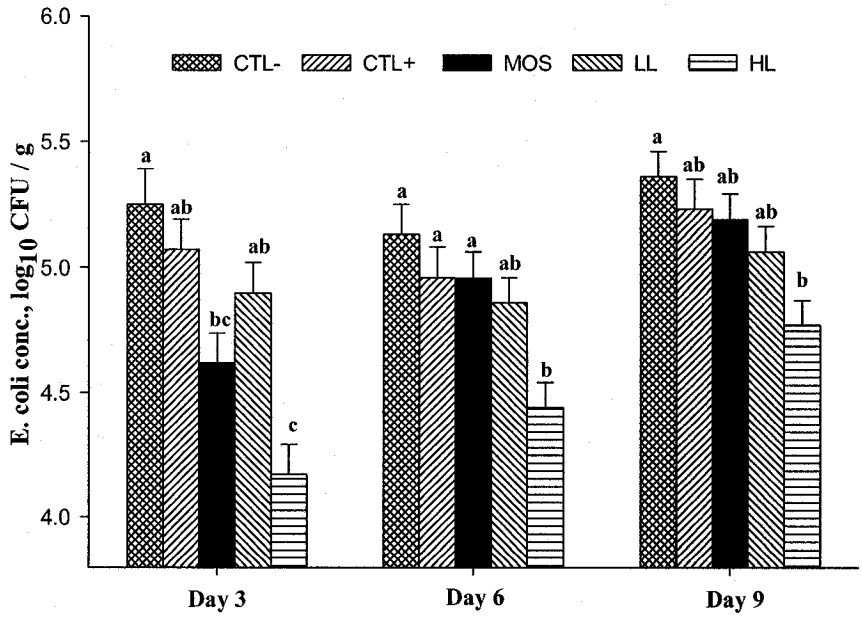
[a,b,c,d] Values with different superscript within a group are different (Bonferroni t-test, $P < 0.05$).

FIGURE 4.2. Concentrations (log_{10} CFU / g) of *Bifidobacteria* in the ceca of broiler chickens fed CTL- (antibiotic free diet); CTL+ (commercial diet containing 11 mg / kg virginiamycin); MOS: (CTL- + 0.2 % and 0.1 % Bio-Mos in the starter (1 to 21 d) and grower feed (22 to 38 d), respectively); LL: (CTL- + 1.25 % Alcell lignin); HL: (CTL- + 2.5 % Alcell lignin) diets.



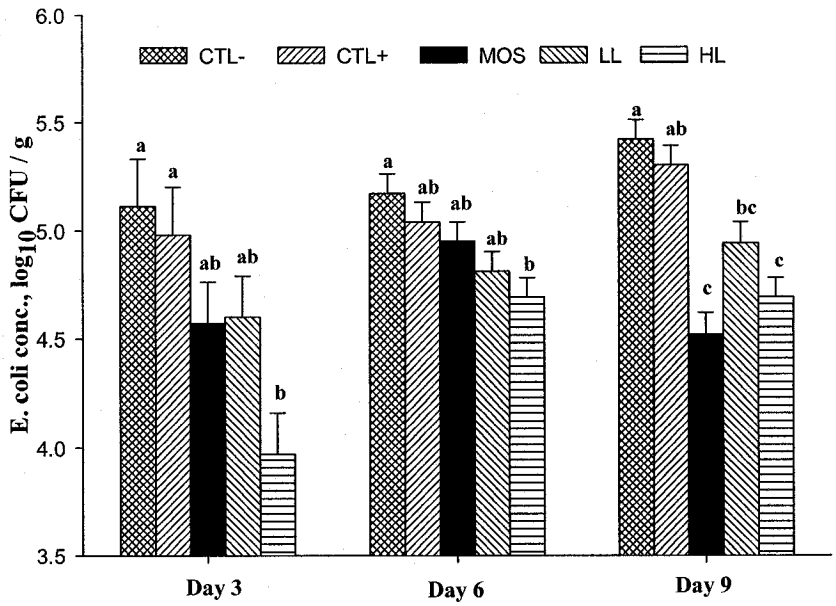
[a,b,c] Values with different superscript within a group are different (Bonferroni t-test, $P < 0.05$).

FIGURE 4.3. Concentrations (log_{10} CFU / g) of *E. coli* in the ceca of PBS gavaged broiler chickens fed CTL- (antibiotic free diet); CTL+ (commercial diet containing 11 mg / kg virginiamycin); MOS: (CTL- + 0.2 % and 0.1 % Bio-Mos in the starter (1 to 21 d) and grower feed (22 to 38 d), respectively); LL: (CTL- + 1.25 % Alcell lignin); HL: (CTL- + 2.5 % Alcell lignin) diets.



[a,b,c] Values with different superscript within a group are different (Bonferroni t-test, $P < 0.05$).

FIGURE 4.4. Concentrations (log_{10} CFU / g) of *E. coli* in the ceca of *E. coli* challenged broiler chickens fed CTL- (antibiotic free diet); CTL+ (commercial diet containing 11 mg / kg virginiamycin); MOS: (CTL- + 0.2 % and 0.1 % Bio-Mos in the starter (1 to 21 d) and grower feed (22 to 38 d), respectively); LL: (CTL- + 1.25 % Alcell lignin); HL: (CTL- + 2.5 % Alcell lignin) diets.



[a,b,c] Values with different superscript within a group are different (Bonferroni t-test, $P < 0.05$).

CHAPTER 5:

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

Under the conditions of this study, conducted in a newly-constructed experimental poultry facility, there was no beneficial effect of an antibiotic growth promotant (virginiamycin) on production performance (growth rate, feed intake and feed conversion ratio) of broilers. Furthermore, the addition of MOS or lignin to AGP-free diets did not alter broiler performance. The dietary additives, in experiment 1, depressed feed intake and this probably depressed growth rate; however, in experiment 2, feed intake was not altered. Differences between the two batches of feed and ingredient inclusion rates might have contributed to the differential responses in feed intake in the two experiments.

