Mushroom and Herb Polysaccharides as Alternative for Antimicrobial Growth Promoters in Poultry
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MUSHROOM AND HERB POLYSACCHARIDES AS
ALTERNATIVE FOR ANTIMICROBIAL GROWTH
PROMOTERS IN POULTRY

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Abstract

Antibiotics are widely used as therapeutics agents and also as growth promoters in poultry production. The possibility of developing resistant populations of bacteria and the side effects of using antibiotics as growth promoters in the farm animals has led to the recent EU-ban on the use of several antibiotics as growth promoters in poultry diets. Therefore, there is an intensive search for alternatives such as probiotics, prebiotics and other feed additives. Immuno-active polysaccharides derived from two mushrooms, *Tremella fuciformis* (*TreS*) and *Lentinus edodes* (*LenS*), and the herb *Astragalus membranaceae Radix* (*AstS*), seem to be potential alternatives for antimicrobial growth and health promoters. These products were considered to play an important role in strengthening the animals’ defense system by improving the physical conditions of gut ecosystem and enhancing functions of the immune system of chickens. The results presented in this dissertation demonstrated that intact mushroom and herb materials and their polysaccharide extracts showed differences in their physico-chemical properties, therefore, these products showed differences in fermentability and led to significant shifts in the bacterial community when fermented *in vitro*. These medicinal mushroom and herb materials, particularly their polysaccharide extracts, show promise in altering microbial activities and composition in chicken ceca. The polysaccharide extracts showed a slightly significant effect on growth performance and had no effects on weights of immune and GIT organs in normal broilers. However, the polysaccharide extracts significantly enhanced body growth and manipulated cecal microbial ecosystem such as viscosity and microbial species in *Avian mycoplasma Gallisepticum* infected chickens. And potential beneficial bacteria were significantly increased by the polysaccharide extracts. The polysaccharide extracts showed significant effects on body growth, immune responses as well as growth of immune organs and development of GIT fragments in coccidian-infected chickens, and particularly when they were used in conjunction with vaccine. The use of the mushroom and herb polysaccharide extracts might enhance T-cell immune responses, characterized by IFN-γ and IL-2 secretion, against coccidiosis in chickens. Supplementation of mushroom and herb extracts resulted in enhancement of resistance to *E. tenella* probably by enhancing both cellular and humoral immune responses against *E. tenella* in chickens.

*Keywords*: mushroom and herb polysaccharides, antimicrobial growth promoters, chickens
Preface

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For Wankun and Tian
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GENERAL INTRODUCTION
General Introduction

ANTIBIOTICS, POULTRY PRODUCTION AND PUBLIC HEALTH

It is generally agreed that requirement of nutrients such as amino acids, vitamins, minerals, fats, fibers and carbohydrates have been well established in many animal species. Feed additives, such as mold inhibitors, antioxidants, antibiotics and organic acids have been extensively used in farm animals all over the world since 1950. However, there has been growing concern about public health risks resulting from antibiotic resistance, carcinogenic responses and other side effects of residues in animal products.

Until recently antibiotics as anti-microbial growth promoters are widely used in the poultry feed industry. Growth promoting antibiotics (low levels of antibiotics) are used as manipulators of the gastrointestinal microbiota, resulting in an improvement of growth and feed efficiency (a.o., Ensminger, 1990; Peterson et al., 1991; Rosen, 1995). However, during the last few decades, concerns on the use of antibiotic growth promoters in farm animals have been raised. The ready availability of antibiotics resulted in their widespread use as growth promoters for farm animals and the possible emergence of antibiotic-resistant strains of bacteria. Moreover, the number of antibiotics available for effective use in human medicine is declining. Until recently, the concern has been principally with regard to the loss of efficacy of antibiotics as growth stimulants and the difficulty of controlling an outbreak of bacterial diseases (Newman, 2002; Wilson and Kenyon, 2002). Some early studies showed that the continuous feeding of antibiotics to chickens in the same environment resulted in a decrease or even loss of growth response (a.o., Wiese and Peterson, 1959; Nelson et al., 1963). However, supplementation of new antibiotics had no significant effect on growth, mortality or feed conversion ratio in broilers (Proudfoot et al., 1990). Worst of all, in some cases, the use of antibiotics in the farm animals result in a dramatic increase in the deaths and illness associated with antibiotic resistance, Newman, 2002.

The extensive use of antibiotic growth promoters in poultry industry has resulted in rapid appearance of resistant forms of microorganisms less sensitive to antibiotics. A study of Ma De Cesare et al. (2002) demonstrated that 19 and 81% of the poultry meat and environmental isolates analyzed were resistant to at least one of the antibiotic molecules tested (enrofloxacin, ciprofloxacin, nalidixic acid, tetracycline and erythromycin). The population of antibiotic-resistant bacteria, which was established during the time when
antibiotics were used routinely, has survived from generation to generation for over 60 years even in the absence of antibiotic exposure (Langlois et al., 1986).

Following the discovery of resistance-transfer factors in 1963, the problem has attracted attention to a broader area of public health. The most important potential route by which humans become infected with resistant bacteria is via the food chain, of which meat is the most significant source although other animal products, such as milk and eggs may be involved (Hinton, 1988). In the light of the fact that millions of birds are consumed in the world each year (according to FAO, the worldwide production of poultry meat in 2002 was 71.7 million tons), even a low incidence of cross infection could be highly significant. Hinton (1988) reported that resistant bacteria, particularly those derived from the gastro-intestinal tract (pathogenic or non-pathogenic), may be transmitted from animals to man. Once antibiotic resistance is established in a bacterial population, it is maintained for long periods of time even in the absence of the selective pressure of antibiotics (Newman, 2002). Some people are allergic to antibiotics and these drug residues could be a source of risk if the animal is sent to slaughter before the withdrawal period has elapsed.

How do bacteria become resistant to antibiotics? MacManus (1997) described that microorganisms can demonstrate either an intrinsic or an acquired resistance to antibiotics. An intrinsic resistance occurs as a result of a point mutation in the bacterial DNA in the selective pressure of antibiotic treatment. An acquired antibiotic resistance may occur in the absence of antibiotic treatment, which results from the transfer of resistant genes that may confer resistance to one or several different antibiotics from one bacterium species to another. The speed of resistance development differs with regard to the bacteria involved, the selective pressure imposed by the use of anti-microbials, and the availability of resistance genes (Schwarz et al., 2002).

Therefore, the EU has banned the use of most antibiotic growth promoters in farm animals, including poultry and is strictly controlling their therapeutic use (European Commission/Scientific Steering Committee, 1999). Non-EU countries are facing the same threat and the same strong industry opposition, but lag far behind in their response. The potential for the same restriction was found in the United States (US Newswire, 2002) and in China (Policy and Laws, Ministry of Agriculture PRC, 2002). China has established a residue control system for food-commodities of animal origin in 1999 in reference to the relevant directives of EC 96/22/EC and 96/23/EC.

The restriction on antimicrobial growth promoters will have consequences for the health and production status of farm animals, especially young pigs and broiler chickens. It has
been reported that the removal of antibiotic growth promoters has led to an increase in enteric conditions leading to increased mortality, bird and animal welfare problems, and an increase in prescribing therapeutic antibiotics (Andreason, 2000). This stimulates an intensive search for alternatives for antibiotic growth and health promoters in farm animals.

**ALTERNATIVES TO ANTIBIOTICS IN POULTRY**

The health status of animals with a high growth performance and a good quality of animal products is a predominant argument in the choice of feed additives. The following tools are being considered to fill the role of alternatives, as reviewed by Verstegen and Schaafsma (1999):

- Probiotics
- Prebiotics / non digestible Oligo- and polysaccharides
- Organic acids
- Enzymes
- Natural medicinal products: Herbs, botanicals and other related substances
  
  The present study is focused on natural medicinal products.

**Probiotics**

Probiotics are live microbial feed supplements that beneficially affect the host animal by improving its intestinal balance (Fuller, 1989). The stable microbiota in the intestine helps the animals to resist infections, particularly in the intestinal tract by bacterial interactions (Jensen, 1993). At present, 19 microorganism-preparations are (provisionally) authorized in the EU as feed additives, of which 7 were used in poultry. These organisms belong to the bacterial genera of *Enterococcus, Bacillus* and *Pediococcus* (Simon and Jadamus, 2002).

Some studies indicate that the effects of probiotic preparations are rather speculative. For example, Subrata-sarker (1997) compared the effect of feeding yeast and antibiotics (aureomycin, chlortetracycline) in broilers. Live weight gain, feed intake, feed conversion efficiency and carcass parameters did not differ between the treatments. However, the majority of studies on poultry revealed improvements of weight gain, feed efficiency and diarrhea reduction in animals receiving probiotics (a.o., Thana *et al.*, 2001; Demir and Eser, 2002; Fulton *et al.*, 2002). The most frequently used probiotics are *Lactobacilli*, *Bifidobacteria, Enterococci, E. coli, Bacilli* and yeasts.
Prebiotics

Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health (Gibson and Roberfroid, 1995). Among the food ingredients, non-digestible carbohydrates (oligo- and polysaccharides), some peptides and proteins, and certain lipids are considered as potential prebiotics. It was suggested that inclusion of non-digestible carbohydrates or dietary fiber in the diet not only provide nutrient sources for bacteria but also influence the entire gastro-intestinal ecosystem (a.o., Buddington and Weiher, 1999; Langhout, 1999; Rolfe, 2000).

Non-digestible carbohydrates that have been studied for beneficial effect on host animals are transgalactosylated oligosaccharides (Tanaka et al., 1983), soybean oligosaccharides (Hayakawa et al., 1990), fructooligosaccharides (FOS) (Damien and Chantal, 1986) and mannoligosaccharides (MOS) (Lyons, 1994). It had been shown that non-digestible oligosaccharides fed animals like pigs and cows had fewer diarrheas and an enhanced growth performance (Katta et al., 1993). In poultry, similar responses, such as an improved weight gain and feed efficiency and a reduced Salmonella load in the gastrointestinal tract, have been noted with inclusion of these products in the diets (a.o., Stanley et al., 1997; Spring et al., 2000; Fernandez et al., 2000; Demir and Eser, 2002).

Organic Acids

Organic acids, such as fumaric, citric or propionic acid, can be used for microflora stabilizers in young pigs, calves and poultry. In Europe, organic acids are used widely to inhibit pathogens like Salmonella in both raw materials and finished feed (Raddiffe, 2000). Supplementation of the diet with organic acids (between 0.5 and 3.0%) has been shown to consistently improve feed conversion efficiency for young pigs and calves, while having little or no effect on growth rate (Giesting and Easter, 1985). Inclusion of organic acids in poultry diets showed a significantly lower incidence of infections and mortality in chickens (Huyghebaert, 1999).
Enzymes

Exogenous enzyme supplementation has been introduced in the poultry feed industry since 1980. As reviewed by several authors (Bedford and Schulze, 1998; Verstegen and Schaafisma, 1999), many beneficial effects have been reported from adding feed enzymes such as cellulase, amylase, arabinase, pectinase, phytase, protease, lipase, xylanase, δ-galactosidase and β-glucosidase. The major effect of these enzymes is the improvement of the availability of nutrients and a reduced anti-nutritive activity. Non-starch polysaccharides were known to increase viscosity of ingesta, and to depress digestion of fats, carbohydrates and proteins. It has been suggested that enzymes, which act on fibrous components in pig and poultry feeds, can reduce viscosity in the gut. Several studies demonstrated that enzyme preparations tested in a wheat or barley based diet improved growth performance, digestibility of carbohydrates in young broiler chicks (a.o., Rutkowski et al., 1999; Brufau et al., 2002; Gopal, 2002), and increased egg mass production and feed conversion in laying hens (Mathlouthi et al., 1999).

Natural Medicinal Products

Natural medicinal products, such as herbs, botanicals and other related substances, have a wide range of activities, and these products are popularly used in both human and animal populations in China. An estimation of the number of Chinese natural medicine ranges between 6,000 and 8,000, of which 77% originate from plants (including fungi and algae), 16% from animals (insects, fish, birds, reptiles, amphibians and beasts) and 7% from minerals (Zhou, 1993). In China, natural medicines were used as feed additives over 2,000 years ago, and many practices and prescriptions have been developed and used since then (Li, 1998; Wang et al., 2002). Natural medicine preparations are increasingly being used in animal diets as growth and health promoters since 1970, due to the fact that a growing consumer demand for “green food products”. As reviewed by Li (1998), about 200 natural medicinal feed additives, single products, combinations and extracts have been developed and extensively used in the animal feed industry of China since 1970. These natural products have been demonstrated to play a role in optimizing productive performance, enhancing health of stressed animals or improving quality of animal products.

The most recent studies worldwide showed that some herbs, spices and extracts can stimulate feed intake and endogenous secretions or may have antimicrobial, coccidiostatic or anthelmintic activities (Wenk, 2002). It was well documented that phytogenic extracts (a
combination of phytogetic active substances, capsaicin, carvacrol, and cinnamic aldehyde) enhanced growth performance of broilers (a. o., Jamroz et al., 2002; Korösi, 2002; Samarasinhe and Wenk, 2002; Wenk and Messikommer, 2002).

The bioactive components in natural medicines are quite complex and the mode of action is unclear. Natural products may serve to provide animals with nutrients such as protein, small peptides, essential amino acids, oligosaccharides, fatty acids, starch, vitamins and organic trace minerals, and may also show many bio-activities such as anti-microbial activities, immune enhancement and stress reduction (Wang et al., 2002). Some of the components from natural medicines have already been isolated and identified, such as saccharides (mono-, oligo- and poly-saccharides), glycosides or hetersides, alkaloid, lactone, volatile oils and organic acids, tannin, resin, phytochrome (Yang and Feng, 1998).

A current estimation of the number of immuno-active Chinese natural medicines ranges between 200-300 with most of them originating from plants and fungus (Li, 2000). It was found that the immuno-active components in medicinal mushrooms and plants include polysaccharides, glycosides, alkaloids, volatile oils and organic acids, of which polysaccharides are considered to be most important (Xie and Niu, 1996). As reviewed by Xue and Meng (1996), polysaccharides were generally derived from spermatophyta and pteridophyta (a.o., Ginseng Radix, Codonopsis Radix, Astragalus membranaceae Radix, Epimedi Herba, Lígustri Fructus, Eleutherococci Radix, and Eleutherococci Radix), fungi (a.o., Polypori scierotium, Lentinus edodes, Tremella fuciformis, and Corilus versicolor) and algae (a.o., Laminariae Thallus and Sargassi Herba).

The immuno-modulating activities of polysaccharides, which were derived from medicinal plants and fungi are well documented in humans and rats (a.o., Miles and Chang, 1997; Lizuka et al., 2000; Zhong et al., 2001; Shon et al., 2002). These polysaccharides were considered to function by stimulating growth of immune organs, and by inducing both cell-mediated and humoral immune response of the host. It was demonstrated that these polysaccharides could play an important role in maintaining health in humans, and have been used for assistant treatment of immune depression or deficiency caused by cancers, hepatitis, radiotherapy and chemical treatments (a.o., Zhang et al., 1998; Wu and Wang, 1999; Wargovich et al., 2001).

Research on immuno-active plants and fungus in humans provides a theoretical basis on using these components for enhancing productive performance and increasing economic efficiency in animals. Immuno-active polysaccharides from medicinal plants and fungus might be potential growth and health promoters in poultry. Certain polysaccharides derived
from mushrooms, *Tremella fuciformis* and *Lentinus edodes*, and an herb, *Astragalus membranaceus Radix*, have been studied in chickens. It has been demonstrated that these polysaccharides show anti-viral (Cheng *et al.*, 1998; Yang *et al.*, 1998; Yu and Zhu, 2000), anti-bacterial (Fan *et al.*, 1993; Yuan *et al.*, 1993) and anti-parasitic activities (Hu *et al.*, 1998; Pang *et al.*, 2000) in poultry, as immune enhancers. However, their mode of actions related to gut health were not well investigated.

**A Pilot Study: The Use of a Chinese Herbal Medicine Formulation in Broilers’ Diets**

A pilot study of Guo *et al* (2000) showed that a Chinese herbal medicine (CHM) formulation, which consists of 14 herbs, was considered to be a potential antibiotic growth promoter in broilers. Four dietary levels (0.25, 0.5, 1 and 2 g/kg) of a CHM formulation were used as alternatives to virginiamycin (VRG, 20 mg/kg). A total of 720 female broiler chicks were used to test the effects of VRG and CHM on BW gain, feed intake, feed conversion ratio and viability at 7 to 28 days of age. At the end of the experiment, a total of 72 birds, with 4 birds from each replicate of three treatments (the non-supplemented group, the VRG and 0.5 g/kg CHM groups), were killed for the dissection analysis. Liver color and the relative weight of liver and intestinal tract organs including duodenum-pancreas-jejunum, ileum and ceca were determined to study their mechanisms.

In this experiment, CHM dietary treatments had significantly increased BW gain at 7 to 21 days of age but not at 21 to 28 days of age, and the birds fed with 0.5 g/kg CHM showed higher BW gain at the first two weeks of the experiments (Figure 1). Feed intake was significantly increased by the CHM treatments at 21 to 28 days of age, as a result, FCR in those groups was significantly increased. The dissection results showed that dietary CHM and VRG had no significant effects on liver and intestinal tract weights. However liver color of the birds fed with CHM was more uniform than those fed with VRG and the un-supplemented birds. In the first experiment, CHM showed a promising effect on performance of chickens.

However, the effective components from CHM were unknown and the mechanisms for enhanced growth performance are not well understood. Therefore, the following experiments was carried out to further investigate the bio-active components (polysaccharides) from natural medicines mainly from two mushrooms, *Lentinus edodes* and *Tremella fuciformis*, and an herb, *Astragalus membranaceus Radix*, and their mode of action related to the gut ecosystem and immune stimulation in poultry.
Figure 1. Effect of CHM supplemental levels on BW gain of female broiler chicks from day 7 to day 21, which was studied in the pilot experiment. Results are given as means (n=6) ± SE; CHM concentrations are 0, 0.25, 0.5, 1 and 2g/kg CHM. The quadratic regression function for BW gain from day 7 to 21: $Y = 48.85 + 2.57 - 1.28 (P = 0.023; R^2 = 24)$

SCOPE OF THE STUDY

Antibiotics are widely used as therapeutic agents and growth promoters in the poultry feed industry. However, antibiotics will cause a disturbed gut eco-system and may develop a resistant population of bacteria in birds. The possibility of developing resistant populations of bacteria and the side effects for humans of using antibiotics as growth promoters in farm animals has led to the recent EU-ban on the use of several antibiotics in poultry diets. Therefore, there is an intensive search for alternatives such as probiotics, prebiotics and other feed additives. Until now, only a few antibiotic alternatives have been investigated. On the basis of Chinese literature, polysaccharides from two mushrooms, Tremella fuciformis and Lentinus edodes, and the herb Astragalus membranacea Radix, seem to be potential alternatives for antibiotic growth and health promoters. These products, used as immune enhancers, showed antimicrobial activity in chickens, and are easily available on the market.

Mushroom and herb polysaccharides are considered to become an adequate alternative for antibiotics due to strengthening of the animals’ defense system against the invasion by infectious organisms. However, the physico-chemical properties of these polysaccharides are not well known since the molecular structure of these polysaccharides is quite complex. The effect of the polysaccharides on growth performance and their mode of actions related to gut health and immuno-modulation in farm animals, like poultry, are not yet well studied.
It is hypothesized in this project that mushroom and herb polysaccharides (as a prebiotic) may selectively stimulate growth and/or activity of one or a limited number of favorite bacterial species of large intestine, and as such beneficially affect gut health of the bird. In addition, mushroom and herb polysaccharides may enhance immune response of chickens. The objectives of this research are:

- To isolate and identify polysaccharides from mushrooms and herbs and to study their physico-chemical properties;
- To study the fermentation characteristics of mushroom and herb polysaccharides *in vitro*;
- To study immune modulating effects of mushroom and herb polysaccharides against coccidiosis in chickens;
- To investigate the effects of mushroom and herb polysaccharides on performance in chickens;
- To elucidate possible modes of action related to the gut ecosystem and immune system of chickens.

**OUTLINE OF THE THESIS**

The thesis describes both *in vitro* and *in vivo* trials that were conducted with polysaccharide extracts from two mushrooms, *Tremella fuciformis* (TreS), *Lentinus edodes* (LenS) and the herb *Astragalus membranaceus* Radix (AstS). The results of these experiments are reported in 9 chapters.

Chapter 1 gives a review on immuno-activities, medicinal properties of mushroom and herb polysaccharides as alternatives for anti-microbials in poultry diets. Aims of this chapter are (1) to classify the origin of Chinese natural medicine species and focus on their polysaccharide fractions with respect to the physico-chemical properties, (2) to describe the procedure of isolation and identification of the polysaccharide fractions, and (3) to summarize the immune activities of these polysaccharides and their effects in poultry nutrition as potential anti-microbial promoters. Moreover, two mushrooms, *Lentinus edodes* and *Tremella fuciformis*, and an herb, *Astragalus membranaceus* Radix, are discussed in more detail.

Chapter 2 describes the preparation of the polysaccharide extracts of mushrooms, *Tremella fuciformis* (TreE), and *Lentinus edodes* (LenE), and the herb *Astragalus membranaceae* Radix (AstE). Qualitative chemical analysis such as HPAEC was used for total sugar content and sugar composition of these polysaccharide extracts.
Chapter 3 and 4 are two *in vitro* trials that were designed to test the prebiotic effects of the mushroom and herb materials and their polysaccharide extracts. In Chapter 3, the fermentation characteristics of intact mushroom and herb materials, *LenS*, *TreS* and *AstS*, and their polysaccharide extracts, *LenE*, *TreE* and *AstE*, were studied in an *in vitro* trial. In this trial, the *in vitro* cumulative gas production technique was used, with chicken cecal contents as an inoculum. Gas production was measured kinetically and pH, VFA and NH₃ production was measured 72 hrs after incubation. In order to further study the effects of these products on the diversity of cecal bacteria after *in vitro* fermentation, specific PCR amplification of 16S rDNA gene fragments in combination with denaturing gradient gel electrophoresis (DGGE) was used. The DGGE band patterns of chicken cecal microflora are described in Chapter 4.

Chapter 5 describes an *in vivo* trial in which the effects of dietary mushroom and herb polysaccharides (*LenE*, *TreE* and *AstE*) were assumed relative to an antibiotic growth promoter (virginiamycin). A dose response for each of the extracts was investigated as well. Growth of immune organs and development of gastro-intestinal tracts were also determined to study possible mechanism for a potential enhanced growth performance in broilers.

An *in vivo* trial (Chapter 6) further demonstrated the results of the two *in vitro* trials described in Chapters 3 and 4. The experiment was conducted to investigate growth and changes of the cecal ecosystem (celal pH, viscosity and predominant microbial species) of the birds fed with the mushroom and herb polysaccharide extracts, as compared with an antibiotic (Apramycin). In this experiment, birds were naturally infected with *Avian Mycoplasma Gallisepticum*. Cecal predominant microbial species of chickens were measured using culture-based microbiology techniques. In additions, the dose response of the potential polysaccharide extracts were also investigated.

Chapters 7 and 8 describe an *in vivo* assessment of dietary mushroom and herb polysaccharides against a coccidial infection (*Eimeria tenella*). The mushroom and herb extracts were used as feed supplements or as adjuvants of a live, attenuated oocyst vaccine. The immune response of chickens, including growth, cecal lesions, as well as growth of immune organs and development of the gastrointestinal tract, was measured post infection (Chapter 7). The production of the specific parasite cytokines, IFN-γ and IL-2, were measured at 7 and 14 days post-infection (Chapter 8).

In Chapter 9, the effects of the mushroom and herb polysaccharide extracts on both systemic and mucosal immune response in chickens, infected with *E. tenella*, including parasite-specific antibody production by ELISA, specific T-lymphocyte proliferation,
erythrocyte rosette production of T, B lymphocytes were studied.

Finally, the general discussion provides an overview of effects of the studied mushroom and herb polysaccharide extracts, as alternatives for antimicrobials, on growth and health of chickens, and their mode of actions related to the gut ecosystem and the immune system of chickens.

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Chapter 1

IMMUNOACTIVE, MEDICINAL PROPERTIES OF MUSHROOM
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ANTIMICROBIALS IN POULTRY DIETS

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Imunoactive, Medicinal Properties of Mushroom and Herb Polysaccharides: Alternative for Antimicrobials in Poultry Diets


Abstract

Until recently, antibiotics as anti-microbial growth promoters were widely used in the poultry feed industry. However, the possibility of developing resistant populations of bacteria and the side effects of using antibiotics as growth promoters in farm animals has lead to the recent EU-ban on the use of several antibiotics in poultry diets. Therefore, there is an intensive search for alternatives such as probiotics, prebiotics and other feed additives. The polysaccharides derived from medicinal mushrooms and herbs are considered as potential alternatives. In an extensive review of the literature, different natural species of mushrooms and herbs were detected with different physico-chemical properties and immune activities. Immune activities of the polysaccharides from two mushrooms, Lentinus edodes and Tremella fuciformis, and an herb, Astragalus membranaceus Radix, are reported, based on studies in rats, humans, and chickens. These immuno-active polysaccharides generally can stimulate growth of immune organs, enhance number and activities of immunocytes, and induce both cellular and humoral immune response in these species. They have been used as either adjuvants of vaccines, therapeutic agents or feed supplements, and shown significant anti-viral, anti-bacterial and anti-parasitic activities in poultry.

Keywords: mushroom, herb, polysaccharides, growth, physico-chemical, immunity, poultry
INTRODUCTION

It is well documented that Chinese natural medicinal products as feed supplements have been used as growth and health promoters in farm animals in China for centuries (Li, 1998; Wang et al., 2002). The bio-active components in the natural medicinal products are quite complex, of which the most common components are saccharides, glycosides or heterosides, alkaloids, lactones, volatile oils and organic acids, tannins, resins, phytochromes, proteins, and inorganic salts (Yang and Feng, 1998). Not all of these components are bio-active with respect to the immune response. It was found that the immuno-active components of the natural medicinal products include polysaccharides, glycosides, alkaloids, volatile oils and organic acids, of which polysaccharides were considered to be the most important components (Xue and Meng, 1996; Li, 2000). Polysaccharides may act as immune enhancers or immunomodulators and, according to Xue and Meng (1996), these components may display anti-bacterial, anti-viral, anti-parasitic, anti-cancer, anti-radiation and anti-ageing activity, reducing stress-related compounds and lowering the blood cholesterol level. Generally, the polysaccharides could affect both innate and adaptive immunity including cellular and humoral responses (Lien and Gao, 1990; Xue and Meng, 1996). Polysaccharides act as biological modifiers and mainly affect the reticulo-endothelial system and populations of macrophages, lymphocytes, leukocytes and natural killer (NK) cells (Zhao, 1993; Wu et al., 1996; Zhong et al., 2001, 1998; Li et al., 2001). By enhancing the synthesis of DNA, RNA and protein of immune cells, and regulating the cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) level in the body, these polysaccharides can enhance production of specific antibodies and cytokines (a.o., Mao, 1989; Cheng, 1998; Qun et al., 1999; Lizuka et al., 2000). The polysaccharides were also shown to enhance the activities of complement system as a critical component of innate immunity (Fan et al., 1993).

Research in East Asian regions related with the beneficial effects of the immuno-active mushroom and herb polysaccharides in vitro and in vivo may provide a theoretical basis for future use of these components to enhance production, health status and economic efficiency in farm animals. It is well documented that certain mushroom and herb polysaccharides as immune enhancers can be used in prevention of animal diseases such as bacterial (Yuan et al., 1993), viral (a.o., Cheng et al., 1998; Yu and Zhu, 2000) and parasitic diseases (Hu et al., 1998; Pang et al., 2000) in poultry. In addition, polysaccharides may escape enzymatic digestion and selectively stimulate growth and/or activity of a limited
number of favorite bacterial species, and as such beneficially affect gut health of the bird, as suggested by Gibson and Roberfroid (1995). However, such prebiotic effects of these polysaccharides have not been investigated in depth. The objective of the present review is (1) to classify the origin of Chinese natural medicine species and focus on their polysaccharide fractions with respect to the physico-chemical properties, (2) to describe the procedure of isolation and identification of the polysaccharide fractions, and (3) to summarize the immune activities of these polysaccharides and their effects on poultry nutrition as potential anti-microbial promoters. Moreover, two mushrooms, *Lentinus edodes* and *Tremella fuciformis*, and an herb, *Astragalus membranaceus Radix*, are discussed in more detail.

**ORIGIN OF CHINESE NATURAL MEDICINE SPECIES**

An estimation of the number of Chinese natural medicine ranges between 6,000 and 8,000, of which 77% originating from plants (include fungi and algae), 16% from animals (insects, fish, birds, reptiles, amphibians and beasts) and 7% from minerals (Zhou, 1993). It is estimated that around 500 species are used in common practices in China, and 82% of these species (413 species) come from plants (Zhou, 1993) (Table 1). There are over 600 state production bases and 13,000 private farms specialized in the production of intact herbal materials in the mainland China. More than 860,000 acres of lands are devoted to the cultivation of medicinal plants (Zhou, 1998).

<table>
<thead>
<tr>
<th>Classification</th>
<th>Number of family</th>
<th>Number of species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algae</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Fungi</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Pteridophyta</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Spermatophyta</td>
<td>110</td>
<td>398</td>
</tr>
<tr>
<td>Gymnospermae</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Angiospermae</td>
<td>105</td>
<td>390</td>
</tr>
<tr>
<td>Total</td>
<td>123</td>
<td>413</td>
</tr>
</tbody>
</table>

A current estimation of the number of immuno-active Chinese natural medicine ranges between 200-300 with most of them originating from plants (Li, 2000). As reviewed by Xue and Meng (1996), polysaccharides were generally derived from spermatophyta and pteridophyta (*a.o.*, *Ginseng Radix, Angelicae sinensis Radix, Codongopsis Radix, *...*).
Astragalus membranaceae Radix, Epimedi Herba, Ligustri Fructus, Eleutherococci Radix, and Eleutherococci Radix), fungi (a.o., Polypori scierotium, Ganoderm lucidum, Lentinus edodes, Tremella fuciformis, and Corilus versicolor) and algae (a.o., Laminariae Thallus and Sargassi Herba).

**ISOLATION, IDENTIFICATION AND PHYSICO-CHEMICAL PROPERTIES OF THE POLYSACCHARIDES**

The determination of the structure and sugar composition of the polysaccharide fractions usually includes, as a first step, the isolation of the polysaccharides from their original sources by removal the other constituents such as protein, fat and starch, without modifying the polysaccharide materials. Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are generally used in the analysis of rather simple polysaccharides or polysaccharide fragments. Chemical and specific enzymatic degradation is used to obtain fragments that fit within the analytical range of NMR and MS analyses (Huisman et al., 1998).

The relationship between the polysaccharide structure and its bio-active function is not well understood. The majority of polysaccharides have no bio-active functions, because their molecular structure is a simply D-glucose polymer with a long straight chain by α-1-4 glycoside linkages like starch and cellulose (Xue and Meng, 1996). However, polysaccharides from medicinal mushrooms and herbs generally do have bio-active effects. Xue and Meng (1996) therefore suggested that bio-active polysaccharides might be characterized by the following: (1) a polymer with different mono-saccharide composition with β-glycoside linkages; (2) a complex three dimensional structure; (3) the molecular weight between 10^4 and 10^7 Dalton. Table 2 shows the structural characteristics of some polysaccharides.

Some polysaccharides, like those from the herb, Astragalus membranaceus Radix, and the mushrooms, Lentinus edodes and Tremella fuciformis, have been studied more extensively (Figure 1).
Table 2. The structure of some polysaccharides

<table>
<thead>
<tr>
<th>Polysaccharides</th>
<th>Origin</th>
<th>Glycoside linkages</th>
<th>Molecular weights (Daltons)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lentinan</td>
<td><em>Lentinus edodes</em></td>
<td>β-1, 3 glucan,</td>
<td>5 × 10^5</td>
<td>Liu <em>et al.</em>, 1999c</td>
</tr>
<tr>
<td>Glucan</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>β-1, 6 glucan</td>
<td>1 × 10^4</td>
<td>Blagoeva <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>Krestin</td>
<td><em>Coriolus versicolor</em></td>
<td>β-1, 3; β-1, 4, β-1, 6 glucan</td>
<td>1 × 10^5</td>
<td>Zhang <em>et al.</em>, 2001</td>
</tr>
<tr>
<td>Schizophyllan</td>
<td><em>Schizophyllum commune</em></td>
<td>β-1, 3 and β-1, 6 glucan</td>
<td>1 × 10^4 - 1×10^6</td>
<td>Mol <em>et al.</em>, 1988</td>
</tr>
<tr>
<td>Glucomannan</td>
<td><em>Amorphophallus Konjak</em></td>
<td>β-1, 4 D-mannose and D- glucose</td>
<td>1- 2×10^6</td>
<td>Shimahara, 1975</td>
</tr>
<tr>
<td>Fructan</td>
<td><em>Gomphrena macrocephala</em></td>
<td>β-2, 6 fructan polymer</td>
<td>5.5×10^5- 1×10^4</td>
<td>Shiomi <em>et al.</em>, 1996</td>
</tr>
</tbody>
</table>

Figure 1. The roots of *Astragalus* (left), the fruit body of *Lentinus edodes* (middle) and the *Tremella fuciformis* (right).
These products are regularly used as either food ingredients (the two mushrooms) or a food additive (the herb, a daily use dose, 9-16 g) in Chinese and Japanese cuisine for maintaining health (Satonori et al., 1999; Lee et al., 2000). Moreover they are easily available and might be useful for their inclusion in poultry diets as growth promoters. Table 3 shows some characteristics such as chemical composition, medicinal purpose, cultivation technology, and the production and cost price of these products.

The advances in cultivation technology, in conjunction with increased consumer demands, account for reduce in price and increased availability. There are over 2,000 types of Astragalus which exist worldwide, of which around 130 Chinese varieties have been extensively used medicinally (Ma, et al, 2002). The annual production of Astragalus from 50’s to 70’s was around 3,000 tons. However in 1983 the production of Astragalus reached around 21,000 tons, which is almost 7 times that of 50’s to 70’s (Zhao et al., 1995). China is the major producer of edible mushrooms (3.9 million tons with a production value of about 9.7 billion USD, which is about 64% of the world supply) (Stamets, 2000). The popularity of the culinary characteristics and medicinal purpose offered by Lentinus and Tremella mushrooms bode well for continued growth and development of the industry worldwide. For instance, the United States produced 3,707 tons of Lentinus in 2000, and annual increases in Lentinus production in the United State have averaged over 20% since 1987 (Stamets, 2000).