The addition of MOS to AGP-free diets significantly increased villi height and goblet cell number in the jejunum, and increased the populations of *Lactobacilli* and *Bifidobacteria* in the cecal digesta. The LL diet increased the cecal populations of *Lactobacilli* and *Bifidobacteria* when compared to the AGP diet, but there were no benefits when compared to the AGP-free diet. Results also indicated that AGP and the high level of lignin (2.5 % of DM) strongly inhibited the cecal growth of *Lactobacilli* and *Bifidobacteria*.

Results from the challenge study with pathogenic strains of *E. coli* (serotypes O2 and O88) indicated that MOS and LL had comparable advantage over AGP, in lowering the cecal population of total *E. coli*. The high level of lignin (HL) had a more pronounced effect in reducing *E. coli* load, but this treatment also caused a major reduction in the cecal populations of *Lactobacilli* and *Bifidobacteria*, which is not desired. Rather than using AGP, MOS and LL represent better dietary strategies to reduce the intestinal loads of *E. coli*, as a means of minimizing food-borne illnesses from poultry products.

The results of this study also indicate that the dietary addition of MOS, and to a lesser extent lignin, significantly reduced the proliferation of *E. coli* in poultry litter when compared to diets containing AGP. The prebiotic additives (MOS and lignin) provide, therefore, an opportunity to control cellulitis, a major cause of poultry carcass condemnation and economic losses in Canada.

The poultry industry could benefit from the use of MOS and lignin as alternatives to antibiotics as these natural additives attempt to address issues of economic efficiency, food safety, and antibiotic-resistance in bacteria of human and chicken concerns.

LIMITATIONS OF THE STUDY

1. A limitation of this study is the failure to obtain consistency in broiler performance between the two experiments. This probably arose as a result of the differential responses in feed intake in the two experiments. These experiments were conducted with two batches of feed and the ingredient composition varied due to least cost formulation by the commercial feed manufacturer.

2. *E. coli* was not enumerated from the cecal digesta in experiment 1, but *E. coli* was enumerated in experiment 2. This lack of consistency in *E. coli* results may demonstrate limitations of the methodology used for *E. coli* enumeration. Rather than using the total plate count method, the PCR technique would have been the more appropriate method to enumerate cecal *E. coli* in experiment 1. Furthermore, due to possibility of low levels of microbial contamination of the newly-constructed experimental poultry facility, the need for the more sensitive PCR technique would have been greater in experiment 1 than in experiment 2.

AREAS FOR FUTURE RESEARCH

1. A dose response study of the effects of MOS on gut integrity and intestinal microbiology (*Lactobacilli*, *Bifidobacteria*, *E. coli* and *Salmonella*) in broilers to determine the optimal dietary inclusion rate.

2. The effects of combining MOS and lignin on gut integrity and intestinal microbiology in broilers might be an interesting area to explore.

3. The effects of MOS and lignin on intestinal concentrations of *Campylobacter* and *Salmonella*, other principal pathogenic bacteria causing food-borne illnesses, need to be determined.

4. A major benefit of dietary addition of AGP is the control of *Clostridium perfringens*, intestinal pathogenic bacteria that causes enteritidis, leading to serious economic losses in poultry production. The efficacy of MOS and lignin to control intestinal *C. perfringens*, therefore, needs to be assessed.

5. A comparative study of the antimicrobial effects of the poly-phenolic fragments of Alcell lignin with mono-phenolic compounds, such as carvacrol, thymol and cinnamaldehyde, would provide useful information on any benefits of a poly-phenolic compound on the intestinal microbial ecology of chickens.

REFERENCES

Aabo, S., J. P. Christensen, M. S. Chadfield, B. Carstensen, T. K. Jensen, M. Bisgaard, and, J. E. Olsen. 2000. Development of an *in vivo* model for study of intestinal invasion by *Salmonella enterica* in chickens. Infect. and Immun. 68(12):7122 – 7125.

Aarestrup, F. M., A. M. Seyfarth, H. D. Emborg, K. Pedersen, R. S. Hendriksen, and F. Bager. 2001. Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. Antimicrob. Agents Chemother. 45(7):2054-2059.

Albright, M. L., T. C. Schoroeder, and M. R. Langemeier. 1994. Determinants of cattle feeding cost-of-gain variability. J. Prod. Agric. 7:206-210.

Amagase, H., B. L. Petesch, H. Matsuura, S. Kasuga, and Y. Itakura. 2001. Intake of garlic and its bioactive components. J. Nutr. 131(3S):955S-962S.

Ammerman, E., C. Quarles, and P. Twining. 1988. Broiler response to the addition of dietary fructo oligosaccharides. Poult. Sci. 67:46(Suppl. 1) (Abs.).

Aptekmann, K. P., S. M. Baraldi Arton, M. A. Stefanini, and M. A. Orsi. 2001. Morphometric analysis of the intestine of domestic quails (*Coturnix coturnix japonica*) treated with different levels if dietary calcium. Anat. Histol. Embryol. 30:277-280.

Ballou, C. E. 1970. A study of immunochemistry of three yeast mannans. J. Biol. Chem. 245(5):1197-1203.

Belay, T. and R. G. Teeter. 1996. Virginiamycin and caloric density effects on live performance, blood serum metabolite concentration, and carcass composition of broilers reared in thermoneutral and cycling ambient temperatures. Poult. Sci. 75(11):1383-1392.

Blomberg, L., H. C. Krivan, P. S. Cohen, and P. L. Conway. 1993. Piglet ileal mucus protein and glycolipid (Galactosylceramide) receptors specific for *Escherichia coli* K88 fimbriae. Infect. Immun. 61(6):2526-2531.

Bozin, B., N. Mimica-Dukic, N. Simin, and G. Anackov. 2006. Characterization of the volatile composition of essential oils of some lamiaceae spices and the antimicrobial and antioxidant activities of the entire oils. J. Agri. Food Chem. 54(5):1822-1828.

Burch, D. 2006. Anticipated effects of the withdrawal of antibiotic growth promoters (AGPs) from pigs in the European Union on 1st January 2006. http://www.octagon-services.co.UK/articles/withdrawalAGP.htm Accessed August 2006.

Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phillips. 2003. The European ban on growth promoting antibiotics and emerging consequences for human and animal health. J. Antimicrob. Chemother. 52(2):159-161.

CAST. 1999. Animal agriculture and global food supply. Task force report no 135, Council for Agricultural Science and Technology, Ames, Iowa, USA.

CEAS (Centre for European Agricultural Studies). 1991. The impact on animal husbandry in the European community of the use of growth promoters. Pages 1-319 in Growth promoters in animal feed. Report to the European commission.

Chambers, J. R., J. L. Spencer, and H. W. Modler. 1997. The influence of complex carbohydrates on *Salmonella typhimurium* colonization, pH and density of broiler ceca. Poult. Sci. 76:445-451.

Collignon, P. 2004. Antibiotic growth promoters. J. of Antimicrob. Chemother. 54(1):272.

Cook, M. E. 2000. Interplay of management, microbes, genetics, immunity affects growth, development. Feedstuffs 3:11-12.

Cromwell, G. L. 1999. Safety issues, performance benefits of antibiotics for swine examined. Feedstuffs 7:18-21.

Dafwang, I. I., M. E. Cook, M. L. Sunde, and H. R. Bird. 1985. Bursal, intestinal, and spleen weights and antibody response of chicks fed sub-therapeutic levels of dietary antibiotics. Poult. Sci. 64:634-639.

Denev, S. A., I. Dinev, I. Nikiforov, and V. Koinarski. 2005. Effects of mannan oligosaccharides on composition of cecal microflora and performance of broiler chickens. Pages 351-353 in 15th European Symp. Poult. Nut. Balatonfüred, Hungary: WPSA.

Doyle, E. M. 2001. Alternatives to antibiotic use for growth promotion in animal husbandry. Food Research Institute, University of Wisconsin, Madison, US.

Edens, F. W., C. R. Parkhurst, I. A. Casas, and W. J. Dobrogosz. 1997. Principles of *ex ovo* competitive exclusion and *in ovo* administration of *Lactobacills reuteri*. Poult. Sci. 76:179-196.

Elwinger, K., E. Berndtson, B. Engstrom, O. Fossum, and L. Waldenstedt. 1998. Effect of antibiotic growth promoters and anticoccidials on growth of Clostridium perfringens in the caeca and on performance of broiler chickens. Acta Vet. Scand. 39(4):433-441.

Emborg, H., A. K. Ersboll, O. E. Heuer, and H. C. Wegener. 2001. The effect of discontinuing the use of antimicrobial growth promoters of the productivity in the Danish broiler production. Prev. Vet. Med. 50:53-70.

Engberg, R. M., M. S. Hedemann, T. D. Leser, and B. B. Jensen. 2000. Effect of zinc bacitracin and salinomycin on intestinal microflora and performance of broilers. Poult. Sci. 79:1311-1319.

Engberg, R. M., M. S. Hedemann, S. Steenfeldt, and B. B. Jensen. 2004. Influence of whole wheat and xylanase on broiler performance and microbial composition and activity in the digestive tract. Poult. Sci. 83:925-938.

Engster, H., D. Marvil, and B. Stewart-Brown. 2002. The effect of withdrawing growth promoting antibiotics from broiler chickens: a long term commercial industry study. J. of Appl. Poult. Res. 11:431-436.

Eren, M., G. Deniz, H. Birickik, S. S. Gezen, I. I. Turkemen, and H. M. Yavuz. 1999. Effects of supplementation of zinc bacitracin, mannanoligosaccharide, probiotic in broiler feeds on fattening performance. Vet. Fak. Derg. Ulu. Iniv. 18:73-84.

Fairchild, A. S., J. L. Grimes, F. T Jones, M. J. Wineland, F. W. Edens, and A. E. Sefton. 2001. Effects of Hen age, Bio-Mos and Flavomycin on poult susceptibility to oral *Escherichia coli* challenge. Poult. Sci. 80:562-571.

Ferket, P. R. 2000. Practical nutritional perspective on gut health and development. Pages 74-86 in Proc. 27th Carolina Poult. Nut. Conf. and Soybean Meal Symp., Research Triangle Park, NC, USA.

Ferket, P. R., C. W. Parks, and J. L. Grimes. 2002. Benefits of dietary antibiotic and mannanoligosaccharide supplementation for poultry. 22 Pages in Proc. Multi-State Poult. Feeding and Nutr. Conf., Indianapolis, Indiana, USA.

Ferket, P. R. 2004. Alternatives to antibiotics in poultry production: responses, practical experience and recommendations. In Re-imaging the feed industry. Pages 56-67 in Proc. Alltech's 20th Ann. Symp. T. P. Lyons and K. A. Jacques, ed. Nottingham Univ. Press, Nottingham, UK.

Fernandez, M. I. and B. W. Woodward. 1999. Comparison of conventional and organic beef production systems. I. Feedlot performance and production costs. Livestock Prod. Sci. 61:213-223.

Fernandez, F., M. Hinton, and B. Van Gils. 2002. Dietary mannan oligosaccharides and their effect on chicken caecal microflora in relation to *Salmonella enteritidis* colonization. Avian Path. 31: 49-58.

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. J. Food Prot. 62(3):229-233.

Fuller, R. 1989. Probiotics in man and animals. J. App. Bacteriol. 66(5):365-378.

Garrido, M. N., M. Skjervheim, H. Oppegaard, and H. Sorum. 2004. Acidified litter benefits the intestinal flora balance of broiler chickens. Appl. Environ. Microbiol. 70(9):5208-5213.

Gaskins, H. R., C. T. Collier, and D. B. Anderson. 2002. Antibiotics as growth promotants mode of action. Anim. Biotechnol. 13(1):29-42.

Gast, R. K. and S. T. Benson. 1995. The comparative virulence for chicks of *Salmonella enteritidis* phage type 4 isolates and phage types commonly found in poultry in the United States. Avian Dis. 39(3):567-574.

Gauthier, R. 2002. Organic acids and essential oils. A realistic alternative to antibiotic growth promoters in pigs and poultry. 10 Pages in Today's feed additives in animal feeding: Productivity and compliance. 18th Annual convention Philsan. Makai, Philippines.