Polysaccharides can be isolated and purified from rots of Astragalus (Huang et al., 1982), and from both the mycelia (Wu et al., 1984; Jin et al., 1991; Liu et al., 1999c) and the fruiting bodies (Xia and Cheng 1988; Yang et al., 1999, 2001) of Lentinus and Tremella. The physico-chemical properties of the polysaccharide fractions are summarized in Table 4. It can be seen from Table 4 that two glucans (AG-1, AG-2) and two heterosaccharides (AH-1 and AH-2) were isolated and purified from rots of Astragalus. The polysaccharide-protein complexes were obtained from both the mycelia (LenP_m-A, LenP_m-B) and the fruiting bodies (LenP_f-A, LenP_f-B and LenP_f-C) of Lentinus, and a total of four heteropolysaccharides were obtained from the mycelia (TreP_m-A, TreP_m-B, TreP_m-C) and the fruiting bodies (TreP_f) of Tremella (Table 4). It can be concluded that the polysaccharides from different species of the mushroom and herbal species and even from the same species are different in their physico-chemical properties such as the molecular weights, sugar composition and structure. The structure of these polysaccharides is not thoroughly investigated.
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Substrates</th>
<th>Contents</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family and common name</td>
<td><em>AstS</em></td>
<td><em>Leguminosae</em> family; “Huang Qi”</td>
<td>Zhou, 1993; Xu, 1990</td>
</tr>
<tr>
<td></td>
<td><em>LenS</em></td>
<td><em>Tricholomataceae</em> family; “Shiitake”</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>TreS</em></td>
<td><em>Tremellaceae</em> family; “Silver Ear”, “White Jerry Leaf”, “Snow Ear”, “Ying Er”</td>
<td>Chandra, 1989; Singer, 1961</td>
</tr>
<tr>
<td>Cultivation</td>
<td><em>AstS</em></td>
<td>Seminal reproduction, stem grafting or branch separating</td>
<td><a href="http://www.agri.ac.cn/agri_net/">http://www.agri.ac.cn/agri_net/</a></td>
</tr>
<tr>
<td></td>
<td><em>LenS</em></td>
<td>Synthetic and natural logs</td>
<td>Royse <em>et al.</em>, 1985</td>
</tr>
<tr>
<td></td>
<td><em>TreS</em></td>
<td>Synthetic and natural logs</td>
<td>Huang, 1982</td>
</tr>
<tr>
<td>Composition</td>
<td><em>AstS</em></td>
<td>Crude saponin (5.81%), astragaloside (0.14%), and polysaccharide (23%), flavonoids, triterpene glycosides, 22 kinds of amino acids and 14 kinds of micro-minerals.</td>
<td>Qi, 1987; Zheng <em>et al.</em>, 1998</td>
</tr>
<tr>
<td></td>
<td><em>TreS</em></td>
<td>Dietary non-starch polysaccharides (70%) such as acidic polysaccharides and glucuronoxylomannan, and vitamin D.</td>
<td>Min, 1996</td>
</tr>
<tr>
<td>Traditional use</td>
<td><em>AstS</em></td>
<td>White color, sweets and warm; Night sweats and deficiency of chi (fatigue, weakness, and loss of appetite), and diarrhea.</td>
<td>Zhou, 1993</td>
</tr>
<tr>
<td></td>
<td><em>LenS</em></td>
<td>Brown to black brown color, Steak-like with a pungent flavor; Asian cuisine as health maintaining food.</td>
<td>Zhou, 1993</td>
</tr>
<tr>
<td></td>
<td><em>TreS</em></td>
<td>White and translucent, sour, sweet and bland; Chinese cuisine, chronic tracheitis and other cough-related conditions such as asthma and dry cough.</td>
<td>Zhou, 1993</td>
</tr>
<tr>
<td>Modern use</td>
<td><em>AstS</em></td>
<td>Cancers, common cold/sore throat, heart attack, hepatitis, infections, immune suppression and dysfunction.</td>
<td>Qun <em>et al.</em>, 1999</td>
</tr>
<tr>
<td></td>
<td><em>TreS</em></td>
<td>Anti-tumor, lower cholesterol, anti-diabetes, calcium absorption, liver protection, and prevention of osteoporosis, anti-inflammation and slowing of the aging process.</td>
<td>Xia and Cheng, 1988</td>
</tr>
<tr>
<td>Production and price</td>
<td><em>AstS</em></td>
<td>1.5 million tons (stock), 1.5 to 1.9 USD/kg</td>
<td><a href="http://www.888888.org/rj.gif">http://www.888888.org/rj.gif</a></td>
</tr>
<tr>
<td></td>
<td><em>LenS</em></td>
<td>80,000 tons (production), 1.9-2.5 USD/kg</td>
<td><a href="http://www.888888.org/rj.gif">http://www.888888.org/rj.gif</a></td>
</tr>
<tr>
<td></td>
<td><em>TreS</em></td>
<td>12,500 tons (production), 1.9-2.5 USD/kg</td>
<td><a href="http://www.888888.org/rj.gif">http://www.888888.org/rj.gif</a></td>
</tr>
</tbody>
</table>
Table 4. Physico-chemical properties of mushroom and herb polysaccharides

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Polysaccharides¹</th>
<th>Characteristics²</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical properties</td>
<td>AstP</td>
<td>AG-1</td>
<td>Water-soluble glucan</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AG-2</td>
<td>Water-insoluble glucan</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AH-1</td>
<td>Acidic heterosaccharide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AH-2</td>
<td>Water-soluble heterosaccharide</td>
</tr>
<tr>
<td></td>
<td>LenP</td>
<td>LenPₘ₋A</td>
<td>Water-soluble polysaccharide-protein complex</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LenPₘ₋B</td>
<td>Water-soluble polysaccharide-protein complex</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LenPᵢ₋A</td>
<td>Polysaccharides complex (GlcA, 24.1%; protein, 2.0%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LenPᵢ₋B</td>
<td>Polysaccharides complex (GlcA, 34.8%; protein, 7.4%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LenPᵢ₋C</td>
<td>Polysaccharides complex (GlcA, 40.1%; protein, 25.3%)</td>
</tr>
<tr>
<td></td>
<td>TreP</td>
<td>TrePₘ₋A</td>
<td>Neutral heterosaccharide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TrePₘ₋B</td>
<td>Acidic heterosaccharide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TrePₘ₋C</td>
<td>Acidic heterosaccharide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TrePᵢ</td>
<td>Water-soluble heterosaccharide</td>
</tr>
<tr>
<td>Molecular weights</td>
<td>AstP</td>
<td>AG-1</td>
<td>1.23 × 10⁴</td>
</tr>
<tr>
<td>(Dalton)</td>
<td></td>
<td>AG-2</td>
<td>3.46 × 10⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AH-1</td>
<td>3.63 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>LenP</td>
<td>LenPₘ₋A</td>
<td>5.08 × 10⁵</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LenPₘ₋B</td>
<td>1.05 × 10⁵</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LenPᵢ₋A</td>
<td>9.54 × 10⁵</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LenPᵢ₋B</td>
<td>9.0 × 10⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LenPᵢ₋C</td>
<td>1.4 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>TreP</td>
<td>TrePₘ₋A</td>
<td>7.6 × 10⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TrePₘ₋B</td>
<td>7.6 × 10⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TrePₘ₋C</td>
<td>7.6 × 10⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TrePᵢ</td>
<td>1.15 × 10⁵</td>
</tr>
<tr>
<td>hSugar composition</td>
<td>AstP</td>
<td>AG-1</td>
<td>Glu</td>
</tr>
<tr>
<td>-------------------</td>
<td>------</td>
<td>------</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>AG-2</td>
<td>Glu</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AH-1</td>
<td>HexA: Glu; Rha: Ara = 1: 0.04: 0.02: 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AH-2</td>
<td>Glu: Ara = 1: 0.15</td>
<td></td>
</tr>
<tr>
<td>LenP</td>
<td>LenP&lt;sub&gt;α&lt;/sub&gt;-A</td>
<td>2,4-di-: 2,4,6-tri-: 2, 3, 4, 6-tetra-O-Me-glucose = 1: 2: 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LenP&lt;sub&gt;α&lt;/sub&gt;-B</td>
<td>Gal, Man, Xyl, Fru = 3.1: 4.8: 1.9: 1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LenP&lt;sub&gt;β&lt;/sub&gt;-A</td>
<td>Ara: Xyl: Man: Gal: Glu = 0.39: 0.46: 1.0: 0.93: 14.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LenP&lt;sub&gt;β&lt;/sub&gt;-B</td>
<td>Ara: Xyl: Man: Gal: Glu = 0.19: 0.41: 1.0: 0.93: 10.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LenP&lt;sub&gt;β&lt;/sub&gt;-C</td>
<td>Ara: Xyl: Man: Gal: Glu = 0.31: 0.47: 1.0: 1.15: 8.9</td>
<td></td>
</tr>
<tr>
<td>TreP</td>
<td>TreP&lt;sub&gt;α&lt;/sub&gt;-A</td>
<td>L-Fru: L-Ara: D-Xyl: D-Man: D-Gal: D-Glu = 1.06: 1.0: 0.33: 0.29: 0.037: 0.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TreP&lt;sub&gt;α&lt;/sub&gt;-B</td>
<td>L-Fru: L-Ara: D-Xyl: D-Man: D-Glu: GlcA = 0.16: 1.0: 0.28: 0.73: 0.036: 0.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TreP&lt;sub&gt;α&lt;/sub&gt;-C</td>
<td>L-Fru: L-Ara: D-Xyl: D-Man: D-Glu: GlcA = 0.086: 1.0: 0.37: 0.75: 0.058: 0.37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TreP&lt;sub&gt;β&lt;/sub&gt;</td>
<td>L-Fru: L-Ara: D-Xyl: D-Man: D-Glu: GlcA = 0.92: 0.49: 0.18: 1.0: 1.15: 0.57</td>
<td></td>
</tr>
<tr>
<td>Structure</td>
<td>AstP</td>
<td>AG-1</td>
<td>α-1, 4 and α-1, 6 glucan linkage = 5: 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AG-2</td>
<td>α-1, 4 glucan</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LenP</td>
<td>β-1, 3 D-glucan backbone, β-1, 6 D-glucan side chain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LenP&lt;sub&gt;α&lt;/sub&gt;-A</td>
<td>α- the glycoside linkages</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LenP&lt;sub&gt;α&lt;/sub&gt;-B</td>
<td>α- the glycoside linkages</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LenP&lt;sub&gt;α&lt;/sub&gt;-C</td>
<td>β- the glycoside linkages</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TreP</td>
<td>Repeat unit: D-GlcA-D-xyl-D-man; an α-1, 3 Mannan backbone with β- linked Xyl side chains and GlcA</td>
</tr>
</tbody>
</table>

1. *AstP* = *Astragalus membranaceus* polysaccharides with four fractions AG-1, AG-2, AH-1 and AH-2; *LenP* = *Lentinus edodes* polysaccharides derived from mycelia (LenP<sub>α</sub>-A and LenP<sub>α</sub>-B) and body fruit (LenP<sub>β</sub>-A, LenP<sub>β</sub>-B and LenP<sub>β</sub>-C) and *TreP* = *Tremella fuciformis* polysaccharides derived from mycelia (TreP<sub>α</sub>-A, TreP<sub>α</sub>-B, and TreP<sub>α</sub>-C) and body fruit (TreP<sub>β</sub>).

2. Fru = fructose; Ara = arabinose; Xyl = xylose; Man = mannose; Gal = galactose; Glu = glucose; Rha = rhamnose; GlcA = glucuronic acid; HexA = Hexuronic acid.
IMMUNE ACTIVITIES OF MUSHROOM AND HERB POLYSACCHARIDES

The immuno-modulating activities of the polysaccharides, which were derived from medicinal plants and fungi, are well documented in humans and rats (a.o., Xia and Cheng, 1988; Wei, 1991; Wu et al., 1996; Miles and Chang, 1997; Wang et al., 1997; Wang, 1998; Lizuka et al., 2000; Zhong et al., 2001; Shon et al., 2002). The polysaccharides could play an important role in the health maintaining of humans (Wargovich et al., 2001), and these polysaccharides were considered to function by (1) promoting activities of antigen non-specific immune NK cells that are able to rapidly and effectively kill cancerous cells; (2) placing a premium on the production of interferons that effectively prevent virus reproduction; (3) increasing the activities of complement C3 that enhances the animals’ disease resistance; (4) increasing the number and activities of the phagocytes that release H2O2 dissolving cancerous cells; and (5) preventing the reduction of leukocytes (Yuan and Shi, 2000; Wargovich et al. 2001). Immune activities of the most extensively studied polysaccharides, which were derived from Astragalus, Lentinus and Tremella, are presented in Table 5.

As shown in Table 5, immune response to the different treatments in the publications very tremendously due to difference in used polysaccharides, treated species and immune parameters that were taken into account. Thus, in general, it can be concluded from these studies that polysaccharides derived from the mushroom (Lentinus and Tremella) and herb (Astragalus), which were used as immune enhancers or immunodulators, can play important roles in: (1) Stimulating the growth of immune organs such as spleen, thymus and bursa; (2) Increasing the number and activities of many interdependent cell types such as T, B lymphocytes, macrophage, NK cells and lymphokine-activated killer (LAK) cells that collectively protect the body from bacterial, parasitic, fungal and viral infections and from the growth of tumor cells; (3) Enhancing T-cell mediated immune response, increased rate of T-lymphocyte transformation and proliferation and agglutination of red cells, while decreased inhibiting effects of serum, macrophage and suppressor T-cells (Ts) on T-cells’ function and against deformation and necrosis of lymphocytes in spleen, thymus and lymph nodes; (4) Enhancing humoral immune response by increasing spleen and serum antibody production, the number of hemolytic plague forming cells (PFC) and hemolysin production; (5) Inducing secretion and production of cytokines and complements of T-cells and NK cells activated by antigens, which responsible for clonal T-cell proliferation and antibody production of B-cells, proliferation and activity of
macrophages and NK cells.

The immuno-modulating effects of the mushroom and herb polysaccharides may result from an increase of the biological effect regulators, cAMP and cGMP, which play a role in regulating proliferation, differentiation and secretion of immune cells (Lee et al., 2002). It was reported that plasma cAMP and cGMP concentrations can be regulated by the mushroom and herb polysaccharides, such as TreP_m (Fan et al., 1993) and AstE (Mao, 1989; Liang, 1996; Wu and Wang, 1999). This indicates that the polysaccharides enhance immune function most probably through influencing cAMP and cGMP concentrations.

EFFECTS OF THE POLYSACCHARIDES AS HEALTH PROMOTERS IN POULTRY

Chinese natural medicinal products are thought to act by strengthening the animals’ defense system against invasion by infectious organisms, and in addition, they can display some hormone-like and vitamin-like effects (Li, 1998; Wang et al., 2002). It is well documented that the polysaccharides derived from Astragalus membranacea Radix, Lentinus edodes and Tremella fuciformis, have been used as immune enhancers against bacterial (Yuan et al., 1993), viral (a.o., Wei et al., 1997; Liu et al., 1999b; Yu and Zhu, 2000) and parasitic diseases (Hu et al., 1998; Pang et al., 2000) in chickens. However, there are only a few reports on using the mushroom and herb polysaccharides as dietary growth promoters in poultry.

Viral Infections

The mushroom and herb polysaccharides (LenP, AstP and TreP_m), which were used as immune enhancers, significantly reduced incidence of the viral diseases and mortality of chickens as result of viral infections, such as Marek’s disease (Wei et al., 1997; Liu et al., 1999b), Avian myelocytic leukemia (Qu et al., 1998), and infectious bursal disease (Yu and Zhu, 2000), (Table 6).
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Polysaccharides&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Dosage (mg/kg bw)&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Species</th>
<th>Response to treatments&lt;sup&gt;3&lt;/sup&gt;</th>
<th>References</th>
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<tbody>
<tr>
<td><strong>Immune organs</strong></td>
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<tr>
<td>Spleen weights</td>
<td>AstP</td>
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<td>chickens</td>
<td>166&lt;sup&gt;a&lt;/sup&gt;; 125</td>
<td>Zhang et al., 1998a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>250 × 5d</td>
<td>rats</td>
<td>146</td>
<td>Liang et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000</td>
<td>mice</td>
<td>123</td>
<td>Mao et al., 1988</td>
</tr>
<tr>
<td></td>
<td>LenP&lt;sub&gt;f&lt;/sub&gt;</td>
<td>200 × 3d</td>
<td>chickens</td>
<td>162&lt;sup&gt;a&lt;/sup&gt;; 123</td>
<td>Zhang et al., 1998a</td>
</tr>
<tr>
<td></td>
<td>TreP&lt;sub&gt;f&lt;/sub&gt;</td>
<td>100 × 7d</td>
<td>mice</td>
<td>148</td>
<td>Xia and Cheng, 1988</td>
</tr>
<tr>
<td></td>
<td>TreP&lt;sub&gt;m&lt;/sub&gt;</td>
<td>100 × 7d</td>
<td>rats</td>
<td>156</td>
<td>Xia and Cheng, 1988</td>
</tr>
<tr>
<td>Bursa weights</td>
<td>AstP</td>
<td>200 × 3d</td>
<td>chickens</td>
<td>183&lt;sup&gt;b&lt;/sup&gt;; 122</td>
<td>Zhang et al., 1998a</td>
</tr>
<tr>
<td></td>
<td>LenP&lt;sub&gt;f&lt;/sub&gt;</td>
<td>200 × 3d</td>
<td>chickens</td>
<td>162&lt;sup&gt;b&lt;/sup&gt;; 108</td>
<td>Zhang et al., 1998a</td>
</tr>
<tr>
<td>Thymus weights</td>
<td>AstP</td>
<td>250 × 5d</td>
<td>rats</td>
<td>146</td>
<td>Liang et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000</td>
<td>mice</td>
<td>98</td>
<td>Mao et al., 1988</td>
</tr>
<tr>
<td></td>
<td>TreP&lt;sub&gt;f&lt;/sub&gt;</td>
<td>100 × 7d</td>
<td>rats</td>
<td>108</td>
<td>Xia and Cheng 1988</td>
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<td><strong>Immune cells</strong></td>
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<td>Number of NK cells</td>
<td>LenP&lt;sub&gt;f&lt;/sub&gt;</td>
<td>2 × 5d</td>
<td>man</td>
<td>91 to 131&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Shinya et al., 1991</td>
</tr>
<tr>
<td>Activity of NK cells</td>
<td>AstP</td>
<td>20/b × 3d</td>
<td>chickens</td>
<td>129</td>
<td>Tang et al., 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40/b × 3d</td>
<td>chickens</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LenP&lt;sub&gt;f&lt;/sub&gt;</td>
<td>1/m² × 12d</td>
<td>man</td>
<td>139&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Wu et al., 1996</td>
</tr>
<tr>
<td>Number of LAK cells</td>
<td>LenP&lt;sub&gt;f&lt;/sub&gt;</td>
<td>2 × 5d</td>
<td>man</td>
<td>110 to 122&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Shinya et al., 1991</td>
</tr>
<tr>
<td>Activity of LAK cells</td>
<td>AstP</td>
<td>20/rat</td>
<td>rats</td>
<td>300</td>
<td>Wang 1992</td>
</tr>
<tr>
<td>Phagocytosis of M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>AstP</td>
<td>500</td>
<td>rats</td>
<td>188</td>
<td>Li et al., 1989</td>
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<tr>
<td></td>
<td>LenP&lt;sub&gt;m-B&lt;/sub&gt;</td>
<td>20 × 1d</td>
<td>mice</td>
<td>175</td>
<td>Jin et al., 1991</td>
</tr>
<tr>
<td></td>
<td>TreP&lt;sub&gt;m&lt;/sub&gt;</td>
<td>100 × 7d</td>
<td>rats</td>
<td>103</td>
<td>Xia and Cheng, 1988</td>
</tr>
<tr>
<td></td>
<td>TreP&lt;sub&gt;m&lt;/sub&gt;</td>
<td>100 × 7d</td>
<td>rats</td>
<td>102</td>
<td>Xia and Cheng, 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200 × 7d</td>
<td>rats</td>
<td>254</td>
<td>Fan et al., 1993</td>
</tr>
<tr>
<td>Activity of bursa lymphocytes</td>
<td>AstP</td>
<td>40 × 7d</td>
<td>chickens</td>
<td>(P&lt;0.05, P&lt;0.01)</td>
<td>Zhong et al., 1998</td>
</tr>
<tr>
<td>Activity of spleen and thymus lymphocytes</td>
<td>LenP&lt;sub&gt;f&lt;/sub&gt;</td>
<td>2 × 7d</td>
<td>chickens</td>
<td>(P&lt;0.05, P&lt;0.01)</td>
<td>Zhong et al., 1998</td>
</tr>
<tr>
<td>Number of CD4 cells</td>
<td>LenP&lt;sub&gt;f&lt;/sub&gt;</td>
<td>2 × 5d</td>
<td>man</td>
<td>115&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Shinya et al., 1991</td>
</tr>
<tr>
<td>Number of CD8 cells</td>
<td>LenP&lt;sub&gt;f&lt;/sub&gt;</td>
<td>2 × 5d</td>
<td>man</td>
<td>109&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Shinya et al., 1991</td>
</tr>
<tr>
<td>E-rosette production</td>
<td>TreP&lt;sub&gt;f&lt;/sub&gt;</td>
<td>50ug/rat</td>
<td>rats</td>
<td>159</td>
<td>Xia and Cheng, 1988</td>
</tr>
<tr>
<td></td>
<td>TreP&lt;sub&gt;m&lt;/sub&gt;</td>
<td>50ug/rat</td>
<td>rats</td>
<td>208</td>
<td>Fan et al., 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 × 3d</td>
<td>chickens</td>
<td>136</td>
<td></td>
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<tr>
<td><strong>Cellular Immunity</strong></td>
<td></td>
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<td>Lymphocytes proliferation</td>
<td>AstP</td>
<td>35 × 7d</td>
<td>chickens</td>
<td>127 to 185&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Liu et al., 1999b</td>
</tr>
<tr>
<td></td>
<td>LenP&lt;sub&gt;f&lt;/sub&gt;</td>
<td>8 × 7d</td>
<td>chickens</td>
<td>111 to 118&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Liu et al., 1999b</td>
</tr>
<tr>
<td></td>
<td>TreP&lt;sub&gt;f&lt;/sub&gt;</td>
<td>100ug/rat</td>
<td>rats</td>
<td>211&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Xia and Cheng, 1988</td>
</tr>
<tr>
<td></td>
<td>TreP&lt;sub&gt;m&lt;/sub&gt;</td>
<td>100ug/rat</td>
<td>rats</td>
<td>220&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Xia and Cheng, 1988</td>
</tr>
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<td></td>
<td></td>
<td>50 × 4d</td>
<td>chickens</td>
<td>170</td>
<td>Fan et al., 1993</td>
</tr>
<tr>
<td>Agglutination of red cell</td>
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<td>500</td>
<td>mice</td>
<td>113</td>
<td>Mao et al., 1989</td>
</tr>
<tr>
<td></td>
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<td>93</td>
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### Humoral immunity

<table>
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<tbody>
<tr>
<td>Number of hemolytic plaque forming cells</td>
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<td>20 mice</td>
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<td>50 x 5d mice</td>
<td>178</td>
</tr>
<tr>
<td></td>
<td>AstP</td>
<td>100 x 5d mice</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td>treP&lt;sub&gt;m&lt;/sub&gt;</td>
<td>50 x 7d rats</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>AstP</td>
<td>50 x 7d rats</td>
<td>321</td>
</tr>
<tr>
<td>Number of spleen cells</td>
<td>AstP</td>
<td>500 rats</td>
<td>127</td>
</tr>
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<td></td>
<td>treP&lt;sub&gt;m&lt;/sub&gt;</td>
<td>1000 rats</td>
<td>96</td>
</tr>
<tr>
<td>Number of B-cells</td>
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<td>120</td>
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<tr>
<td>Antibody titers</td>
<td>treP&lt;sub&gt;m&lt;/sub&gt;</td>
<td>200 chickens</td>
<td>P&lt;0.05&lt;sup&gt;d&lt;/sup&gt;</td>
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### Cytokines

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<th>Value</th>
<th>Species</th>
<th>Reference</th>
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</thead>
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<td>AstP</td>
<td>35 x 7d chickens</td>
<td>112 to 137</td>
</tr>
<tr>
<td></td>
<td>LenP&lt;sub&gt;f&lt;/sub&gt;</td>
<td>8 x 7d chickens</td>
<td>117 to 152</td>
</tr>
<tr>
<td></td>
<td>treP&lt;sub&gt;m&lt;/sub&gt;</td>
<td>100 x 4d rats</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>treP&lt;sub&gt;m&lt;/sub&gt;</td>
<td>200 x 4d rats</td>
<td>148</td>
</tr>
<tr>
<td>TNF production</td>
<td>AstP</td>
<td>50 x 9d rats</td>
<td>112&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>IFN titers</td>
<td>AstP</td>
<td>50 x 9d rats</td>
<td>139&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>treP&lt;sub&gt;m&lt;/sub&gt;</td>
<td>11/b x 7d chickens</td>
<td>118 to 155&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>treP&lt;sub&gt;m&lt;/sub&gt;</td>
<td>200 x 3d rats</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>treP&lt;sub&gt;m&lt;/sub&gt;</td>
<td>400 x 4d rats</td>
<td>129</td>
</tr>
<tr>
<td>mRNA levels of IL-1α, LenP&lt;sub&gt;m&lt;/sub&gt;-A</td>
<td>AstP</td>
<td>20 mice marked increase</td>
<td>Liu et al., 1999a</td>
</tr>
<tr>
<td>IL-1β, TNF-α, IFN-γ</td>
<td>treP&lt;sub&gt;m&lt;/sub&gt;</td>
<td>200 x 4d rats</td>
<td>P&lt;0.05</td>
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### Complements

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<tr>
<th>Parameter</th>
<th>Value</th>
<th>Species</th>
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<tbody>
<tr>
<td>AstP</td>
<td>500 rats</td>
<td>P&lt;0.05</td>
<td>Fan et al., 1993</td>
</tr>
<tr>
<td>treP&lt;sub&gt;m&lt;/sub&gt;</td>
<td>100 x 3d chickens</td>
<td>107</td>
<td>Fan et al., 1993</td>
</tr>
</tbody>
</table>

<sup>1</sup> AstP = Astragalus membranaceus Radix polysaccharides; LenP = Lentinus edodes polysaccharides derived from mycelia (LenP<sub>m</sub>, LenP<sub>m</sub>-A) and body fruit (LenP<sub>f</sub>) and TreP = Tremella fuciformis polysaccharides derived from mycelia (treP<sub>m</sub>) and body fruit (treP<sub>f</sub>).

<sup>2</sup> d = number of successive days of treatments executed.

<sup>3</sup> Response to the treatments was based on 100 of the negative control (without superscripts) or the positive control (with superscripts in which<sup>a</sup> = cyclophosphamide; <sup>b</sup> = chemical therapy; <sup>c</sup> = cancer patients; <sup>d</sup> = Marek’s disease; <sup>e</sup> = phythahematoagglutinin; <sup>f</sup> = the vaccination group).

The polysaccharides were shown to significantly reduce the impact of viral infections in chickens through influencing immune response and the cellular antioxidant defense mechanism (Hassan, 1988). It was demonstrated that the use of AstP and LenP significantly (P<0.01, P<0.05) stimulated the growth of the immune organs in Newcastle disease infected chickens (Zhang et al., 1998b), and enhanced T-lymphocyte proliferation (P<0.01) in Marek’s disease infected chickens (Liu et al., 1999b). It was reported that TreP<sub>m</sub> (Cheng et al., 1998) and the polysaccharide isolated from corn pollen (Yang et al., 1998) significantly
(P<0.05) enhanced serum antibody titers of Marek’s disease, Newcastle disease and Egg Drop Syndrome virus in chickens. Thus, mushroom and herb polysaccharides may enhance both cellular and humoral immune response of infected chickens. In addition, several studies showed that the mushroom (LenP) and herb (AstP) polysaccharides play an important role in reducing the lipid peroxide concentrations (Wei et al., 1997), while enhancing anti-oxidation activity of superoxide dismutase (Chi et al., 1998; Qu et al., 1997) in organs of Marek’s disease and avian myelocytic leukemia virus infected chickens. This indicates the mushroom and herb polysaccharides might participate in the phenomena of the anti-oxidation action of superoxide dismutase, and thus reduce cellular pathological changes of organs in infected animals.

<table>
<thead>
<tr>
<th>Viral diseases</th>
<th>Substrates</th>
<th>Dosage(^2) (mg/kg bw)</th>
<th>Relative incidence(^3) (%)</th>
<th>Relative mortality(^3) (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marek’s disease</td>
<td>AstP</td>
<td>35 × 7d</td>
<td>52</td>
<td>47</td>
<td>Liu et al., 1999b</td>
</tr>
<tr>
<td></td>
<td>LenP</td>
<td>40 × 7d</td>
<td>65</td>
<td>38</td>
<td>Wei et al., 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 × 7d</td>
<td>86</td>
<td>62</td>
<td>Wei et al., 1997</td>
</tr>
<tr>
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<td>8 × 7d</td>
<td>59</td>
<td>65</td>
<td>Liu et al., 1999b</td>
</tr>
<tr>
<td>Avian myelocytic leukemia</td>
<td>AstP</td>
<td>40 × 7d</td>
<td>74</td>
<td>73</td>
<td>Qu et al., 1998</td>
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<tr>
<td></td>
<td>LenP</td>
<td>2 × 7d</td>
<td>78</td>
<td>79</td>
<td>Qu et al., 1998</td>
</tr>
<tr>
<td>Infectious bursal disease</td>
<td>AstP</td>
<td>20 × 7d</td>
<td>-</td>
<td>27</td>
<td>Yu and Zhu, 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40 × 7d</td>
<td>-</td>
<td>33</td>
<td>Yu and Zhu, 2000</td>
</tr>
</tbody>
</table>

1 LenP = Lentinus edodes polysaccharide; AstP = Astragalus membranaceus Radix polysaccharide;

2 \(d\) = number of successive days of treatments;

3 Relative incidence or mortality was calculated based on 100 % incidence or mortality for the infected groups.

**Parasitic Infections**

The mushroom (TreP\(_m\)) and herb (AstP) polysaccharides, which were used in conjunction with vaccine, have shown preliminary promise in controlling experimental coccidia infections (Hu et al., 1998; Pang et al., 2000). The mushroom polysaccharide (TreP\(_m\)) with vaccine significantly enhanced immune response and body weight gain of coccidial infected chickens, and the optimum dosage of TreP\(_m\) was 200mg/kg body weight (Table 7). TreP\(_m\) enhanced T-cell immune responses, characterized by IFN-\(\gamma\) secretion,
against coccidiosis in chickens (Hu et al., 1996).

**Table 7.** Efficacy of *Tremella fuciformis* mycelia polysaccharide (*TreP*<sub>m</sub>) against *Eimeria tenella* infection in chickens (Hu et al., 1998)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose</th>
<th>BWG&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Mortality</th>
<th>Lesion score&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Oocyst output&lt;sup&gt;1&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Challenge</td>
<td>5 × 10&lt;sup&gt;6&lt;/sup&gt; oocyst b&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Vaccine</td>
<td>75 oocyst b&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>87</td>
<td>90</td>
<td>67</td>
<td>25</td>
</tr>
<tr>
<td><em>TreP</em>&lt;sub&gt;m&lt;/sub&gt;</td>
<td>200 × 7 mg kg&lt;sup&gt;-1&lt;/sup&gt; d</td>
<td>78</td>
<td>139</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td><em>TreP</em>&lt;sub&gt;m&lt;/sub&gt;+Vaccine</td>
<td>135</td>
<td>0</td>
<td>67</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> BWG, mortality, lesion score and oocyst output were expressed relative to 100% of the challenge group.

**Bacterial Infections**

The mushroom polysaccharide (*TreP*<sub>m</sub>) can be used as an adjuvant of avian *Pasteurellosis* vaccine in chickens (Yuan et al., 1993). A dose response trial of Yuan et al. (1993) showed that the mortality of the groups immunized with *TreP*<sub>m</sub> (80, 120, 150, 200 and 300 mg/kg bw) and the vaccine was 50, 37, 22, 37 and 0% respectively after avian *Pasteurellosis* (MLD C48<sup>-1</sup>) infection. The mortality of the groups that received only the vaccine was 70%. Thus, the protection rate of the vaccine was increased 67-160% using *TreP*<sub>m</sub> as an adjuvant. A further experiment conducted by Yuan et al., (1993) showed that the mortality of the groups immunized with *TreP*<sub>m</sub> (300 mg/kg bw) with the vaccine were 14, 44, and 42% after successive *Pasteurellosis* infections at 29, 78 and 105 days of age, and the mortality of the groups which received the vaccine only was 32, 46, and 58% respectively. The protection rate of the vaccine increased 3.7-27% in conjunction with *TreP*<sub>m</sub>, indicating that *TreP*<sub>m</sub> has anti-bacterial activity.

**PREBIOTIC EFFECTS**

Recent studies have demonstrated that non-digestible (fermentable) carbohydrates or dietary fiber can play an important role in animal production and human health. Gibson and Roberfroid (1995) described these ingredients as prebiotics, because they are not digestible by mammalian enzymes, but they could selectively stimulate growth and (or) metabolism of beneficial bacteria in the hindgut. These characteristics may results in the exclusion of pathogens in the gut. The prebiotics described to date range in size from small sugar alcohols and disaccharides to oligosaccharides and large polysaccharides (Rolfe, 2000).
The majority of bacterial species that colonize the intestinal tract require fermentable carbohydrates as an energy source (Salyers et al., 1977). The inclusion of non-digestible carbohydrates in the diets not only serves to provide nutrient sources for bacteria but also influences the entire gastrointestinal ecosystem (Buddington and Weiher, 1999; Langhout, 1999).

CONCLUSION

In this review of the literature, it was reported that polysaccharides are widely distributed in natural medicinal products, particularly plants and fungi. Polysaccharides from different species of these products and even from the same species showed different physico-chemical properties such as molecular weights, sugar composition and structure. The link between the structure of the polysaccharides and their bio-activities is not well understood. It can be concluded that the polysaccharides particularly those derived from mushrooms (*Lentinus, Tremella*) and the herb (*Astragalus*) have been used as immune enhancers or immuno-dulators in rats and humans and partly in chickens, activating both innate and adaptive or specific immunity including the cellular and humoral immunity. These mushroom and herb polysaccharides were also shown to significantly reduce the impacts of bacterial, viral and parasitic infections by enhancing the immune response of chickens. They have been used as either adjuvants of vaccines, therapeutic agents or feed supplements, and shown anti-viral, anti-bacterial and anti-parasitic activities in chickens.

Today’s poultry producers are confronted by numerous techniques that have been proposed as possible therapies to prevent outbreak of diseases. The immuno-active polysaccharides from mushrooms and herbs might be good candidates as growth and health promoting feed additives in poultry diets. Thus, further studies are needed to focus on the prebiotic effects of bio-active components to achieve optimum productive performance in poultry, and to elucidate the possible mode of action related to the gut ecosystem and the immune response.

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Chapter 2

EXTRACTION AND IDENTIFICATION OF WATER-SOLUBLE POLYSACCHARIDES FROM TWO MUSHROOMS AND AN HERB

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To be submitted
Extraction and Identification of Water-Soluble Polysaccharides from Two Mushrooms and An Herb

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Abstract

The aim of the present study was to isolate and characterize water-soluble polysaccharide fractions from two mushrooms, 

\textit{Lentinus edodes (LenS)} and \textit{Tremella fuciformis (TreS)}, and an herb, \textit{Astragalus membranaceus Radix (AstS)}, so as to study their bio-activities \emph{in vitro} and \emph{in vivo}. The dried and grounded intact mushroom and herb materials (\textit{LenS}, \textit{TreS} and \textit{AstS}) were sequentially extracted with hot water, according to the general procedure of water-soluble polysaccharide extraction, and protein was partially removed with an equal volume of 10\% tri-chloroacetic acid. Finally three volumes 96\% ethanol was used to precipitate polysaccharide-containing materials, and the polysaccharide precipitate was collected and freeze-dried for dry matter, protein and total sugar content measurements. Sugar composition of the intact materials and their extracts was analyzed using a High-Performance Anion Exchange Chromatography (HPAEC). The results showed that the two mushrooms gave lower yields (8-10\%) of polysaccharides than that of the herb (31\%) with polysaccharides making up over 60\% of the DM of the extract. The results of sugar composition analysis showed that \textit{LenS} contains fructose, galactose, glucose, mannose, xylose and glucuronic acid with molar ratio of 0.1: 0.2: 9.1: 0.1: 0.5: 0.1; \textit{TreS} contains fructose, galactose, glucose, mannose, xylose and glucuronic acid with molar ratio of 0.9: 0.1: 3.5: 1.5: 4.1: 0.5; and \textit{AstS} contains rhamnose, galactose, glucose, mannose, xylose and galacturonic acid with molar ratio of 0.6: 0.2: 8.3: 0.4: 0.1: 0.3. Of the three extracts, \textit{AstE} contains only glucose, and \textit{TreE}, however, contains fructose, galactose, glucose, mannose, xylose, and glucuronic acid with a molar ratio of 0.8: 0.2: 3.0: 1.6: 4.5: 0.2; \textit{LenE} contains galactose, glucose, and xylose with a molar ratio of 0.8: 8.8: 0.5. Intact mushroom and herb materials and their polysaccharide extracts showed differences in their physico-chemical properties in terms of protein and polysaccharide contents, as well as the sugar compositions.

Keywords: mushroom, herb, extraction, polysaccharides, composition
INTRODUCTION

A current estimation of the number of immuno-active natural medicine ranges between 200-300, with most of them originating from plants (Li, 2000). It was found that the immuno-active components of these natural medicinal products include polysaccharides, glycosides, alkaloids, volatile oils and organic acids, of which polysaccharides were considered to be the most important components (Xue and Meng, 1996). As reviewed by Xue and Meng (1996), polysaccharides were generally derived from plants mainly spermatophyta and pteridophyta, fungus, and algae.

Some polysaccharides from herbs, such as Astragalus membranaceus Radix, and mushrooms, such as Lentinus edodes and Tremella fuciformis, have been studied more extensively. Polysaccharides were isolated and purified from roots of Astragalus (Huang et al., 1982), and from both the mycelia (Wu et al., 1984; Jin et al., 1991; Liu et al., 1999) and the fruit body (Xia and Cheng 1988; Yang et al., 1999, 2001) of Lentinus and Tremella. The polysaccharides from different mushroom and herbal species and even from the same species are different in their physico-chemical properties such as the molecular weights, sugar composition and structure, as reviewed by Guo et al. (accepted). These components may act as immune enhancers or immunodulators and, according to Xue and Meng (1996), and may display multiply bio-activities.

The determination of the structure and sugar composition of the polysaccharide fractions usually includes, as a first step, the isolation of the polysaccharides from their original sources by removal the other constituents such as protein, fat and starch, without modifying the polysaccharide materials. Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are generally used in the analysis of rather simple polysaccharides or polysaccharide fragments. Chemical and specific enzymatic degradation is used to obtain fragments that fit within the analytical range of NMR and MS analyses (Huisman et al., 1998). An improved chromatographic technique known as High-Performance Anion Exchange Chromatography (HPAEC) was developed to separate carbohydrates. Coupled with pulsed amperometric detection (PAD), it permits direct quantification of carbohydrates at low picomole levels with minimal sample preparation and clear-up (Stroop et al., 2002).

In this study, the water-soluble polysaccharides from two mushrooms, Lentinus edodes and Tremella fuciformis, and an herb, Astragalus Membranaceus Radix, were isolated and characterized in order to study their bio-activities in vitro and in vivo. The physico-chemical
properties of the polysaccharide extracts, in terms of protein, total polysaccharide contents and their sugar composition, were determined using quantified chemical methods, such as HPAEC (PAD).

**MATERIALS AND METHODS**

*Mushroom and Herb Polysaccharide Preparation*

The two mushrooms, *Lentinus edodes* (*LenS*) and *Tremella fuciformis* (*TreS*), and the herb, *Astragalus membranaceus* (*AstS*), were purchased from a local source (*LenS* and *TreS* produced in Zhejiang China were from Oriental b. v., Nieuwgraaf 302, 6921 RS Duiven, Netherlands; *AstS* produced in Gansu China was from Po Chai Tong, Waterlooplein 13, 1011 NV Amsterdam, Netherlands). *LenS*, *TreS* and *AstS* were dried overnight at 45°C and ground through a 1mm sieve prior to the extraction of their polysaccharide fractions (*LenE*, *TreE* and *AstE*), according to the general procedure of water-soluble polysaccharide extraction of Liu *et al.* (1999) with minor modifications. In brief, two 50g batches of ground *LenS*, *TreS* and *AstS* were boiled in de-mineralized water (500 ml) for 1.5 h. After cooling, the material was centrifuged at 2500g for 10 min. The supernatant was decanted and saved, and the residue extracted twice more in boiling water. The supernatants were combined and then reduced to 2/3 of the original volume by partial freeze-drying. The supernatant was slowly added to three volumes of 96% ethanol and stored at 4°C overnight to precipitate the polysaccharide containing fractions, and then centrifuged at 2500g for 10 min and the supernatant discarded. The precipitate was then washed with a cold mixture of water and ethanol (96%) solution (1:3) and centrifuged again. The precipitate was then re-suspended in water to dissolve it completely. Protein was partially removed by adding an equal volume of 10 % tri-chloroacetic acid. After standing for 2 h, the protein precipitate was removed by centrifugation at 2500g for 20 minutes. The supernatant was collected and slowly added to three volumes of 96% ethanol to precipitate polysaccharides (stored overnight at 4 °C). The solution was centrifuged at 2500g for 10 min. The precipitate was washed with a mixture of water and ethanol (96%) solution (1:3) and centrifuged again. The polysaccharide precipitate was then collected, frozen and freeze-dried.
Dry matter, Protein and Total Sugar Contents

The dry matter contents of both intact materials and the extracts were determined by freeze-drying, until no further decrease in weight was observed. Protein content was determined by the semi-automated Kjeldahl method (ISO, 1997). Total sugar content of the extracts was determined using a spectrophotometric method based on the reaction of the phenol-sulphuric acid with carbohydrates (Dubois et al., 1956).

Sugar Composition

This hydrolysis method is referred to as Saeman hydrolysis (Garleb et al., 1989) with some modifications. Samples (40.0 mg) of the three intact mushroom and herb materials (LenS, TreS and AstS) and their extracts (LenE, TreE and AstE) as well as the residues after hot water extraction (LenR, TreR and AstR) were hydrolyzed by weighing into Kimax tubes. The tubes were put in an ice-bath and added with 0.45 ml 72% (w/w) H₂SO₄. The tubes were put in a heating block at 30 °C for 1h. After this pretreatment, distilled water (4.95ml) was added to the mixture and mixed well on a vortex mixer. The tubes were heated at 100 °C for 3 h in the heating block and then let them cool to room temperature. After cooling, the hydrolyzates (120 μl) were added with water (2.4 ml) and were neutralized with 1mol Ba(OH)₂ (480 μl), and then filtered through a 0.2 μm-nylon filter. Samples were stored at 4°C for sugar composition analysis, such as fructose, rhamnose, arabinose, xylose, mannose, galactose and glucose as well as galacturonic acid and glucuronic acid.

HPAEC of the samples was performed in a Dionex DX-500 Bio-LC system (Dionex Corp., Sunnyvale, CA), using a CarboPac PA 10 column (250 × 4 mm), and detection was made using a pulsed amperometric detector (Dionex Corp., Sunnyvale, CA). Data were collected and analyzed on computers equipped with the Dionex PeakNet software.

RESULTS

Mushroom and Herb Composition

The dry matter contents for LenS, TreS and AstS were very similar (Table 1). Of the three intact materials, TreS had the lowest crude protein content, about half that of LenS and 2/3 of AstS. The two mushrooms gave lower yields (8-10%) of polysaccharides than that of
the herb (31%) with polysaccharides making up over 60% of the DM of the extract. The dry matter contents for LenE, TreE and AstE were not different. The crude protein content of LenE was almost double that of TreE and AstE.

Table 1. Chemical analyses of the air-dry intact mushroom and herb materials and their polysaccharide extracts

<table>
<thead>
<tr>
<th>Compositions</th>
<th>Intact mushroom and herb materials&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LenE</td>
</tr>
<tr>
<td>Dry matter (DM) (g/kg)</td>
<td>952</td>
</tr>
<tr>
<td>Crude protein (g/kg DM)</td>
<td>114</td>
</tr>
<tr>
<td>Yields of polysaccharides (%)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>8.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Polysaccharide extracts&lt;sup&gt;3&lt;/sup&gt;</th>
<th>LenE</th>
<th>TreE</th>
<th>AstE</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM (g/kg)</td>
<td>632</td>
<td>687</td>
<td>647</td>
</tr>
<tr>
<td>Crude protein (g/kg DM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total sugar content (g/kg DM)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Yields of the polysaccharide are expressed as the percentage of the air-dry matter.