Gibson, G. R. 1998. Dietary modulation of human gut microflora using prebiotics. Br. J. Nutr. 80(4): S209-S212.

Gibson, G. R. and X. Wang. 1994. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. J. Appl. Bacteriol. 77(4):412-420.

Gibson, G. R. and M. B. Roberfroid. 1995. Dietary modulation of the human colonic microbiotica: Introducing the concept of prebiotics. J. Nutr. 125(6):1404-1412.

Gordon, H. A. and E. Bruckner-Kardoss. 1961. Effect of normal microflora on intestinal surface area. Am. J. Physiol. 201:175-178.

Guillemot, D., C. Carbon, B. Balkau, P. Geslin, H. Lecoeur, F. Vauzelle-kervroedan, G. Bouvenot, and E. Eschwege. 1998. Low dosage and long treatment duration of beta-lactam: risk factors for carriage of penicillin-resistant *Streptococcus pneumoniae.* JAMA 279(5):365-370.

Gunal, M., G. Yayli, O. Kaya. N. Karahan, and O. Sulak. 2006. The effects of Antibiotic Growth Promoter, Probiotic or Organic Acid supplementation on performance, intestinal microflora and tissue of broilers. Inter. J. Poult. Sci. 5(2):149-155.

Gustafson, R. H. and R. E. Bowen. 1997. Antibiotic use in animal agriculture. J. Appl. Microbiol. 83(5):531-541.

Helander, I. M., H. L. Alakomi, K. Latva-Kala, T. Mattila-Sandhol, I. Pol, E. J. Smid, L. G. M. Gorris, and A. Von Wright. 1998. Chracterization of the action of selected essential oil components on gram-negative bacteria. J. Agri. Food Chem. 46:3590-3595.

Henry, P. R., C. B. Ammerman, and R. D. Miles. 1986. Influence of virginiamycin and dietary manganese on the performance, manganese utilization, and intestinal tract weight of broilers. Poult. Sci. 65:321-324.

Heyndrickx, M., D. Vandekerchove, L. Herman, I. Rollier, K. Grijspeerdt, and L. De Zutter. 2002. Routes for *Salmonella* contamination of poultry meat: epidemiological study from hatchery to slaughterhouse. Epidemiol. Infect. 129(2):253-265.

Hidaka, H. and M. Hirayama. 1991. Useful characteristics and commercial applications of fructo oligosaccharides. Biochem. Soc. Transact. 19:561-565.

Hogberg, A., J. E. Lindberg, T. Leser, and P. Wallgren. 2004. Influence of cereal non-starch polysaccharides and enzymes supplementation on digestion site and gut environment in weaned piglets. Acta Vet. Scand. 45(1-2):87-98.

Hooge, D. M. 2004. Meta-analysis of broiler chicken pen trials evaluating dietary mannan oligosaccharides, 1993-2003. Inter. J. Poult. Sci. 3(3):163-174.

Hughes, P. and J. Heritage. 2004. Antibiotic growth-promoters in food animals. in Assessing quality and safety of animal feeds. FAO Animal Production and Health.

Humphrey, B. D., E. A. Koutsos, and K. C. Klasing. 2002. Requirements and priorities of the immune system for nutrients. Pages 69-77. in Biotechnology in the Feed and Food Industry, Proc. of Alltech 18th Annu. Symp. T.P. Lyons and K.A. Jacques eds. Nottingham University Press, Nottingham, UK.

Huyghebaert, G. 2003. Replacement of antibiotics in poultry. in Eastern Nutrition Conference, Canada.

Iji, P., A. A. Saki, and D. R. Trivey. 2001. Intestinal structure and function of broiler chickens on diets supplemented with a mannan oligosaccharide. J. Sci. Food Agri. 81:1186-1192.

Janeway, C. A. 1993. How the immune system recognizes invaders. Sci. Am. 269(3):72-79.

JETACAR. 1999. The use of antibiotics in food-producing animals: antibiotic-resistant bacteria in animals and humans. Report of the Joint Expert Advisory Committee on Antibiotic Resistance. Commonwealth of Australia, Canberra, Australia.

Jin, L. Z., Y. W. Ho, N. Abdullah, M. A. Ali, and S. Jalaludin. 1996a. Influence of dried *Bacillus subtillis* and *Lactobacilli* cultures on intestinal microflora and performance in broilers. Asian Astrl. J. Ani. Sci. 9:397-403.

Jin, L. Z., Y. W. Ho, N. Abdullah, M. A. Ali, and S. Jalaludin. 1996b. Antagonistic effects of intestinal *Lactobacillus* isolates on pathogen of chicken. Lett. Appl. Microbiol. 23:67-71.

Jung, H. G. and G. C. Fahey. 1983. Nutritional Implications of Phenolic Monomers and Lignin: A Review. J. Ani. Sci. 57(1):206-219.

Kaldhusdal, M. and E. Skjerve. 1996. Association between cereal contents in the diet and incidence of necrotic enteritis in broiler chickens in Norway. Prev. Vet. Med. 28:1-16.

Kawai, Y., Y. Ishii, K. Arakawa, K. Uemura, B. Saitoh, J. Nishimura, H. Kitazawa, Y. Yamazaki, Y. Tateno, T. Itoh, and T. Saito. 2004. Structural and functional differences in two cyclic bacteriocins with the same sequences produced by Lactobacilli. Appl. Environ. Microbiol. 70(5):2906-2911.

Klasing, K. C. 1988. Nutritional aspects of leukocytic cytokines. J. Nutr. 118(12):1436-1446.

Kumor, L. W., A. A. Olkowski, S. M. Gomis, and B. J. Allan. 1998. Cellulitis in broiler chickens: epidemiological trends, meat hygiene, and possible human health implications. Avian Dis. 42(2): 285-291.

Kumprecht, I. and F. Zobac. 1997. The effect of mannan oligosaccharides in feed mixtures on the performance of broilers. Zivocisca Vyroba 42:117-124.

Langhout, P. 2000. New additives for broiler chickens. Pages 24-27 in Feed Mix Special: Alternatives to antibiotics.

Lee, H. S. and Y. J. Ahn. 1998. Growth-inhibiting effects of cinnamonum cassia bark-derived materials on human intestinal bacteria. J. Agric. Food Chem. 46(1):8-12.

Lee, K. W., H. Everts, and A. C. Beynen. 2004. Essential oils in broiler nutrition. Inter. J. Poult. Sci. 3(12):738-752.

Lees, P. and F. S. Aliabadi. 2002. Rational dosing of antimicrobial drugs: animals versus humans. Inter. J. of Antimicrob. Agents 19:269-284.

Levy, S. B. 2001. Antibiotic resistance: consequences of inaction. Clin. Infect. Dis. 33:S124-S129 (Suppl. 3)

Lora, J. H., A. W. Creamer, L. C. F. Wu, and G. C. Goyal. 1993. Industrial scale production of organosolv lignins: characteristics and applications. Pages 252-256 in Cellusosics: Chemical, biochemical and material aspects. J.F. Kennedy, G.O. Phillips, and P.A. Williams, eds., Ellis Horwoods Ltd, Sussex.

Mallon, D. J., J. E. Corkill, S. M. Hazel, J. S. Wilson, N. P. French, M. Bennett, and C. A. Hart. 2002. Excretion of vancomycin-resistant enterococci by wild mammals. Emerg. Infect. Dis. 8(6):636-638.

Mayrhofer, S., P. Paulsen, F. J. Smulders, and F. Hilbert. 2004. Antimicrobial resistance profile of five major food-borne pathogens isolated from beef, pork and poultry. Int. J. Food Microbiol. 97(1):23-29.

McDonald, L. C., F. J. Chen, H. J. Lo, H. C. Yin, P. L. Lu, C. H. Huang, P. Chen, T. L. Lauderdale, and M. Ho. 2001. Emergence of reduced susceptibility and resistance to fluoroquinolones in *Escherichia coli* in Taiwan and contributions of distinct selective pressures. Antimicrob. Agents Chemother. 45(11):3084-3091.

Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. 5(5):607-625.

Menao, M. C., C. S. A. Ferreira, A. G. M. Castro, T. Knobl, and A. J. Pianntino Ferreira. 2002. Sorogrupos de *Escherichia Coli* isolados de frangos com doenca respiratoria cronica. Arq. Inst. Biol. 69(4):15-17.

Miles, R. D. and R. H. Harms. 1984. Influence of virginiamycin on broiler performance, uniformity and litter quality. Nut. Reports Inter. 29:971-975.

Miles R. D., G. D. Butcher, P. R. Henry, and R. C. Littell. 2006. Effect of antibiotic growth promoters on broiler performance, intestinal growth parameters and qualitative morphology. Poult. Sci. 85:476-485.

Mirelman, D., G. Altmann, and Y. Eshdat. 1980. Screening of bacterial isolates for mannose-specific lectin activity by agglutination of yeasts. J. Clinical Microbiol. 11(4):328-331.

Mitjans, M., L. Garcia, E. Marrero, and M. P. Vinardell. 2001. Study of Ligmed-A, an antidiarrheal drug based on lignin, on rat small intestine enzyme activity and morphometry. J. of Vet. Pharmacol. Ther. 24:349-351.

Mitsuoka, T., H. Hidaka, and T. Eida. 1987. Effect of fructo oligosaccharides on intestinal microflora. Die Nahrung 31:427-436.

Moharrery, A. and M. Mahzonieh. 2005. Effect of malic acid on visceral characteristics and coliform counts in small intestine in the broiler and layer chickens. Inter. J. Poult. Sci. 4(10):761-764.

Moore, P. R., A. Evenson, T. D. Luckey, E. McCoy, C. A. Elvehjem, and E. B. Hart. 1946. Use of sulfasuxidine, streptothricin and streptomycin in nutritional studies with the chick. J. Biol. Chem. 165:437-441.

Morrell, V. 1997. Antibiotic resistance: Road of no return. Sci. 278(5338):575-576.

Murakami, H., Y. Akiba, and M. Horiguchi. 1994. A modified bioassay for energy utilization in newly hatched chicks.1. Determination of optimum duration for feed withdrawal and excreta collection. Poult. Sci. 73:1094-1106.

Murry, A. C., A. Jr. Hinton, and R. J Buhr. 2004. Effect of a probiotic containing two *Lactobacillus* strains on growth performance and population of bacteria in the ceca and carcass rinse of broiler chickens. Poult. Sci. 83(Suppl.1):322. (Abstr.).

Nelson, J. L., J. W. Alexander, L. Gianotti, C. L. Chalk, and T. Pyles. 1994. Influence of dietary fiber on microbial growth in vitro and bacterial translocation after burn injury in mice. Nutrition 10(1):32-36.

Newman, K. 1994. Mannan-oligosaccharides: Natural polymers with significant impact on the gastrointestinal microflora and the immune system. Pages 167-174 in Proc. Alltech's 10th Ann. Symposium: Biotechnology in the feed industry. T.P. Lyons and K.A. Jacques, eds. Nottingham University Press, Nottingham, UK.

NRC (National Research Council). 1980. The effects on human health of subtherapeutic use of antimicrobials in animal feeds. National Academy of Sci., Washington, DC.

NRC (National Research Council). 1994. Nutrient Requirements of Poultry. 9th rev. ed. National Academy Press, Washington, DC.

Odetallah, N. H., C. W. Parks, and P. F. Ferket. 2002. Effect of wheat enzyme preparation on the performance characteristics of tom turkeys fed wheat-based rations. Poult. Sci. 81:987-994.

Odetallah, N. H., J. J. Wang, J. D. Garlich, and J. C. H. Shih. 2003. Keratinase in starter diets improves growth of broiler chicks. Poult. Sci. 82:664-670.

Ofek, I., D. Mirelman, and N. Sharon. 1977. Adherence of *Escherichia Coli* to human mucosal cells mediated by mannose receptors. Nature 265:623-625.

Oussalah, M., S. Caillet, and M. Lacroix. 2006. Mechanism of action of Spanish oregano, Chinese cinnamon, and savory essential oils against cell membranes and walls of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. J. Food. Prot. 69(5):1046-1055.

Page, S. W. 2003. The role of enteric antibiotics in livestock production. in A review of published literature. Avcare Limited, Canberra, Australia.