<sup>2</sup> LenS = Lentinus edodes; TreS = Tremella fuciformis; AstS = Astragalus membranaceus;

<sup>3</sup> LenE = Lentinus edodes polysaccharide extract; TreE = Tremella fuciformis polysaccharide extract; AstE = Astragalus membranaceus polysaccharide extract.

**Sugar Composition**

Sugar composition of the intact mushroom and herb materials and their polysaccharide extracts, as well as the residues after hot water extraction is presented in Table 2. Of the three intact materials, LenS contains fructose, galactose, glucose, mannose, xylose and glucuronic acid with molar ratio of 0.1: 0.2: 9.1: 0.1: 0.5: 0.1; TreS contains fructose, galactose, glucose, mannose, xylose and glucuronic acid with molar ratio of 0.9: 0.1: 3.5: 1.5: 4.1: 0.5; and AstS contains rhamnose, galactose, glucose, mannose, xylose and galacturonic acid with molar ratio of 0.6: 0.2: 8.3: 0.4: 0.1: 0.3. Both LenS and AstS contain a higher amount of glucose compared with TreS (91 and 83 mol% vs. 35 mol%), but AstS contain rhamnose and galacturonic acid that were not present in both LenS and TreS.

Of the three extracts, AstE contains pure glucose, and TreE, however, contains fructose, galactose, glucose, mannose, xylose, and glucuronic acid with a molar ratio of 0.8: 0.2: 3.0: 1.6: 4.5: 0.2; LenE contains galactose, glucose and xylose with a molar ratio of 0.8: 8.8: 0.5. The residues contain a high amount of sugars except the LenR.
Table 2. Sugar composition of the air-dry intact mushroom and herb materials and their polysaccharide extracts

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Sugar composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fru</td>
</tr>
<tr>
<td><strong>LenS</strong></td>
<td>0 (1)</td>
</tr>
<tr>
<td><strong>TreS</strong></td>
<td>4 (9)</td>
</tr>
<tr>
<td><strong>AstS</strong></td>
<td>3 (6)</td>
</tr>
<tr>
<td><strong>LenE</strong></td>
<td></td>
</tr>
<tr>
<td><strong>TreE</strong></td>
<td>3 (8)</td>
</tr>
<tr>
<td><strong>AstE</strong></td>
<td></td>
</tr>
</tbody>
</table>

1. *LenS* = *Lentinus edodes*; *TreS* = *Tremella fuciformis*; *AstS* = *Astragalus membranaceus*; *LenE* = *Lentinus edodes* polysaccharide extract; *TreE* = *Tremella fuciformis* polysaccharide extract; *AstE* = *Astragalus membranaceus* polysaccharide extract. *LenR* = *Lentinus edodes* residue; *TreR* = *Tremella fuciformis* residue; *AstR* = *Astragalus membranaceus* residue.

2. Fru = fructose; Rha = rhamnose; Ara = arabinose; Gal = galactose; Glu = glucose; Man = mannose; Xyl = xylose; GalA = galacturonic acid; GlcA = glucuronic acid.

3. w/w%: percentages of the sample weights.

4. mol%: percentages of the molar weights.

**DISCUSSION**

The bioactive components in herbs (including medicinal mushrooms) are quite complex. The common components derived from herbs and mushrooms include saccharides (mono-, oligo- and poly-saccharides), glycosides or heterosides (flavonoid glycoside, anthraquinone, cardiac glycoside, coumarin, saponin, phenolis glycoside, and cyanogenetic glycoside), alkaloid, lactone, volatile oils and organic acids, tannin, resin, phytochrome, protein, amino acids and inorganic salts (Yang and Feng, 1998). The portion of the plant used medicinally for *AstS* is the four- to seven-year-old dried roots collected in the spring (Xu, 1990), and for *LenS* and *TreS* is the fruiting bodies (Yang et al., 1999; Huang 1982). There are many publications concerning the composition of the mushroom and herb species studied above. Of the available literature, it seems that the composition of *Lentinus edodes* has been studied...
most extensively. *Lentinus edodes* contains proteins, fats, carbohydrates, fiber, vitamins, and minerals and a variety of bioactive constituents such as *Lentinan*, a heteroglucan, guanosine 5’–monophosphate (Yang et al., 1999). *Tremella fuciformis* contains more than 70% dietary fibers such as acid polysaccharides, glucurono-xylomannan, and vitamin D (Min, 1996). The hairy roots of the large-scale cultured *Astragalus membranaceus* contained 5.81% crude saponin, 0.14% astragaloside, 23% total polysaccharide (acidic 8.29% and soluble 14.88%) (Zheng et al., 1998). Active constituents of *Astragalus membranaceus* include saponins, flavonoids, polysaccharides, triterpene glycosides, 22 different amino acids and 14 micro-minerals (Qi, 1987).

Some physico-chemical properties of the polysaccharide fractions derived from the two mushrooms (*LenS* and *TreS*) and the herb (*AstS*) in literature was summarized in Table 3. Two kinds of glucan polysaccharides and two kinds of complex polysaccharides were isolated from *Astragalus membranaceus* (Huang et al., 1982). There are three kinds of polysaccharide-protein complexes obtained from the fruit body of *LenS* (Yang et al., 1999, 2001), and a heterosaccharide was isolated from *TreS* (Xia and Cheng, 1988). Thus, there were probably a variety of complex carbohydrates in the crude extracts obtained in this study.

It was found in this study that *AstE* was composed of pure glucose, which is consistent with a study of Huang et al. (1982) that two kinds of water-soluble glucans were isolated from *AstS* (Table 3). However it seems a water-soluble heterosaccharide, which was composed of glucose and arabinose, was not obtained from *AstS*, according to this literature. A study of Xia and Cheng (1988) showed that a polysaccharide composed of fructose, arabinose, xylose, mannose, glucose and glucuronic acid, was isolated from *TreS*, which fits well the sugar composition of *TreE* in this study, except that galactoses were also found in *TreE*. There are three kinds of polysaccharide-protein complexes obtained from the fruit body of *LenS*, which were composed of different ratio of arabinose, xylose, mannose, galactose and glucose (Yang et al., 1999, 2001). This is not consistent with the present study, in which both arabinose and mannose, however, were not present in *LenE*. Arabinoses were not found in the intact material (*LenS*) either, and mannose in the intact material was quite low (1%) which may not be detectable by HPAEC.
### Table 3. Physico-chemical properties of mushroom and herb polysaccharides according to literature

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Polysaccharides</th>
<th>Characteristics</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical properties</td>
<td><em>AstP</em></td>
<td><em>AstP</em>-1, water-soluble glucan; <em>AstP</em>-2, water-insoluble glucan; <em>AstP</em>-3, acidic heterosaccharide; <em>AstP</em>-4, water-soluble heterosaccharide</td>
<td>Huang <em>et al.</em>, 1982</td>
</tr>
<tr>
<td>Molecular weights (Dalton)</td>
<td><em>AstP</em></td>
<td><em>AstP</em>-1, 1.23 × 10&lt;sup&gt;4&lt;/sup&gt;; <em>AstP</em>-2, 3.46 × 10&lt;sup&gt;4&lt;/sup&gt;; <em>AstP</em>-3, 3.63 × 10&lt;sup&gt;4&lt;/sup&gt;;</td>
<td>Qi, 1987</td>
</tr>
<tr>
<td></td>
<td><em>TreP</em></td>
<td>water-soluble heterosaccharide</td>
<td>Xia and Cheng, 1988</td>
</tr>
<tr>
<td>Sugar composition</td>
<td><em>AstP</em></td>
<td><em>AstP</em>-1, Glu; <em>AstP</em>-2, Glu; <em>AstP</em>-3, HexA: Glu: Rha: Ara = 1: 0.04: 0.02: 0.01; <em>AstP</em>-4, Glu: Ara = 1: 0.15</td>
<td>Huang <em>et al.</em>, 1982</td>
</tr>
<tr>
<td></td>
<td><em>LenP</em></td>
<td><em>LenP</em>-1, Ara: Xyl: Man: Gal: Glu = 0.39: 0.46: 1.0: 0.93: 14.1; <em>LenP</em>-2, Ara: Xyl: Man: Gal: Glu = 0.19: 0.41: 1.0: 0.93: 10.7; <em>LenP</em>-3, Ara: Xyl: Man: Gal: Glu = 0.31: 0.47: 1.0: 1.15: 8.9</td>
<td>Yang <em>et al.</em>, 1999, 2001</td>
</tr>
<tr>
<td></td>
<td><em>TreP</em></td>
<td>L-Fru: L-Ara: D-Xyl: D-Man: D-Glu: GlcA = 0.92: 0.49: 0.18: 1.0: 1.15: 0.57</td>
<td>Xia and Cheng, 1988</td>
</tr>
</tbody>
</table>

<sup>1</sup> *AstP* = *Astragalus membranaceus* polysaccharides with four fractions (*AstP*-1, *AstP*-2, *AstP*-3 and *AstP*-4); *LenE* = *Lentinus edodes* polysaccharides derived from body fruit with three fractions (*LenP*-1, *LenP*-2 and *LenP*-3) and *TreP* = *Tremella fuciformis* polysaccharides derived from body fruit.

<sup>2</sup> Fructose = fructose; Ara = arabinose; Xyl = xylose; Man = mannose; Gal = galactose; Glu = glucose; Rha = rhamnose; GlcA = glucuronic acid; HexA = Hexuronic acid.
There are some variations in sugar composition and the molar ratio of the polysaccharides in the literature and in the present study. It is not surprising since polysaccharide fractions in the mushroom and herb extracts were not purified in this study, which were around 60% of the extracts based on dry matter contents. However, the polysaccharides reported in the literature were purified and identified. The two mushrooms gave lower yields (8-10%) of polysaccharides than that of the herb (31%). Although water extraction is a crude process, water-soluble polysaccharides made up over 60% of the extracted dry matter in all these products. As indicated below, proteins and other unidentified compounds were also present in the water extracts.

Intact materials differed in their protein content. TreS had the lowest protein content, whereas LenS had the highest protein content. Mushrooms tend to have a higher protein content when they are younger and growing rapidly. It seems that part of the intact substrate protein was extracted along with the polysaccharides, possibly due to the presence of a polysaccharide-protein complex. For example, Liu et al. (1999) isolated a polysaccharide-protein complex from LenS that consisted of 94.2% carbohydrate and 5.8% protein. Uncharacterized materials were also extracted along with the polysaccharides.

In brief, the two mushrooms (LenS and TreS) gave lower yields (8-10%) of polysaccharides than that of the herb (AstS) (31%) with polysaccharides making up over 60% of the dry matter of the extract. The protein was partly removed from extracts. Sugar composition was differed in different intact mushroom and herb materials and their polysaccharide extracts. Further studies are needed to purify the polysaccharide fractions from these products and elucidate their physico-chemical properties such as the sugar composition, molecular weights and the structure.

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Chapter 3

IN VITRO FERMENTATION CHARACTERISTICS OF TWO MUSHROOM SPECIES AND AN HERB, AND THEIR POLYSACCHARIDE FRACTIONS, USING CHICKEN CECAL CONTENTS AS INOCULUM

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Poultry Science (in press)
In Vitro Fermentation Characteristics of Two Mushroom Species and An Herb, and their Polysaccharide Fractions, Using Chicken Cecal Contents as Inoculum

F.C. Guo, B.A. Williams, R.P. Kwakkel, and M.W.A. Verstegen

Abstract

In vitro fermentabilities of two mushrooms (Lentinus edodes – LenS; Tremella fuciformis – TreS), an herb (Astragalus membranaceus – AstS), and their polysaccharide fractions (LenE, TreE and AstE), were investigated using microflora from chicken ceca. Polysaccharides were extracted using the hot water method. The mushrooms had lower polysaccharide yields (8-10%) than the herb (31%). Fermentation kinetics were determined using the in vitro cumulative gas production technique. End-products such as gas, volatile fatty acids (VFA) and ammonia were also determined. The gas profiles of intact materials were similar for AstS and LenS. TreS had a biphasic digestion pattern. The extracts had similar profiles to the intact materials, though gas production rates were faster. Intact materials tended to produce less VFA than the extracts, though LenS and AstE had the highest total VFA production overall. Intact materials contained more protein than the extracts, and therefore resulted in more branched-chain fatty acids and ammonia. Fermentation kinetics and end-point products demonstrated differences in availability of substrates between the mushrooms and herb. These medicinal mushroom and herb materials, particularly their polysaccharide extracts, show promise in altering microbial activities and composition in chicken ceca. In vivo experiments are necessary for confirmation of this hypothesis.

Key words: mushroom, herb, polysaccharides, fermentation kinetics, fermentation end-products
INTRODUCTION

There is increasing interest in gastro-intestinal fermentation by the intestinal microflora in relation to both gut and overall health of the host. The intestinal environment can be influenced by diet composition and by dietary additives such as antibiotics, prebiotics and other feed additives, and may thus change the composition and activity of the microbiota of animals (Gardiner et al., 1993). Certain plant polysaccharides are now recognized as having prebiotic activity (Verstegen and Schaaftsma, 1999; Cummings and Macfarlane, 2002). Prebiotics are defined as non-digestible food ingredients that beneficially affect the host, by selectively stimulating the growth and/or activity of one or a limited number of bacterial species in the colon and thus improving host health (Gibson and Roberfroid, 1995). In poultry, fermentation occurs mainly in the ceca. Ceca provide a stable environment for microorganisms and as a result, contain the largest and most complex microbial community. Some bacteria in the large intestine can hydrolyze plant polysaccharides, producing small molecular weight carbohydrates from large polymers in humans and animals (Sunvold et al., 1995). Fermentation of carbohydrates leads to the production of mainly straight-chain fatty acids, and will result in the net utilization of ammonia for growth of bacteria. Increased volatile fatty acids (VFA) and decreased ammonia are perceived as being beneficial for long-term host health (Williams et al., 2000). Hence, fermentable carbohydrates, such as oligo- and polysaccharides, can be considered as potential additives (prebiotics) to animal diets.

It was found that the immunologically active components in medical mushrooms and plants may include polysaccharides, glycosides, alkaloid, volatile oils and organic acids (Yang and Feng, 1998). A current estimation of the number of immunologically active plants and fungi ranges between 200-300, and, of these, polysaccharides were considered to be one of the most important components in this regard (Xie and Niu, 1996). Most research on microbial degradation of plant cell walls was done in ruminants with forage crops and grasses (Burrit et al., 1984; Engels, 1996), alfalfa (Titgemeyer et al., 1991), straw (Chesson, 1981), and soybeans (Van Laar et al., 1999), all of which could influence the composition of the bacterial populations in the hindgut of host animals. Certain polysaccharides from mushrooms and herbs that have been used as immune enhancers have shown anti-bacterial, anti-viral and anti-parasitic activities in chickens (Xue and Meng, 1996). Such mushrooms and herbs may also act as prebiotics and thereby enhance colonization resistance of the host gut to potential pathogens. However, little is known about the effects of these
polysaccharides on the microbial community and its activities, nor about the microbial ecosystem within the gut of chickens.

The long-term goal of this work was to investigate whether specific mushrooms and herbs, or their water-soluble extracts, may have a prebiotic and/or immune-enhancing effect in chickens. The specific objectives of the experiment reported here, were to determine the in vitro fermentability of intact mushroom and herbal substrates and their polysaccharide extracts using microflora from the hindgut of chickens as an inoculum. Endpoint pH, VFA and ammonia were also measured to determine the effect of the substrates on the fermentation end-products, in order to use the most fermentable mushroom and herb substrates as potential ingredients for future inclusion in experimental poultry diets.

MATERIALS AND METHODS

Mushroom and Herb Polysaccharide Preparation

The two mushrooms, Lentinus edodes (LenS) and Tremella fuciformis (TreS), and the herb, Astragalus membranaceus (AstS), were purchased from a local source (LenS and TreS produced in Zhejiang China were from Oriental b. v., Nieuwgraaf 302, 6921 RS Duiven, Netherlands; AstS produced in Gansu China was from Po Chai Tong, Waterlooplein 13, 1011 NV Amsterdam, Netherlands). LenS, TreS and AstS were dried overnight at 45°C and ground through a 1mm sieve prior to the extraction of their polysaccharide fractions (LenE, TreE and AstE), according to the general procedure of water-soluble polysaccharide extraction of Liu et al. (1999) with minor modifications. In brief, two 50g batches of ground LenS, TreS and AstS were boiled in de-mineralized water (500 ml) for 1.5 h. After cooling, the material was centrifuged at 2500g for 10 min. The supernatant was decanted and saved, and the residue extracted twice more in boiling water. The supernatants were combined and then reduced to 2/3 of the original volume by partial freeze-drying. The supernatant was slowly added to three volumes of 96% ethanol and stored at 4°C overnight to precipitate the polysaccharide containing fractions, and then centrifuged at 2500g for 10 min and the supernatant discarded. The precipitate was then washed with a cold mixture of water and ethanol (96%) solution (1:3) and centrifuged again. The precipitate was then re-suspended in water to dissolve it completely. Protein was partially removed by adding an equal volume of 10 % tri-chloroacetic acid. After standing for 2 h, the protein precipitate was removed by centrifugation at 2500g for 20 minutes. The supernatant was collected and slowly added to
three volumes of 96% ethanol to precipitate polysaccharides (stored overnight at 4°C). The solution was centrifuged at 2500g for 10 min. The precipitate was washed with a mixture of water and ethanol (96%) solution (1:3) and centrifuged again. The polysaccharide precipitate was then collected, frozen and freeze-dried.

*Substrate Analyses*

The dry matter contents of both intact materials and the extracts were determined by freeze-drying, until no further decrease in weight was observed. Protein content was determined by the semi-automated Kjeldahl method (ISO, 1997). Total sugar content of the extracts was determined using a spectrophotometric method based on the reaction of the phenol-sulphuric acid with carbohydrates (Dubois et al., 1956).

*Substrates, Inoculum and Method of Incubation*

Fermentation kinetics was assessed using the *in vitro* cumulative gas production technique as described by Theodorou et al., (1994), using an automated system (Davies et al., 2000). Approximately 1.0 g of each substrate was weighed accordingly into a 100-ml serum bottle. To this was added 82 ml of semi-defined medium (modified from Lowe et al., 1985). This medium was originally developed to support growth of rumen microorganisms and should supply all requirements in terms of minerals, vitamins and co-factors, except for energy, which in this case was supplied by the test substrate. All bottles were inoculated with 5 ml of the diluted cecal contents prepared as described below. Bottles were then incubated for 72 h at 39± 0.5 °C.

Chicken cecal contents were collected from a total of 40 slow-growing broilers (81-day old) fed with a commercial diet free of added antibiotics. Cecal contents were pooled and kept in a warmed thermos flask filled with CO₂. The flask was sealed and transported to the lab (~ one hour transport). The inoculum was diluted 1:4 with saline (9 g 1⁻¹ NaCl), mixed using a hand-blender for 60 seconds, and filtered through a double-layer cheesecloth under a constant stream of CO₂.

Three replicate bottles were used for each of the intact materials (*LenS*, *TreS* and *AstS*), while four replicates were used for the three extracts (*LenE*, *TreE* and *AstE*). Two replicate bottles containing only medium and cecal inoculum were used as negative controls.
**VFA and Ammonia Analysis**

After 72 h incubation, pH was measured and samples for VFA and ammonia analysis taken from every bottle. Samples for VFA analysis (10 ml) were added to bottles containing 0.5 ml of phosphoric acid (85%) and frozen (-20°C) until analysis. Additional samples (5.0 ml) were added to bottles containing 5 ml of 10% tri-chloroacetic acid and were frozen until analysis for ammonia.

VFA contents: acetic (AAC), propionic (APR), butyric (ABU), valeric (AVAL), iso-butyric (AIBU), and iso-valeric (AIVAL) acids, were determined by GLC (Fisons HRGC Mega 2; CE Instruments, Milan, Italy, glass column filled with chromasorb 101, carrier gas N₂ saturated with methanoic acid, 190 °C with iso-caproic acid as the internal standard). Total VFA concentration (MMTOT), total VFA production (ATOT) in units of acetic acid equivalents, and the branched-chain ratio (BCR) were calculated according to Bauer et al. (2001). Acetic acid equivalents (Henry, 1981) allow a comparison to be made between acids, on the basis of their carbon content.

Ammonium-N content was determined according to the method described by Scheiner (1976). The supernatants were deproteinized using 10% trichloride acetic acid.

**Curve Fitting of Gas Production Profiles**

For each bottle, cumulative gas production (ml g⁻¹ DM) was fitted to a multi-phasic Michaelis-Menten equation (Groot et al., 1996). The mono-phasic model was used unless the diphasic model significantly improved the fitting (Motulsy and Ransnas, 1987). The model equation was:

\[ Y = \sum_{i=1}^{n} \frac{A_i}{1 + \left( \frac{C_i}{t} \right)^{B_i}} \]

Where
- \( Y \) = the cumulative gas production (ml g⁻¹ DM);
- \( A_i \) = asymptotic gas production for phase i (ml);
- \( B_i \) = Smoothness factor for phase i;
- \( C_i \) = time at which half of the asymptote gas has been produced for phase i (h);
- \( i \) = number of phase in gas production;
- \( t \) = time (h).
The maximum rate of gas production ($R_{\text{max}}$) and the time at which it occurs ($T_{R_{\text{max}}}$) were calculated according to the following equations (W.J.J., Gerrits, Animal Nutrition Group, Wageningen University- personal communication):

$$R_{\text{max}} = (Ax (C^B) x B x (T_{R_{\text{max}}} \cdot (B-1)))/(1+(C^B) x (T_{R_{\text{max}}} \cdot (B)))^2$$

$$T_{R_{\text{max}}} = C x (((B-1)/(B+1))^{(1/B)})$$

**Statistical Analyses**

Gas production parameters, pH, VFA and ammonia values were subjected to statistical analysis using the GLM procedure in SAS (Statistical Analyses System 6.11, 1995). The significance difference between the different substrates was tested using Tukey's studentized range test of multiple comparisons (Steel and Torrie, 1980).

**RESULTS**

**Mushroom and Herb Composition**

The dry matter contents for LenS, TreS and AstS were very similar (Table 1). Of the three intact materials, TreS had the lowest crude protein content, about half that of LenS and 2/3 of AstS. The two mushrooms gave lower yields (8-10%) of polysaccharides than that of the herb (31%) with polysaccharides making up over 60% of the DM of the extract. The dry matter contents for LenE, TreE and AstE were not different. The crude protein content of LenE was almost double that of TreE and AstE.

**Gas Production**

Figure 1 shows representative cumulative gas production profiles for the three intact substrates and their polysaccharide extracts. Gas production of the three extracts was faster than that of their intact substrates. Of the three intact substrates, most gas was produced from LenS. It can be seen from Figure 1, that the gap of the gas production between the extracts and the intact materials were greater for Ast (AstS and AstE) than for Len (LenS and LenE) and Tre (TreS and TreE). Gas production was similar for Len and Ast materials in terms of
following mono-phasic kinetics. Both the intact material and extract of *Tre* showed diphasic fermentation kinetics. However, fermentation of *TreS* had not gone to its fullest extent after 72-hour incubation.

**Table 1.** Chemical analyses of the air-dry intact mushroom and herb materials and their polysaccharide extracts

<table>
<thead>
<tr>
<th>Compositions</th>
<th>Intact mushroom and herb materials&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Polysaccharide extracts&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LenS</td>
<td>TreS</td>
</tr>
<tr>
<td>Dry matter (DM) (g/kg)</td>
<td>957</td>
<td>958</td>
</tr>
<tr>
<td>Crude protein (g/kg DM)</td>
<td>208</td>
<td>103</td>
</tr>
<tr>
<td>Yields of polysaccharides (%)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>8.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

<sup>1</sup> Yields of the polysaccharide are expressed as the percentage of the air-dry matter.

<sup>2</sup> LenS = *Lentinus edodes*; TreS = *Tremella fuciformis*; AstS = *Astragalus membranaceus*;

<sup>3</sup> LenE = *Lentinus edodes* polysaccharide extract; TreE = *Tremella fuciformis* polysaccharide extract; AstE = *Astragalus membranaceus* polysaccharide extract.

Table 2 shows gas production kinetic parameters 72 h after incubation of the different substrates. Except for the *TreS*, there were no differences between substrates in terms of half-time (C) to the asymptotic gas production or of time to maximum rate of gas production (T<sub>Rmax</sub>). The polysaccharide fractions tended to be fermented more rapidly and to a greater extent compared to the intact substrates. The three extracts showed higher maximum rates of gas production (R<sub>max</sub>) and tended to produce more gas compared with their intact substrates. *AstE* had the fastest R<sub>max</sub> and the largest total gas production. The initial pH of the inoculum was 6.84 and the final pH of the incubated blanks was 6.82.

Table 3 shows VFA production 72 h after incubation. In general, total VFA production (MMTOT and ATOT) from the intact materials was lower than that of the extracts, except for LenS, where there was no difference in total VFA production between the intact material and the extract. More branched-chain VFA were produced from the intact materials compared to the extracts as could be seen from the higher BCR. Acetate production was similar for all substrates, with slightly more acetate being produced from TreE compared with AstS. Polysaccharide extracts produced significantly more propionate than the intact materials.
The amount of propionate was smallest from the *AstS* and highest from the *TreE*. Butyrate production was more variable. Least butyrate was produced from the *TreS* and *TreE* substrates and the greatest amounts of butyrate were produced from the *AstS* and *AstE* substrates.

**Table 2.** *In vitro* fermentation gas production kinetics of the intact mushroom and herb materials and their polysaccharide extracts

<table>
<thead>
<tr>
<th>Substrates²</th>
<th><em>T</em>&lt;sub&gt;r max&lt;/sub&gt; (h)</th>
<th>C (g/L)</th>
<th><em>R</em>&lt;sub&gt;r max&lt;/sub&gt; (ml/h)</th>
<th>DMCV (ml/g DM)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>LenS</em></td>
<td>1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>235.5&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>6.3&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>TreS</em></td>
<td>ND&lt;sup&gt;6&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;6&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;6&lt;/sup&gt;</td>
<td>184.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.1&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>AstS</em></td>
<td>0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.2&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>188.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>LenE</em></td>
<td>3.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>33.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>273.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.1&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>TreE</em></td>
<td>13.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>214.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.2&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>AstE</em></td>
<td>2.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>65.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>332.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>MSD³</td>
<td>3.0</td>
<td>8.3</td>
<td>4.3</td>
<td>24.2</td>
<td>0.1</td>
</tr>
<tr>
<td><em>P</em>-values⁴</td>
<td>0.0001</td>
<td>0.0008</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

1. Values are means of 3 determinations for the intact materials and 4 determinations for the extracts.
2. *LenS* = *Lentinus edodes*; *TreS* = *Tremella fuciformis*; *AstS* = *Astragalus membranaceus*; *LenE* = *Lentinus edodes* polysaccharide extract; *TreE* = *Tremella fuciformis* polysaccharide extract; *AstE* = *Astragalus membranaceus* polysaccharide extract.
3. MSD = minimum significant difference.
4. *P*-values = Significant effects of different substrates (P<0.001).
5. C = the half-time to the asymptotic gas production; *T*<sub>r max</sub> = the time when there is the maximum rate of gas production; *R*<sub>r max</sub> = the maximum rate of gas production; DMCV = total gas production.
6. ND indicates poor data fit using both mono- and biphasic models.
7. <sup>abc</sup>: Means within columns with no common superscript differ significantly (P<0.05).

**VFA and Ammonia Production**

Ammonia concentration of the inoculum was 206.1 mg/l and the blanks contained 144.3 mg/l ammonia after incubation. At the end of fermentation, ammonia concentrations were higher for the intact materials than for the extracts (Table 3).
Figure 1. Gas production profiles from fermentation of the intact mushroom and herb materials and their polysaccharide extracts. Values are means of 3 determinations for the intact materials (●), LenS = Lentinus edodes; TreS = Tremella fuciformis; AstS = Astragalus membranaceus, and 4 determinations for the polysaccharide extracts (■), LenE = Lentinus edodes extract; TreE = Tremella fuciformis extract; AstE = Astragalus membranaceus extract.
Table 3. Volatile fatty acids (VFA), branched-chain ratio (BCR) and ammonia (NH₃) production measured at the end of fermentation of intact mushroom and herb materials and their extracts

<table>
<thead>
<tr>
<th>Substrates²</th>
<th>AAC³</th>
<th>APR³</th>
<th>ABU⁵</th>
<th>AIBU⁵</th>
<th>AIVAL⁵</th>
<th>AVAL⁵</th>
<th>MMTOT⁵</th>
<th>ATOT⁵</th>
<th>BCR⁶</th>
<th>NH₃</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg AAE⁷/g DM</td>
<td>mg AAE⁷/g DM</td>
<td>mmol/g DM</td>
<td>mg AAE⁷/g DM</td>
<td>mg AAE⁷/g DM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LenS</td>
<td>251.4ᵃᵇ</td>
<td>178.8ᵇ</td>
<td>114.9ᵃᵇ</td>
<td>7.8ᵃᵇ</td>
<td>15.6ᵃ</td>
<td>21.4ᵇᶜ</td>
<td>7.5ᵇᶜ</td>
<td>590ᵇ</td>
<td>0.082ᵃ</td>
<td>419.1ᵃ</td>
</tr>
<tr>
<td>TreS</td>
<td>236.3ᵃᵇ</td>
<td>173.5ᶜ</td>
<td>60.5ʰᵈ</td>
<td>7.0ᵇ</td>
<td>9.0ᵇᶜ</td>
<td>16.7ᵇᶜ</td>
<td>6.6ᶜᵈ</td>
<td>503ᶜ</td>
<td>0.069ᵇ</td>
<td>322.0ᵇ</td>
</tr>
<tr>
<td>AstS</td>
<td>217.3ᵇ</td>
<td>93.1ᵈ</td>
<td>120.1ᵃᵇ</td>
<td>8.1ᵃᵇ</td>
<td>16.3ᵃ</td>
<td>20.5ᵇᶜ</td>
<td>6.0ᵈ</td>
<td>475ᶜ</td>
<td>0.104ᵃ</td>
<td>399.2ᵃ</td>
</tr>
<tr>
<td>LenE</td>
<td>263.4ᵃᵇ</td>
<td>213.4ᵇ</td>
<td>90.9ᶜ</td>
<td>3.2ᵉᵈ</td>
<td>5.6ᵉᵈ</td>
<td>12.3ᶜ</td>
<td>7.7ᵃᵇ</td>
<td>589ᵇ</td>
<td>0.037ᶜ</td>
<td>245.6ᶜ</td>
</tr>
<tr>
<td>TreE</td>
<td>275.0ᵈ</td>
<td>237.8ᵃ</td>
<td>57.3ᵈ</td>
<td>5.9ᵇᶜ</td>
<td>4.2ᵈᵉ</td>
<td>15.3ᶜ</td>
<td>7.9ᵃᵇ</td>
<td>596ᵇ</td>
<td>0.045ᶜ</td>
<td>253.0ᶜ</td>
</tr>
<tr>
<td>AstE</td>
<td>254.4ᵃᵇ</td>
<td>232.0ᵃᵇ</td>
<td>165.4ᵃ</td>
<td>1.2ᵈ</td>
<td>1.9ᵉ</td>
<td>23.0ᵃᵇ</td>
<td>8.4ᵃ</td>
<td>678ᵃ</td>
<td>0.040ᵈ</td>
<td>144.7ᵈ</td>
</tr>
</tbody>
</table>

MSD³ | 16.7 | 8.1 | 8.0 | 1.2 | 1.3 | 3.3 | 0.3 | 25 | 0.021 | 23.5 |

P-values⁴ | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 |

¹ Values are means of 3 determinations for the intact materials and 4 determinations for the extracts.
² LenS = Lentinus edodes; TreS = Tremella fuciformis; AstS = Astragalus membranaceus; LenE = Lentinus edodes polysaccharide extract; TreE = Tremella fuciformis polysaccharide extract; AstE = Astragalus membranaceus polysaccharide extract.
³ MSD = minimum significant difference.
⁴ P-values = Significant effects of different substrates (P<0.001).
⁵ AAC = acetic acid; APR = propionic acid; ABU = butyric acid; AVAL = valeric acid; AIBU = iso-butyric acid; AIVAL = iso-valeric acid; MMTOT = total VFA concentration; ATOT = total VFA production.
⁶ BCR = (AIBU + AIVAL + AVAL)/(AAC + APOR + ABUT).
⁷ AAE = Acetic acid equivalents.

ᵃᵇᶜ : Means within columns with no common superscript differ significantly (P<0.05).
Of the three intact materials, TreS had the lowest ammonia concentration. Ammonia concentrations were similar for LenE and TreE and were lowest for AstE. Ammonia concentrations of the blanks after fermentation were 377.2 mg/l, which is very much higher than that of three polysaccharides and TreS, and slightly lower than LenS and AstS.

**DISCUSSION**

**Substrate Composition**

The two mushrooms gave lower yields (8-10%) of polysaccharides than that of the herb (31%). Although water extraction is a crude process, water-soluble polysaccharides made up over 60% of the extracted DM in all these products. As indicated below, proteins and other unidentified compounds were also present in the water extracts and may also have influenced fermentation kinetics. Mushrooms tend to have a higher protein content when they are younger and growing rapidly. It seems that part of the intact substrate protein was extracted along with the polysaccharides, possibly due to the presence of a polysaccharide-protein complex. Liu et al. (1999) isolated a polysaccharide-protein complex from LenS that consisted of 94.2% carbohydrate and 5.8% protein. Uncharacterized materials were also extracted along with the polysaccharides.

**Gas Production and Fermentation**

Gas production kinetics for intact materials and extracts of Len and Ast were similar in fermentation in terms of showing mono-phasic kinetics. As would be expected, the fermentation of the extracts was more rapid and occurred to a larger extent since the extracts contain more easily fermentable carbohydrates compared to the intact materials. Both the intact and extracted material of Tre showed diphasic fermentation kinetics. This suggests that there may be two distinct fractions in Tre with greatly different rates of fermentation, which was then reflected in the distinctly different fermentation profiles. However, whether these are differences in carbohydrate structure, protein-carbohydrate linkages or the effects of the other compounds, are not known.

This would suggest that either the non-extracted components present in AstS are not fermentable, or that the fermentable components in AstS stimulated species that do not produce gas. The difference between LenS and LenE was small in terms of the total gas
production, and fermentation of LenE was more rapid compared to LenS. For TreS and TreE, it seems that the main difference was in the fermentation kinetics (fermentation of TreS had not reached completion after 72 h incubation.). The relatively rapid and extensive in vitro fermentation of the extracts means that micro-organisms in those cultures are more effective at breaking down a complex carbohydrate matrix. An active microflora in the large intestine reduces the chances for pathogens to proliferate due to an improved colonization resistance (Raibaud, 1992). Although water extracts are not well defined and contain other materials that may also influence microbial fermentation, it is known that the predominant component in the water extracts of these materials are polysaccharides (> 60%). The relatively rapid and extensive fermentation of the extracts was expected, since they contain a large amount of carbohydrates that seem to be readily fermentable. The difference in the fermentation characteristics between the original substrates is most likely a direct indicator of the difference in polysaccharides, protein and fiber contents. Of these substrates, the rate of fermentation of AstS was slower than for LenS, though this was reversed with the extracts for which AstE had the highest R_max and total gas production. Irrespective of the kinetic phases, fermentation of Tre was slower than the other materials. Although TreE had much lower protein values than LenE and AstE, the ammonia value of TreE was still high, suggesting that there may be unique structural features of TreE that limited the availability of carbohydrates. Houdijk (1998) reported that fermentation of carbohydrates in the large intestine has beneficial health effects. The fermentation of protein, which occurs in the large intestine if there is not enough energy for micro-organisms, may have deleterious effects on animal health, which might be reduced by preferential fermentation of carbohydrates.

**VFA and Ammonia Production**

The VFA are important energy sources for both ruminant and monogastric animals (France and Siddons, 1993). Fermentation in the rumen and hindgut produces volatile fatty acids such as acetic, propionic and butyric acids, which can be metabolized by the animal. The amount and proportion of these volatile fatty acids produced can influence the metabolism of the animals. In ruminants/dairy cows, acetic acid can be used for production of milk fat (France and Siddons, 1993), whereas propionic acid can be used as a precursor for the synthesis of glucose (Van Houtert, 1993). Generally, in ruminants, rapidly fermentable substrates have a relatively higher propionic acid production, whereas more slowly fermentable and cellulose-rich substrates have acetic acid-directed fermentation. This is
caused by differences in intracellular conditions of the microbes (pH, electron donor/acceptor-ratio), which favor the production pathway of propionic acids when fermenting rapidly fermentable materials (Van Houtert, 1993).

Fermentation of carbohydrates leads to the production of mainly straight-chain acids, while the fermentation of protein results in production of branched-chain acids (e.g. from amino acids such as valine, leucine, and iso-leucine) (Macfarlane et al., 1992; Getachew et al., 1998). Also, the fermentation of carbohydrates will more likely result in the net utilization of NH₃ for growth of bacteria, while the fermentation of protein as a source of energy will lead to net production of NH₃ which then diffuses across the intestinal wall into the bloodstream. In terms of animal health, increase of VFA and decreased NH₃ are perceived as being beneficial for long-term host health, while the opposite is true for low VFA and increasing NH₃ (Williams et al., 2000).

Choct (2001) reported that the digestibility of non-starch polysaccharides (NSP) in pigs and poultry is affected by many factors including the physical chemical characteristics of the polysaccharides, the cell wall structure of the plant from which they are derived, and the level of the dietary NSP. There are some vague correlations between the type of carbohydrates and their fermentation products in pigs and poultry. For example, fermentation of soluble pectin produces approximately 80% acetate and only a small amount of butyrate, whereas guar gum produces less acetate and more butyrate (Choct, 2001). However, Canibe et al., (1997) were unable to detect any differences in the molar ratio of short chain fatty acids in different segments of the large intestine of pigs, despite the fact that individual sugars had vastly different rate of fermentation. The intact mushroom and herb materials had higher protein contents and thus tended to have a higher BCR compared with the extracts. The highest concentration of acetate was produced from TreE. Propionate production was greater for the extracts than for the intact materials. Butyrate and valerate production was generally lower for the extracts, except for AstE, which generally had significantly higher levels of all of the straight chain VFA. This was consistent with the greater substrate disappearance seen for in vitro fermentation of these substrates. AstE had the lowest level of branched-chain fatty acids, which, in combination with the high degree of fermentation and lower ammonia levels, would suggest that there could have been more incorporation of nitrogen into microbial cells, though this was not measured. Ammonia levels were significantly higher for the intact materials, which had higher protein contents and presumably less carbohydrate available for fermentation. It can be seen that the ammonia concentration is linked with the BCR, i.e., lower ammonia of the extracts is associated with a
lower BCR (Figure 2). This would suggest that one should avoid feeding animals a large amount of non-digestible N (amino acids) in the absence of appropriately fermentable carbohydrates, to achieve optimal gut health.

![Graph showing the relationship between ammonia concentration and branch chain ratio (BCR)](image)

**Figure 2.** Correlation between the ammonia concentration and the branch chain ratio (BCR = (AIBU + AIVAL + AVAL)/(AAC + APRO + ABUT)) measured at the end of fermentation of intact mushroom and herb materials and their extracts. Values are means of 3 determinations for the intact materials, LenS (▲) = *Lentinus edodes*; TreS (●) = *Tremella fuciformis*; AstS (■) = *Astragalus membranaceus*, and 4 determinations for the polysaccharide extracts, LenE (△) = *Lentinus edodes* extract; TreE (○) = *Tremella fuciformis* extract; AstE (□) = *Astragalus membranaceus* extract.