Parks, C. W., J. L. Grimes, and P. R. Ferket. 2005. Effects of virginiamycin and a mannan-oligosaccharide-virginiamycin shuttle program of the growth and performance of large white female turkeys. Poult. Sci. 84:1967-1973.

Patterson, J. A., J. I. Orban, A. L. Sutton, and G. N. Richards. 1997. Selective enrichment of bifidobacteria in the intestinal tract of broilers by thermally produced ketoses and effect on broiler performance. Poult. Sci. 46:497-500.

Patterson, J. A. and K. M. Burkholder. 2003. Application of prebiotics and probiotics in poultry production. Poul. Sci. 82:627-631.

Pedroso, A. A., J. F. Menten, M. R. Lambais, A. M. Racanicci, F. A. Longo, and J. O. Sorbara. 2006. Intestinal bacterial community and growth performance of chickens fed diets containing antibiotics. Poult. Sci. 85(4):747-752.

Pelicano, E. R. L., P. A. Souza, H. B. A. Souza, D. F. Figueiredo, M. M. Boiago, S. R. Carvalho, and V. F. Bordon. 2005. Intestinal mucosa development in broiler chickens fed natural growth promoters. Rev. Bras. Cienc. Avic. 7(4): 221-229.

Pfizer Animal Health. 1999. Anitibiotic use is animals. The Poultry Informed Professional 27:1-8.

Phillip, L. E., E. S. Idziak, and S. Kubow. 2000. The potential use of Lignin in animal nutrition, and in modifying microbial ecology of the gut. in Eastern Nut. Conf., Animal Nutrition Association of Canada, QC, Canada.

Phillips, I. 1999. Assessing the evidence that antibiotic growth promoters influence human infections. J. Hosp. Infect. 43:173-178.

Phillips, I., M. Casewell, T. Cox, B. De Croot, C. Friis, R. Jones, C. Nightingale, R. Preston, and J. Waddell. 2004. Does the use of antibiotics in food animals pose a risk to human health?. A critical review of published data. J. Antimicrob. Chemother. 53(1):28-52.

Pye, K. 1996. The production of non-wood papermaking fibres by the Alcell process. Pages 1-17 in Proc. PIRA Conf. PIRA International. Peterborough, UK.

Rada, V., K. Sirotek, and J. Petr. 1999. Evaluation of selective media for Bifidobacteria in poultry and rabbit caecal samples. J. Vet. Med., 46:369-373.

Rastall, R. A., G. R. Gibson, H. S. Gill, F. Guarner, T. R. Klaenhammer, B. Pot, G. Reid, I. R. Rowland, and M. E. Sanders. 2005. Modulation of the microbial ecology of the human colon by probiotics, prebiotics, and symbiotics to enhance human health: An overview of enabling science and potential applications. FEMS Microbiol. Ecol. 52:145-152.

Revington, B. 2002. Feeding poultry in the post-antibiotic era. Pages 1 -14. in Proc. Multi-State Poult. Feeding and Nutr. Conf., Indianapolis, Indiana, USA.

Ricke, S. C., P. J. Van der Aar, G. C. Fahey, and L. Berger. 1982. Influence of dietary fibres on performance and fermentation characteristics of gut contents from growing chicks. Poult. Sci. 61:1335-1343.

Rodriguez-Morales, S., R. L. Compadre, R. Castillo, P. J. Breen, and C. M. Compadre. 2005. 3D-QSAR, synthesis, and antimicrobial activity of 1-alkylpyridinium compounds as potential agents to improve food safety. Eur J Med Chem. 40(9):840-9.

Rolfe, R. D. 2000. The role of probiotic cultures in the control of gastrointestinal health. J. Nutr. 130(2S Suppl):396S-402S.

Rosen, G. D. 1995. Antibacterials in poultry and pig nutrition. Pages 143-172 in Biotechnology in animal feeds and animal feeding. R.J. Wallace and A. Chesson, eds. VCH Verlagsgesellschaft mbH, Weinheim, Germany.

Rosen, G. D. 2001. Multi-factorial efficacy evaluation of alternatives to antimicrobials in pronutrition. Proc. BSAS Meeting, York, UK.

Ross, D. F., B. T. Cartwright, M. R. Doshi, and H. S. Dugal. 1986. Ultrafiltration of kraft black liquor. Pages 1 – 15 in IPC Technical Paper Series No. 199. The Institute of paper chemistry, Appleton, Wisconsin.

Russell. J. B. and F. Diez-Gonzales. 1998. The effects of fermentation acids on bacterial growth. Adv. Micro. Physiol. 39:205-234.

Rybolt, M. L., R. W. Wills, and R. H. Bailey. 2005. Use of secondary enrichment for isolation of *Salmonella* from naturally contaminated environmental samples. Poult. Sci. 84(7):992-997.

Sackey, B. A., P. Mensah, E. Collison, and E. Sakyi-Dawson. 2001. *Campylobacter*, *Salmonella*, *Shigella* and *Escherichia coli* in live and dressed poultry from metropolitan accra. Int. J. Food Microbiol. 71(1):21-28.

Safalaoh, A. C. L. 2006. Body weight gain, dressing percentage, abdominal fat and serum cholesterol of broilers supplemented with a microbial preparation. African J. Food Agri. Nutri. and Develop. (AJFAND), 6(1): 1-10.

Salminen, S., C. Bouley, M. C. Boutron-Ruault, J. H. Cummings, A. Franck, G. R. Gibson, E. Isolauri, M. C. Moreau, M. Roberfroid, and I. Rowland. 1988. Functional food science and gastrointestinal physiology and function. Br. J. Nutr. 80:147-171.

SAS Institute, 2003. SAS User's Guide. Version 9.1 ed. SAS Institute Inc., Cary, NC.

Savage, T. F. and E.I. Zakrzewska. 1996. The performance of male turkeys fed a starter diet containing a mannan oligosaccharide (Bio-Mos) from day old to eight weeks of age. Pages 47-54 in Proc. Alltech's 12th Ann. Symposium: Biotechnology in the Feed Industry. T.P. Lyons and K.A. Jacques, eds. Nottingham University Press, Nottingham, UK.