The intact mushroom and herb materials had a greater protein content and thus tended to have greater concentrations of branched chain VFA and therefore higher BCR than the extracts. These treatments also had numerically higher valeric acid concentrations. Thus, VFA patterns differed between substrates, possibly due to selection by specific microbial species which were best able to ferment specific compounds.

Thus, gas production kinetics, final pH, VFA and NH$_3$ demonstrated differences in availability of substrates for fermentation between the different mushroom and herb materials and their extracts. The fermentation of these polysaccharide extracts resulted in the production of mainly straight-chain fatty acids and decreased NH$_3$. These medicinal mushrooms and the herb, and more particularly their polysaccharide extracts, may influence
the activity and composition of microbial population after fermentation, both in terms of fermentation kinetics and changes in end-products. However, it is still not clear whether these substrates selectively stimulated the growth and activity of a number of beneficial bacteria species in the hindgut of chickens. Further experiments will be carried out to analyse changes in the species present in the bacterial community after in vitro fermentation. In addition, it must be remembered that this study was carried out in vitro. Whether these mushroom and herb polysaccharide extracts can be used as potential additives (prebiotics) to poultry diets is still unknown. Therefore, future in vivo experiments are needed to further test the prebiotic effects of these polysaccharides as growth and health promoters in living chickens.

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Chapter 4

THE EFFECT OF MUSHROOM AND HERB POLYSACCHARIDE FRACTIONS ON DGGE BAND PATTERNS OF CHICKEN CECAL MICROFLORA IN VITRO AS DETERMINED BY 16S RDNA ANALYSIS

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The Effect of Mushroom and Herb Polysaccharide Fractions on DGGE Band Patterns of Chicken Cecal Microflora in Vitro as Determined by 16s rDNA Analysis

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Abstract

The effects of two mushrooms (Tremella fuciformis - TreS; Lentinus edodes - LenS) and an herb (Astragalus membranaceus - AstS), and their polysaccharide fractions (TreE, LenE and AstE) as prebiotics on cecal bacterial community of chickens were investigated in vitro. Specific PCR amplification of 16S rDNA gene fragments in combination with denaturing gradient gel electrophoresis (DGGE) were used to analyze the microbial community before and after in vitro fermentation, using chicken cecal contents as the original inoculum. The similarity indices of the DGGE band pattern of the intact substrates compared with the inoculum and blanks ranged from 40 to 58%, of these, TreS showed the lowest similarity, whereas AstS showed the highest. The similarity indices of the band pattern of the extracts compared with the inoculum and blanks ranged from 23 to 80%, of which LenE showed the lowest similarity, whereas AstE showed the highest. The similarity indices of DGGE were significantly lower in the LenE samples than in the LenS samples compared with the inoculum and blanks, whereas it was higher in the AstE samples than in the AstS samples. However, there were no significant differences between TreS and TreE. Both intact mushroom and herb materials and their polysaccharide extracts led to significant shifts in the bacterial community when fermented in vitro. This suggests that certain species were selected for, as the presence of these materials as sole substrate acted as an enrichment culture. It will be important to establish whether this selection may also occur in vivo, if the same substrates are included in poultry diets.

Keywords: mushroom, herb, polysaccharides, DGGE band pattern, 16S rDNA
INTRODUCTION

The indigenous microflora of monogastric animals makes up a complex ecosystem where the number of bacteria and composition of the microflora vary in different parts of the gastrointestinal tract (GIT) (Jensen, 1999). Bacterial populations in the stomach are kept low by a low pH, whereas in the upper part of the small intestine, transit of digesta is considered to be too rapid to allow for extensive microbial growth. As digesta transit time begins to slow down in the proximal part of the small intestine (ileum) and in the large intestine (cecum, colon and rectum), gut bacteria have more time to multiply, resulting in a large and active microbial community. In poultry, fermentation occurs mainly in the ceca. The ceca provide a stable environment for microorganisms and, as a result, contains the largest and most complex ecosystem. There is a complex community of aerobes, facultative anaerobes and strictly anaerobes in the intestinal tract of poultry. The majority of the community are anaerobes however, and they seem to be primarily responsible for digestion of any nutrients that reach the large intestine (Langhout, 1998). The density of the microbial community in the cecum and colon of monogastric species is around $10^{10} - 10^{11}$ viable cells per gram of digesta, comprising of more than 500 different species (Jensen, 1999). According to Barnes et al. (1972), the total number of bacteria is around $10^{11}$ cells /g (wet weight) in ceca of chickens, which is mainly anaerobic ($10^5$-$10^9$ cells/g).

The intestinal environment can be influenced by diet composition and by dietary additives such as antibiotics, and prebiotics, and may thus change the composition and activity of the microbiota (Gardiner et al., 1993, Jensen, 1993). Some bacteria in the large intestine are more specialized in the hydrolysis of plant polysaccharides, producing small molecular weight carbohydrates from large polymers (Sunvold et al., 1995). Certain plant polysaccharides are now recognized as having prebiotic activity (Verstegen and Schaafsma, 1999; Cummings and Macfarlane, 2002). Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selective stimulation of the growth and/or activity of one or a limited number of bacterial species in the colon while may improve host health (Gibson and Roberfroid, 1995).

Polysaccharides are considered to be the important immuno-active components in medicinal mushrooms and herbs (Xie and Niu, 1996). Certain polysaccharides from mushrooms and herbs, which were used as immune enhancers, have shown anti-bacterial, anti-viral and anti-parasitic activities (Xue and Meng, 1996). They may also have prebiotic effects in terms of altering microbial composition and activity in the hindgut of host animals,
though this has not yet been reported in literature. In terms of describing a very complex microflora in the GIT, a molecular technique based on 16S ribosomal DNA (16S rDNA) was developed to study microbial diversity in ecosystems (Amann et al., 1995; Muyzer et al., 1996; Vaughan et al., 1999). Denaturing gradient gel electrophoresis (DGGE) is a genetic fingerprinting technique that examines microbial diversity based upon the electrophoresis of PCR-amplified gels containing a linear gradient of DNA denaturants (Muyzer et al., 1993, 1996). This technology has been extensively used to study a complex microflora of GIT in humans (Satokari et al., 2001; Zoetendal et al., 2001, 2002; Heilig et al., 2002), pigs (Simpson et al., 1999, 2000) and more recently, in chickens (van der Wielen et al., 2002; Zhu et al., 2002).

In recent years, *in vitro* studies have been used to investigate the microbial activities of GIT in animals by analyzing the fermentation kinetics and their end-products such as volatile fatty acids and ammonia (Williams et al., 2000). However, the population dynamics of microbial communities in such systems are only starting to be studied. In a recent study, the cumulative gas production technique (Theodorou et al., 1994), an existing *in vitro* method, was used to assess the fermentation kinetics of the intact mushroom and herb materials, *Tremella fuciformis* - TreS, *Lentinus edodes* - LenS, and *Astragalus membranaceus* - AstS, and their polysaccharide extracts, TreE, LenE and AstE (Guo et al., accepted). The work reported here describes a study carried out alongside the kinetic study, in which the effect of their respective polysaccharides extracts on enrichment of certain species *in vitro* was investigated. This was done by PCR-amplification of V6 to V8 regions of 16S rDNA and comparison of DGGE patterns derived from a 16S rDNA amplicons.

**MATERIALS AND METHODS**

**Mushroom and Herb Polysaccharide Preparation**

Intact mushroom and herb materials were purchased from a local source (*Lentinus edodes* and *Tremella fuciformis* produced in Zhejiang China were from Oriental b. v., Nieuwgraaf 302, 6921 RS Duiven, The Netherlands; *Astragalus membranaceus* produced in Gansu China was from Po Chai Tong, Waterlooplein 13, 1011 NV Amsterdam, The Netherlands). The intact mushroom and herb materials were dried overnight at 45 °C, and ground through a 1mm sieve prior to water-soluble polysaccharide extraction (Liu et al., 1999). The dry matter content of the extracts was determined by freeze-drying. Protein
content was determined by the semi-automated Kjeldahl method (ISO, 1997). Total sugar content of the extracts was determined with the phenol-sulphuric method (Dubois et al., 1956). The yields of the polysaccharide fractions and their chemical analyses are presented in Table 1.

**Substrates, Inoculum and Method of Incubation**

Fermentation kinetics was assessed using the *in vitro* cumulative gas production technique as described by Theodorou et al., (1994), using an automated system (Davies et al., 2000). Approximately 1.0 g of each substrate was weighed accordingly into a 100-ml serum bottle. A semi-defined medium (82 ml), modified from Lowe et al. (1985), was added. This medium was originally developed to support growth of microorganisms and should supply all requirements in terms of minerals, vitamins and co-factors, except for energy, which in this case was supplied by the test substrate. All bottles were inoculated with 5 ml of the diluted cecal contents prepared as described below. Bottles were then incubated for 72 h at 39 ± 0.5°C.

**Table 1.** Chemical analyses of the air-dry intact mushroom and herb materials and their polysaccharide extracts

<table>
<thead>
<tr>
<th>Compositions</th>
<th>Intact mushroom and herb materials¹</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LenS</td>
<td>TreS</td>
</tr>
<tr>
<td>Dry matter (DM) (g/kg)</td>
<td>957</td>
<td>958</td>
</tr>
<tr>
<td>Crude protein (g/kg DM)</td>
<td>208</td>
<td>103</td>
</tr>
<tr>
<td>Yields of polysaccharides (%)¹</td>
<td>8.0</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>LenE</td>
<td>TreE</td>
</tr>
<tr>
<td>DM (g/kg)</td>
<td>952</td>
<td>955</td>
</tr>
<tr>
<td>Crude protein (g/kg DM)</td>
<td>114</td>
<td>47</td>
</tr>
<tr>
<td>Total sugar content (g/kg DM)</td>
<td>632</td>
<td>687</td>
</tr>
</tbody>
</table>

¹ Yields of the polysaccharide are expressed as the percentage of the air-dry matter.

² LenS = *Lentinus edodes*; TreS = *Tremella fuciformis*; AstS = *Astragalus membranaceus*;

³ LenE = *Lentinus edodes* polysaccharide extract; TreE = *Tremella fuciformis* polysaccharide extract; AstE = *Astragalus membranaceus* polysaccharide extract.

Chicken cecal contents were collected from a total of 40 slow-growing broilers (81-day old) fed with a commercial diet, free of added antibiotics. Cecal contents were pooled and
kept in warmed thermos flasks filled with CO₂. The flask was sealed and transported to the lab (~ one hour transport). The inoculum was diluted 1:4 with saline (9 g l⁻¹ NaCl), mixed using a hand-blender for 60 seconds, and filtered through a double-layer cheesecloth under a constant stream of CO₂.

Three replicate bottles were used for each of the intact materials (LenS, TreS and AstS) and the three extracts (LenE, TreE and AstE). Two replicate bottles containing only medium and cecal inoculum were used as negative controls (blanks).

**Sample Collection and DNA Isolation**

A sample of mixed cecal contents was weighted (0.1 g) prior to dilution (this was the “inoculum” sample), and mixed with 1 ml of 0.05 M potassium phosphate buffer (pH 7.0). Blank bottles were sampled (2 ml) before and after incubation as well as all bottles after incubation. All the samples were then centrifuged at 10,000×g, 4 °C for 5min. The pellets were resuspended in 1 ml of Tris-EDTA buffer (pH 8) (Sambrook *et al.*, 1989). DNA was isolated from the inoculum and the culture fluids by bead beating, phenol-chloroform extraction, and ethanol precipitation as described previously by Zoetendal *et al.* (1998).

**PCR Amplification**

DNA isolated from *in vitro* samples was consequentially used as template to amplify the V6-V8 regions of the bacterial 16S rDNA with primers U968-GC and L1401 (Zoetendal *et al.*, 1998). PCR was performed by a PE Applied Biosystems GenAmp PCR system 9700 (Foster City, California), using the Taq DNA polymerase kit from Life Technologies (Gaithesburg, Md., USA). The samples were amplified by using the following program: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 56 °C for 20 s, and 68 °C for 40 s; and finally, 68 °C for 7 min. The aliquots of 5μl were analyzed by electrophoresis on a 1.2% (wt/vol) agarose gel containing ethidium bromide to check the sizes, purity and amounts of the amplicons as described by Zoetendal *et al.*(1998).
Denaturing Gradient Gel Electrophoresis (DGGE)

The DGGE system (Bio-Rad Laborartories, Docode, Hercules California) was used for sequence-specific separation of the PCR products of V6-V8 region of the bacterial 16S rDNA. DGGE analyses of the amplicons were performed on 8% polyacrylamide gels containing a urea-formamide gradient from 38 to 48% (a 100% urea-formamide solution consists of 7 M urea and 40% [vol/vol] formamides) according to the specifications of Muyzer (1993) and Zoetendal et al., 2002. After completion of the electrophoresis, the gels were stained with AgNO₃ according to the method of Sanguinetti et al. (1994).

Statistical Analysis

The gels were scanned with a Sharp JX-330 scanner using Corel Photopaint 97. The gel images were analyzed using the Molecular Analysis Program, version 2.15 (Magurran, 1988; Gillan, 1998; Simpson, 1999). In this model, Sorenson’s index was used to differentiate samples according to the following equation:

\[ Cs = \frac{2j}{(a + b)} \times 100 \]

Where: Cs = the pairwise similarity coefficient (similarity index); a = the number of DGGE bands in lane 1; b = the number of DGGE bands in lane 2; and j = the number of common DGGE bands. If the two profiles are identical, Cs has a value of 100% and if the profiles are completely different, Cs has a value of 0%.

All the similarity data were subjected to statistical analysis by use of SPSS 8.0 (Statistical Package for the Social Science, 1997). Duncan’s multiple range test was used to test the significance between the similarity indices of the different substrates. A pairwise t-test was then used to compare each intact material and its polysaccharide extract (per inoculum or blank).

RESULTS

DGGE profiles of fluids at the end of fermentation of the intact substrates (Figure 1) and the extracts (Figure 2), were generally characterized by the presence of a large number of both dominant and faint bands. The diversity of these banding patterns, both in terms of the
number and density of bands, was much greater compared with that of the initial inoculum and the blanks at the end of fermentation. Specific bands, which either appeared or became dominant in samples from the intact and extracted substrates, but were not detected for the inoculum and blanks, have been indicated in Figures 1 and 2.

Statistical analysis of the DGGE band patterns after fermentation of the intact substrates (Figure 1) are shown in Table 2. Compared with the inoculum or blanks, the similarity indices of DGGE of the intact substrates ranged from 39.9% to 57.7%, of which TreS had the lowest similarity index, and AstS the highest. However, the only significant difference between the intact materials was found for TreS, which was least similar to the blank before incubation.

Table 2 also shows the statistical analysis of DGGE band patterns after fermentation of the extracted substrates. Compared with the inoculum and blanks, the similarity indices of DGGE of the samples after fermentation ranged from 23.3% to 79.8%. Of these, LenE was least similar, and AstE most similar to the inoculum and blanks. There were significant differences between the similarity indices of all three extracts.

### Table 2. Similarity indices of the band pattern for the intact mushroom and herb substrates and their extracts after in vitro fermentation

<table>
<thead>
<tr>
<th>Enrichment comparisons</th>
<th>Similarity indices (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LenS</td>
</tr>
<tr>
<td>Inoculum</td>
<td>46.2±2.6\textsuperscript a</td>
</tr>
<tr>
<td>Blanks before incubation</td>
<td>57.7±1.9\textsuperscript b</td>
</tr>
<tr>
<td>Blanks after incubation</td>
<td>44.8±2.7\textsuperscript a</td>
</tr>
<tr>
<td></td>
<td>LenE</td>
</tr>
<tr>
<td>Blanks after incubation</td>
<td>23.3±1.9\textsuperscript a,***</td>
</tr>
</tbody>
</table>

\footnotetext[1]{Results are given as means (n=6) ± SE; Intact mushroom (LenS = Lentinus edodes; TreS = Tremella fuciformis) and herb (AstS = Astragalus membranaceous) materials and their extracts (LenE = Lentinus edodes polysaccharide extract; TreE = Tremella fuciformis polysaccharide extract; AstE = Astragalus membranaceous polysaccharide extract).}

\textsuperscript abc: Means within rows with no common superscript differ significantly (P<0.05).

\textsuperscript *, **, ***: Means differ significantly between each intact material and its polysaccharide extract (per inoculum or blank (*P<0.05, **P<0.01, ***P<0.001)).
Figure 1. DGGE band pattern of bacterial community profiles of the samples containing the intact materials. DGGE profile lanes: 1, 2, 3: *Lentinus edodes* (*LenS*); 4, 5, 6: *Tremella fuciformis* (*TreS*); 7, 8, 9: *Astragalus membranaceus* (*AstS*) 10, 11: the inoculum; 12, 13: blank before incubation; 14, 15: blank after incubation. Solid arrowheads indicate the positions where the bands appeared (a) or become dominant (b) of each substrate as compared with inoculum and blanks.

Figure 2. DGGE band pattern of bacterial community profiles of the samples containing the extracts. DGGE profile lanes: 1, 2, 3: *Lentinus edodes* extract (*LenE*); 4, 5, 6: *Tremella fuciformis* extract (*TreE*); 7, 8, 9: *Astragalus membranaceus* extract (*AstE*); 10, 11: inoculum; 12, 13: blank before incubation; 14, 15: blank after incubation. Brackets indicate the most variation areas in band pattern. Solid arrowheads indicate the position where the bands appeared (a) or become dominant (b) as compared with inoculum and blanks.
If one compares the intact and extracted substrates, it can be seen that in relation to the inoculum and blanks, *LenE* had greater diversity than *LenS* in terms of similarity indices. There was no significant difference between *TreS* and *TreE* in terms of diversity of samples after fermentation. For *AstS* and *AstE* however, there were significant differences, whereby *AstE* was more similar to the inoculum and blank profiles than *AstS*.

**DISCUSSION**

One of the limitations of using traditional culture-based techniques, is that only the readily cultivable organisms are counted. In terms of describing the species present within a complex microflora, techniques examining 16S rDNA such as PCR and DGGE are increasingly being used to analyze changes in the bacterial population of human GIT (Zoetendal et al., 1998, 2002; Satokari et al., 2001; Heilig et al., 2002). In this study, DGGE profiles were characterized by a large number of both strong and weak bands from the *in vitro* samples after fermentation of specific substrates, suggesting a higher number of bacterial species present in those samples. All of these bacterial species must have been present in the inoculum and blanks, but were most likely present in such low numbers that they were not detectable by DGGE. Apparently, the mushroom and herb substrates had selectively stimulated these species in the inoculum, so that more bands were detected after fermentation of these substrates.

Five hundred different species have been isolated from the ceca of chickens (Jensen, 1999), but it was clear that the number of bands in these DGGE profiles did not reflect this number. This is not surprising, firstly because not all isolated bands are likely to be present in animals all the time anyway, and secondly, because the PCR/DGGE methodology only reveals the dominant bands. This finding is consistent with van der Wielen (2002), who reported that the total DGGE bands does not represent the total number of species present in the chicken GIT. This has also been observed in other studies (Raskin et al., 1997; Simpson et al., 1999). However, it is well known that many of the GIT species are only minor constituents of the total population, and are therefore below the detection limits of the total template DNA and PCR amplification with semi-conserved primer sets in the GIT (Simpson et al., 1999). In addition, some bands may not necessarily represent individual species, but rather groups that have the same migration rate along the DGGE gel. Further limitations of DGGE techniques, as described by Muyzer and Smalla (1998), include separation of relatively small fragments (less than 500bp), co-migration of DNA fragments, and
limitations in total number of resolvable fragments.

Notwithstanding these limitations, however, the diversity revealed by the DGGE banding patterns after fermentation of the intact mushroom and herb substrates was much higher compared with the inoculum and blanks. This greater diversity may indicate that larger populations of more bacterial species are present in these cultures following the in vitro fermentation. As indicated in the literature, the intact mushroom and herb substrates contain nutrients such as proteins, fats, carbohydrates, fibers, vitamins and minerals, as well as a variety of bioactive constituents (Min, 1996; Yang et al., 1999; Zheng et al., 1998; Qi, 1987). These organic compounds must be fermented to varying extents under anaerobic conditions by various bacterial species from the cecal inoculum. These results indicate that the use of these substrates has led to an enrichment of different microorganisms, though, a phylogenetic identification of these bands was beyond the scope of this study.

DGGE analysis of samples after fermentation of the intact and extracted substrates showed that there was a shift in the microbial community, particularly for LenE and TreS. Given that the microbial diversity appears to have increased in the presence of the substrates, it suggests that species which were previously present but undetectable, had been enriched. Particularly for those species enriched following fermentation of the extracted substrates, it is likely that they were species specialized in the hydrolysis of plant polysaccharides, producing small molecular weight carbohydrates from large polymers (Sunvold et al., 1995).

The variation between the substrates themselves and their extracts was probably a reflection of the large variations in physico-chemical properties of these polysaccharides such as sugar composition, molar weights and structures (Yang et al., 1999; Xia and Cheng, 1988; Pang, 1995), which then provide a range of substrates for different microbial species. The stimulation of these different species are apparently detectable using DGGE. In this study, protein was only partially removed from the extracts and the total carbohydrate content was around 60% (Table 1). This indicates that proteins and other unidentified compounds were also present in the polysaccharide extracts and may have also influenced the bacterial community. It would be interesting to identify these remaining compounds and ferment them individually, to determine the extent of their fermentability and their effect on the microbial population.

In vitro fermentation of these polysaccharide extracts resulted in the production of mainly straight-chain fatty acids and decreased NH$_3$ (Guo et al., accepted). It was therefore concluded that these medicinal mushrooms and the herb, and more particularly their polysaccharide extracts, did seem to affect both the activity and composition of this
microbial population after fermentation, both in terms of fermentation kinetics (gas production) and changes in end-product patterns.

It can be concluded that intact mushroom and herb materials and their extracts led to a shift in the bacterial community fingerprint as seen after in vitro fermentation, suggesting that mushroom and herbal substrates had enriched the growth of certain bacterial species. Fermentation of the two mushrooms and their extracts led to a higher microbial diversity compared with the herb materials. The approach reported here could be useful to evaluate how specific dietary components could influence a microbial community in vitro. It may also helpful to sequence the bacteria which have been enriched, though the database for poultry is still comparatively small. Future work should consider whether these (or other) substrates which lead to such shifts in vitro, may also have such properties in vivo, and then whether such shifts can lead to improvements in animal health.

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Chapter 5

EFFECTS OF MUSHROOM AND HERB POLYSACCHARIDES, AS ALTERNATIVES FOR AN ANTIBIOTIC, ON GROWTH PERFORMANCE IN BROILERS

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Effects of Mushroom and Herb Polysaccharides, as Alternatives for an antibiotic, on Growth Performance in Broilers

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Abstract

The present in vivo trial was conducted to study the effects of polysaccharide extracts of two mushrooms, *Lentinus edodes* (LenE) and *Tremella fuciformis* (TreE), and an herb, *Astragalus membranaceus* (AstE), on growth performance, and the development of organs and the gastro-intestinal tract (GIT) of broiler chickens. Three extracts (LenE, TreE and AstE) were supplemented at levels of 0.5, 1, 2, 3 and 4 g/kg from 7 to 14 days of age, and compared with an antibiotic treatment group (20 mg/kg, virginiamycin-VRG) as well as a group of non-supplemented birds. BW gain, feed intake and feed conversion ratio of the extract-supplemented groups were not significantly different from those of the antibiotic group. Significant effects of type of extract and level on growth performance were found from 7 to 28 days of age. Generally, the birds fed with LenE showed higher BW gain and lower feed conversion ratio than those fed with TreE and AstE from 7 to 28 days of age, and 2 g/kg LenE was considered the optimal level of supplementation for enhanced broiler growth. However, the extracts did not show a significant effect on the relative weights of organs and GIT compared with the antibiotic group.

Keywords: mushroom, herb, polysaccharides, antibiotics, growth, broilers
INTRODUCTION

Until recently, antibiotics as antimicrobial growth promoters were widely used in the poultry feed industry. The advantages of using antibiotics as feed supplements in terms of growth stimulation and improvement of feed efficiency are well documented (a. o., Bird, 1980; Miles et al., 1984, 1984a; Ensminger, 1990; Peterson et al., 1991). However, the possibility of developing resistant populations of bacteria and side effects of using antibiotics as growth promoters in farm animals has been of concern, which has been principally with regard to the loss of efficacy of antibiotics as growth stimulants and the difficulty of controlling an outbreak of bacterial diseases (Hinton, 1988; Newman, 2002). Some early studies showed that continuous feeding of antibiotics to chickens resulted in a decreased growth response (Waibel et al., 1954; Sherman et al., 1959; Wiese and Peterson, 1959; Nelson et al., 1963). The possible emergence of antibiotic-resistant strains of bacteria on the premises where growing birds are fed on low levels of antibiotics was reported (Narayanankutty et al., 1992; Aarestrup et al., 2000). Following the discovery of resistance-transfer factors, there has been growing concern about public health risks resulting from antibiotic resistance, carcinogenic responses and other side effects of residues in food. The most important potential route by which humans become infected with resistant bacteria is via the food chain such as meat, milk, and eggs (Hinton, 1988). In the light of the fact that poultry meat is one of the most important sources of animal protein for several populations of the world, even a low incidence of cross infection could be highly significant.

The possibility of developing resistant populations of bacteria and side effects of using antibiotics as growth promoters in farm animals, has led to the recent EU-ban on the use of most antibiotics on the farm animals as growth promoters (European Commission/Scientific Steering Committee, 1999). This will have consequences for growth performance of birds in the poultry industry. Moreover, a lack of antibiotics in the diet will probably impair gut health in terms of rate of occurrence of nutritional disorders caused by bacteria, viruses and parasites. Therefore, an intensive search for alternatives such as probiotics, prebiotics and other feed additives has been started in the last decade (Jensen, 1999; Rolfe, 2000; Thana et al, 2001; Fulton et al., 2002).

Non-digestible carbohydrates (oligo- and polysaccharides) play an important role in animal production and human health by changing the microbial ecosystem in the gut (Rolfe, 2000). It had been shown that animals that have been fed non-digestible oligosaccharides had fewer diarrheas and an enhanced growth performance (Fukuyasu and Oshida, 1986; Katta et
al., 1993 Stanley et al., 1997; Spring et al., 2000). Natural medicinal products have been largely used as feed supplements in China for many years (Li, 1998). As reviewed by Xue and Meng (1996), some mushroom and herb polysaccharides have been used as immune enhancers or immunomodulators and show anti-bacterial, anti-viral, anti-parasitic and anti-carcinogenic bioactivities. However, the effect of mushroom and herb polysaccharides on performance in poultry is not yet fully investigated.

The present in vivo experiment was conducted to study the effects of polysaccharide extracts of two mushrooms, Lentinus edodes (LenE) and Tremella fuciformis (TreE) and an herb, Astragalus membranaceus (AstE), on growth performance in broiler chickens. In addition, dose-responses of the polysaccharide extracts were investigated, and weights of organs and individual segments of the gastro-intestinal tract (GIT) (relative to BW) were determined to study the possible mechanism for a potential enhanced growth performance in chickens.

MATERIALS AND METHODS

Chickens and Management

At 7 days of age, a total of 2,040 Arbor Acres broiler chickens (purchased from Hualong Poultry Breeding Company, Gansu, China) were randomly allocated to 17 experimental treatments. Each treatment consisted of 6 replicate pens with 20 birds each. Each pen was 120 cm × 90 cm (540 cm² per bird). The birds were reared in floor pens using sawdust as litter at Wei Liping’s Poultry Farm, Lanzhou, China.

The lighting program was set at 40-60 watt /20 m² during the first two weeks and 15 watt /20 m² at the third and fourth week, with 24 h light per day. The temperature was set at 35 °C to 32 °C during the first week and gradually declined with 2 °C per week until 20 °C was reached. Relative humidity was about 65-70%. The birds were free access to water.

Polysaccharide Preparation

Intact mushroom and herb materials were purchased from a local source (Lentinus edodes and Tremella fuciformis had been produced in Zhejiang, China, and Astragalus membranaceus had been produced in Gansu, China and were purchased from Gansu Huanghe Pharmacy Market, Lanzhou, China). The intact mushroom and herb materials were dried overnight at 45 °C and ground through a 1mm sieve for polysaccharide extraction,
according to the general procedure of water-soluble polysaccharide extraction (Liu et al., 1999). The yields of the polysaccharide fractions and their compositions are presented in Table 1.

**Experimental Design and Animal Diets**

The experiment was a randomized design with a 3 x 5 factorial arrangement for 3 extracts (LenE, TreE and AstE) and 5 supplemental levels (0.5, 1, 2, 3, and 4 g/kg of the diet). There were two extra control groups, one group had virginiamycin (VRG) (Lanzhou Shunda Vet. Pharmacy, China) in the diet (positive control), the other group had not (negative control). The level of VRG supplementation (20 mg/kg) was considered to be optimal for growth enhancement and improvement of feed efficiency (Odunsi et al., 1999). Polysaccharide extracts were only supplemented for one week (from 7 to 14 days of age) based on traditional Chinese medicine practices (Hu et al., 1998). The entire experiment lasted for 3 weeks (from 7 to 28 days of age).

The birds were fed ad libitum with the same starter (from 0 to 7 days of age) and grower (from 8 to 28 days of age) diet; the latter was supplemented with different levels of polysaccharide extracts or VRG, according to the experimental design. The diets, free of any (other) antibiotic, were based on maize and soybean as the main ingredients. The composition of the diets is presented in Table 2.

**Table 1.** Yields and chemical analyses of the mushroom and herb polysaccharide extracts

<table>
<thead>
<tr>
<th>Compositions</th>
<th>LenE</th>
<th>TreE</th>
<th>AstE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yields of polysaccharides (%)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>6.8</td>
<td>16.0</td>
<td>10.1</td>
</tr>
<tr>
<td>Dry matter (g/kg)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>958</td>
<td>965</td>
<td>967</td>
</tr>
<tr>
<td>Total sugar content (g/kg DM)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>655</td>
<td>613</td>
<td>687</td>
</tr>
</tbody>
</table>

<sup>1</sup> LenE = *Lentinus edodes* polysaccharide extract; TreE = *Tremella fuciformis* polysaccharide extract; AstE = *Astragalus membranaceus* polysaccharide extract.

<sup>2</sup> Yields of polysaccharides were expressed on basis of air-dry matter of the intact mushroom and herb materials.

<sup>3</sup> The dry matter contents of the extracts were determined by freeze-drying.

<sup>4</sup> Total sugar content of the extracts was determined using the phenol-sulphuric method (Dubois et al., 1956).
Data Collection

Bird performance was assessed by measuring feed intake and BW, and BW gain and feed conversion ratio (FCR) was calculated accordingly. BW and feed consumption per pen were recorded at 7, 14, 21 and 28 days of age. Mortality of each pen was recorded during the experiment.

At 28 days of age, 6 birds, one bird per pen, were randomly selected from one of the supplemented groups of each extract, which had shown best growth rate, as well as the two control groups and killed for dissection purposes. Organs (spleen, thymus, bursa, heart and liver) and the GIT were removed from each bird. The digestive tract was divided into the following sections: crop + oesophagus, proventriculus + gizzard, duodenum, jejunum, ileum and ceca, which were measured after the contents had been removed by gentle manual squeezing. Weight of the organs and individual segments of the GIT was expressed as percentage of BW.

Table 2. Composition of the experimental diets (g/kg)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Starter diet</th>
<th>Grower diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>570</td>
<td>635.3</td>
</tr>
<tr>
<td>Soybeans</td>
<td>320</td>
<td>270</td>
</tr>
<tr>
<td>Fish meal</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Animal fat</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Limestone</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>Salt (NaCl)</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Calcium hydrophosphate (CaHPO₄)</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>2.6</td>
<td>2.1</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Premix¹</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Calculated analysis (g/kg)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Starter diet</th>
<th>Grower diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>210</td>
<td>190</td>
</tr>
<tr>
<td>Metabolisable energy (kcal/kg)</td>
<td>3,000</td>
<td>3,050</td>
</tr>
<tr>
<td>Calcium</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Available phosphate</td>
<td>4.5</td>
<td>4.3</td>
</tr>
<tr>
<td>Lysine</td>
<td>11.7</td>
<td>10.5</td>
</tr>
<tr>
<td>Methionine</td>
<td>6.0</td>
<td>5.1</td>
</tr>
</tbody>
</table>

¹ Supplied for per kg of diet: vitamin A (retinal acetate) 8500 IU, cholechalciferol 1800 IU, vitamin E (DL-a-tophorol acetate) 13 mg, vitamin K₃, 3.4 mg, riboflavin 1.8 mg, niacinamide 38 mg, D-pantothenic acid 9.8 mg, folic acid 0.9 mg, cyanocobalamin 18 μg, biotin 80 μg, choline chloride 200 mg, Na₂SeO₃·5H₂O 0.15 mg, FeSO₄·7H₂O 80 mg, MnO₂ 100 mg, KI 0.3 mg, ethoxyquin 100 mg, carrier corn meal.
Statistical Analysis

All data were subjected to statistical analysis by use of SPSS 8.0 (Statistical Package for the Social Sciences, 1997) using GLM. Significant differences between the treatment groups were analyzed using Tukey multiple range test. In addition, a 3 x 5 factorial arrangement was made for the three extracts and their five supplemental levels by analysis of variance using the following ANOVA model:

\[ Y_{ijk} = \mu + E_i + Dose_j + Dose_j^2 + E*Dose_{ij} + E*Dose_{ij}^2 + e_{ijk} \]

Where: \( Y_{ijk} \) = observed trait; \( \mu \) = overall mean; \( E_i \) = the effect of the extracts (\( i = LenE, TreE \) and \( AstE \)), as a fixed factor; \( Dose_j \) and \( Dose_j^2 \) = a linear or quadratic effect of the dietary levels (\( j = 0.5, 1, 2, 3, \) and \( 4 \) g/kg), as covariates; \( E*Dose_{ij} \) and \( E*Dose_{ij}^2 \) = a linear or quadratic effect of the extract x level interactions; \( e_{ijk} \) = random error.

A dose-response relationship for each of the three extracts (\( LenE, TreE \) and \( AstE \), respectively) at the different periods (day 7-14, day 15-21, day 22-28 and day 7-28, respectively) was fitted to a linear [1] or quadratic [2] regression function:

\[ Y = b_0 + b_1 X \]  \hspace{1cm} [1]

\[ Y = b_0 + b_1 X + b_2 X^2 \]  \hspace{1cm} [2]

Where \( Y \) = predicted response; \( X \) = the dose of the extract; \( b_0 \) = intercept (i.e., BW gain, feed intake or FCR on the basal diet), \( b_1 \) and \( b_2 \) = a linear or quadratic regression coefficient.

The significance of the addition of the quadratic component to the linear model was assessed by an F-test.

RESULTS

Performance

From day 22 to 28 and day 7 to 28, no differences in BW gain, feed intake and FCR could be detected between different treatments (Table 3). In the first two weeks of the trial, birds fed VRG gained significantly more weight and had a slightly better FCR than the non-supplemented group. The birds fed with \( LenE \) had significantly higher BW gain in the
first two weeks and a significant lower FCR was found in birds fed with LenE in the second week compared to the non-supplemented group. The birds fed with TreE showed significantly higher BW gain than the non-supplemented group in the first week only.

Feed intake in the first week increased if the birds were fed TreE and AstE as compared to VRG, and FCR also significantly increased when these extracts were fed.

Of the three extracts, LenE showed significantly highest BW gain and lowest FCR from day 15 to 21, and there was no significant difference either BW gain or FCR between TreE and AstE.

A tendency of an extract effect (P<0.10) on BW gain was found for the period 7 to 14 days of age only (Table 4). A significant dietary level effect on BW gain was found in broilers from day 15 to 21 (both linear and quadratic, P<0.05) and day 7 to 28 (quadratic, P<0.10) (Table 4). Significant differences of the extract x dose interaction on BW gain were not found at all periods.

The parameters for fitted linear and quadratic functions for each of the three extracts in each period are presented in Table 5. A significant dose response was only found in the LenE supplemented groups from day 7 to 14, day 15 to 21 (P<0.10), and from day 7 to 28 (P<0.05). In all other regressions, the addition of a quadratic component did not improve the linear model.

A tendency of an extract effect (P<0.10) on feed intake was found from day 7 to 14 (Table 6). A significant dietary level effect on feed intake was found for the period 15 to 21 days of age, which showed a significant linear effect (Table 6). In addition, a significant effect of the extract x dose interaction on feed intake was found for the period of day 7 to 14 and the entire period (day 7 to 28).

The parameters for fitted linear and quadratic functions for each of the three extracts in each period are presented in Table 7. A significant dose response was found in groups of LenE for the period day 15 to 21 (linear) and day 7 to 28 (quadratic), of TreE for day 22 to 28 (linear), and of AstE for day 7 to 14 (linear).

Significant extract or dose effects on FCR were not found (Table 8). However, a significant effect of the extract x dose interactions was found at day 7 to 14 and day 7 to 28, which showed a more quadratic response (Table 8).

The parameters for fitted linear and quadratic functions for each of the three extracts in each period are presented in Table 9. A significant dose response was found in groups of AstE for the period of day 7 to 14 (linear), of TreE for day 22 to 28 (linear) and of LenE for day 15 to 21 (linear), and day 7 to 14, day 22 to 28 and day 7 to 28 (quadratic).
Table 3. BW gain, feed intake and feed conversion ratio (FCR) of broiler chickens supplemented with the mushroom and herb extracts

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day 7-14</th>
<th>Day 15-21</th>
<th>Day 22-28</th>
<th>Day 7-28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BW gain (g b⁻¹ d⁻¹)</td>
<td>FI (g b⁻¹ d⁻¹)</td>
<td>FCR</td>
<td>BW gain (g b⁻¹ d⁻¹)</td>
</tr>
<tr>
<td>Non-suppl.</td>
<td>31.8ᵇ</td>
<td>47.1ᵇ</td>
<td>1.51ᵃᵇ</td>
<td>32.2ᶜ</td>
</tr>
<tr>
<td>VRG</td>
<td>32.8ᵃ</td>
<td>46.8ᵇ</td>
<td>1.43ᶜ</td>
<td>35.0ᵃᵇ</td>
</tr>
<tr>
<td><em>LenE</em></td>
<td>32.3ᵃ</td>
<td>48.4ᵃᵇ</td>
<td>1.52ᵃᵇ</td>
<td>36.3ᵃ</td>
</tr>
<tr>
<td><em>TreE</em></td>
<td>32.6ᵃ</td>
<td>52.7ᵃ</td>
<td>1.62ᵃ</td>
<td>32.9ᵇᶜ</td>
</tr>
<tr>
<td><em>AstE</em></td>
<td>31.9ᵃᵇ</td>
<td>51.1ᵃᵇ</td>
<td>1.60ᵃ</td>
<td>32.8ᵇᶜ</td>
</tr>
</tbody>
</table>

SEM² | 0.1 | 0.4 | 0.01 | 0.3 | 0.4 | 0.03 | 0.3 | 0.3 | 0.01 | 0.1 | 0.3 | 0.01

¹ Results are given as means (n=6) for the non-supplemented and VRG (virginiamycin) groups and as mean (n = 30) for *LenE* (*Lentinus edodes* extract), *TreE* (*Tremella fuciformis* extract) and *AstE* (*Astragalus membranaceus* extract) groups.

² SEM: Standard error of the mean.