SCAN. 1998. Opinion of the scientific committee for animal nutrition on the immediate and long term risk to the value of streptogamins in human medicine posed by the use of virginiamycin as an animal growth promoter.

Schrader, J. S., R. S. Singer, and E. R. Atwill. 2004. A propective study of management and litter variables associated with cellulitis in California broiler flocks. Avian Dis. 48(3): 522-530.

Shafey, T. M., S. Al-Mufarej, M. I. Shalaby, and A. J. Jarelnabi. 2001. Effects of mannan oligosaccharides on antibody response to infectious bronchitis, infectious bursal disease and Newcastle disease in chickens. J. Appl. Anim. Res. 19:117-127.

Sims, M. D., K. A. Dawson, K. E. Newman, P. Spring, and D. M. Hooge. 2004. Effects of mannan oligosaccharide, bacitracin methylene disalicyclate, or both on live performance and intestinal microbiology of turkeys. Poult. Sci. 83:1148-1154.

Smirnov, A., R. Perez, E. Amit-Romach, D. Sklan, and Z. Uni. 2005. Mucin dynamics and microbial populations in chicken small intestine are changed by dietary probiotic and antibiotic growth promoter supplementation. J. Nutr. 135(2):187-192.

Smith, D. L., J. A. Johnson, A. D. Harris, J. P. Furuno, E. N. Perencevich, and J. G. Morris. 2003. Assessing risks for a pre-emergent pathogen: virginiamycin use and the emergence of streptogramin resistance in *Enterococcus faecium*. Lancet Infect. Dis. 3(4):241-249.

Soil Association. 2001. Antibiotics – use and mis-use. in: Soil Association Library. Bristol, UK.

Solis de los Santos, F., M. B. Farnell, G. Tellez, J. M. Balog, N. B. Anthony, A. Torres-Rodriguez, S. Higgins, B. M. Hargis, and A. M. Donoghue. 2005. Effect of prebiotic on gut development and ascites incidence of broilers reared in a hypoxic environment. Poult. Sci. 84:1092-1100.

SOU. 1997. Antimicrobial feed additives. in Swedish commission on antimicrobial feed additives, Ministry of Agriculture, Stockholm.

Spring, P., C. Wenk, K. A. Dawson, and K. E. Newman. 2000. The effects of dietary mannan-oligosaccharides on cecal parameters and the concentrations of Enteric bacteria in the ceca of *Salmonella* challenged broiler chicks. Poult. Sci. 79:205-211.

Stanley, V. G., C. Brown, and A. E. Sefton. 2000. Comparative evaluation of a yeast culture, mannan-oligosaccharide and an antibiotic on performance of turkeys. Poult. Sci. 79(Suppl. 1):117.(Abstr.).

Stappenbeck, T. S., L. V. Hooper, and J. I. Gordon. 2002. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. Proc. Natl. Acad. Sci. U.S.A. 99(24):15451-15455.

Stutz, M. W., S. L. Johnson, and F. R. Judith. 1983. Effects of diet, bacitracin, and bodyweight restrictions on the intestine of broiler chicks. Poult. Sci. 62:1626-1632.

Stutz, M. W. and G. C. Lawton. 1984. Effects of diet and antimicrobials on growth, feed efficiency, intestinal *Clostridium perfringens*, and ileal weight of broiler chicks. Poult. Sci. 63:2036-2042.

Tazume, S., A. Ozawa, T. Yamamoto, Y. Takahashi, K. Takeshi, S. M. Sairi, C. G. Ichorob, and P. G. Waiyaki. 1993. Ecological study on intestinal bacterial flora of patients with diarrhoea. Clin. Infect. Dis. 16(2):S77-S82 (Suppl.).

Thomke, S. and K. Elwinger. 1998. Growth promotants in feeding pigs and poultry. I. Growth and feed efficiency responses to antibiotic growth promotants. Annales de Zootechnie 47:85-97.

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 Dis. 41(2):433-437.

Truscott, R. B. and A. Al-Sheikhly. 1977. The production and treatment of necrotic enteritis in broilers. Am. J. Vet. Res. 38(6):857-861.

Union of Concerned Scientists. 2001. Hogging It!: Estimates of Antimicrobial Abuse in Livestock. http://www.ucsusa.org/food_environment/antibiotics_and_food/hogging-it-estimates-of-antimicrobial-abuse-inlivestock.html. Accessed September 2006.

Valencia, Z. and E. R. Chavez. 1997. Lignin as a purified dietary fiber supplement for piglets. Nutr. Res. 17(10):1517-1527.

Van den Bogaard, A. E., L. B. Jensen, and E. E. Stobberingh. 1997. Vancomycin-resistant enterococci in turkeys and farmers. N. Engl. J. Med. 337(21):1558-1559.

Van den Bogaard, A. E., N. London, C. Driessen, and E. E. Stobberingh. 2001. Antibiotic resistance of fecal *E. coli* in poultry, poultry farmers and poultry slaughters. J. Antimicrob. Chemother. 47(6):763-771.

Van der Wielen, P. W., L. J. Lipman, F. Van Knapen, and S. Biesterveld. 2002. Competitive exclusion of *Salmonella* enterica serovar *Enteritidis* by *Lactobacillus crispatus* and *Clostridium lactatifermentans* in a sequencing fed-batch culture. Appl. Environ. Microbiol. 68(2):555-559.

Van Immerseel, F., J. De Buck, I. De Smet, F. Pasmans, F. Haesebrouck, and R. Ducatelle. 2004. Interactions of butyric acid- and acetic acid-treated *Salmonella* with chicken primary cecal epithelial cells in vitro. Avian Dis. 48(2):384-391.

Vijaya Kumar, S. G., S. K. Singh, P. Goyal, N. Dilbaghi, and D. N. Mishra. 2005. Beneficial effects of probiotics on human health. Pharmazie 60(3):163-171.

Vo, A. T., E. Van Duijkeren, A. C. Fluit, W. J. Wannet, A. J. Verbruggen, H. M. Maas, and W. Gaastra. 2006. Antibiotic resistance, integrons and *Salmonella* genomic island 1 among non-typhoidal *Salmonella* serovars in The Netherlands. Int. J. Atimicrob. Agents. 28(3):172-179.