ᵃᵇᶜ Means with different superscripts within the column are significantly different (P< 0.05).
<table>
<thead>
<tr>
<th>Day</th>
<th>Extract&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Dietary level of extracts (g/kg)</th>
<th>Sources of variance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Day 7-14</td>
<td>LenE</td>
<td>31.7</td>
<td>32.1</td>
</tr>
<tr>
<td></td>
<td>TreE</td>
<td>33.2</td>
<td>32.0</td>
</tr>
<tr>
<td></td>
<td>AstE</td>
<td>32.0</td>
<td>32.1</td>
</tr>
<tr>
<td>SEM</td>
<td>.34 .25 .22 .23 .24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 15-21</td>
<td>LenE</td>
<td>34.8</td>
<td>37.8</td>
</tr>
<tr>
<td></td>
<td>TreE</td>
<td>32.3</td>
<td>33.7</td>
</tr>
<tr>
<td></td>
<td>AstE</td>
<td>32.3</td>
<td>31.7</td>
</tr>
<tr>
<td>SEM</td>
<td>.52 .79 .59 .59 .63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 22-28</td>
<td>LenE</td>
<td>44.4</td>
<td>45.0</td>
</tr>
<tr>
<td></td>
<td>TreE</td>
<td>46.1</td>
<td>45.8</td>
</tr>
<tr>
<td></td>
<td>AstE</td>
<td>45.1</td>
<td>46.3</td>
</tr>
<tr>
<td>SEM</td>
<td>.76 .64 .54 .72 .51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7-28</td>
<td>LenE</td>
<td>37.0</td>
<td>37.8</td>
</tr>
<tr>
<td></td>
<td>TreE</td>
<td>37.1</td>
<td>37.4</td>
</tr>
<tr>
<td></td>
<td>AstE</td>
<td>36.5</td>
<td>36.7</td>
</tr>
<tr>
<td>SEM</td>
<td>.30 .27 .36 .30 .26</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> Results are given as means (n = 6) of the extract supplemental groups.

<sup>2</sup> Extracts: LenE = *Lentinus edodes* extract; TreE = *Tremella fuciformis* extract; AstE = *Astragalus membranaceus* extract

<sup>3</sup> SEM: Standard error of the mean. P<0.10 are printed in bold.
Table 5. Parameter estimates and asymptotic SE (in parenthesis) of BW gain, described by linear and quadratic functions in broilers

<table>
<thead>
<tr>
<th>Type of model</th>
<th>Extracts</th>
<th>b0</th>
<th>b1</th>
<th>b2</th>
<th>SE</th>
<th>P-value</th>
<th>R²</th>
<th>F (1, 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 7-14</td>
<td>LenE</td>
<td>32.05</td>
<td>0.13</td>
<td>1.06</td>
<td>.414</td>
<td>.02</td>
<td></td>
<td>5.41</td>
</tr>
<tr>
<td></td>
<td>TreE</td>
<td>32.72</td>
<td>-0.07</td>
<td>1.16</td>
<td>.683</td>
<td>.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AstE</td>
<td>32.16</td>
<td>-0.11</td>
<td>1.01</td>
<td>.452</td>
<td>.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linear</td>
<td>LenE</td>
<td>36.54</td>
<td>-0.11</td>
<td>1.85</td>
<td>.670</td>
<td>.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TreE</td>
<td>32.92</td>
<td>-0.03</td>
<td>2.63</td>
<td>.935</td>
<td>.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AstE</td>
<td>32.18</td>
<td>0.31</td>
<td>1.88</td>
<td>.263</td>
<td>.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 15-21</td>
<td>LenE</td>
<td>45.76</td>
<td>-0.77</td>
<td>3.22</td>
<td>.105</td>
<td>.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TreE</td>
<td>46.27</td>
<td>-0.49</td>
<td>2.34</td>
<td>.154</td>
<td>.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AstE</td>
<td>45.78</td>
<td>-0.33</td>
<td>2.47</td>
<td>.354</td>
<td>.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 22-28</td>
<td>LenE</td>
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¹ Models: Linear: Y = b0 + b1 X; Quadratic: Y = b0 + b1 X + b2 X².
² Extracts: LenE = Lentinus edodes extract; TreE = Tremella fuciformis extract; AstE = Astragalus membranaceus extract. ³ P <0.10 are printed in bold.
⁴ F (1, 27) represents the significance of adding a quadratic component to the linear model. Critical F (1, 27) – values: 4.21 (P<0.05) and 10.19 (P<0.01).
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<th>Sources of variance</th>
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1 Results are given as means (n = 6) of the extract supplemental groups.
2 Extracts: LenE = Lentinus edodes extract; TreE = Tremella fuciformis extract; AstE = Astragalus membranaceus extract.
3 SEM: Standard error of the mean. P < 0.10 are printed in bold.
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<th>b2</th>
<th>SE</th>
<th>P-value</th>
<th>R²</th>
<th>F (1, 27)</th>
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<tr>
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1 Models: Linear: Y = b0 + b1 X; Quadratic: Y = b0 + b1 X + b2 X².
2 Extracts: LenE = Lentinus edodes extract; TreE = Tremella fuciformis extract; AstE = Astragalus membranaceus extract.
3 P < 0.10 are printed in bold.
4 F(1, 27) represents the significance of adding a quadratic component to the linear model. Critical F (1, 27) -values: 4.21 (P<0.05) and 10.19 (P<0.01).
<table>
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<th>Day</th>
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<th>Sources of variance</th>
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1 Results are given as means (n = 6) of the extract supplemental groups.
2 Extracts: LenE = *Lentinus edodes* extract; TreE = *Tremella fuciformis* extract; AstE = *Astragalus membranaceus* extract.
3 SEM: Standard error of the mean. P < 0.10 were printed in bold.
Table 9. Parameter estimates and asymptotic SE (in parenthesis) of feed conversion ratio, described by linear and quadratic functions in broilers

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<th>P-value</th>
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<tr>
<td></td>
<td>Day 7-28</td>
<td>LenE</td>
<td>1.90 (0.06)</td>
<td>-0.15 (0.06)</td>
<td>0.041 (0.014)</td>
<td>.09</td>
<td>.002</td>
<td>.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TreE</td>
<td>1.87 (0.05)</td>
<td>0.04 (0.06)</td>
<td>-0.005 (0.013)</td>
<td>.09</td>
<td>.411</td>
<td>.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AstE</td>
<td>1.91 (0.06)</td>
<td>-0.01 (0.07)</td>
<td>0.003 (0.016)</td>
<td>.11</td>
<td>.821</td>
<td>.01</td>
</tr>
</tbody>
</table>

1 Models: Linear: \( Y = b0 + b1X \); Quadratic: \( Y = b0 + b1X + b2X^2 \).
2 Extracts: LenE = Lentinus edodes extract; TreE = Tremella fuciformis extract; AstE = Astragalus membranaceus extract.
3 P < 0.05 are printed in bold.
4 F (1, 27) represents the significance of adding a quadratic component to the linear model. Critical F (1, 27) – values: 4.21 (P<0.05) and 10.19 (P<0.01).
Dissection Tracts

Organs

The relative weights of some organs that were dissected at 28 days of age are presented in Table 10. Although the relative weights of thymus (0.44 vs. 3.37 and 0.39) and bursa (0.15 vs. 0.14 and 0.13) were somewhat larger in a numerical sense for the three extracts as compared with the VRG and non-supplemented group, no statistical differences could be found among the groups.

Table 10. Weights of organs of broilers supplemented with mushroom and herb extracts at 28 days of age

<table>
<thead>
<tr>
<th>Treatments†</th>
<th>Heart (per 100g BW)</th>
<th>Liver</th>
<th>Thymus</th>
<th>Bursa</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-suppl.</td>
<td>0.55</td>
<td>2.21</td>
<td>0.39</td>
<td>0.13</td>
<td>0.11</td>
</tr>
<tr>
<td>VRG</td>
<td>0.55</td>
<td>2.07</td>
<td>0.37</td>
<td>0.14</td>
<td>0.10</td>
</tr>
<tr>
<td>LenE (2 g/kg)</td>
<td>0.60</td>
<td>2.19</td>
<td>0.44</td>
<td>0.16</td>
<td>0.11</td>
</tr>
<tr>
<td>TreE (1g/kg)</td>
<td>0.55</td>
<td>2.10</td>
<td>0.41</td>
<td>0.18</td>
<td>0.10</td>
</tr>
<tr>
<td>AstE (2 g/kg)</td>
<td>0.62</td>
<td>2.17</td>
<td>0.48</td>
<td>0.12</td>
<td>0.12</td>
</tr>
</tbody>
</table>

SEM² | 0.013 | 0.058 | 0.088 | 0.008 | 0.004 |

† Results are given as means (n=6) for the non-supplemented group, VRG = virginiamycin group, LenE = Lentinus edodes extract, TreE = Tremella fuciformis extract, AstE = Astragalus membranaceus extract.

² SEM: Standard error of the mean.

Means within each column are not significantly different (P>0.05).

GIT

The relative weights of GIT segments that were dissected at 28 days of age are presented in Table 11. The expected significant difference of GIT weights among the groups was not found. However, the relative weights of proventriculus+gizzard (3.15 vs. 2.85 and 2.84) and duodenum (0.62 vs. 0.57 and 0.56) were numerically larger in groups fed with the three extracts than both VRG and non-supplemented groups.
Table 11. Development of GIT of broilers supplemented with the mushroom and herb extracts at 28 days of age

<table>
<thead>
<tr>
<th>Treatments¹</th>
<th>Prov + giz²</th>
<th>Crop + Eso³</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Ceca</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-suppl.</td>
<td>2.84</td>
<td>0.62</td>
<td>0.56</td>
<td>1.16</td>
<td>0.93</td>
<td>0.21</td>
</tr>
<tr>
<td>VRG</td>
<td>2.85</td>
<td>0.57</td>
<td>0.57</td>
<td>1.17</td>
<td>1.05</td>
<td>0.19</td>
</tr>
<tr>
<td>LenE (2 g/kg)</td>
<td>3.16</td>
<td>0.67</td>
<td>0.63</td>
<td>1.16</td>
<td>1.05</td>
<td>0.19</td>
</tr>
<tr>
<td>TreE (1g/kg)</td>
<td>3.10</td>
<td>0.61</td>
<td>0.61</td>
<td>1.19</td>
<td>0.96</td>
<td>0.20</td>
</tr>
<tr>
<td>AstE (2 g/kg)</td>
<td>3.19</td>
<td>0.73</td>
<td>0.62</td>
<td>1.29</td>
<td>1.00</td>
<td>0.20</td>
</tr>
<tr>
<td>SEM²</td>
<td>.075</td>
<td>0.03</td>
<td>0.02</td>
<td>0.03</td>
<td>0.03</td>
<td>0.01</td>
</tr>
</tbody>
</table>

¹ Results are given as means (n=6) for the control, VRG = virginiamycin, LenE = Lentinus edodes extract, TreE = Tremella fuciformis extract, AstE = Astragalus membranaceus extract.

² SEM: Standard error of the mean.

³ Prov + Giz = relative weights of Proventriculus + gizzard; Crop + Eso = relative weights of Crop + esophagus.

Means within each column are not significantly different (P>0.05).

DISCUSSION

Performance

Usually feed additives are supplemented to a diet for the whole period of production. In the present trial, the polysaccharide extracts were supplemented for only one week (from 7 to 14 days of age), which was considered to be the critical period of broilers with a fast growth rate and adequate development of the immune system, and bacterial populations appears to be established in the gut ecosystem (Smith, 1965). A study of Hu et al. (1998) showed the immune response of chickens was significantly increased by a mushroom polysaccharide supplement from 7 to 14 days of age, and its efficacy could be observed in the third week after feed supplementation.

It was found in this broiler trial that VRG significantly enhanced BW gain in the first two weeks (from 7 to 21 days of age) and FCR was slightly lower, as compared with the non-supplemented birds. VRG is known to be active against the Gram-positive bacteria in the gut, and it subsequently showed to have additional potential as a growth promoter in chickens when used at subtherapeutic levels (a.o., Eyssen and De Somer, 1963; Armstrong, 1986; Veltmann and Weideman, 1987). A number of reports had provided evidence for
enhancement of growth rates and FCR by dietary inclusion of VRG ranging from 10 to 22 mg/kg of feed (March et al., 1978; Buress et al., 1986; Odunsi et al., 1999). However, a study of Proudfoot et al. (1990) showed that the inclusion of VRG in a broiler diet failed to improve BW at either 21 or 42 days of age, and VRG supplementation had no effect on mortality or feed conversion efficiency either, regardless of the mode of administration. This was partly consistent with the present study where VRG did not result in a growth stimulation from day 22 to 28, and it had no significant effects on feed intake and FCR either. A lack of response of VRG in the present trial could have a consequence of the short period of VRG supplementation.

In this study, a significant effect of both VRG and the extracts on performance was found only in the first two weeks, and especially during the supplemental period (from day 7 to 14). This suggests that possible beneficial effects might have been more pronounced, if the supplementation period had been prolonged. The mushroom and herb polysaccharides have been demonstrated anti-bacterial (Yuan et al., 1993), anti-viral (Yang et al., 1998) or anti-parasitic activity (Hu et al., 1998; Pang et al., 2000) in chickens, when given to the birds for a short period or as an adjuvant of vaccines. Until now, the use of polysaccharides as growth promoters in poultry is quite limited.

In the present study, a significant effect of the extract x dose interactions on BW gain, feed intake and FCR was found from 7 to 28 days of age following the supplementation of the extracts. LenE supplemented birds showed better BW gain and lower FCR than TreE and AstE supplemented birds, and the later showed higher feed intake during the first week of supplementation. The differences in response between the different extracts seems logical, since there were large variations in physico-chemical properties of these polysaccharides such as sugar composition, molar weights and structures (Huang et al., 1982; Xia and Cheng, 1988; Pang et al., 1995; Yang et al., 1999). Of the three extracts, LenE supplemented birds showed a significant dose response of BW gain from 7 to 28 days of age, a quadratic increase with increase of LenE dosage. A significant dose response on feed intake was found in groups of LenE (day 15 to 21 and day 7 to 28), TreE (day 22 to 28), and AstE (day 7 to 14), as a result, significant dose response effects on FCR in these groups were also found. In vitro fermentation of these polysaccharide extracts resulted in the production of mainly straight-chain fatty acids and decreased NH₃ (Guo et al., accepted). The polysaccharide extracts seem to affect both the activity and composition of this microbial population after fermentation, both in terms of fermentation kinetics (gas production) and changes in end-product patterns. Further analysis of the microbial community in these fermentation
samples showed that of the three extracts, LenE led to a more significant shift in the bacterial community when fermented in vitro (Guo et al., submitted).

**Dissection Tracts**

Several studies showed that mushroom and herb polysaccharides, used as immune enhancers, significantly increased growth of immune organs such as thymus, bursa and spleen weights in both normal or immune inhibitor treated animals as chickens and rats (Mao et al., 1988; Xia and Cheng, 1988; Liang et al., 1994; Zhang et al., 1998). In this study, both thymus and bursa weights of the birds were slightly increased by the mushroom and herb extracts, although the expected statistically significant effect on the relative weights of the organs was not found.

The reduction in intestinal weight as a result of feeding antibiotics is considered to be one of the mechanisms by which nutrient absorption is improved (Gordon and Bruckner-Kardoss, 1961a, 1961b). The experiment conducted by Henry (1986) showed that intestinal tract weight was decreased from 3.34 to 2.69 g per 100g BW when 12 mg/kg VRG was fed, and the decrease in intestinal tract weight was a result of thinning of the intestinal wall. However, the expected significant difference of GIT weights between the birds fed with the antibiotic and the non-supplemented birds could not be detected in this experiment, and an expected difference between the extract supplemented groups and the non-supplemented group was not found either. It was thought that too few birds were selected from these groups for dissection, which result in large variations in the GIT organs in this experiment.

Conclusively, supplementation of VRG in the feed significantly enhanced BW gain and slightly increased feed efficiency from 7 to 21 days of age, but it failed to improve growth performance and feed efficiency from 22 to 28 days of age. The birds fed the extracts showed better growth than non-supplemented birds, but it was not significantly different from those fed VRG. Of the three extracts, LenE demonstrated to be a potential growth promoter with a significant quadratic response. Future experiments are need to be conducted to find out whether the mushroom and herb extracts can be used as alternatives for antibiotic growth promoters in challenged birds, and further experiments are also required to study the mechanisms for potential enhanced growth performance in poultry.
ACKNOWLEDGEMENTS

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preparations to a Newcastle disease inactivated vaccine on the development of the immune 
Chapter 6

EFFECTS OF MUSHROOM AND HERB POLYSACCHARIDES, AS ALTERNATIVES FOR AN ANTIBIOTIC, ON CECAL MICROBIAL ECOSYSTEM IN CHICKENS

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Submitted to: Poultry Science
Effects of Mushroom and Herb Polysaccharides, as Alternatives for an Antibiotic, on Cecal Microbial Ecosystem in Chickens

F.C. Guo, B.A. Williams, R.P. Kwakkel, H.S. Li, X.P. Li, J.Y. Luo, W.K. Li and M.W.A. Verstegen

Abstract

An in vivo experiment was conducted to study the potential prebiotic effect of the mushroom and herb polysaccharide extracts, Lentinus edodes extract (LenE), Tremella fuciformis extract (TreE), and Astragalus membranaceus (AstE), on chicken growth and the cecal microbial ecosystem, as compared to the antibiotic (Apramycin-APR). This investigation was carried out in the term of a dose response study. The chickens were naturally infected with Avian Mycoplasma Gallisepticum (AMG) prior to the experiment. BW gain, cecal pH, viscosity and predominant microbial populations were measured one week after the extract and APR treatments. Both the extracts and APR significantly stimulated growth of the chickens infected with AMG. BW gain of the average of the groups fed with the extracts was significantly lower compared with that of the antibiotic. The extracts had no significant effect on cecal pH. However, cecal viscosity and microbial populations were significantly affected by feeding both the extracts and the antibiotic. In contrast to APR, the extracts stimulated the number of the potentially beneficial bacteria (Bifidobacteria, and Lactobacilli), while reducing the number of the potentially harmful bacteria (Bacteroides and E.coli). Of the three extracts, LenE was associated with the highest numbers of cecal Bifidobacteria and Lactobacilli. With each increase in the LenE dose, birds tended to have higher BW gain and total aerobe and anaerobe counts. Numbers of predominant cecal bacteria, in particular, E.coli, Bifidobacteria and Lactobacilli, were significantly increased with increases in the LenE dose. It would seem that the use of these specific mushroom and herb polysaccharide extracts holds some promise as potential modifiers of intestinal microbiota in diseased chickens.

Keywords: mushroom and herb polysaccharides extracts, chickens, growth, cecal microbial ecosystem
INTRODUCTION

The ceca can be described as the location for a very complex microbial ecosystem, although other parts of the digestive tract of chickens might also be important sites for microbial colonization. Ceca are one of the areas of greatest microbial activities in the gastro-intestinal tract (GIT) of chickens. Relative to other parts of the GIT, ceca provide a stable environment for micro-organisms, resulting in a large microbial population due to the slower transit time. According to Barnes et al., (1972), the total number of bacteria is around 10^{11} cells/g (wet weight) in the ceca of chickens, with anaerobic (10^8-10^9 cells/g). The intestinal microflora is known to play an important role in the health status of host animals. In general, intestinal bacteria may be divided into species that exert either harmful (pathogenic) or beneficial effects on host health (Macfarlane and Cummings, 1991). Therefore, a common approach to maintain host health is to increase the number of desirable bacteria in order to inhibit colonization of invading pathogens (Rolfe, 1991; Brown, 1996).

The composition and activity of intestinal microbiota can be altered by diet composition and dietary manipulations such as the use of feed additives and antibiotics (Coates, 1981; Jensen, 1993). Antibiotic therapies have been reported as major factors in the etiology of gut health disorders (Gardiner et al., 1993; Solomons 1993). Increasing insight into the potentially beneficial activities of the gastrointestinal microbiota and increasing public concern about antibiotic resistance and residues in animal products, have resulted in the search for alternatives, such as prebiotics, probiotics and other feed additives.

Certain plant polysaccharides are now recognized as having a prebiotic effect (Verstegen and Schaařsma, 1999; Cummings and Macfarlane, 2002). Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selective stimulation of the growth and/or activity of one or a limited number of bacterial species in the colon, thus benefiting host health (Gibson and Roberfroid, 1995). Carbohydrates, especially oligo- and poly-saccharides, have been used as prebiotics to influence the composition of the bacterial populations in the large intestine of a number of animal species (Baily et al., 1991; Newman, 1995; Grizard and Barthomeuf, 1999; Jensen, 1999; Hughes et al., 2000; Breves et al., 2001; Rycroft et al., 2001; Zimmermann et al., 2001; Korakli et al., 2002).

Natural medicinal products originating from fungus and herbs have been used as feed additives for farm animals in China for centuries, and show many bio-activities such as anti-microbial activities, immune enhancement and stress reduction (Wang et al., 2002). The bio-active components of these products are quite complex (Yang and Feng, 1998), but of
these polysaccharides are considered to be the most important immuno-active components (Xie and Niu, 1996; Xue and Meng, 1996). It is well documented that the polysaccharides derived from Astragalus membranaceae Radix, Lentinus edodes and Tremella fuciformis, which have been used as immune enhancers, also show anti-bacterial (Yuan et al., 1993), anti-viral (Wei et al., 1997; Cheng et al., 1998; Liu et al., 1999b; Yu and Zhu, 2000) and anti-parasitic activities (Hu et al., 1998; Pang et al., 2000) in chickens. It has been hypothesized that some of these effects are actually the results of a prebiotic effect by which the polysaccharides stimulate growth of the beneficial bacterial populations in the large intestine, and thus increase resistance to pathogens. However, the effects of mushroom and herb polysaccharides on the microbial ecosystem in the large intestine of animals have not been well investigated as such. Also, it is not yet known what an optimal inclusion level of these mushroom and herb polysaccharides could be, when used as modifiers of the intestinal microbiota.

The present in vivo experiment was conducted to study the prebiotic effects of the mushroom and herb polysaccharide extracts, Lentinus edodes extract - LenE and Tremella fuciformis extract - TreE, and Astragalus membranaceus extract - AstE, as alternatives for an antibiotic (Apramycin - APR), on the growth and cecal microbial ecosystem of chickens, and to investigate the optimum supplemental level of the potential prebiotic for enhanced growth and beneficial intestinal microbiota.

MATERIALS AND METHODS

Animal Husbandry and Diets

A total of 200 day-old female Huangyu broiler chicks (A local slow-growing breed from Gansu Poultry Breeding Company, China) were reared in horizontal battery brooders using wood sawdust as litter, with a density of 10 birds/m². The brooder temperature was set at 30 °C to 33 °C during the first week and gradually decreased by 2 °C per week, until 28 °C was reached by the third week. Relative humidity was between 65-70%. The lighting program was 24h light throughout the experiments.

All birds were feed ad libitum. The diet was based on maize and soybean meal and was used throughout the experiment. The diet composition is shown in Table 1.
Avian Mycoplasma Gallisepticum (AMG) Infection

Birds were proved to be naturally infected with AMG prior to the experiment. A few birds showed respiratory symptoms at 3 days of age, and the disease then spread to the whole flock one week later. Mortality reached 8% at 13 days of age. Presumptive diagnosis was based on the occurrence of typical signs (mucous discharge from the mouth and nostrils, increased respiratory rate, and swollen head at the time of death) and lesions (airsacculitis, sinusitis and synovitis) together with differential diagnosis (isolation and identification of the causative organisms), and a final diagnosis was based on serological tests (Gansu Province Animal Health Severce, China).

Polysaccharide Preparation

Intact mushroom and herb materials were purchased from a local source (Gansu Huanghe Pharmacy Market, Lanzhou China). The intact mushroom and herb materials were dried overnight at 45 °C and ground through a 1mm sieve for polysaccharide extraction, according to the general procedure of water-soluble polysaccharide extraction (Liu et al., 1999a). The yields of the polysaccharide fractions and their total sugar contents are shown in Table 2.

Experimental Design

At 14 days of age, a total of 135 birds, after exclusion of extreme weights, were randomly assigned to nine dietary treatments: addition of LenE at level of 1, 2, 3, 5 and 10 g/kg of the diet, TreE and AstE both at level of 2 g/kg, antibiotic, and control. The antibiotic used was Apramycin (APR) sulfate soluble powder (C_{21}H_{41}N_{5}O_{11}) at 20 mg/kg BW (Antibiotic Lab, CAAS Lanzhou China). Polysaccharide extracts and APR were administered orally, twice daily from 15 to 21 days of age. Appropriate proportions were prepared so that each chicken received the intended dose, directly into the crop, using oral gavage. Each treatment consisted of 3 pens with 5 birds per pen. A pen was considered as the replicate experimental unit.
### Table 1. Composition of the diets (g/kg)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Starter diet</th>
<th>Grower diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>570</td>
<td>635.3</td>
</tr>
<tr>
<td>Soybeans (d)</td>
<td>320</td>
<td>270</td>
</tr>
<tr>
<td>Fish meal</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Animal fat</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Limestone</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>Salt (NaCl)</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Calcium hydrophosphate(CaHPO4)</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>2.6</td>
<td>2.1</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Premix(^1)</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

**Calculated analysis (g/kg)**

- Crude protein: 210
- Metabolisable energy (kcal/kg): 3,000
- Calcium: 10
- Available phosphate (P): 4.5
- Lysine: 11.7
- Methionine: 6.0

\(^1\) Supplied for per kg of diet: vitamin A (retinal acetate), 8,500 IU; cholecalciferol, 1,800 IU; DL-α-topherol acetate, 13.0 IU; vitamin K₃, 3.4 mg; thiamin, 1.8 mg; riboflavin, 5.2 mg; D-pantothenic acid, 9.8 mg; niacinamide, 38 mg; folic acid, 0.90 mg; pyridoxine, 3.60 mg; vitamin B₁₂, 18 μg; d-biotin, 80 μg; choline chloride, 200 mg; Fe, 80 mg; Zn, 75 mg; Mn, 100 mg; Se, 0.15 mg; I, 0.3 mg; carrier maize.

### Table 2. Yields and sugar contents of the mushroom and herb polysaccharide extracts\(^1\)

<table>
<thead>
<tr>
<th>Compositions</th>
<th>LenE</th>
<th>TreE</th>
<th>AstE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yields of polysaccharides (%)(^2)</td>
<td>6.8</td>
<td>16.0</td>
<td>10.1</td>
</tr>
<tr>
<td>Dry matter (g/kg)(^3)</td>
<td>958</td>
<td>965</td>
<td>967</td>
</tr>
<tr>
<td>Total sugar content (g/kg DM)(^4)</td>
<td>655</td>
<td>613</td>
<td>687</td>
</tr>
</tbody>
</table>

\(^1\) *LenE = Lentinus edodes* polysaccharide extract; *TreE = Tremella fuciformis* polysaccharide extract; *AstE = Astragalus membranaceus* polysaccharide extract.

\(^2\) Yields of polysaccharides were based on airy-dry matter of the intact materials.

\(^3\) The dry matter contents of the extracts were determined by freeze-drying.

\(^4\) Total sugar content was determined using the phenol-sulphuric method (Dubois et al., 1956).
Data Collection

Body weight and mortality per pen were recorded at 14 and 21 days of age. At the end of the experiment, all birds were killed by cervical dislocation in a germ-free isolation chamber sterilized by ultraviolet radiation. The ceca were then removed from each bird and the fresh excreta of the ceca gently squeezed and carefully collected in sterilized 25ml tubes, each of which was pooled for five birds (per pen).

Three grams of fresh cecal samples were diluted with 10 ml distilled water and vortexed before measuring pH (pH meter, pHB-5A, Lida, Shanghai, China) and viscosity (NZ-6 type conical viscosity meter, Shanghai, China, with shearing rate 50-1). One gram of wet sample was diluted with 10 ml of sterilized distilled water, of which 1 ml was transferred into 9ml of sterilized distilled water. Samples were serially diluted from $10^{-1}$ to $10^{-7}$. One-tenth ml of each diluted sample was plated on the appropriate medium for enumeration of microbial populations. Bacterial counts were performed using the appropriate dilution and plate culture techniques under aerobic or anaerobic conditions according to Barnes and Impey (1970), and the results were expressed as colony forming units (cfu) log10 per g fresh sample.

The bacterial groups and species determined included total anaerobes (reinforced clostridial agar), total aerobes (nutrient blood agar), Lactobacilli (citromalic acid-enriched MRS agar), Bacteroides (eosin methylene blue EMB agar), Enterococci (esculin-crystal violet- aeculine azide agar), E. coli (MacConkey agar), and Bifidobacteria (Bifidobacterium agar composed of tomato juice 400ml, dissoluble amylum 0.5g, peptone 15g, yeast extract 2g, glucose 20g, sodium chloride 5g, Tween-80 1ml, 5% cysteine 0.5ml, liver extract 80ml, agar powder 20g, and distilled water 520ml; pH=7.0, at 37°C for 72 hrs) (Institute of Biological Products, China Hygiene Ministry).

Statistical Analysis

The data was subjected to statistical analysis by analysis of variance using SPSS 8.0 (Statistical Package for the Social Sciences, 1997). Orthogonal contrasts were used to assess: 1) the effect of antibiotic, examining the contrast between the antibiotic and control groups; 2) the effect of polysaccharide extracts, examining the contrast between the overall extracts ($LenE$, $TreE$ and $AstE$ at level of 0.2%) and the antibiotic group; 3) the difference between the three extracts, examining the contrasts, $LenE$ versus $TreE$, $AstE$ versus $LenE$ and $TreE$.

Differences of BW gain, cecal pH, viscosity and microbial counts between the different
dietary levels of LenE (1, 2, 3, 5 and 10 g/kg) were tested by Tukey multiple range test.

The dose response of LenE was also fitted to both linear [1] and quadratic [2] regression functions:

\[ Y = b0 + b1 \times x \]  \hspace{1cm} [1]

\[ Y = b0 + b1 \times x + b2 \times x^2 \]  \hspace{1cm} [2]

Where \( Y \) = predicted response; \( x \) = the dose of the extract; \( b0 \) = intercept (i.e., BW gain, cecal pH, viscosity or bacterial populations on the basal diet), \( b1 \) and \( b2 \) = a linear or quadratic regression coefficient. The significance of the linear and quadratic models was assessed by an F-test.

**RESULTS**

*Effects of LenE, TreE and AstE*

The BW gain of the birds fed with APR was significantly higher compared with the non-supplemented birds (Table 3). The overall mean of the BW gain of the groups fed with the extracts was significantly lower than the antibiotic group. There was no significant difference in BW gain between the groups fed the extracts. The pH value of the different treatments was not significantly different. The birds fed with APR showed significantly higher cecal viscosity than the non-supplemented birds. The overall mean of the cecal viscosity of the groups fed with the extracts was not significantly different from the antibiotic group. Of the three extracts, the TreE group showed the highest cecal viscosity.

The total aerobe counts of the antibiotic group were significantly higher than that of the control group, and of the groups fed with the extracts (Table 3). Total anaerobic counts were not significantly different between the antibiotic and control groups, as well as between the extracts and the antibiotic group. Of the three extracts, the LenE group had significantly highest anaerobe counts.

The number of both Bacteroides and E. coli of the birds fed with APR was significantly higher compared with that of the non-supplemented birds and the overall mean of the birds fed with the extracts (Table 3). Of the three extracts, the TreE group showed the lowest Bacteroides and E. coli counts. The antibiotic group had significantly lower Enterococci
counts than the control group, but it was not significantly different from the extracts. The antibiotic group showed significantly lower Bifidobacteria and Lactobacilli counts compared with the control group. The overall mean of both Bifidobacteria and Lactobacilli counts of the groups fed with the extracts was significantly higher compared with the antibiotic group. Of the three extracts, the LenE group showed highest Bifidobacteria and Lactobacilli counts, whereas the TreE group showed the lowest counts.

**Dietary Level Effect of LenE**

BW gain increased with increased LenE dose in the diet (Table 4). The 5 g/kg LenE showed the highest BW gain. However, cecal pH and viscosity between the different levels of LenE was not significantly different. However there is a slight increase in cecal viscosity with increase of LenE dose.

Total aerobe and anaerobes increased with increased LenE dose, and LenE at a dose of 1 g/kg at the diet showed significantly lowest total aerobe and anaerobe counts (Table 4). Both 1 and 5 g/kg levels of LenE had significantly lower Bacteroides than 3 and 10 g/kg levels of LenE, and 3 g/kg LenE had significantly highest number of Bacteroides. The number of Enterococci decreased with increased LenE dose (from 1 to 5 g/kg), and the highest and lowest doses (1 and 10 g/kg) of LenE showed the significantly highest Enterococci counts.

The number of E.coli increased with increase of LenE dose, and 5 and 10 g/kg LenE levels showed the significantly highest counts. The number of Bifidobacteria and Lactobacilli increased with increases of the LenE dose. A level of 2 g/kg LenE showed the highest Bifidobacteria and Lactobacilli counts.
Table 3. BW gain, cecal pH, viscosity and microbial counts of chicken fed with the mushroom and herb polysaccharide extracts

<table>
<thead>
<tr>
<th>Treatments²</th>
<th>BW gain (g b⁻¹d⁻¹)</th>
<th>pH</th>
<th>Viscosity (cPm)⁶</th>
<th>Aerobes</th>
<th>Anaerobes</th>
<th>Bacteroides</th>
<th>E. coli</th>
<th>Enterococci</th>
<th>Bifidobacteria</th>
<th>Lactobacilli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.7</td>
<td>7.0</td>
<td>0.88</td>
<td>5.93</td>
<td>9.52</td>
<td>6.70</td>
<td>6.93</td>
<td>7.36</td>
<td>8.55</td>
<td>7.54</td>
</tr>
<tr>
<td>APR</td>
<td>25.6</td>
<td>7.2</td>
<td>1.29</td>
<td>9.08</td>
<td>9.39</td>
<td>8.72</td>
<td>9.69</td>
<td>7.25</td>
<td>8.12</td>
<td>6.58</td>
</tr>
<tr>
<td>LenE – 2 g/kg</td>
<td>17.6</td>
<td>6.8</td>
<td>1.17</td>
<td>6.19</td>
<td>9.53</td>
<td>6.38</td>
<td>6.58</td>
<td>7.29</td>
<td>9.09</td>
<td>8.33</td>
</tr>
<tr>
<td>TreE – 2 g/kg</td>
<td>18.9</td>
<td>7.0</td>
<td>1.22</td>
<td>5.99</td>
<td>9.09</td>
<td>5.74</td>
<td>6.14</td>
<td>7.16</td>
<td>8.69</td>
<td>8.16</td>
</tr>
<tr>
<td>AstE – 2 g/kg</td>
<td>21.6</td>
<td>7.0</td>
<td>1.17</td>
<td>6.18</td>
<td>9.26</td>
<td>6.45</td>
<td>6.73</td>
<td>7.23</td>
<td>9.03</td>
<td>8.21</td>
</tr>
<tr>
<td>SEM³</td>
<td>1.84</td>
<td>0.13</td>
<td>0.03</td>
<td>0.32</td>
<td>0.05</td>
<td>0.27</td>
<td>0.03</td>
<td>0.02</td>
<td>0.10</td>
<td>0.096</td>
</tr>
</tbody>
</table>

Contrast statements⁴

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Probability levels of contrasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vs APR</td>
<td>&lt; .000</td>
</tr>
<tr>
<td>Extracts⁵ vs APR</td>
<td>.001</td>
</tr>
<tr>
<td>LenE vs TreE</td>
<td>.558</td>
</tr>
<tr>
<td>AstE vs LenE &amp; TreE</td>
<td>.066</td>
</tr>
</tbody>
</table>

¹ Results are given as means (n = 3).
² APR = Apramycin; LenE = Lentinus edodes polysaccharide extract; TreE = Tremella fuciformis polysaccharide extract; AstE = Astragalus membranaceus polysaccharide extract.
³ SEM = Mean of the standard error
⁴ Orthogonal contrasts
⁵ Extracts = all extracts (n = 9).
⁶ cPm = cycles per minutes.
⁷ The colony-forming unit (cfu) is expressed as log₁₀ colonies per g cecal contents.
Table 4. BW gain, cecal pH, viscosity and microbial counts of chicken fed with different levels of LenE\textsuperscript{1}

<table>
<thead>
<tr>
<th>LenE\textsuperscript{2} (g/kg)</th>
<th>BW gain\textsuperscript{3} (g b\textsuperscript{1} d\textsuperscript{-1})</th>
<th>pH</th>
<th>Viscosity (cPm)\textsuperscript{4}</th>
<th>Aerobes</th>
<th>Anaerobes</th>
<th>Bacteroides (cfu)\textsuperscript{5} log10</th>
<th>Enterococci</th>
<th>E. coli</th>
<th>Bifidobacteria</th>
<th>Lactobacilli</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.4\textsuperscript{b}</td>
<td>7.2</td>
<td>1.10</td>
<td>5.57\textsuperscript{b}</td>
<td>8.80\textsuperscript{b}</td>
<td>6.25\textsuperscript{c}</td>
<td>7.58\textsuperscript{a}</td>
<td>6.32\textsuperscript{c}</td>
<td>8.53\textsuperscript{a}</td>
<td>7.91\textsuperscript{d}</td>
</tr>
<tr>
<td>2</td>
<td>17.6\textsuperscript{b}</td>
<td>6.8</td>
<td>1.17</td>
<td>6.09\textsuperscript{a}</td>
<td>9.10\textsuperscript{ab}</td>
<td>6.38\textsuperscript{bc}</td>
<td>7.45\textsuperscript{b}</td>
<td>6.63\textsuperscript{b}</td>
<td>9.08\textsuperscript{a}</td>
<td>8.49\textsuperscript{a}</td>
</tr>
<tr>
<td>3</td>
<td>21.0\textsuperscript{ab}</td>
<td>6.3</td>
<td>1.16</td>
<td>6.14\textsuperscript{a}</td>
<td>9.17\textsuperscript{a}</td>
<td>6.87\textsuperscript{a}</td>
<td>7.20\textsuperscript{c}</td>
<td>6.78\textsuperscript{ab}</td>
<td>8.69\textsuperscript{d}</td>
<td>8.19\textsuperscript{c}</td>
</tr>
<tr>
<td>5</td>
<td>26.5\textsuperscript{a}</td>
<td>6.4</td>
<td>1.18</td>
<td>6.21\textsuperscript{a}</td>
<td>9.20\textsuperscript{a}</td>
<td>6.35\textsuperscript{c}</td>
<td>7.13\textsuperscript{c}</td>
<td>6.92\textsuperscript{a}</td>
<td>8.81\textsuperscript{bc}</td>
<td>8.30\textsuperscript{b}</td>
</tr>
<tr>
<td>10</td>
<td>22.5\textsuperscript{ab}</td>
<td>6.7</td>
<td>1.15</td>
<td>6.00\textsuperscript{a}</td>
<td>9.13\textsuperscript{a}</td>
<td>6.58\textsuperscript{b}</td>
<td>7.57\textsuperscript{a}</td>
<td>6.96\textsuperscript{a}</td>
<td>8.93\textsuperscript{ab}</td>
<td>8.37\textsuperscript{b}</td>
</tr>
</tbody>
</table>

SEM\textsuperscript{3} 1.09 0.13 0.01 0.07 0.05 0.06 0.05 0.07 0.05 0.05
Dietary effects .001 .246 .075 .013 .012 <.001 <.001 <.001 <.001 <.001

\textsuperscript{1} Results are given as means (n=3) ± SE.

\textsuperscript{2} LenE = Lentinus edodes polysaccharide extract;

\textsuperscript{3} SEM = Mean of the standard error

\textsuperscript{4} cPm = cycles per minutes.

\textsuperscript{5} The colony-forming unit (cfu) is expressed as log\textsubscript{10} colonies per g cecal contents.

\textsuperscript{abcd} Means with different superscripts within the column are significantly different (P<0.05).
<table>
<thead>
<tr>
<th>Type of model</th>
<th>Observed traits</th>
<th>b0</th>
<th>b1</th>
<th>b2</th>
<th>SE</th>
<th>P-value</th>
<th>R²</th>
<th>F (1, 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear</td>
<td>BW gain</td>
<td>17.92</td>
<td>0.69</td>
<td>0.35</td>
<td>3.68</td>
<td>.037</td>
<td>.29</td>
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<tr>
<td></td>
<td>PH</td>
<td>6.79</td>
<td>-0.03</td>
<td>0.53</td>
<td>0.04</td>
<td>.433</td>
<td>.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Viscosity</td>
<td>1.14</td>
<td>0.002</td>
<td>0.04</td>
<td>0.28</td>
<td>.318</td>
<td>.08</td>
<td></td>
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<tr>
<td></td>
<td>Aerobes</td>
<td>5.90</td>
<td>0.02</td>
<td>0.28</td>
<td>0.17</td>
<td>.120</td>
<td>.18</td>
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<tr>
<td></td>
<td>Anaerobes</td>
<td>8.99</td>
<td>0.02</td>
<td>0.17</td>
<td>0.17</td>
<td>.001</td>
<td>.59</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bacteroides</td>
<td>6.41</td>
<td>0.02</td>
<td>0.24</td>
<td>0.20</td>
<td>.187</td>
<td>.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enterococci</td>
<td>7.36</td>
<td>0.06</td>
<td>0.20</td>
<td>0.17</td>
<td>.113</td>
<td>.18</td>
<td></td>
</tr>
<tr>
<td>Quadratic</td>
<td>BW gain</td>
<td>10.98</td>
<td>4.65</td>
<td>-0.35</td>
<td>2.35</td>
<td>&lt;.001</td>
<td>.73</td>
<td>19.81</td>
</tr>
<tr>
<td></td>
<td>PH</td>
<td>7.46</td>
<td>-0.42</td>
<td>0.034</td>
<td>0.47</td>
<td>.107</td>
<td>.31</td>
<td>4.74</td>
</tr>
<tr>
<td></td>
<td>Viscosity</td>
<td>1.09</td>
<td>0.03</td>
<td>-0.003</td>
<td>0.03</td>
<td>.048</td>
<td>.40</td>
<td>7.64</td>
</tr>
<tr>
<td></td>
<td>Aerobes</td>
<td>5.42</td>
<td>0.30</td>
<td>-0.002</td>
<td>0.21</td>
<td>.009</td>
<td>.54</td>
<td>12.14</td>
</tr>
<tr>
<td></td>
<td>Anaerobes</td>
<td>8.71</td>
<td>0.18</td>
<td>-0.014</td>
<td>0.13</td>
<td>.006</td>
<td>.57</td>
<td>10.98</td>
</tr>
<tr>
<td></td>
<td>Bacteroides</td>
<td>6.26</td>
<td>0.10</td>
<td>-0.007</td>
<td>0.24</td>
<td>.450</td>
<td>.12</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>Enterococci</td>
<td>7.83</td>
<td>-0.26</td>
<td>0.024</td>
<td>0.05</td>
<td>&lt;.001</td>
<td>.94</td>
<td>200.80</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>6.13</td>
<td>0.25</td>
<td>-0.017</td>
<td>0.09</td>
<td>&lt;.001</td>
<td>.89</td>
<td>32.56</td>
</tr>
<tr>
<td></td>
<td>Bifidobacteria</td>
<td>8.65</td>
<td>0.06</td>
<td>-0.003</td>
<td>0.21</td>
<td>.388</td>
<td>.15</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>Lactobacilli</td>
<td>7.98</td>
<td>0.12</td>
<td>-0.008</td>
<td>0.19</td>
<td>.135</td>
<td>.28</td>
<td>1.70</td>
</tr>
</tbody>
</table>

1. LenE = Lentinus edodes polysaccharide extract.
2. Models: Linear: $Y = b_0 + b_1 X$; Quadratic: $Y = b_0 + b_1 X + b_2 X^2$.
3. P <0.05 are printed in bold.
4. F (1, 12) represents the significance of adding a quadratic component to the linear model. Critical F (1, 12) values: 4.75 (P<0.05) and 9.33 (P<0.01).
Compared with the linear model, the quadratic model significantly improved the regression relationship for the observed traits (Table 5). There was a significant ($P < 0.001$) increased quadratic regression relationship between the LenE supplemental levels and the BW gain, Enterococci and E.coli counts. The data of total aerobes and anaerobes fitted well to the quadratic model ($P<0.01$). The cecal anaerobes fitted well to the quadratic model ($P<0.01$). The cecal viscosity also showed a slightly significant ($P<0.05$) quadratic function. However, the pH value, Bacteroides, Bifidobacteria and Lactobacilli counts all fitted poorly to both the linear and quadratic functions ($P>0.05$). The estimated parameters for regression functions of the LenE dose response are shown in Table 5.

DISCUSSION

**BW Gain, Cecal pH and Viscosity**

In this study, the broiler chicks became naturally infected with Avian mycoplasma Gallisepticum (AMG) prior to the experiment. Therefore, feeding both APR and the extracts might have significantly improved the health status of the infected birds, as indicated by the growth values. BW gain of the birds given either APR or the extracts was significantly improved as compared with the untreated birds which showed very poor growth. Furthermore, BW gain was increased linearly ($P < 0.001$) with increasing LenE dose.

The advantages of using antibiotics as feed supplements in terms of growth stimulation and improvement of host health and feed efficiency have been well documented (Bird, 1980; Miles et al., 1984; Armstrong, 1986; Ensminger, et al., 1990; Peterson et al., 1991). The efficacy of antibiotics is more obvious in diseased birds than normal birds. For example, a study of Eyssen and De Somer (1963) demonstrated that birds had a better response to antibiotic growth promoters under dirty housing conditions than under clean housing conditions.

Few reports exist comparing mushroom and herb polysaccharides with antibiotic growth promoters in poultry. However, it has been well documented that the polysaccharides derived from Astragalus membranaceae Radix, Lentinus edodes and Tremella fuciformis, significantly decreased mortality and incidence of bacterial (Yuan et al., 1993), viral (a.o., Wei et al., 1997; Liu et al., 1999b; Yu and Zhu, 2000) and parasitic diseases (Hu et al., 1998; Pang et al., 2000) in chickens. Yuan et al. (1993) reported that TreP m, a polysaccharide
extract isolated from mycelia of *Tremella fuciformis*, can be a good adjuvant for vaccines. A dose response trial of Yuan *et al.* (1993) showed that the protection rate of *Pasteurellosis* vaccine increased 67 to 160% with increases of the *TreP* level in chickens post infection of *Pasteurellosis* (MLD C48^{-1}). This is similar to the current study in which BW gain was significantly increased with increases in *TreP* dose.

Although the pH value showed numerically decreased with increased *LenE* dose, the expected significant lower cecal pH by feeding the mushroom and herb polysaccharide extracts was not observed in this study. This is not consistent with the *in vitro* fermentation test (Guo *et al.*, accepted). To some extents, it was surprising given that fermentation of carbohydrates leads to the production of straight-chain acids, and the fermentation of protein results in production of branched-chain acids (e.g. from amino acids such as valine, leucine, and iso-leucine), both of which can lower intestinal pH (Cummings, 1981; Macfarlane *et al.*, 1992). Unlike *in vitro* fermentation, acid concentrations in ceca were probably relatively low since these fermentation end products may have already absorbed in lumen, when polysaccharides were fermented by microbial *in vivo*.

The viscosity of cecal contents of the birds fed with the extracts was significantly higher compared with the control group. This is consistent with other studies (Hughes *et al.*, 2000; Iji *et al.*, 2001), which showed that inclusion of non-starch polysaccharides in broiler’s diets raised intestinal viscosity and excreta moisture. According to these authors, viscosity is dependent on several factors including the size of the polysaccharide molecule, whether it is branched or linear, the presence of charged groups, the surrounding structures and the concentration. In this experiment *TreE* had more influence on viscosity of cecal contents compared with *LenE* and *AstE*. This may be mainly caused by difference in physico-chemical properties of the extracts, although other unidentified substances may also influence the viscosity of the cecal contents. It has been reported that sugar composition, molecular weights and structure of these polysaccharides are all different (Xia and Cheng, 1988; Pang *et al.*, 1995; Yang *et al.*, 1999, 2001).

**Predominant Intestinal Microbiota**

Non-digestible carbohydrates (oligo-and polysaccharides) are potential prebiotics which could selectively enrich for beneficial bacterial species. Due to their chemical structure, these compounds are not hydrolyzed by digestive enzymes nor absorbed in the upper part of the gastrointestinal tract. Such ingredients therefore enter the large intestine and
may serve as substrates for the endogenous colonic bacteria (Salyers, 1979; Eastwood, 1992; Gibson and Roberfroid, 1995). Intestinal bacteria may be grouped into those species that may have harmful or pathogenic influences on host health such as *Proteus, Staphylococci, Clostridia* and *Veillonellae*, those that may have beneficial affects such as *Lactobacilli* and *Bifidobacteria*, and those that may have both effects such as *Enterococci, E. coli, Streptococci* and *Bacteroides* (Macfarlane and Cummings, 1991).

There were significant changes in microbial counts for animals fed the APR and the polysaccharide extract diets, with an increase in the total number of aerobes and anaerobes. Hesselman and Aman (1986) reported that viscosity was increased in the presence of water-soluble non-starch polysaccharides, which therefore decreased digesta passage rate. Thus, the highest viscosity which resulted from feeding APR and the polysaccharide extracts may have slowed down the digesta transit time in the lower part of the small intestine (ileum) and in the large intestine (cecum and rectum) of chickens. This could have meant that intestinal bacteria had sufficient time to multiply, resulting in an increase in the microbial population. Surprisingly, *TreE* showed the highest viscosity yet the cecal microbial counts of the *TreE* group were significantly lower compared with the *LenE* and *AstE* groups. This may be due to confounding factors, since the bacterial populations and activities can fluctuate in response to substrate availability and pH, as well as the viscosity of digesta in the intestines (Cummings and Macfarlane, 1991).

The mushroom and herb extract diets were largely associated with reduced *Bacteroides, Enterococci* and *E.coli* numbers, but increased numbers of *Bifidobacteria*, and *Lactobacilli* relative to the control and antibiotic groups. As demonstrated by several studies (Macy and Probst, 1979; Baily et al., 1991; Gibson and Roberfroid, 1995; Sunvold et al. 1995; Newman, 1995; Langhout, 1999), some bacteria in the large intestine are more specialized in the hydrolysis of large molecular carbohydrates such as oligo- and polysaccharides, producing small molecular weight carbohydrates from large polymers and then fermenting them, which can lead to greater bacterial numbers. Fermentation end-products such as short-chain fatty acids, lower intestinal pH, which can depress harmful bacteria and stimulate beneficial bacteria.

There are large variations in number of *Bacteroides* among the *LenE* levels, as a result, which didn’t fit either linear or quadratic regression functions. Although the *Bifidobacteria* and *Lactobacili* counts increased with increased *LenE* dose, both fitted poorly to the linear or quadratic function. It may be associated with the exceptionally highest *Bifidobacteria* and *Lactobacili* counts of the 2 g/kg *LenE* group.
Of the three extracts, the LenE and AstE groups were associated with the highest cecal microbial populations. The different response of birds to the three extracts may be related to the physico-chemical properties of these polysaccharide fractions and to their fermentation characteristics in the large intestine of chickens. According to an in vitro study, AstE and LenE were rapidly degraded and highly fermentable, whereas TreE was less readily fermented (Guo et al., accepted). Fermentation of these polysaccharides resulted in great shift of cecal microbial community of chickens (Guo et al., submitted).

Unlike the extracts, APR treatment significantly increased the number of Bacteroides, Enteriococci and E. coli, but inhibited growth of Enteriococci, Bifidobacteria and Lactobacilli in ceca. It was reported that growth-promoting antibiotics lower the number and activity of intestinal microbes (Rosen, 1995). Cecal Enterococci of chickens were largely reduced by feeding APR. It has been shown that microorganisms responsible for sub-clinical infections may be reduced or eliminated by antibiotic treatments (Jensen, 1993).

In summary, both the mushroom and herb polysaccharide extracts and APR stimulated growth of the chickens naturally infected with AMG after one-week treatments. The average BW gain of the groups fed with the extracts was significantly lower compared with those fed APR-containing diets. However, cecal viscosity and microbial population counts were significantly higher in chickens fed the extracts and APR. Unlike APR, presence of the extracts seems to have stimulated the number of potential beneficial bacteria such as Bifidobacteria, and Lactobacilli, while reducing the number of harmful bacteria such as Bacteroides and E.coli. Birds had different responses to the three extracts, with LenE showing the greatest potential as a prebiotic. Increases of the LenE dose was associated with birds which tended to have higher BW gain and increased cecal microbial counts. Thus, the mushroom and herb polysaccharide extracts, LenE, TreE and AstE, may have some prebiotic effect leading to shifts in the intestinal microbial populations of chickens. One of the limitations of using traditional culture-based microbiology techniques is that only easily cultivable organisms are counted, which does not represent the total number of bacterial species present in the GIT of the chickens in terms of describing the species present within a complex microflora. This experiment was conducted using diseased birds, and the results suggest that in infected birds, these extracts had a positive effect. However, it is not yet known whether a similar effect might be found for non-infected birds. Further work is required to investigate this.
ACKNOWLEDGMENTS

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Chapter 7

EFFICACY OF MUSHROOM AND HERB POLYSACCHARIDES AGAINST AVIAN COCCIDIOSIS: IMMUNIZATION WITH OR WITHOUT LIVE OOCYST

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Submitted to: British Poultry Science
Efficacy of Mushroom and Herb Polysaccharides Against Avian Coccidiosis:
Immunization with or without Live Oocyst


Abstract

An experiment was conducted to investigate the effects of polysaccharide extracts (E) of two mushrooms, *Lentinus edodes* (LenE) and *Tremella fuciformis* (TreE), and an herb, *Astragalus membranaceus* (AstE), on the immune responses of the chickens infected with *Eimeria tenella*. A total of 180 broiler chickens were assigned to nine groups: three groups fed with each of the extracts (LenE, TreE and AstE), three groups fed with the extracts and immunized with live oocyst vaccine (LenE+V, TreE+V and AstE+V), a group immunized with the vaccine only, and two controls (*E. tenella* infected and non-infected groups). The oocyst vaccine was given at 4 days of age and the extracts (1 g/kg of the diet) were supplemented from 8 to 14 days of age. At 18 days of age, all birds except those of the non-infected group were infected with 9 x 10⁴ sporulated oocysts. The results showed that at 7 days post infection (p.i.), birds fed the extracts had lower BW gain than those given the vaccine only, however, the extracts in conjunction with the vaccine significantly enhanced BW gain. Of the three extracts, LenE and TreE showed a better growth promoting effect. The extracts largely increased oocyst excretion during the primary response post vaccination. The cecal peak oocyst output and lesion scores were higher in groups fed the extracts than in the group immunized with the vaccine only, whereas those of the extract plus vaccine groups were not different from the vaccine group. Of the three extracts, both LenE and AstE-fed groups showed lower cecal oocyst output. The birds immunized with vaccine showed significantly higher thymus and bursa weights but lower liver and spleen weights compared with the birds fed the extracts. As compared with the vaccine group, the birds fed with the extracts showed significantly higher GIT weights, however, the birds fed with the extracts plus vaccine showed lower GIT weights at day 7 p.i. Thus, the vaccine showed better effects on immune response of the infected chickens than the extracts. The polysaccharide extracts may prove useful against avian coccidiosis, and particularly when they used in conjunction with vaccine, have shown preliminary promise against the experimental coccidial infection.

*Keywords*: mushroom, herb, polysaccharides, *E. tenella*, growth, immune response chickens
INTRODUCTION

Coccidiosis, caused by protozoan parasites of genus *Eimeria*, is a significant problem to the poultry industry throughout the world. The protozoan parasites multiply in intestinal epithelial cells of chickens, thus, the pathology is largely associated with the destruction of the epithelial lining of the infected part of the intestine, which results in a reduced ability for digestion and absorption of nutrients by the bird (Long, 1982). It was estimated that annual costs of anticoccidial drugs were 720 million US dollars in China (Suo and Li, 1998) and 60 million US dollars in USA (Long, 1984). In the United Kingdom, the total costs of coccidiosis in chickens were estimated to have been at least 54.9 million US dollars in 1995, of which 98.1% involved broilers (Williams, 1999). Coccidiosis is responsible for 6-10% of all the broiler mortality, and the global economic losses, as a result of a reduction in growth rate, feed intake and feed conversion efficiency, are estimated at 1 billion US dollars annually (Banfield *et al.*, 1999).

Currently, chemotherapy is extensively used and the disease has largely been controlled through the use of anti-coccidial drugs added directly to the feed (Chapman, 2001; Rana and Tikaram. 2001b; Conway *et al.*, 2002). However, intensive use of anti-coccidial drugs has led to the rapid appearance of resistant strains of the disease (Choi *et al.*, 1988; Chapman, 1997; Rana and Tikaram, 2001a). The problems faced in using therapeutic drugs are further complicated by the fact that a public is increasingly concerned residues of the drugs left in the poultry products (Newman, 2002). Increasing development of drug-resistant coccidia species has stimulated searches for alternative control methods, one of which is vaccination. In recent years, there is an increasing interest to develop vaccines for the control of coccidiosis (Rai *et al.*, 1996; Lillehoj, 2000; Yun, 2000; Vermeulen *et al.*, 2001; Chapman *et al.*, 2002; Williams, 2002). Approaches have focused on the use of live, attenuated vaccines as reviewed by Shirley and Bednrik (1997) and recombinant vaccines as reviewed by Vermeulen (1998). Both of these approaches are producing promising results, but each, too, has its problems. Live vaccines still bear the risk of inducing pathology, while recombinant vaccines are still in the early stages of development.

Natural medicinal products are considered to fill in the role of strengthening the birds’ defense system against invasion by infectious organisms (Li, 1998). Natural medicinal products such as fungus and herbs have been used as feed additives in farm animals in China for centuries, and shown many bioactivities such as anti-microbial activities, immune enhancement and stress reduction (Wang *et al.*, 2002). It is well documented that certain
polysaccharides derived from medicinal mushrooms and herbs have been used as immune enhancers and shown anti-parasitic activities (Hu et al., 1998; Pang et al., 2000) in chickens.

The present experiment was designed to evaluate the effects of specific immuno-active mushroom and herb polysaccharide extracts, *Lentinus edodes* extract - *LenE*, *Tremella fuciformis* extract - *TreE* and *Astragalus membranaceus* extract - *AstE*, which were used either as feed supplements or adjuvant, on immune responses of chickens against coccidiosis.

**MATERIALS AND METHODS**

**Birds and Experimental Design**

A total of 180, 3-days-old Sanhuang broiler chickens (a local slow-growing breed from the Institute of Animal Science, CAAS, Beijing) of both sexes were used. Animals, not infected with coccidia, were randomly assigned to 9 groups with 20 birds each: Groups 1 to 3 were three extract supplemented groups (*LenE, TreE* and *AstE*); Groups 4 to 6 were three groups fed with extracts, and immunized with live oocyst vaccine (*LenE+V, TreE+V* and *AstE+V*); Group 7 was the immunized with oocyst vaccine only; Groups 8 and 9 were the *E. tenella* infected and non-infected groups, respectively.

**Diets and Management**

The composition of the diet is presented in Table 1. The maize and soybean based diet, without coccidiostat additives, was provided *ad libitum* to the birds. The diet was heated to 80 °C for 60 min before feeding. Birds were reared under coccidia-free conditions in horizontal battery pens using wood sawdust as litter, and 20 birds in each pen with a density of 10 birds/m². The formal experiment was conducted from 4 to 31 days of age (a total of 28 days). The temperature was set at 30°C to 33 °C during the first week and gradually declined by 2 °C per week until 20 °C was reached. Relative humidity was about 60-80%. The lighting program was 24h light throughout the experiment.
Table 1. Composition of the experimental diet

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Basal diet (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>555</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>326.6</td>
</tr>
<tr>
<td>Fish meal</td>
<td>40</td>
</tr>
<tr>
<td>Soya oil</td>
<td>40</td>
</tr>
<tr>
<td>Monocalciumphosphate</td>
<td>13</td>
</tr>
<tr>
<td>Limestone</td>
<td>11.3</td>
</tr>
<tr>
<td>Salt (NaCl)</td>
<td>3</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>1</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.12</td>
</tr>
<tr>
<td>Vitamin and mineral mix</td>
<td>10</td>
</tr>
</tbody>
</table>

Calculated analysis (g/kg)

| Crude protein                       | 210               |
| Crude fat                           | 62.4              |
| Crude fibre                         | 30.9              |
| Ash                                 | 55.8              |
| Metabolisable energy (kcal/kg)      | 3,000             |
| Available phosphorus (P)            | 4.5               |
| Calcium                             | 9.0               |
| Sodium (Na)                         | 1.5               |
| Potassium (K)                       | 8.2               |
| Chlorine (Cl)                       | 2.7               |
| Lysine                              | 11.7              |
| Methionine                          | 6.0               |

1 Supplied for per kg of diet: vitamin A (retinal acetate) 9000 IU, cholecalciferol 1800 IU, vitamin E (DL-α-tocopherol acetate) 20 mg, vitamin K₃ 3.4 mg, thiamin 1.8 mg, riboflavin 5.2 mg, D-pantothenic acid 9.8 mg, niacinamide 36 mg, folic acid 0.90 mg, pyridoxine 3.60 mg, cyanocobalamin 18 μg, biotin 80 μg, choline chloride 200 mg, FeSO₄•7 H₂O 80 mg, ZnSO₄•4 H₂O 75 mg, MnO₂ 100 mg, Na₂SeO₄•5H₂O 150 μg, KI 300 μg, carrier maize.

Polysaccharide Preparation and Supplementation

Intact mushroom and herb materials were purchased from a local source (Lentinus edodes and Tremella fuciformis produced in Zhejiang, China; Astragalus membranaceus produced in Gansu, China). The intact mushroom and herb materials were dried overnight at 45 °C and ground through a 1mm sieve for water-soluble polysaccharide extraction, according to Liu et al. (1999). The yields of the polysaccharides and their chemical analyses
are presented in Table 2.

LenE, TreE and AstE were mixed with the animal diet separately according to the experimental design and supplemented for one week (from 8 to 14 days of age). The supplemental level was 1 g/kg of the diet, which was considered to be the optimum level for enhanced health in coccidial infected chickens, according to Hu et al. (1998).

**Live Oocyst Vaccination**

An attenuated live vaccine (a lower dose of live oocysts, Laboratory of Parasitology and Parasitological Diseases, China Agricultural University) consisted of a mixture of oocysts of *E. tenella* and diluents (2% K$_2$Cr$_2$O$_7$ solution). At 4 days of age, appropriate proportions were prepared so that each chicken received the intended dose of the oocyst mixture (200 live oocysts, 0.2 ml per animal), directly into the crop, using an oral gavage.

| Table 2. Yields and chemical analyses of mushroom and herb polysaccharide extracts$^1$ |
|---------------------------------|-----|-----|-----|
| Compositions                     | LenE | TreE | AstE |
| Yields of polysaccharides (%)$^2$| 8    | 10   | 31   |
| Dry matter (g/kg)$^3$            | 952  | 955  | 940  |
| Crude protein (g/kg DM)$^4$      | 114  | 47   | 67   |
| Total sugar content (g/kg DM)$^5$| 632  | 687  | 647  |

$^1$ LenE = *Lentinus edodes* polysaccharide extract; TreE = *Tremella fuciformis* polysaccharide extract; AstE = *Astragalus membranaceus* polysaccharide extract.

$^2$ The yield of polysaccharides was based on the air-dry matter of the intact materials.

$^3$ The dry matter content of the extracts was determined by freeze-drying.

$^4$ Protein content was determined by the semi-automated Kjeldahl method (ISO, 1997).

$^5$ Total sugar content was determined by the phenol-sulphuric method (Dubois et al., 1956).

**Parasite Preparation and Infection**

The parasite used in this experiment was a laboratory *E. tenella* strain maintained by periodic passage through young chickens at the facilities of the Laboratory of Parasitology and Parasitological Diseases, China Agricultural University.

At 18 days of age, all experimental birds except those of the non-infected control group were infected with $9 \times 10^4$ sporulated oocysts prepared above in 0.3 ml of water, directly into
the crop, using an oral gavage. After infection, clinic syndromes and blood in faeces were observed, and oocysts from droppings were counted every day using a McMaster’s chamber (Hodgson, 1970).

Clinic syndromes, mortality and blood in faeces were observed post infection (p.i.). Oocyst excretion from droppings of each treatment was daily counted post vaccination using a McMaster’s chamber (Hodgson, 1970).

Data Collection

BW gain

Birds from each treatment were weighed individually at day 7 (one day before the extract supplementation), day 18 (Eimeria infection), day 24 (day 7 p.i.) and day 31 (day 14 p.i.). BW gain was calculated accordingly.

Cecal lesion score and oocyst output

Seven days post-infection, 10 birds from each treatment were randomly selected and killed by cervical dislocation. The ceca were removed and opened. The infected ceca were examined and scored according to the method described by Suo and Li (1998), and then grounded with a meat grinder together with the contents. The total number of oocysts in each collection of ceca was determined from duplicate counts of diluted aliquots of homogenates using a McMaster’s chamber (Hodgson, 1970).

Relative weights of immune organs and gastro-intestinal tract (GIT) fragments

The same 10 birds, which were used for examining cecal lesions at 7 days post-infection, were used for dissection, and at 14 days post-infection, the rest 10 birds were killed for dissection. The immune organs, thymus, spleen, bursa and liver, were removed from each bird and weighed. The GIT was also removed from each bird and divided into the following sections: crop+oesophagus, proventriculus+gizzard, duodenum, jejunum, ileum and ceca. All GIT organs except for ceca were measured after the contents were gently squeezed from them. The relative weights of the organs and the GIT were expressed as a percentage of body weight.
**Statistical Analysis**

All the data were subjected to statistical analysis by use of SPSS 8.0 (Statistical Package for the Social Science, 1997). A bird was considered as an experimental unit. Orthogonal contrasts were used to assess: 1) the effect of the coccidial infection, examining contrast between the infected and non-infected groups; 2) the effect of a vaccination, examining the contrast between the vaccine and infected groups; 3) the effect of extract supplementation solely, examining the contrast between the overall mean of the groups fed with the extracts (E) and the vaccine group; 4) the effect of extract supplement plus vaccination, examining the contrast between the overall mean of the groups fed the extracts and immunized with the vaccine (E+V) and the vaccine group.

Tukey’s multiple range test (HSD) was used to test significance between the three extract supplemented groups (LenE, TreE and AstE) as well as between the extract plus vaccine groups (LenE+V, TreE+V and AstE+V).

As the oocyst yields were not normally distributed, they were transformed into In (x+1). Ceca lesion scores were analyzed by a Chi-square test (Kruskal Willis test).

**RESULTS**

**BW Gain**

A significant difference of BW gain was found in different treatments from day 7 to 17 and day 18 to 24 (Table 3). From 7 to 17 days of age, the infected-birds immunized with the live oocyst vaccine had a significant higher BW gain compared with the infected but non-immunized birds. The overall mean of BW gain of the extract supplemented groups were significantly lower compared with the vaccine group, however, that of the E+V groups was not significantly different from the vaccine group. Both AstE+V and LenE+V groups had significantly higher BW gain than the TreE+V group.

From 18 to 24 days of age, BW gain of the infected group was significantly lower compared with both the vaccinated and non-infected groups. The overall mean BW gain of the extract-supplemented groups was significantly lower compared with the vaccine group. Of the three extracts, LenE and TreE showed a significant better effect on BW gain. The overall mean BW gain of the E+V groups was significantly higher compared with the vaccine group. Of the three E+V groups, the LenE+V group showed the significantly highest BW gain.
Table 3. BW gain of *Eimeria tenella* infected chickens fed with the polysaccharide extracts

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>BWG (g b⁻¹ d⁻¹)¹</th>
<th>Day 7-17</th>
<th>Day 18-24</th>
<th>Day 25-30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infected</td>
<td>10.7 ± 0.3</td>
<td>22.4 ± 0.5</td>
<td>15.6 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>11.3 ± 0.2</td>
<td>17.0 ± 0.7</td>
<td>17.4 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Vaccinated</td>
<td>11.8 ± 0.2</td>
<td>20.3 ± 0.6</td>
<td>18.6 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Mean extracts (E)²</td>
<td>11.1 ± 0.1</td>
<td>17.6 ± 0.7</td>
<td>20.2 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>AstE</td>
<td>11.5 ± 0.2</td>
<td>14.6 ± 0.8b</td>
<td>19.2 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>LenE</td>
<td>10.9 ± 0.2</td>
<td>19.5 ± 1.2a</td>
<td>21.1 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>TreE</td>
<td>11.2 ± 0.3</td>
<td>18.8 ± 1.1a</td>
<td>20.5 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>Mean (E+V)³</td>
<td>11.9 ± 0.2</td>
<td>22.8 ± 0.4</td>
<td>17.1 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>AstE+V</td>
<td>12.2 ± 0.3A</td>
<td>21.5 ± 0.8b</td>
<td>17.6 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>LenE+V</td>
<td>12.3 ± 0.2A</td>
<td>24.0 ± 0.5A</td>
<td>16.6 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>TreE+V</td>
<td>11.2 ± 0.2B</td>
<td>22.8 ± 0.5AB</td>
<td>17.2 ± 0.9</td>
<td></td>
</tr>
</tbody>
</table>

Contrast statements

<table>
<thead>
<tr>
<th>Probability level of contrasts⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected vs Non-infected</td>
</tr>
<tr>
<td>Vaccinated vs Infected</td>
</tr>
<tr>
<td>E vs Vaccinated</td>
</tr>
<tr>
<td>E+V vs Vaccinated</td>
</tr>
</tbody>
</table>

¹ Results are given as means (n = 20 for Day 7-17 and Day 18-24; n = 10 for Day 25-30) ± SEM.

² Mean E: results are given as overall mean of three extract groups (LenE = *Lentinus edodes* extract; TreE = *Tremella fuciformis* extract; AstE = *Astragalus membranaceus* extract) ± SEM

³ Mean E+V: results are given as overall mean of the extract plus vaccine groups (LenE+V = *Lentinus edodes* extract plus vaccine; TreE+V = *Tremella fuciformis* extract plus vaccine; AstE+V = *Astragalus membranaceus* extract plus vaccine) ± SEM

⁴ P < 0.05 are printed in bold.

abc, ABC: means with different superscripts within the three extracts differed significantly (P<0.05).

**Oocyst Excretion from Droppings**

Figure 1 shows the oocyst excretion from droppings after live oocyst vaccination and infection in broiler chicks. First peak oocyst excretion occurred at 7 days after vaccination when the daily logged oocyst output of the LenE+V, TreE+V, AstE+V groups was 6.9, 5.7 and 5.6, respectively, which were much higher than that of the vaccine group (5.5).

The second peak oocyst excretion from droppings occurred 18 days after vaccination when the daily logged oocyst output of the LenE+V, TreE+V, AstE+V groups was 5.4, 4.6
and 6.6, respectively, which were much lower than the vaccine group (7.5).

The highest oocyst production occurred at 7 days post infection, when the daily logged oocyst output of the LenE TreE and AstE groups were 8.6, 8.8 and 7.8, respectively and the vaccine group 8.2, which were higher than the LenE+V, TreE+V and AstE+V groups (7.8, 6.2 and 7.2, respectively). The infected group had the highest logged oocyst output (9.8).

![Graph A](image)

**Figure 1.** The daily logged oocyst output from droppings post-vaccination and infection for the extract plus vaccine groups (LenE+V = *Leninus edodes* extract plus vaccine; TreE+V = *Tremella fuciformis* extract plus vaccine; AstE+V = *Astragalus membranaceus* extract plus vaccine) (A) and for the extract supplemented groups (LenE = *Leninus edodes* extract; TreE = *Tremella fuciformis* extract; AstE = *Astragalus membranaceus* extract) (B).
Cecal Oocyst Output and Lesion Score

As expected, the non-infected group had no cecal output and lesion score was zero. Therefore, these figures are not included in Table 4. The infected group had significantly (P<0.01) higher cecal oocyst output compared with the vaccine group at day 7 p.i. (Table 4). The overall mean oocyst output of the extract-supplemented groups was significantly (P<0.05) higher compared with the vaccine group, whereas that of the E+V groups was not significantly different from the vaccine group. Of the three extracts, the LenE and AstE groups had significantly lower cecal oocyst output. However, the AstE+V group had significantly higher cecal oocyst output than the TreE+V group.

The cecal lesion score of the different treatments at day 7 p.i. is presented in Figure 2. The infected group showed the highest frequency of Score 3 and 4, and it also showed the significantly highest lesion score (Table 4). The overall mean lesion score of the extract-supplemented groups was significantly higher compared with the vaccine group, whereas that of the E+V groups was not significantly different. Cecal lesion score was not significantly different among the three extract supplemented groups as well as among the three E+V groups.

Weights of Immune Organs

Table 5 shows the relative weights of the immune organs as a percentage of BW in broiler chickens at 7 and 14 days post-infection. Relative to the non-infected birds, the infected birds had significantly higher liver weights but lower thymus and bursa weights at 7 days p.i. The vaccine group showed significantly higher thymus and bursa (except day 14 p.i.) weights but lower liver and spleen weights compared with the infected group at both 7 and 14 days p.i. The same result was found when the vaccine group was compared with the overall mean of the extract-supplemented groups and the E+V groups at both 7 and 14 days p.i. Compared with the infected birds, the birds fed with the extracts showed slightly higher thymus and bursa weights but lower liver weights. Of the E+V groups, the AstE+V group showed the significantly highest thymus and liver weights at 7 days p.i.
Table 4. Cecal oocyst output and lesion score of *Eimeria* infected chickens fed with the polysaccharide extracts

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Cecal oocyst output</th>
<th>Lesion score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ln (x+1)</td>
<td>(mean rank)</td>
</tr>
<tr>
<td>Infected</td>
<td>14.93 ± 0.15</td>
<td>3.4 ± 0.2 (73.7)</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>13.29 ± 0.41</td>
<td>1.5 ± 0.3 (35.8)</td>
</tr>
<tr>
<td>Mean extracts (E)(^2)</td>
<td>14.55 ± 0.10</td>
<td>2.7 ± 0.1 (60.1)</td>
</tr>
<tr>
<td>AstE</td>
<td>14.42 ± 0.12(^b)</td>
<td>2.7 ± 0.3 (59.1)</td>
</tr>
<tr>
<td>LenE</td>
<td>14.25 ± 0.15(^b)</td>
<td>2.8 ± 0.3 (62.0)</td>
</tr>
<tr>
<td>TreE</td>
<td>14.99 ± 0.11(^a)</td>
<td>2.7 ± 0.3 (59.1)</td>
</tr>
<tr>
<td>Mean (E+V)(^3)</td>
<td>12.51 ± 0.34</td>
<td>1.6 ± 0.2 (37.3)</td>
</tr>
<tr>
<td>AstE+V</td>
<td>13.46 ± 0.58(^A)</td>
<td>1.7 ± 0.4 (39.3)</td>
</tr>
<tr>
<td>LenE+V</td>
<td>12.69 ± 0.53(^{AB})</td>
<td>1.6 ± 0.3 (37.5)</td>
</tr>
<tr>
<td>TreE+V</td>
<td>11.38 ± 0.48(^B)</td>
<td>1.5 ± 0.4 (35.2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Contrasts</th>
<th>Probability level of contrasts(^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinated vs. Infected</td>
<td><strong>.003</strong></td>
</tr>
<tr>
<td>E vs. Vaccinated</td>
<td><strong>.014</strong></td>
</tr>
<tr>
<td>E+V vs. Vaccinated</td>
<td>.145</td>
</tr>
</tbody>
</table>

\(^1\) Results are given as means (n =10) ± SEM.

\(^2\) Mean E: results are given as overall mean of three extract groups (*LenE = Lentinus edodes* extract; *TreE = Tremella fuciformis* extract; *AstE = Astragalus membranaceus* extract) ± SEM.

\(^3\) Mean E+V: results are given as overall mean of the extract plus vaccine groups (*LenE+V = Lentinus edodes* extract plus vaccine; *TreE+V = Tremella fuciformis* extract plus vaccine; *AstE+V = Astragalus membranaceus* extract plus vaccine) ± SEM.

\(^4\) The nonparametric test (Kruskal Willis test) was used to test lesion scores of the different treatment groups: Chi-Square = 48.4; P < .001.

\(^5\) P < 0.05 are printed in bold.

\(^a\), \(^b\), \(^c\), \(^ABC\): means with different superscripts within the three extracts differed significantly (P<0.05).
**Figure 2.** Lesion score of ceca in chickens at 7 days post *Eimeria* infection. The infected ceca (n=10) were examined and scored on a scale of lesion from Score 0 (no obvious lesion) to 4 (most serious lesion). Score 1: Very few scattered petechiae on the caecal wall, no thickening of the caecal walls, normal caecal contents. Score 2: Lesions more numerous with noticeable blood in the caecal contents, caecal wall is somewhat thickened, normal caecal contents. Score 3: Large amounts of blood or caecal cores present, caecal walls greatly thickened, little, if any, faecal contents in caeca. Score 4: Cecal walls greatly distended with blood or large caseous cores, faecal debris lacking included in cores.

**Weights of GIT**

Table 6 shows the relative weights of the GIT organs of broiler chickens at 7 and 14 days after *Eimeria* infection. Relative to the non-infected birds, the infected birds showed significantly lower duodenum and ileum weights at 7 days p.i. and slightly lower jejunum weight at both 7 p.i. At 14 days p.i., this observation was counterbalanced. However, the infected birds showed a significantly higher jejunum weight and slightly higher duodenum and ileum compared with the non-infected birds. The vaccine group significantly increased crop+oesophagus (at day 14 p.i.), jejunum (at day 7 p.i.) and ileum weights (at day 7 p.i.), but reduced jejunum (at day 14 p.i.), proventriculus+gizzard and cecum weights (at day 7 p.i.) compared with the infected group.