Waldroup, P. W., E. O. Oviedo-Rondon, and C. A. Fritts. 2003a. Comparison of Bio-Mos and antibiotic feeding programs in broiler diets containing copper sulphate. Inter. J. Poult. Sci. 2(1):28-31.

Waldroup, P. W., C. A. Fritts, and F. Yan. 2003b. Utilization of Bio-Mos mannan oligosaccharide and Bioplex copper in broiler diets. Inter. J. Poult. Sci. 2(1):44-52.

Walton, J. R. 1988. The modes of action and safety aspects of growth promoting agents. Pages 92-97 in Proc. Maryland Nut. Conf., University of Maryland, College Park, MD.

Weese, J. S. 2002. Microbiologic evaluation of commercial probiotics. J. Am. Vet. Med. Assoc. 220(6):794-797.

Williams, P. and R. Losa. 2001. The use of essential oils and their compounds in poultry nutrition. World's Poult. Sci. 17.14-15.

Wireup, M. 2001. The Swedish Experience of the 1986 year ban of antimicrobial growth promoters, with special reference to animal health, disease prevention, productivity, and usage of antimicrobial. Microb. Drug Resist. 7(2):183-190.

Witte, W. 1999. Antibiotic resistance in Gram-positive bacteria: epidemiological aspects. J. Antimicrob. Chemother. 44:A1-9 (suppl.).

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. Poult. Sci. 82:1030-1036.

Zemek, J., B. Kosikova, J. Augustin, and D. Joniak. 1979. Antibiotic properties of Lignin components. Folia Microbiol. 24(6):483-486.

Zhao, C., B. Ge, J. De Villena, R. Sudler, E. Yeh, S. Zhao, D. G. White, D. Wagner, and J. Meng. 2001. Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the Greater Washington, D.C., area. Appl. Environ. Microbiol. 67(12):5431-5436.

Zimmerman, B., E. Bauer, and R. Mosenthin. 2001. Pro- and prebiotics in pig nutrition – potential modulators of gut health?. J. Anim. Feed Sci. 10:47-56.

Zinniel, D. K., P. Lambretch, N. B. Harris, Z. Feng, D. Kuczmarski, P. Higley, C. A. Ishimaru, A. Arunakumari, R. G. Barletta, and A. K. Vidaver. 2002. Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. Appl. Environ. Microbiol. 68(5):2198-2208.

APPENDICES

Appendix I. Effects of antibiotics, mannanoligosaccharide, and lignin on relative gut (without gizzard) weight, and weight of empty duodenum, jejunum, ileum and ceca (g / Kg body weight) of broiler chickens[1]

	Treatments[2]					
Age	CTL -	CTL +	MOS	LL	HL	SEM
			Gut			
Day 14	151.74	140.96	144.41	136.57	154.37	4.41
Day 28	108.81	103.44	97.26	108.39	102.25	5.49
Day 42	75.01	64.04	78.38	80.96	81.29	4.02
			Duodenum			
Day 14	7.92	8.45	8.25	7.84	7.22	1.09
Day 28	4.02	3.48	4.04	4.13	4.34	0.32
Day 42	3.09	2.49	3.32	3.15	3.25	0.41
			Jejunum			
Day 14	16.08	15.09	17.55	16.04	17.11	0.70
Day 28	10.76	10.79	10.64	12.45	11.68	0.71
Day 42	8.68	7.25	8.78	8.55	9.85	1.60
			Ileum			
Day 14	13.36	12.37	13.38	12.32	13.28	0.79
Day 28	10.90	11.23	10.78	12.58	10.73	0.59
Day 42	8.86	8.61	9.39	9.22	9.48	0.69
			Ceca			
Day 14	4.64	4.61	4.11	3.89	4.27	0.33
Day 28	3.43	3.10	3.34	3.47	3.42	0.19
Day 42	2.92	2.97	3.18	3.21	3.39	0.20

[1]Mean of four replicates

[2]CTL-: antibiotic free diet; CTL+: commercial type diet with 11 mg / kg virginiamycin; MOS: antibiotic free diet supplemented with 0.2 % and 0.1 % Bio-Mos in the starter (1 to 21 d) and in the grower feed (22 to 42 d), respectively; LL and HL: antibiotic free diet supplemented with 1.25 % or 2.5 % Alcell lignin, respectively

Appendix II. Effects of antibiotics, mannanoligosaccharide, and lignin on pH of duodenum, jejunum, ileum and cecal digesta of broiler chickens[1]

	Treatments[2]					
Age	CTL -	CTL +	MOS	LL	HL	SEM
			Duodenum			
Day 14	6.28	6.23	6.33	6.05	6.08	0.13
Day 28	6.28	6.30	6.28	6.25	6.33	0.02
Day 42	6.28	6.28	6.18	6.25	6.25	0.03
			Jejunum			
Day 14	6.08	6.15	6.13	6.05	6.15	0.07
Day 28	6.30	6.33	6.28	6.23	6.30	0.05
Day 42	6.23	6.18	6.13	6.23	6.13	0.10
			Ileum			
Day 14	7.15	7.38	6.70	6.50	7.28	0.16
Day 28	7.45	7.38	7.10	7.23	7.23	0.33
Day 42	7.33	6.95	6.93	6.95	6.73	0.13
			Ceca			
Day 14	6.08	5.80	5.85	6.13	5.43	0.20
Day 28	6.93	6.60	6.50	6.63	6.48	0.14
Day 42	6.90	6.75	6.70	6.78	6.73	0.08

[1]Mean of four replicates

[2]CTL$^-$: antibiotic free diet; CTL+: commercial type diet with 11 mg / kg virginiamycin; MOS: antibiotic free diet supplemented with 0.2 % and 0.1 % Bio-Mos in the starter (1 to 21 d) and in the grower feed (22 to 42 d), respectively; LL and HL: antibiotic free diet supplemented with 1.25 % or 2.5 % Alcell lignin, respectively

APPENDIX III: ANIMAL CARE PROTOCOL