As compared with the vaccine group, the extract-supplemented groups had significantly higher duodenum, ileum and cecum weights at day 7 p.i but lower crop+oesophagus at day 14 p.i. The E+V groups showed significantly lower crop-oesophagus, proventriculus+gizzard, jejunum and ileum weights at day 7 p.i.
### Table 5. The weights of immune organs in *Eimeria* infected chickens at 7 and 14 days post infection (%BW)

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Thymus</th>
<th>Thymus</th>
<th>Liver</th>
<th>Liver</th>
<th>Spleen</th>
<th>Spleen</th>
<th>Bursa</th>
<th>Bursa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 d p.i.</td>
<td>14 d p.i.</td>
<td>7 d p.i.</td>
<td>14 d p.i.</td>
<td>7 d p.i.</td>
<td>14 d p.i.</td>
<td>7 d p.i.</td>
<td>14 d p.i.</td>
</tr>
<tr>
<td>Non-infected</td>
<td>0.83</td>
<td>0.69</td>
<td>3.01</td>
<td>2.92</td>
<td>0.19</td>
<td>0.31</td>
<td>0.44</td>
<td>0.30</td>
</tr>
<tr>
<td>Infected</td>
<td>0.43</td>
<td>0.53</td>
<td>3.70</td>
<td>3.26</td>
<td>0.20</td>
<td>0.33</td>
<td>0.32</td>
<td>0.35</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>0.71</td>
<td>0.88</td>
<td>2.85</td>
<td>2.42</td>
<td>0.14</td>
<td>0.18</td>
<td>0.44</td>
<td>0.42</td>
</tr>
<tr>
<td>Mean extracts (E)²</td>
<td>0.59</td>
<td>0.62</td>
<td>3.48</td>
<td>2.72</td>
<td>0.20</td>
<td>0.27</td>
<td>0.36</td>
<td>0.30</td>
</tr>
<tr>
<td>AstE</td>
<td>0.55</td>
<td>0.67</td>
<td>3.44</td>
<td>2.73</td>
<td>0.19</td>
<td>0.31</td>
<td>0.34</td>
<td>0.31</td>
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<tr>
<td>LenE</td>
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<td>0.60</td>
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<td>2.47</td>
<td>0.20</td>
<td>0.21</td>
<td>0.36</td>
<td>0.27</td>
</tr>
<tr>
<td>TreE</td>
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<td>0.59</td>
<td>3.56</td>
<td>2.95</td>
<td>0.22</td>
<td>0.28</td>
<td>0.37</td>
<td>0.31</td>
</tr>
<tr>
<td>Mean (E+V)³</td>
<td>0.48</td>
<td>0.59</td>
<td>3.06</td>
<td>2.72</td>
<td>0.17</td>
<td>0.24</td>
<td>0.36</td>
<td>0.30</td>
</tr>
<tr>
<td>AstE+V</td>
<td>0.72</td>
<td>0.57</td>
<td>3.35A</td>
<td>2.75</td>
<td>0.19</td>
<td>0.26</td>
<td>0.34</td>
<td>0.26</td>
</tr>
<tr>
<td>LenE+V</td>
<td>0.39</td>
<td>0.58</td>
<td>3.10AB</td>
<td>2.77</td>
<td>0.17</td>
<td>0.22</td>
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<td>0.32</td>
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<tr>
<td>TreE+V</td>
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<td>0.63</td>
<td>2.73B</td>
<td>2.63</td>
<td>0.16</td>
<td>0.24</td>
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<tr>
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**Contrast statements**

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<td>Infected vs Non-infected</td>
</tr>
<tr>
<td>Vaccinated vs Infected</td>
</tr>
<tr>
<td>E vs Vaccinated</td>
</tr>
<tr>
<td>E+V vs Vaccinated</td>
</tr>
</tbody>
</table>

1 Results are given as means (n = 10).
2 Mean E: results are given as overall mean of three extract groups (LenE = *Lentinus edodes* extract; TreE = *Tremella fuciformis* extract; AstE = *Astragalus membranaceus* extract).
3 Mean E+V: results are given as overall mean of the extract plus vaccine groups (LenE+V = *Lentinus edodes* extract plus vaccine; TreE+V = *Tremella fuciformis* extract plus vaccine; AstE+V = *Astragalus membranaceus* extract plus vaccine).
4 SEM = the standed error mean of the treatments.
5 P <0.05 are printed in bold.
6abc, ABC: means with different superscripts within the three extracts differed significantly (P<0.05).
<table>
<thead>
<tr>
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<td>0.95</td>
<td>0.99</td>
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<td>3.9</td>
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<td>0.84</td>
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<td>1.01</td>
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<td>2.4</td>
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<td>0.85</td>
<td>1.38</td>
<td>1.45</td>
<td>1.27</td>
<td>1.12</td>
<td>0.89</td>
<td>0.68</td>
</tr>
<tr>
<td>LenE</td>
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<td>3.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.9</td>
<td>0.92</td>
<td>0.84</td>
<td>1.40</td>
<td>1.70</td>
<td>1.09</td>
<td>1.21</td>
<td>1.01</td>
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<tr>
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<td>3.6</td>
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<td>1.24&lt;sup&gt;A&lt;/sup&gt;</td>
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<td>0.81</td>
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<td>1.03</td>
<td>0.83</td>
<td>0.64</td>
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<td>3.7</td>
<td>0.80</td>
<td>0.84</td>
<td>1.01&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.23</td>
<td>0.71</td>
<td>1.01</td>
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<td>0.01</td>
<td>0.09</td>
<td>0.02</td>
<td>0.02</td>
<td>0.03</td>
<td>0.04</td>
<td>0.03</td>
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Contrast statements

<table>
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<th>Probability level of contrasts&lt;sup&gt;5&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected vs Non-infected</td>
<td>.083  .252  .554  .318 &lt;.001 .011  .167  .426  .029 &lt;.001 .200 .952 .905</td>
</tr>
<tr>
<td>Vaccinated vs Infected</td>
<td>.920  .064  .265 &lt;.001 .101  .575  .018  .015 .001 .338 .015 .341</td>
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<td>E vs Vaccinated</td>
<td>.298  .013  .205 &lt;.001 .003  .526  .398  .140 .038 .202 .001 .953</td>
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<td>E+V vs Vaccinated</td>
<td>.001  .021  .018 &lt;.001 .535  .384 .003  .654 .002 .802 .574 .456</td>
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</table>

<sup>1</sup> Results are given as means (n =10).

<sup>2</sup> Mean E: results are given as overall mean of three extract groups (LenE = Lentinus edodes extract; TreE = Tremella fuciformis extract; AstE = Astragalus membranaceus extract).

<sup>3</sup> Mean E+V: results are given as overall mean of the extract plus vaccine groups (LenE+V = Lentinus edodes extract plus vaccine; TreE+V = Tremella fuciformis extract plus vaccine; AstE+V = Astragalus membranaceus extract plus vaccine).

<sup>4</sup> SEM = the standred error mean of the treatments.

<sup>5</sup> P < 0.05 are printed in bold.

<sup>abc, ABC</sup> means with different superscripts within the three extracts differed significantly (P<0.05).
The birds of the E+V groups had relatively lower weights of GIT organs compared with the birds fed with the extracts. Of the three extracts, AstE showed a significantly increased weight of proventriculus+gizzard at day 7 p.i.. The LenE +V group had significantly more weight of proventriculus+gizzard than the TreE+V group at 7 days p.i. AstE+V showed significantly increased jejunum weight than TreE+V at day 7 p.i..

DISCUSSION

BW Gain and Mortality

Usually feed additives are given to the birds during the whole period of production. In the present trial, the polysaccharide extracts were supplemented for one week (from 7 to 14 days of age). This period was considered to be the critical period for broilers with a fast growth rate and adequate development of the immune system, at a level of 1g/kg of the birds’ diet, according to a study of Hu et al. (1998).

In this study, the birds immunized with the live oocyst vaccine showed better growth compared with the non-infected birds. Some studies (Williams et al., 1999; Williams and Gobbi, 2002) showed that birds vaccinated with a live attenuated anticoccidial vaccine had significantly (P<0.001) higher growth rate than birds treated with anticoccidial drugs, particularly where drug resistance might result in failure to control disease. The efficacy of the live, attenuated vaccines against coccidiosis in chickens is well documented (Lillehoj, 2000; Yun, 2000; Vermeulen et al., 2001; Chapman et al., 2002). As reviewed by Shirley and Bednik (1997), the use of live, attenuated vaccines gives promising results with significantly increased immune response and relieved the reduction of growth performance in coccidial infected birds.

It was found from this study that BW gain was higher in birds immunized with the live oocyst vaccine than the birds fed with the extracts. However, when used as adjuvant, the extracts significantly enhanced BW gain in the coccidial infected birds. This was consistent with a study of Hu et al. (1998) (Table 7). His experiment showed that the chickens fed with TreEm, a polysaccharide derived from mycelia of Tremella fuciformis, when used as a adjuvant, significantly (P<0.001) enhanced BW gain and reduced the mortality compared with the vaccine only, and the oocyst output in coccidial infected chickens was largely reduced by feeding TreEm (Table 7).

It was found from this study that LenE and TreE, whether used as feed supplements or
vaccine adjuvant, showed a better effect on growth rate of the infected chickens than AstE. The difference in magnitude of the response between the extracts may be due to the difference in their physico-chemical properties.

Table 7. Efficacy of Tremella fuciformis mycelia polysaccharide (TreEₘ) against Eimeria tenella infection in chickens (Hu et al., 1998)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose</th>
<th>BWG¹</th>
<th>Mortality¹</th>
<th>Lesion score¹</th>
<th>Oocyst output¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Challenge</td>
<td>5 × 10⁴ oocyst b⁻¹</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Vaccine</td>
<td>75 oocyst b⁻¹</td>
<td>87</td>
<td>90</td>
<td>67</td>
<td>25</td>
</tr>
<tr>
<td>TrePₘ</td>
<td>200 × 7 mg kg⁻¹ d</td>
<td>78</td>
<td>139</td>
<td>100</td>
<td>50</td>
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<tr>
<td>TrePₘ+Vaccine</td>
<td>135</td>
<td>0</td>
<td>67</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

¹ BWG, mortality, lesion score and oocyst output were expressed relative to 100% of the challenge group.

Some mortality occurred after E. tenella infection in this experiment (on average of 3%). As reported by Long et al. (1986), E. tenella infections become severe before the immune response develops fully and mortality due to this species may reach unacceptable levels. As reported by Hu et al. (1998), the mortality of the chickens reached up to 53% at 7 days after E. tenella (5 x 10⁴) infection. In this study, the birds were infected with 9 x 10⁴ sporulated oocysts that could have resulted in high mortality after infection, but this didn’t occur. As reviewed by Suo and Li (1998), many factors such as sex, age, species and immune status of the bird could affect the sensitivity of birds to coccidiosis. The small response of the birds to the coccidial infection in this study was most probably a result from the coccidial strain used in this experiment, that was an attenuated laboratory strain, and thus has less pathogenic to the chickens compared with a pathogenic wild form. The less susceptibility of the birds to Eimeria infection may also be related to the genotype. The breed used in this experiment is a local slow-growing breed that may have a higher resistance to a coccidial infection compared with fast-growing purebred. It was demonstrated by Rodriguez et al. (1997) that of the risk factors investigated, genotype showed a significant effect on mortality, and the local crossbred had more resistance to coccidiosis than the purebred.

**Oocyst Output and Cecum Lesions**

In this experiment, the first peak oocyst production from droppings occurred at 7 days after live oocyst vaccination. Oocyst production ceased at 14 days post-vaccination. If at
this point, birds receive a severe infection, they would be refractory to the infection (Stiff and Bafundo, 1993; Long et al., 1986). It was shown in this experiment that the oocyst production reached a maximum at 7 days after coccidial infection. The extracts significantly increased the oocyst excretion in droppings during the primary response post vaccination (during the period of the extract supplementation), which might bear a risk of inducing pathology. However, the extracts had largely suppressed the oocyst excretion of the infected chickens during the extract withdrawal period. During the time that the parasite is multiplying, it is dependent on the host for supplying essential nutrients, gaining them either directly from the host cell or from the surrounding environment (Long, 1982). Non-digestible carbohydrates (oligo-and polysaccharides), due to their chemical structure, are not hydrolyzed by digestive enzymes and absorbed in the upper part of the gastrointestinal tract. Such ingredients therefore enter the large intestine and may serve as substrates for endogenous colonic bacteria, thus indirectly providing the host with energy, metabolic substrates and essential micronutrients (Salyers, 1979; Eastwood, 1992; Gibson and Roberfroid, 1995). Thus, these polysaccharide extracts might indirectly serve to provide small amounts of nutrients for the parasite multiplying. Matsuzawa (1979) found that feeding carbohydrates such as lactose, sucrose, starch and D-fructose to chicken increased excretion of oocysts, and glucose, maltose and branch starch greatly increased excretion of oocysts.

The extracts might have increased viscosity which decreased digesta passage rate, and they may also have increased moisture of digesta or litter, and, therefore, increased oocyst production. Our study carried out earlier (Guo et al., unpublished) showed that cecal viscosity was significantly increased by the extracts. Several studies (Hesselman and Aman, 1986; Hughes et al., 2000; Iji et al., 2001) demonstrated that inclusion of non-starch polysaccharides in broiler’s diets raised intestinal viscosity and excreta moisture. An increase in digesta viscosity levels would have the opposite effect on a coccidial infection (Banfield et al., 1999, 2002).

**Immune Organs**

In the present study, the weights of immune organs (thymus and bursa) of *Eimeria* infected chickens were significantly reduced p.i. The immune status of the host is known to play an important role in the resistance to various infections. The protective immunity to infection by *Eimeria* parasites has been demonstrated to be dependent on T-cell mediated
immune responses (Ovington et al. 1995). A study by Breed et al. (1997) showed that the proportion of CD8+-expressing T-cells in the peripheral blood of chickens increased after a primary infection with *E. tenella*, and the increase in the CD8+ population coincided with an increased lymphocyte proliferation upon stimulation with *E. tenella* sporozoite antigen *in vitro*. Antibodies produced systematically in serum, and locally in the intestine may play a role in resistance against coccidial infections (Wallach et al., 1994). A significant production of specific immunoglobulins was measured in intestinal mucosa of chickens with *E. tenella* infection (Girard et al., 1997).

In this experiment, both the extracts and the live oocyst vaccine stimulated growth of thymus and bursa, but both inhibited increase of liver weights of the infected birds, and the birds that received the vaccine had better immune response than the birds fed with the extracts. Live oocyst vaccines for the control of coccidiosis rely upon the development of acquired immunity, adaptive immunity or specific immunity to *Eimeria* parasites (Suo and Li, 1998; Lillehoj and Lillehoj, 2000). As these parasites progress through the different stages of their life cycle in the bird’s intestine, they serve as the mechanism to stimulate the cell-mediated immune response of the chicken (Lillehoj et al., 1989; Martin et al., 1993, 1994; Lillehoj, 1998; Li et al., 2002; Miyamoto et al., 2002).

Majority of the polysaccharides that were derived from medicinal plants and fungus have a function of immune system stimulation, as reviewed by Xue and Meng (1996). Generally, these polysaccharides affect both cellular and humoral immunity (Lien and Gao, 1990). It was well documented that the mushroom and herb polysaccharides stimulated growth of immune organs such as thymus, spleen and bursa, and they had antagonistic action to reduction of the immune organs caused by using immune inhibitors such as cyclophosphamid in chickens (Zhang et al., 1998) and rats (Mao et al., 1988; Liang, 1994; Xia and Cheng, 1988).

**Growth of GIT Organs**

The coccidial infection is initiated by the ingestion of sporulated oocysts. These pass into the proventriculus and gizzard, where the pH and carbon dioxide levels prime the oocyst for excystation. Once in the small intestine trypsin acts on the oocyst cell wall, dissolving a plug through which the sporozoites escape. Host epithelial cells are then rapidly invaded. The parasites go through several stages of multiplication, destroying the host cell, before maturing into male and female gametocytes. The male fertilizes the female
gametocyte to form an oocyst, which grows in size, and finally moves into the lumen of the host intestine before being passed out in the faces (Long, 1982). Therefore, the birds that have succumbed to coccidiosis have distinct pathology in one or more regions of the intestine. Coccidial species are both highly host specific and infect a specific region of the intestine (Suo and Li, 1998). *E. tenella* infects the ceca of the chicken.

As described by Suo and Li (1998), the slightly *Eimeria* infected birds had no sign of thickening of the cecal walls with normal cecal contents, whereas the severe infected birds had large amounts of blood or cecal lesion cores present in the cecal lumen, cecal walls greatly thickened, little, if any, fecal contents in ceca. In more chronic cases, cecal walls were greatly distended with large caseous cecal cores. It was shown in this experiment that relative to the infected birds, the birds which received the live oocyst vaccine showed significantly lower cecum weights at 7 days p.i.. It would be appeared that both the extracts and vaccine lowered cecum weights of the infected birds at day 14 p.i.

For other part of the GIT organs, it would be expected a lower GIT weight in the *Eimeria* infected birds, resulting from a reduced ability to digest and absorb nutrients by infected chickens. It was demonstrated in this experiment that the infected birds which were also ingested a lower amount of live oocysts showed lower weights of the GIT organs in chickens compared with the non-infected birds as well as the birds which received the extracts only. It can be concluded that the parasites not only caused lesions in the specific region of GIT, in this case cecum, but also adversely affected the development of the unparasitic GIT organs.

In conclusion, growth performance of *E. tenella* infected chickens was largely improved by feeding the extracts or giving live oocyst vaccine. The birds immunized with vaccine had better immune responses than the birds fed with the extracts. However, the extracts, when used in conjunction with anti-coccidial vaccine, have shown preliminary promise against experimental infections. Of the three extracts, *LenE* and *TreE* showed a better growth promoting effect in the coccidial infected chickens. In this experiment, the parasite challenge to the birds was an attenuated laboratory strain that resulted in lower mortality and less reduction in growth performance of the chickens. In addition, the inclusion level of the extracts in the present experiment may not be optimal for enhanced growth and health in the coccidial infected chickens. Further experiments should be conducted using a pathogenic wild parasite by inclusion of more polysaccharide supplemental levels to investigate the effect of the extracts on immune responses of the chickens.
ACKNOWLEDGEMENTS

This research was partially supported by International Foundation For Science (No. B/3175-1). The help of Zhao Hui, Laboratory of Parasitology and Parasitological Diseases, China Agricultural University with providing the experimental facilities, reagents and with guiding and helping the practical work for this experiment is highly appreciated.

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1209-1229.
Chapter 8

EFFECTS OF MUSHROOM AND HERB POLYSACCHARIDES ON CYTOKINE PRODUCTION IN CHICKENS INFECTED WITH EIMERIA TENELLA

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Effects of Mushroom and Herb Polysaccharides on Cytokine Production in Chickens Infected with Eimeria Tenella


Abstract

The effects of polysaccharide extracts (E) from two mushrooms, *Lentinus edodes* (*LenE*) and *Tremella fuciformis* (*TreE*), and an herb, *Astragalus membranaceus* (*AstE*), on production of the cytokines IFN-γ and IL-2 were investigated in *Eimeria tenella* infected chickens. A total of 180 broiler chicks were assigned to nine groups: three groups fed with extracts (*LenE*, *TreE* and *AstE*), three groups fed with extracts and immunized with live oocyst vaccine (*LenE+V, TreE+V and AstE+V*), a group immunized with the vaccine, and two controls (yes or no *E. tenella* infected groups). Serum IFN-γ titers and IL-2 production of splenocytes were determined at 7 and 14 days post-infection (p.i.) The overall mean serum IFN-γ of the groups fed with the extracts and immunized with vaccine was significantly (P<0.01) higher compared with the vaccine group at 7 and 14 days p.i., whereas that of the groups fed with the extracts only was not different from the vaccine group. Of the three extracts, *AstE* showed the highest IFN-γ titers at 7 days p.i., and the *TreE+V* group had significantly higher IFN-γ titers compared with both *LenE +V* and *AstE+V* groups. The IL-2 production of splenocytes showed a similar pattern to the serum IFN-γ titers. Seven days p.i., the overall mean IL-2 level of the groups fed with extracts plus vaccine was significantly (P<0.01) higher compared with the group immunized with the vaccine only, but that of the groups fed the extracts were not significantly different from the vaccine group. At 14 days p.i., the overall mean IL-2 production of both the groups fed with the extracts and the groups fed with the extracts plus vaccine was not significantly different from the vaccine group. The use of the mushroom and herb polysaccharide extracts might enhance T-cell immune responses, characterized by IFN-γ and IL-2 secretion, against coccidiosis in chickens, and particularly when they are used in conjunction with vaccine. Supplementation of the mushroom and herb extracts may enhance protective immunity against infections.

Keywords: mushroom, herb, polysaccharides, *E. tenella*, cytokines
INTRODUCTION

The immune status of the host is known to play an important role in its ability of resistance to infections. The cell-mediated immune response and cytokines are thought to play a major role in resistance against various pathogens in poultry (Lillichøj, 1998). Most cytokines are glycoproteins that are secreted by cells of the immune system, and almost all cytokines are pleiotropic effectors showing multiple biological activities. Growing evidence indicates that cytokines are important molecules that control the host immune response to *Eimeria* infections in poultry (Ovington *et al.*, 1995; Lillichøj and Choi, 1998; Min *et al.*, 2001). Five major cytokines have been described in chickens, interferon (IFN-γ) and interleukin (IL-2 and IL-15), transforming growth factor (TGF), and tumor necrosis factor (TNF), of which IFN-γ and IL-2 are assumed to play a critical role in immune protection in chickens (Lillichøj, 1998; Lowenthal *et al.*, 1997, 2000; Min *et al.*, 2001; Miyamoto *et al.*, 2002). IFN-γ is known as a pro-inflammatory cytokine, whereas IL-2 plays a role in anti-inflammatory reactions (Thakur *et al.*, 2002). IFN-γ is produced mainly by T-cells and natural killer cells activated by antigens or mitogens. IFN-γ is involved in regulation, proliferation and differentiation of hematopoietic cells, and it enhances nonspecific immunity to tumors, bacteria, viruses, and parasites (Sellier *et al.*, 1995; Lange *et al.*, 1996; Murray *et al.*, 1998). IL-2, produced and secreted by activated T-cells and natural killer cells, is a major interleukin responsible for T-cell proliferation, and it also exerts effects on antibody production of B-cells, as well as proliferation and activity of macrophages and natural killer cells (Li *et al.*, 2002; Miyamoto *et al.*, 2002).

Certain polysaccharides from medicinal fungi and plants affect both cellular and humoral immunity (Lien and Gao, 1990). They have been used as immune enhancers and shown anti-bacterial, anti-viral or anti-parasitic activities in chickens (Xue and Meng, 1996). Both *in vitro* and *in vivo* studies in mammals provide evidence that certain polysaccharides derived from mushrooms and herbs show cytokine-stimulating activities and could mediate protective immunity against infections and cancers (Xia 1986; Jing, 1989; Zhao *et al.*, 1990; Wei, 1991; Zhao, 1993; Hu *et al.*, 1995a, 1995b; Liang *et al.*, 1995; Liu *et al.*, 1998; Gao *et al.*, 1997). Relatively few publications exist describing work in chickens since only limited information exists concerning the role of various lymphocyte subpopulations and cytokine production in avian infections. It is only recently that some avian cytokines have been cloned and sequenced (Song *et al.*, 1997; Kaiser *et al.*, 1998; Lowenthal *et al.*, 2000).
Chicken coccidiosis, caused by intestinal protozoan parasites of the genus *Eimeria*, is a significant problem for the poultry production throughout the world. The disease can result in reduced productivity, depressed immune responses and/or major losses of livestock (Suo and Li, 1998; Banfield et al., 1999; Lillehoj and Lillehoj, 2000). Cell-mediated immunity has been investigated during coccidiosis in chickens (Lillehoj et al., 1989; Martin et al., 1993, 1994; Lillehoj, 1998). Cytokines, particularly IFN-γ and IL-2, were involved in the induction of protective immunity against coccidial infection (Breed et al., 1997; Yun et al, 2000a, 2000b; Li et al., 2002; Miyamoto et al., 2002), and have been used as indicators of cell-mediated immunity in infected hosts (Martin et al., 1993, 1994; Ottenhoff and Mutis, 1995). It was hypothesized that polysaccharides derived from medicinal mushrooms and herbs might enhance cell-mediated immune responses against invasion by infectious agents such as *coccidia* in chickens. The long-term goal of the study was to investigate the possible immuno-modulating characteristics of mushroom and herb polysaccharides as alternatives for antibiotic growth promoters in chickens. The specific aim of the present study was to test the impacts of polysaccharide extracts (E) from two mushrooms, *Lentinus edodes* (*LenE*) and *Tremella fuciformis* (*TreE*), and an herb, *Astragalus membranaceus* (*AstE*), on production of the cytokines IFN-γ and IL-2 in *E. tenella* infected chickens.

**MATERIALS AND METHODS**

**Birds and Experimental Design**

A total of 180, 3-days-old Sanhuang broiler chickens (a local slow-growing breed from the Institute of Animal Science, CAAS, Beijing) of both sexes were used. Animals, not infected with coccidia, were randomly assigned to 9 groups with 20 birds each: Groups 1 to 3 were three extract supplemented groups (*LenE, TreE* and *AstE*); Groups 4 to 6 were three groups fed with extracts, and immunized with live oocyst vaccine (*LenE+V, TreE+V* and *AstE+V*); Group 7 was the immunized with oocyst vaccine only; Groups 8 and 9 were the *E. tenella* infected and non-infected groups, respectively.

**Diets and Management**

The composition of the diet is presented in Table 1. The maize and soybean based diet, without coccidiostat additives, was provided *ad libitum* to the birds. The diet was heated to
80 °C for 60 min before feeding. Birds were reared under coccidia-free conditions in horizontal battery pens using wood sawdust as litter, and 20 birds in each pen with a density of 10 birds /m². The formal experiment was conducted from 4 to 31 days of age (a total of 28 days). The temperature was set at 30 °C to 33 °C during the first week and gradually declined by 2 °C per week until 20 °C was reached. Relative humidity was about 60-80%. The lighting program was 24h light throughout the experiment.

Table 1. Composition of the experimental diet

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Basal diet (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>555</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>326.6</td>
</tr>
<tr>
<td>Fish meal</td>
<td>40</td>
</tr>
<tr>
<td>Soya oil</td>
<td>40</td>
</tr>
<tr>
<td>Monocalciumphosphate</td>
<td>13</td>
</tr>
<tr>
<td>Limestone</td>
<td>11.3</td>
</tr>
<tr>
<td>Salt (NaCl)</td>
<td>3</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>1</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.12</td>
</tr>
<tr>
<td>Vitamin and mineral mix</td>
<td>10</td>
</tr>
</tbody>
</table>

Calculated analysis (g/kg)

<table>
<thead>
<tr>
<th></th>
<th>210</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td></td>
</tr>
<tr>
<td>Crude fat</td>
<td>62.4</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>30.9</td>
</tr>
<tr>
<td>Ash</td>
<td>55.8</td>
</tr>
<tr>
<td>Metabolisable energy (kcal/kg)</td>
<td>3,000</td>
</tr>
<tr>
<td>Available phosphorus (P)</td>
<td>4.5</td>
</tr>
<tr>
<td>Calcium</td>
<td>9.0</td>
</tr>
<tr>
<td>Sodium (Na)</td>
<td>1.5</td>
</tr>
<tr>
<td>Potassium (K)</td>
<td>8.2</td>
</tr>
<tr>
<td>Chlorine (Cl)</td>
<td>2.7</td>
</tr>
<tr>
<td>Lysine</td>
<td>11.7</td>
</tr>
<tr>
<td>Methionine</td>
<td>6.0</td>
</tr>
</tbody>
</table>

1 Supplied for per kg of diet: vitamin A (retinal acetate) 9000 IU, cholecalciferol 1800 IU, vitamin E (DL-α-tocopherol acetate) 20 mg, vitamin K₃ 3.4 mg, thiamin 1.8 mg, riboflavin 5.2 mg, D-pantothenic acid 9.8 mg, niacinamide 36 mg, folic acid 0.90 mg, pyridoxine 3.60 mg, cyanocobalamin18 μg, biotin 80 μg, choline chloride 200 mg, Fe 80 mg, Zn 75 mg, Mn 100 mg, Se 150 μg, I 300 μg, carrier maize.
Polysaccharide Preparation and Supplementation

Intact mushroom and herb materials were purchased from a local source (Lentinus edodes and Tremella fuciformis produced in Zhejiang, China; Astragalus membranaceus produced in Gansu, China). The intact mushroom and herb materials were dried overnight at 45 °C and ground through a 1mm sieve for water-soluble polysaccharide extraction, according to Liu et al. (1999). The yields of the polysaccharides and their chemical analyses are presented in Table 2.

Table 2. Yields and chemical analyses of the mushroom and herb polysaccharide extracts

<table>
<thead>
<tr>
<th>Composition</th>
<th>LenS</th>
<th>TreS</th>
<th>AstS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter (DM) (g/kg)</td>
<td>957</td>
<td>958</td>
<td>967</td>
</tr>
<tr>
<td>Crude protein (g/kg DM)</td>
<td>208</td>
<td>103</td>
<td>157</td>
</tr>
<tr>
<td>Yields of polysaccharides (%)</td>
<td>8.0</td>
<td>10.0</td>
<td>31.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>LenE</th>
<th>TreE</th>
<th>AstE</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM (g/kg)</td>
<td>952</td>
<td>955</td>
<td>940</td>
</tr>
<tr>
<td>Crude protein (g/kg DM)</td>
<td>114</td>
<td>47</td>
<td>67</td>
</tr>
<tr>
<td>Total sugar content (g/kg DM)</td>
<td>632</td>
<td>687</td>
<td>647</td>
</tr>
</tbody>
</table>

1 Intact mushroom (LenS = Lentinus edodes; TreS = Tremella fuciformis) and herb (AstS = Astragalus membranaceus) materials and their extracts (LenE = Lentinus edodes polysaccharide extract; TreE = Tremella fuciformis polysaccharide extract; AstE = Astragalus membranaceus polysaccharide extract).

2 The dry matter content of the extracts was determined by freeze-drying. Protein content was determined by the semi-automated Kjeldahl method (ISO, 1997). Total sugar content of the extracts was determined the phenol-sulphuric method (Dubois et al., 1956). The yield of polysaccharides was based on percentage of the air-dry matter.

LenE, TreE and AstE were mixed with the animal diet separately according to the experimental design and supplemented for one week (from 8 to 14 days of age). The supplemental level was 1 g/kg of the diet, which was considered to be the optimum level for enhanced health in coccidial infected chickens, according to Hu et al.(1998).

Live Oocyst Vaccination

An attenuated live vaccine (a lower dose of live oocysts, Laboratory of Parasitology and Parasitological Diseases, China Agricultural University) consisted of a mixture of oocysts of E. tenella and diluent (2% K2Cr2O7 solution). At 4 days of age, appropriate
proportions were prepared so that each chicken received the intended dose of the oocyst mixture (200 live oocysts, 0.2ml per animal), directly into the crop, using an oral gavage.

**Parasite Preparation and Infection**

The parasite used in this experiment was a laboratory *E. tenella* strain maintained by periodic passage through young chickens at the facilities of the Laboratory of Parasitology and Parasitological Diseases, China Agricultural University.

At 18 days of age, all experimental birds except those of the non-infected control group were infected with $9 \times 10^4$ sporulated oocysts prepared above in 0.3 ml of water, directly into the crop, using an oral gavage. After infection, clinic syndromes and blood in faeces were observed, and oocysts from droppings were counted every day using a McMaster’s chamber (Hodgson, 1970).

**Cytokine Quantification**

Serum IFN-$\gamma$ titer determination

Blood samples were obtained from 6 chickens of each group at 7 and 14 days post-infection (p.i.) by cardiac puncture and allowed to clot for 4h, and then centrifuged (3000g, for 10min). The sera were aliquoted in 1ml vials and kept at -20 oC for measurement of IFN-$\gamma$ titers.

IFN-$\gamma$ was measured by the cell prohibition efficiency bioassay (Yang and Wang, 1998). In brief, IBRS cells (Lab of Vet. Clinic, CAAS) were cultured in 96-well round-bottom microtitre plates, $4 \times 10^4$ cells/50 $\mu$l per well in RPMI 1640 (GIBCO) at 37 oC in a atmosphere of 5% CO2 for 12 hrs. Serum samples at series of dilutions (from 4-1 to 4-8) with 4 replicates, were added to the appropriate wells at 37 oC for 24 hrs. Cells were then challenged with a 50 $\mu$l (1 $\times$ 102) vesicular stomatitis virus (VSV, Lab of Vet. Clinic, CAAS) suspension with a tissue culture infectious dose 50 (TCID 50) and cultured at 37 oC for 36 hrs. Cell prohibition efficiency (VSV control > 75%, IBRS cell control = 0) was recorded microscopically. The IFN-$\gamma$ titer was calculated as the reciprocal of the highest serum dilution that protects 50% of the cells, which was corrected as the international unit (IU/ml) according to the standard IFN-$\gamma$ titers (Guo and Lei, 1999).
IL-2 production of splenocytes

Spleen samples were obtained from the same chickens as we took the blood samples at 7, 14 days p.i. and snap-frozen in liquid nitrogen and stored at –20 oC pending analysis for IL-2 production. The spleens were gently crushed with a syringe plunger through a 40 μm pore size steel screen. The splenocytes were suspended in Hank’s solution (pH 7.4, Lab of Vet. Clinic, CAAS) and harvested after centrifugation (500g, 20 min at 4 oC). After washing twice with Hank’s, the splenocytes were then resuspended in RPMI-1640 (GIBCO). Spleen cells (5 × 106/ml) 200 μl were cultured in 96-well round-bottom microtitre plates with added PHA (2 μ g/ml, product of Institute Guangzhou Medical Industry, China), at 37 oC in a atmosphere of 5% CO2 for 48 hrs, which was then centrifuged at 500g for 30min. The supernatant was collected for measuring IL-2 activity.

A cell proliferation assay was performed using tritiated thymidine (3H-TdR) incorporation (Yang and Wang, 1998). Samples at series of dilutions ( from 4-1 to 4-8) of 100 μl per well were added to 96-well round-bottom microtitre plates using recombinant mouse IL-2 (Lab of Immunology, Gansu Cancer Institute, China) as the standard with three replicates. To each well were then added IL-2 dependent murine T cells (CTLL-2) (1 × 108 /L, mouse, C57BL/6, Lab of Vet. Clinic, CAAS) 100 μl/well, and incubated at 37 oC, 5% CO2 for 24 hrs. 3H-TdR 20 μl (1.85 × 104 Bq, Institute of Guangzhou Medical Industry, China) was added to each well for the final 6 hrs. Cells were harvested onto GF/A glass fiber mats (Whatman International Ltd., UK) using an automated cell harvester (DYQ-III, Potang Medical Equipment Plant, Shaoxin, China) and the radioactivity was determined in a direct beta counter (Kontron Betamatic V).

Statistical Analysis

All the data were subjected to statistical analysis using SPSS 8.0 (Statistical Package for the Social Science, 1997). One way ANOVA was used to examine: 1) the contrast between the infection and non-infected groups; 2) the contrast between the vaccine and infected groups; 3) the contrasts between the overall mean of the extract groups and the vaccine group; 4) the contrasts between the overall mean of the extract plus vaccine groups (E+V) and the vaccine group. Tukey multiple range test was used to test the significance between the different extracts (LenE, TreE and AstE) and the significance between the different extract plus vaccine groups (LenE+V, TreE+V and AstE+V).
RESULTS

Serum IFN-\(\gamma\) Titers

Serum IFN-\(\gamma\) titers of the oocyst-infected birds were significantly (P<0.001) higher than those of uninfected birds at both 7 and 14 days p.i. (Table 3). The IFN-\(\gamma\) titers of the vaccine group was significantly (P<0.01) higher than the infection group at both 7 and 14 days p.i.. The overall mean of the IFN-\(\gamma\) titers of the groups fed with the extracts was not significantly (P>0.05) different from the vaccine group at 7 and 14 days p.i., whereas that of the groups fed with the extracts and immunized with vaccine was significantly (P<0.05) higher than the vaccine group. The overall mean of IFN-\(\gamma\) titers of the groups fed with the extracts was significantly lower than that of the groups fed with the extracts and immunized with vaccine at both 7 and 14 days p.i.

Figure 1 shows the results of serum IFN-\(\gamma\) titers of the different extract treatments at 7 and 14 days after *E. tenella* infection. Of the three extracts, the *AstE* group showed the highest serum IFN-\(\gamma\) titers at 7 p.i., however, there was no significant difference between the *LenE* and *TreE* groups. Of the groups fed with extracts and immunized with the vaccine, the *TreE+V* group showed significantly higher IFN-\(\gamma\) titers compared with both the *AstE+V* and *LenE+V* groups. Twelve days p.i., there was no significant difference in both the extract groups and groups fed with the extract and immunized with the vaccine.

IL-2 Production of Splenocytes

Table 3 shows the results of IL-2 production of splenocytes. The IL-2 production level of the infection group was significantly higher than the non-infected group at 7 and 14 days p.i., but there was no significant difference between the vaccine and infection groups. Seven days p.i., the overall mean IL-2 production of the groups fed with the extracts was not significantly different from the vaccine groups, whereas that of the groups fed with extracts and immunized with vaccine was significantly (P<0.01) higher than the vaccine group. Twelve days p.i., the overall mean IL-2 production of both the extract groups and the groups fed with extracts and immunized with vaccine was not significantly different from the vaccine group. The overall mean IL-2 production of the groups fed with the extracts was significantly lower than that of the groups fed with the extracts and immunized with vaccine at 7 days p.i., but it was not significantly different at 14 days p.i.
Figure 2 shows the results of IL-2 production of splenocytes induced by PHA in different extract treatments at 7 and 14 days p.i. A significant difference was not found between the different extract supplemented groups and the groups fed with extracts and immunized with vaccine at 7 and 14 days p.i.

Table 3. Effect of mushroom and herb polysaccharide extracts on IFN-γ and IL-2 production at 7 and 14 days after Eimeria tenella infection in chickens

<table>
<thead>
<tr>
<th>Treatments1</th>
<th>IFN-γ (IU/ml)</th>
<th>IL-2 (×10^3 cpm)2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 7</td>
<td>Day 14</td>
</tr>
<tr>
<td>Non-infection</td>
<td>158.7±7.5</td>
<td>188.9±7.9</td>
</tr>
<tr>
<td>Infection</td>
<td>274.5±8.3</td>
<td>360.5±4.6</td>
</tr>
<tr>
<td>Vaccine</td>
<td>329.7±7.0</td>
<td>443.4±13.8</td>
</tr>
<tr>
<td>Extracts</td>
<td>312.8±5.6</td>
<td>478.7±3.3</td>
</tr>
<tr>
<td>Extracts+Vaccine</td>
<td>356.2±5.6</td>
<td>488.0±3.4</td>
</tr>
</tbody>
</table>

Contrast statements | Probability levels of the contrasts3
---|---|
Infection vs. Non-infection | .000 | .000 | .007 | .043 |
Vaccine vs. Infection | .001 | .01 | .354 | .179 |
Extracts vs. Vaccine | .083 | .051 | .306 | .843 |
Extracts+Vaccine vs. Vaccine | .012 | .022 | .009 | .688 |

1 Results are given as means ±SE for the non-infection, infection and vaccine groups; Extracts = overall means ± SE (n = 18) of the extract supplemental groups (Lentinus edodes extract, Tremella fuciformis extract, and Astragalus membranaceus extract); Extracts+Vaccine = overall means ± SE (n=18) of the groups fed with the extracts (Lentinus edodes extract, Tremella fuciformis extract, or Astragalus membranaceus extract) and immunized with the live oocyst vaccine.

2 cpm = counts per min.

3 P < 0.05 are printed in bold.
Figure 1. The effect of mushroom and herb polysaccharides on serum IFN-γ titers of chickens at 7 (gray-colored columns) and 14 days (dark-colored columns) after *Eimeria tenella* infection. Results are given as means ± SE (n=6) for LenE = *Lentinus edodes* extract, TreE = *Tremella fuciformis* extract, and AstE = *Astragalus membranaceus* extract; LenE+V = *Lentinus edodes* extract plus vaccine, TreE+V = *Tremella fuciformis* extract plus vaccine, and AstE+V = *Astragalus membranaceus* extract plus vaccine; ab Mean between two treatments with different superscripts differ significantly (P<0.05).

Figure 2. Effect of mushroom and herb polysaccharides on IL-2 production of splenocytes induced by phytaehematoagglutinin at 7(gray-colored columns) and 14 days (dark-colored columns) after *Eimeria tenella* infection in chickens. Results are given as means ± SE (n = 6) for LenE = *Lentinus edodes* extract, TreE = *Tremella fuciformis* extract, and AstE = *Astragalus membranaceus* extract; LenE+V = *Lentinus edodes* extract plus vaccine, TreE+V = *Tremella fuciformis* extract plus vaccine, and AstE+V = *Astragalus membranaceus* extract plus vaccine; abc Means between two treatments with different superscripts differ significantly (P<0.05).
DISCUSSION

T-cell mediated immune responses are thought to play an essential role in the protection against avian coccidiosis. A study by Breed et al. (1997) showed that the proportion of CD8+-expressing T-cells in the peripheral blood of chickens increased after a primary infection with E. tenella, and the increase in the CD8+ population coincided with an increased lymphocyte proliferation upon stimulation with E. tenella sporozoite antigen in vitro. The protective immunity to infection by Eimeria parasites has been demonstrated to be dependent on T-cell mediated immune responses, and the effects of T cells may be associated with the release of cytokines (Ovington et al. 1995). Breed et al. (1997) investigated the functional activity of these peripheral blood leucocytes by determining both the potential to proliferate and to produce IFN-γ upon stimulation with E. tenella sporozoite antigens. A parasite specific Th-cell subset with the capacity of producing IFN-γ was detected after a primary E. tenella infection, which could be involved in the induction of protective immunity against E. tenella.

Cytokines produced by cells of the immune system are prominent regulators of the immune response. Leukocytic cytokines are released during immune responses and may act in autocrine, paracrine, or endocrine manners (Klasing, 1994). The effect of an inflammatory response is determined by the balance between pro-inflammatory cytokines which are responsible for early responses and anti-inflammatory cytokines (Thakur et al., 2002). IFN-γ is known as a pro-inflammatory cytokine because it accelerates inflammation, and also regulates inflammatory reactions either directly or by their ability to induce the synthesis of cellular adhesion molecules or other cytokines in certain cell types, while IL-2 polymers play a role in anti-inflammatory reactions, stimulate the synthesis of IFN-γ in peripheral leukocytes, and induce the secretion of IL1, TNF-alpha and TNF-beta (Katsikis et al., 2001). However, a given cytokine may behave as a pro- as well as an anti-inflammatory cytokine (Cavaillon, 2001).

IFN-γ was demonstrated to play an essential role in limiting primary parasite multiplication during coccidiosis (Rose et al., 1991; Yun et al., 2000a, 2000b). IFN-γ as an indicator of cellular immunity was used to measure T-cell responses to coccidial antigens in chickens (Martin et al., 1993, 1994). Furthermore it was found that recombination chicken IFN-r was able to protect chicken fibroblasts from virus-mediated lysis (Song et al., 1997; Lowenthal et al., 1998), to induce nitrite secretion from macrophages in vitro (Digby and Lowenthal, 1995), and to enhance major histocompatibility complex class II
antigen expression on macrophages (Lowenthal et al., 1997). IL-2 is a major cytokine of cell-mediated immunity, and IL-2 production after reinfection with Eimeria may be an important factor contributing to the genetic differences in coccidiosis of chickens (Li et al., 2002). A more recent study by Miyamoto et al. (2002) showed that IL-2 plays an important role during the course of primary and secondary E. tenella infections in vivo and in vitro, suggesting that IL-2 mediates cellular immunity during the parasite infections. It is thus becoming common practice to analyze the cytokines produced by helper T cells during coccidial infections to monitor the efficacy of the different treatments.

Serum IFN-\(\gamma\) Titers

In the present study, serum IFN-titers were significantly higher in groups fed with the polysaccharide extracts and immunized with the oocyst vaccine than in the vaccine group. A study of Hu et al. (1996) showed that the polysaccharide extract isolated from Tremella mycelia (TreE\(m\)), which was used as an adjuvant, significantly (P<0.01) increased serum IFN titers compared with the vaccine group. Several studies in mammals indicated that the mushroom and herb polysaccharides enhanced IFN secretion by cells of the immune system. LenE inhibits S-180 sarcoma in rats by indirectly stimulating macrophage activity and inducing IFN production of immune cells (Xia, 1986). A polysaccharide–peptide complex from culture mycelia of Lentinus edodes, enhanced gene expression and production of TNF-alpha of human peripheral mononuclear cells in vitro (Liu et al., 1998). AstE enhanced TNF-\(\alpha\) and TNF-\(\beta\) secretion of peripheral mononuclear cells in vitro in humans (Zhao, 1993). TreE\(m\) significantly increased serum IFN titers in rats (P<0.05) (Hu et al., 1995a; Liang et al., 1995). Thus, these polysaccharides might activate T-cells and natural killer cells to produce and secret IFN-\(\gamma\) against invasion of organisms in humans and animals.

It was found from the present experiment that IFN-\(\gamma\) titers of the birds fed with AstE were higher than the birds fed with LenE and TreE at 7 days p.i.. It may be caused by physico-chemical properties of different polysaccharides such as sugar composition, molecular weight and structure. It was well documented that there are large variation in composition and structure of these mushroom and herb polysaccharide fractions (Yang et al., 1999; Xia and Cheng, 1988; Pang et al., 1995). The correlation between the physico-chemical properties and immune activities of the polysaccharides is not well understood. Although water extracts are not well defined and contain other materials that
may also influence immune activity, it is known that the predominant component in the water extracts of these materials are known to be polysaccharides (more than 60%).

**IL-2 Production of Splenocytes**

The results of IL-2 production of splenocytes induced by PHA showed a similar pattern to that of the serum IFN titers. *E. tenella* infected birds had significantly higher IL-2 production than uninfected birds at 7 and 14 days p.i.. This was consistent with the study by Miyamoto et al. (2002) who observed that IL-2 levels in sera and culture supernatants of spleen lymphocytes stimulated with mitogen or *E. tenella* sporozoites were significantly increased on day 7 post-primary infections compared with uninfected group. The peak of IL-2 production coincided with the time of the maximum cecal lesions.

In the present study, the mushroom and herb polysaccharide used as adjuvant significantly increased IL-2 production of splenocytes at 7 days p.i.. Mushroom and herb polysaccharides were documented to have cytokine-stimulating activities in both humans and animals. Liu et al. (1999) reported that both AstE and LenE significantly increased IL-2 activity (P<0.05) in chickens infected with Marek’s disease. It was reported that AstE enhanced IL-2 production of lymphocytes induced by Con A in rhubarb treated rats (Jing, 1989) as well as in old rats (Wei, 1991). AstE was also demonstrated to function by inducing IL-2 production of lymphokine-activated killer (LAK) cells in both cancerous and healthy people (Zhao, 1993). Liu et al., (1998) found that a polysaccharide–peptide complex from mycelia of *Lentinus edodues* enhanced gene expression and production of IL-2 in vitro. Hu et al. (1995b) reported that oral administration of TreEm significantly (P<0.05) enhanced IL-2 production of spleen cells in both young and old rats. Thus the mushroom and herb polysaccharides may play role in enhancing Th immune response by inducing production and secretion of IL-2 by immune cells.

There was a trend of a dose effect of mushroom and herb polysaccharides according to the literature. A lower dose of LenE (1.0 mg/kg) enhanced secretion of IL-2 by immune cells, whereas a higher dose (1.5 mg/kg) suppressed it (Wu and Wang, 1999). Tang et al. (1998) reported that functional activity of the T-lymphocytes as determined by the potential to proliferate upon stimulation with Con A was enhanced by AstE, and the proliferation rate of T-lymphocytes was increased with increased AstE doses. However, Wang (1989) found that lower doses of AstE promoted cellular immune function but higher doses of AstE suppressed it. Thus, the supplemental level of the mushroom and herb
extracts in the present experiment may not be optimal for enhanced immune response in chickens.

The extent of cellular activities and a particular cytokine production of the immune cells might be influenced considerably by many factors such as the purity of the polysaccharide extracts, physico-chemical properties of the polysaccharides, species of chickens and way of administrations, which, however, was not well investigated.

At the moment, the mechanism for enhanced cytokine secretion using mushroom and herb polysaccharides in animals’ diets is not well understood. It may be related to the biological effect regulators. Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) may play role in regulating proliferation, differentiation and secretion of immune cells (Rong, 1987). The cyclic monophosphate nucleotides are found ubiquitously in mammalian cells and act as second messenger transducers to affect the intracellular actions of a variety of cytokines (Shinji et al., 2002). It was reported that plasma cAMP level was significantly increased by AstE treatment in chickens given delta-hydrocortisone (Liang, 1996). Fan et al. (1993) also reported that injection of TreEm, significantly increased plasma cAMP and cGMP levels in chickens (P<0.05). Thus, mushroom and herb polysaccharides in many diseases may act via induction of regulatory T cell subsets and cytokines, and the immunomodulating capacity of these products may related to the biological effect regulators.

It was concluded from this study that the use of polysaccharide extracts, LenE, TreE and AstE, significantly enhanced levels of the cytokine IFN-γ and IL-2 in chickens infected with E. tenella, and particularly when they used in conjunction with vaccine have shown preliminary promise in immune protection against experimental infections. The results thus demonstrate that polysaccharide extracts as immune enhancer might enhance T-cell immune responses against coccidia, characterized by IFN-γ and IL-2 secretion. In order to use the mushroom and herb extracts in controlling of coccidial infections by mediating host protective immune response to coccidia in chickens, further experiments are needed to investigate the optimum supplemental level of the polysaccharide extracts and their mode of action.

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Effects of mushroom and herb polysaccharides on cytokine production in chickens


Chapter 9

EFFECTS OF MUSHROOM AND HERB POLYSACCHARIDES ON CELLULAR AND HUMORAL IMMUNE RESPONSES OF EIMERIA TENELLA INFECTED CHICKENS

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Effects of Mushroom and Herb Polysaccharides on Cellular and Humoral Immune Responses of Eimeria Tenella Infected Chickens

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Abstract

We investigated the effects of polysaccharide extracts (E) from two mushrooms, *Lentinus edodes* (*LenE*) and *Tremella fuciformis* (*TreE*), and an herb, *Astragalus membranaceus* (*AstE*), on cellular and humoral immune responses of *Eimeria tenella* infected chickens. A total of 150 broiler chicks were assigned to 5 treatment groups: three groups were infected with *E. tenella* and fed with extracts (*LenE*, *TreE* and *AstE*), and two control groups were infected with or without *E. tenella*. The three extracts were given at the level of 1 g/kg of the diet from 8 to 14 days of age. Specific systemic and cecum mucosal antibody production, proliferation of splenocytes, and peripheral T and B lymphocytes were measured during three weeks following the *Eimeria* infection. A significant higher production of specific IgA, IgM (at day 14 and 21 p.i.) and IgG (at day 21 p.i.) were detected in the *Eimeria* infected groups fed with the extracts than in the infected group not fed with the extracts. Of the three extracts, *TreE* stimulated a slightly higher production of specific IgM, and a significantly higher IgG production at day 21 p.i.. The cecal antibody production showed a similar trend to that of serum antibodies. The overall mean levels of cecal specific IgA and IgG of the groups fed with extracts was significantly higher compared with the group not fed with extracts at 14 and 21 days p.i. Of the three extracts, *AstE*-fed group showed the highest IgG production at day 7 p.i. Both *TreE* and *LenE*-fed groups had significantly higher IgM and IgG levels compared with the *AstE* group at day 21 p.i.. The extract-fed groups also showed a significantly higher antigen-specific proliferation of splenocytes compared with the group not fed with the extracts at 14 and 21 days p.i. The overall mean of both ERFC (at day 14 and 21) and EAC (%) (at day 14) of the groups fed with the extracts was significantly higher compared with the group not fed with the extracts. It is concluded from this study that supplementation of mushroom and herb extracts resulted in enhancement of resistance to *E. tenella*, probably by enhancing both cellular and humoral immune responses against *E. tenella* in chickens.

Keywords: mushroom, herb, polysaccharides, *E. tenella*, humoral, and cellular immunity
INTRODUCTION

Natural medicinal products as feed supplements have been widely used as growth and health promoters in farm animals in China (Li, 1998; Wang et al., 2002). A current estimation of the number of immuno-active herbs ranges between 200-300, and most of them originate from plants and fungi (Li, 2000). It was found that the immuno-active components in these plants and fungi include polysaccharides, glycosides, alkaloids, volatile oils and organic acids, of which polysaccharides were considered to be most important (Xue and Meng, 1996; Li, 2000). Polysaccharides may act as immune enhancers or immunodulators and these components may display anti-microbial activity (Xia and Cheng, 1988; Xue and Meng, 1996). Generally, polysaccharides could affect both innate and adaptive immunity including cellular and humoral responses (Lien and Gao, 1990; Xue and Meng, 1996), and such poysaccharides have been used for assistant treatment of immune depression or deficiency caused by cancers, hepatitis, radiotherapy, and chemical treatments in humans (Yuan and Shi, 2000; Wargovich et al., 2001).

Some polysaccharides, like those from the herb Astragalus membranaceus Radix, and the mushrooms Lentinus edodes and Tremella fuciformis, were studied more extensively in poultry. It was reported that these mushroom and herb polysaccharides, which were used as feed supplements or vaccine adjuvant, showed anti-bacterial (Yuan et al., 1993), anti-viral (a.o., Wei et al., 1997; Qu et al., 1998; Liu et al., 1999b; Yu and Zhu, 2000), or anti-parasitic activities (Hu et al., 1996; Pang et al., 2000) in chickens. The immune status of the host is known to play an important role in the resistance to various infections. The mushroom and herb polysaccharides may enhance both cellular and humoral responses of chickens. Therefore they could play an important role in strengthening the animals’ defense system against the invasion by infectious organisms. However, the immune mechanisms affected by the mushroom and herb polysaccharides, and other parameters such as growth and health are not thoroughly investigated in chickens.

Chicken coccidiosis is caused by intestinal protozoan parasites of the genus Eimeria, which results in reduced productivity, a depressed immune system and/or major losses of livestock (Lillehoj and Lillehoj, 2000; Banfield et al., 1999; Suo and Li, 1998). Several studies showed that following a coccidial infection, both cell-mediated and humoral immunity plays a role in resistance. To Eimeria spp. T-cells induce a positive immune response that limits oocyst production in both primary and subsequent infections (Lillehoj et al., 1989; Martin et al., 1993, 1994; Lillehoj, 1998). Antibody produced systematically
in serum, and locally in the intestine may play a part in resistance against coccidial infections (Wallach et al., 1994). A significant production of specific immunoglobulins, IgA, IgM and IgG, was measured in intestinal mucosa of chickens with *E. tenella* infection (Girard et al., 1997).

The objective of the present study was to investigate the impacts of mushroom and herb polysaccharide extracts, *Lentinus edodes* (*LenE*), *Tremella fuciformis* (*TreE*), *Astragalus membranaceus* (*AstE*), on cellular and humoral immune responses of chickens infected with *E. tenella*. Specific systemic and mucosal antibody production, the number of peripheral lymphocytes, as well as proliferation of splenocytes were measured during three weeks following *Eimeria* infection.

**MATERIALS AND METHODS**

*Bird and Experimental Design*

A total of 150, 3-day-old Huangyu female broiler chicks (a local, slow-growing breed which was Purchased from Gansu Poultry Breeding Company, Lanzhou, China), not contaminated with coccidia, were randomly assigned to 5 treatments: three extract supplemented groups (*LenE*, *TreE* and *AstE*) and two control groups (yes or no *E. tenella* infection). Each treatment was comprised of three replicated cages, with 10 birds each.

*Animal Diets and Husbandry*

Maize and soybean based starter (0 - 7 days of age), and grower (7- 34 days of age) diets (Table 1), without coccidiostat additives, were provided ad libitum to the birds. The diet was heated to 80 °C 1 h before feeding. Birds were reared under coccidia-free conditions in horizontal battery isolators using wood sawdust as litter. Ten birds were housed in each isolator with a density of 10 birds /m². The formal experiment was conducted from 8 to 34 days of age. The temperature was set at 30 °C to 33 °C during the first week and gradually declined by 2 °C per week until 20 °C was reached. Relative humidity was about 60-80%. The lighting program was 24h light.
Table 1. Composition of the experimental diets (g/kg)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Starter</th>
<th>Grower</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>579</td>
<td>624</td>
</tr>
<tr>
<td>Soybean meal (50%)</td>
<td>240</td>
<td>200</td>
</tr>
<tr>
<td>Fish meal</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Rape seed meal</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Peanut meal</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Monocalciumphosphate</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Limestone (Ca 38%)</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>Salt (NaCl)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>2.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Premix(^2)</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Calculated analysis (g/kg)

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>210.0</td>
<td>200.0</td>
</tr>
<tr>
<td>Metabolisable energy (kcal/kg)</td>
<td>2,900</td>
<td>3,000</td>
</tr>
<tr>
<td>Calcium</td>
<td>10.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Available Phosphors</td>
<td>5.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Lysine</td>
<td>11.0</td>
<td>10.5</td>
</tr>
<tr>
<td>Methionine</td>
<td>5.0</td>
<td>5.1</td>
</tr>
<tr>
<td>Met + Cys.</td>
<td>9.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

\(^1\) Supplied for per kg of diet: vitamin A (retinal acetate), 10,000 IU; cholecalciferol, 2,000 IU; DL-α-tocopherol acetate, 20 mg; vitamin K\(_3\), 3.4 mg; thiamin, 1.8 mg; riboflavin, 5.2 mg; D-pantothenic acid, 12 mg; niacinamide, 38 mg; folic acid, 0.80 mg; pyridoxine, 3.60 mg; vitamin B\(_{12}\), 15 μg; d-biotin, 80 μg; choline chloride, 200 mg; Fe, 80 mg; Cu 100 mg; Zn, 150 mg; Mn, 100 mg; Se, 0.15 mg; I, 0.3 mg; carrier maize.

Mushroom and Herb Polysaccharide Preparation

Intact mushroom and herb materials were purchased from a local source (*Lentinus edodes* and *Tremella fuciformis* produced in Zhejiang China; *Astragalus membranaceus* produced in Gansu China). The intact mushroom and herb materials were dried overnight at 45 °C and ground through a 1mm sieve for water-soluble polysaccharide extraction according to Liu *et al.* (1999). The yields of the polysaccharide fractions and their chemical analyses are presented in Table 2.

*LenE, TreE* and *AstE* were mixed with the animal diet separately according to the experimental design and supplemented for one week (from 8 to 14 days of age). The
supplemental level was 1g/kg of the diet, which was considered to be the optimum level for enhanced health in chickens (Hu et al., 1996).

**Table 2.** Yields and chemical analyses of the air-dry intact mushroom and herb materials and their polysaccharide extracts

<table>
<thead>
<tr>
<th>Composition</th>
<th>LenS</th>
<th>TreS</th>
<th>AstS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter (DM) (g/kg)</td>
<td>957</td>
<td>958</td>
<td>967</td>
</tr>
<tr>
<td>Crude protein (g/kg DM)</td>
<td>208</td>
<td>103</td>
<td>157</td>
</tr>
<tr>
<td>Yields of polysaccharides (%)</td>
<td>8.0</td>
<td>10.0</td>
<td>31.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>LenE</th>
<th>TreE</th>
<th>AstE</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM (g/kg)</td>
<td>952</td>
<td>955</td>
<td>940</td>
</tr>
<tr>
<td>Crude protein (g/kg DM)</td>
<td>114</td>
<td>47</td>
<td>67</td>
</tr>
<tr>
<td>Total sugar content (g/kg DM)</td>
<td>632</td>
<td>687</td>
<td>647</td>
</tr>
</tbody>
</table>

1. Intact mushroom (LenS = Lentinus edodes; TreS = Tremella fuciformis) and herb (AstS = Astragalus membranaceus) materials and their extracts (LenE = Lentinus edodes polysaccharide extract; TreE = Tremella fuciformis polysaccharide extract; AstE = Astragalus membranaceus polysaccharide extract).

2. The dry matter content of the extracts was determined by freeze-drying. Protein content was determined by the semi-automated Kjeldahl method (ISO, 1997). Total sugar content of the extracts was determined the phenol-sulphuric method (Dubois et al., 1956). The yield of polysaccharides was based on percentage of the air-dry matter.

**Parasite Preparation and Antigen Purification**

The parasite used in these experiments was a laboratory E. tenella strain maintained by periodic passage through coccidian-free chickens at the facilities of the Parasitic Disease Department, Gansu Animal Health Service, China. Unsporulated oocysts were obtained on day 7 post-infection (p.i.) from the cecal contents, purified and allowed to sporulate using standard procedures (Suo, 1998).

The soluble sporozoite antigen was obtained from purified sporulated oocysts that were broken with glass beads, sonicated (23 kHz, 1 min for 10 times) at 0 °C in PBS (pH 7.2), and centrifuged at 10,000g for 10min at 4 °C. The protein content in the supernatant was measured with a Bicinchoninic acid protein assay (Smith et al., 1985) and stored at -20 °C for ELISA and the proliferation assay.

At 15 days of age, all the birds except those of the negative control group were infected with 6 x 10⁴ sporulated oocysts of E. tenella in 0.3 ml of water, directly into the crop, using
oral gavages. After infection, clinic syndromes and blood in faces were observed, and oocysts from droppings were counted every day using a McMaster’s chamber (Hodgson, 1970).

**Sampling**

At 7, 14, and 21 days p.i., six birds from each treatment (2 birds from each replicated cage) were randomly selected, and transferred to a germfree room. Blood samples were obtained from chickens by cardiac puncture and directly aliquoted in 2 ml sterile vials using liquid heparin sodium (1000 USP units ml$^{-1}$, Taozhuang Huabao Pharmacy Co. Ltd.) as anticoagulant to obtain the whole blood for E-rosette tests, or allowing to clot for 4h. After centrifugation (10 min, 3000 g), sera were aliquoted in 1 ml vials and kept at -20°C for serum antibody measurements. Chickens were then killed by cervical dislocation. Spleen samples were removed and stored at -20°C pending for proliferation assay. The ceca were removed and immersed in 10 ml cold Hank’s balanced salt solution (HBSS) containing 500 IU ml$^{-1}$ penicillin and 500 µg ml$^{-1}$ streptomycin (Lab of Vet. Clinic, CAAS, China). Cecal contents were removed by flushing the mucosal surfaces with the HBSS for further culture.

**Culture of the Intestinal Tissue**

The method of culture of intestinal tissue was according to Zigterman et al. (1993). In brief, cecal tissues were cut into small pieces (about 2-3 mm), washed with HBSS penicillin-streptomycin (Lab of Vet. Clinic, CAAS, China), and resuspended in 5ml RPMI 1640 Dutch modified (GIBCO) supplemented with 100 µg ml$^{-1}$ gentamycin, 40mM HEPES (pH 7.2) and 2mM L-glutamine (Lab of Vet. Clinic, CAAS, China). The suspensions were centrifuged (5min, 300g) and 800 µl aliquots of supernatant (t = 0) were taken. The suspended tissue samples were incubated at 41°C, 5% CO2, 95% air in 25cm2 tissue culture flasks for 16 h. The samples were then centrifuged and 800 µl aliquots of supernatant (t = 16) were taken. The specific IgA, IgM and IgG isotypes in aliquots (t = 0, t = 16) were determined by ELISA.
ELISA

The 96 well round-bottom microtitre plates were coated with 11.4 μ g/ml soluble E. tenella antigen in 0.05 mol/l carbonate buffer (pH 9.6)(100 μ l/well) by incubation overnight at 37 oC. Two percent bovine serum albumin (Lanzhou Bio-products Company, China) (200 μ l/well) was then added for 1h at 37 oC. The wells were washed 3 times with 300 μ l PBS-Tween 20 buffer (0.05%, pH 7.2, Lab of Vet. Clinic, CAAS, China), and sera or cultured cecal samples (100 μ l/well) diluted with the same PBS-Tween 20 serially, and incubated for one hour at 37 oC. One of the following polyclonal antibodies (100 μ l) was added: horseradish peroxidase (HRP) conjugated goat anti-chicken IgM (μ chain) (diluted 1:2000), HRP conjugated goat anti-chicken IgA (α chain) (diluted 1:2000), or alkaline phosphatase-conjugated rabbit anti-chicken IgG (Fc fragment) (diluted 1: 10,000) (Immunology Lab, Institute of Lanzhou Vet. Science, CAAS). Plates were incubated for 2 h at 37 oC. Following the washing procedure, plates were incubated for 1 h at 37 oC with corresponding substrate solution (Lab of Vet. Clinic, CAAS, China ) (100 μ l per well): 0.1 μ g/ml solution of 2-2’-azino-bis (3-ethylbenzthiazoline 6-sulfonic acid) diammonium in citrate buffer (pH 4) for IgA and IgM, or a 1 μ g/ml solution of p-nitrophenylphosphate in diethanolamine (pH 9.8) for IgG. The reaction was stopped by addition of 50 μ l 2M H2SO4 (Lab of Vet. Clinic, CAAS, China ). Bound isotypes in the intestinal supernatants and sera were quantified by the absorbance (A) at 450 nm. The cecal antibody released was calculated by subtraction of the A450 value at t = 0 h from the t = 16 h value. For each chicken, the specific caecal antibody production was expressed as the A450 mean values of 3 or 4 intestinal pieces of 1g.

Antigen-specific Lymphoproliferation Assay

The splenocytes were prepared according to Girard et al. (1999). In brief, the spleens were gently crushed with a syringe plunger to a 40 μ m pore size steel screen in 1.1 x PBS (pH 7.4, Lab of Vet. Clinic, CAAS, China) and erythrocytes and cell cluster were separated from effector cells after centrifugation (80 g for 5min at 4 oC). The supernatant was then centrifuged at 170 g for 20 min and the cell pellet was suspended in 1 ml PBS (pH 7.4). The splenocytes were counted by trypan blue exclusion for lymphocyte proliferation assay performed as described by Froebel et al., 1999 with minor modification. Splenocytes were cultured in 96 well round-bottom microtitre plates, 5x105 cells/100
μ l/well in RPMI 1640 (GIBCO) supplemented with L-glutamine (300 μ g/ml), penicillin (100 UI/ml), streptomycin (100 μ g/ml), bovine insulin (5 μ g/ml), 2-β-mercaptoethanol (5x10⁻⁵ M) and 2.5% chicken serum (Lab of Vet. Clinic, CAAS, China) with 100 μ l soluble E. tenella antigen at several concentrations (0, 25, 12.5, 6.25, or 3.12 μ g/ml with 3 replicates each) at 41 °C in a humidified atmosphere of 5% CO₂ for 96 hrs. Tritiated thymidine (50 μ l) (3H-TdR, 3.7 kBq, Institute of Ganzhou Medical Industry) was added to each well for the final 20 hrs. Frozen cells (at -20 °C) were harvested onto GF/A glass fiber mats (Whatman International Ltd., UK) using an automated cell harvester (DYQ-III, Potang Medical Equipment Plant, Shaoxin, China), and radioactivity was determined in a direct beta counter (Kontron Betamatic V.). Results were expressed as mean of counts per min (cpm).

**E-rosette Formation Tests**

The method used was that described by Brain et al., (1970) and modified by Dang et al. (1994). Blood samples were diluted with the equal volume of Hank’s (pH 7.4, Lab of Vet. Clinic, CAAS, China). The diluted blood was slowly added to the same volume of a lymphocyte separation solution (33.9% urografin and 9% Ficoll solution, 10: 24) (Lab of Vet. Clinic, CAAS, China), and the mixture was then centrifuged for 20 min at 400g. The lymphocytes were harvested and resuspended in the Hank’s solution and centrifuged again for 10 min 200g. After washed 3 times with Hank’s, the lymphocytes were diluted with Hank’s and counted (5 x 10⁶ / ml), which were used for:

1. Erythrocyte rosette forming cells (ERFC) or T cell counts. In brief, the diluted lymphocytes (100 μ l) were incubated with a suspension of 20% bovine serum in Hank’s solution (pH: 7.4, Lanzhou Bio-products, China) (100 μ l) and 2% SRBC Hank’s solution (Lab of Vet. Clinic, CAAS China) (100 μ l) at 37 °C for 15 min, and then centrifuged for 5 min at 100g. The cell pellets were harvested and stored at 4 °C for 4 hrs. The cells were differentiated by smear examination (Ranjit Chandra, 1984). ERFC, around which formation of a cluster (rosette) of cells consist of more than three sheep erythrocytes, were counted, and expressed as the mean value of 3 replicates.

2. Erythrocyte-antibody-complemental cells (EAC) or B cell counts: In brief, the diluted lymphocytes (100 μ l) were incubated with a sensitized erythrocyte-antibody-complemental suspension (100 μ l) containing 4% SRBC (Lab of Vet. Clinic, CAAS, China), 1:4000 hemolysin (Yuchengjia Co. Ltd. Shengzhen, China), and 20% mouse serum
(Lanzhou Bio-products, China). The rest procedure was same as that of ERFC described above.

**Statistical Analysis**

Data were analyzed using SPSS 8.0 software (Statistical Package for the Social Science, 1997). All data presented as percentages were transformed to their arcsine square root prior to statistical analysis. Significant differences between treatments were analyzed using orthogonal contrasts to access: 1) the effect of oocyst infection, examining the contrast between the infected group and the control group; 2) the effect of the extracts, examining the contrast between the overall mean of the groups fed with extracts and the infection group; 3) the effect of different extracts, examining the contrast between the LenE group and the TreE group, and the contract between the AstE group and overall mean of the LenE and TreE groups.

**RESULTS**

**Serum Antibodies**

The levels of serum *E. tenella* antigen-binding specific antibodies measured at 7, 14 and 21 days p.i. are shown in Table 3. The birds infected with *E. tenella* oocysts had significantly higher antibody levels than uninfected birds, except for IgA and IgM at day 21 p.i.. The overall levels of *E. tenella* antigen-binding specific antibodies of the groups fed with the extracts were not significantly different from the infection group at day 7 p.i.. At day 14 p.i., both IgA and IgM levels of the groups fed with the extracts were significantly higher compared with the infection group, but that of IgG was not significantly different. At 21 days p.i., specific IgA, IgM and IgG levels of the groups fed with the extracts were significantly higher compared with that of the infection group not fed with the extracts.

Of the three extracts, TreE stimulated a slightly high production of IgM and a significantly high production of specific IgG at day 21 p.i.
Cecal Antibodies

*E. tenella* antigen-binding specific antibodies in cecal wall measured 7, 14 and 21 days post-infection showed a similar trend to that of antibody levels in serum (Table 4). The infected birds had significant higher antibody levels compared with the uninfected birds except for specific IgA (at day 7 and 14) and IgM (at day 21), which were not significantly different. The overall level of *E. tenella* antigen-binding specific antibodies of the groups fed with the extracts was not significantly different from the group which was also infected but not fed with the extracts at 7 days p.i. The specific IgA and IgG in groups fed with the extracts were significantly higher compared with the infection group at 14 and 21 days p.i., whereas IgM was not significantly different either at day 14 or at day 21.

Of the three extracts, the *AstE* group had the significantly highest levels of IgG at day 7 p.i., and both *TreE* and *LenE* groups had significantly high levels of the specific IgM and IgG at day 21 p.i..

### Table 3. Sera specific antibody production of *Eimeria tenella* infected chickens fed with mushroom and herb extracts

<table>
<thead>
<tr>
<th>Treatments¹</th>
<th>Sera specific antibody production (A₄₅₀ nm)²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 7</td>
</tr>
<tr>
<td></td>
<td>IgA</td>
</tr>
<tr>
<td>Control</td>
<td>0.027</td>
</tr>
<tr>
<td>Infection</td>
<td>0.065</td>
</tr>
<tr>
<td><em>LenE</em> + Infected</td>
<td>0.066</td>
</tr>
<tr>
<td><em>TreE</em> + Infected</td>
<td>0.065</td>
</tr>
<tr>
<td><em>AstE</em> + Infected</td>
<td>0.068</td>
</tr>
<tr>
<td>SEM²</td>
<td>0.004</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Contrasts</th>
<th>Probability level of the contrasts³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection vs. control</td>
<td>&lt;.001  &lt;.001  &lt;.001  &lt;.001  &lt;.001  .557   .525   &lt;.001</td>
</tr>
<tr>
<td>Extracts³ vs. Infection</td>
<td>.658   .398   .202   .003   .020   .166   &lt;.001  &lt;.001  &lt;.001</td>
</tr>
<tr>
<td><em>LenE</em> vs <em>TreE</em></td>
<td>.803   .294   .766   .647   .574   .944   .214   .052   &lt;.001</td>
</tr>
<tr>
<td><em>AstE</em> vs <em>LenE &amp; TreE</em></td>
<td>.365   .417   .115   .111   .361   .081   .061   .152   &lt;.001</td>
</tr>
</tbody>
</table>

¹ The results are given as means (n = 3, 2 birds per replicate) for all treatments. *LenE* = *Lentinus edodes* extract, *TreE* = *Tremella fuciformis* extract, and *AstE* = *Astragalus membranaceus* extract.

² SEM = the standard error of the means.

³ Extracts = Means of the three extract groups (n = 9).

⁴ Serum diluted 1: 160 for IgA, 1: 40 for IgM and 1: 640 for IgG. A 450 = absorbance (A) at 450 nm.

⁵ P < 0.05 are printed in bold.
Antigen-specific Proliferation of Splenocytes

The result of the antigen-specific T-cell proliferation is presented in Table 5. The proliferation of splenocytes of the infected birds was significantly higher than that of uninfected birds at 14 or 21 days p.i.. The groups fed with the extracts had a significantly higher proliferation of splenocytes compared with the infection group at 14 and 21 days p.i. However, the proliferation of splenocytes between the different extract groups was not significantly different at 7, 14 and 21 days p.i.

Table 4. Cecal specific antibody production of Eimeria tenella infected chickens fed with mushroom and herb extracts

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day 7 IgA</th>
<th>Day 7 IgM</th>
<th>Day 7 IgG</th>
<th>Day 14 IgA</th>
<th>Day 14 IgM</th>
<th>Day 14 IgG</th>
<th>Day 21 IgA</th>
<th>Day 21 IgM</th>
<th>Day 21 IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.13</td>
<td>0.05</td>
<td>0.04</td>
<td>0.15</td>
<td>0.11</td>
<td>0.07</td>
<td>0.10</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>Infection</td>
<td>0.15</td>
<td>0.16</td>
<td>0.43</td>
<td>0.16</td>
<td>0.21</td>
<td>2.4</td>
<td>0.14</td>
<td>0.20</td>
<td>2.0</td>
</tr>
<tr>
<td>LenE + Infected</td>
<td>0.16</td>
<td>0.18</td>
<td>0.44</td>
<td>0.21</td>
<td>0.26</td>
<td>2.8</td>
<td>0.19</td>
<td>0.24</td>
<td>2.6</td>
</tr>
<tr>
<td>TreE + Infected</td>
<td>0.14</td>
<td>0.17</td>
<td>0.40</td>
<td>0.18</td>
<td>0.24</td>
<td>2.6</td>
<td>0.23</td>
<td>0.27</td>
<td>3.0</td>
</tr>
<tr>
<td>AstE + Infected</td>
<td>0.17</td>
<td>0.20</td>
<td>0.78</td>
<td>0.25</td>
<td>0.29</td>
<td>3.0</td>
<td>0.15</td>
<td>0.21</td>
<td>2.2</td>
</tr>
<tr>
<td>SEM²</td>
<td>0.005</td>
<td>0.02</td>
<td>0.06</td>
<td>0.01</td>
<td>0.01</td>
<td>0.3</td>
<td>0.01</td>
<td>0.02</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Contrasts

<table>
<thead>
<tr>
<th>Probability level of the contrasts³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection vs. control</td>
</tr>
<tr>
<td>Extracts³ vs. Infection</td>
</tr>
<tr>
<td>LenE vs TreE</td>
</tr>
<tr>
<td>AstE vs LenE &amp; TreE</td>
</tr>
</tbody>
</table>

1 The results are given as means (n = 3, 2 birds per replicate) for all treatments. LenE = Lentinus edodes extract; TreE = Tremella fuciformis extract; AstE = Astragalus membranaceus extract.

2 SEM = the standard error of the means.

3 Extracts = Means of the three extract groups (n = 9).

4 Cecum samples were diluted for specific IgA (1:2), IgM (1:1) and IgG (1:8). A 450 = absorbance (A) at 450 nm.

5 P < 0.05 are printed in bold.

ERFC and EAC

Both ERFC and EAC % of the infected birds were significantly higher compared with the uninfected birds at 7, 14, and 21 days p.i. (Table 6). The overall mean of both ERFC and EAC % of the groups fed with the extracts were not significantly different from the
infection group at 7 days p.i., but it was significantly higher at 14 days p.i. At 21 days p.i.,
the overall mean of ERFC % of the groups fed with the extracts was significantly higher
than the infection group. There was no significant difference between the different extract
groups, for either ERFC or EAC %.

Table 5. Effect of mushroom and herb polysaccharide extracts on antigen-specific
splenocyte proliferation

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Proliferation of splenocytes (cpm x10³)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 7</td>
</tr>
<tr>
<td>Control</td>
<td>2.5 ± 0.14</td>
</tr>
<tr>
<td>Infection</td>
<td>9.5 ± 2.5</td>
</tr>
<tr>
<td>LenE + Infected</td>
<td>13.5 ± 3.7</td>
</tr>
<tr>
<td>TreE + Infected</td>
<td>10.3 ± 2.8</td>
</tr>
<tr>
<td>AstE + Infected</td>
<td>13.3 ± 4.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Contrasts</th>
<th>Probability level of the contrasts²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected vs Control</td>
<td>.108</td>
</tr>
<tr>
<td>Extracts² vs. Infection</td>
<td>.430</td>
</tr>
<tr>
<td>LenE vs TreE</td>
<td>.538</td>
</tr>
<tr>
<td>AstE vs LenE &amp; TreE</td>
<td>.811</td>
</tr>
</tbody>
</table>

¹ The results are given as means ± SE (n = 3, 2 birds per replicate) for all treatments. LenE = Lentinus
edodes extract; TreE = Tremella fuciformis extract; AstE = Astragalus membranaceus extract.

² Extracts = Means of the three extract groups (n = 9).
³ cpm = counts per min.
⁴ P < 0.05 are printed in bold.

DISCUSSION

Specific Antibody Production

The present study showed that levels of both systemic and intestinal E. tenella
antigen-binding antibodies of the birds infected with E. tenella was significantly higher
than that of uninfected birds measured at 7, 14 and 21 days p.i.. This is consistent with a
study by Girard et al. (1997) who found a significant production of specific IgA, IgM and
IgG in both intestinal mucosa and sera of chickens infected with E. tenella. The production
of parasite-binding immunoglobulins was significantly higher in the parasitized than in the
unparasitized areas, and in sera of the infected birds, levels of antibodies were higher in
infected birds than in uninfected birds. Specific serum antibodies may play a part in protecting chickens against coccidiosis (Wallach et al., 1994).

In the present study, the antibody levels were, in general, increased at day 7 after *E. tenella* infection. It was largely increased by day 14, and decreased by day 21. A primary *E. tenella* infection may trigger significant specific antibody immune responses. The levels of *E. tenella* antigen-binding specific IgM and IgA in serum were relatively low. The response of IgM was of short duration, and specific IgA increased at 14 days p.i. and slightly decreased at 21 days p.i. The serum contained a large amount of specific IgG. A significant higher production of IgG was measured at both 14 and 21 days p.i. A large amount of specific immunoglobulin was measured in the parasitized area (cecum) in this study, and the cultured ceca of the infected chickens contained considerable amounts of *E. tenella* specific IgG.

### Table 6. Erythrocyte rosette forming cells of peripheral lymphocytes (ERFC and EAC %)\(^1\) in *Eimeria tenella* infected chickens supplemented with mushroom and herb extracts

<table>
<thead>
<tr>
<th>Treatments(^2)</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ERFC (%)</td>
<td>EAC (%)</td>
<td>ERFC (%)</td>
</tr>
<tr>
<td>Control</td>
<td>29.5</td>
<td>13.6</td>
<td>22.4</td>
</tr>
<tr>
<td>Infection</td>
<td>24.4</td>
<td>17.6</td>
<td>25.8</td>
</tr>
<tr>
<td>LenE + Infected</td>
<td>29.5</td>
<td>16.9</td>
<td>30.1</td>
</tr>
<tr>
<td>TreE + Infected</td>
<td>29.4</td>
<td>16.4</td>
<td>30.3</td>
</tr>
<tr>
<td>AstE + Infected</td>
<td>30.5</td>
<td>17.2</td>
<td>31.2</td>
</tr>
<tr>
<td>SEM(^3)</td>
<td>0.57</td>
<td>0.55</td>
<td>1.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Contrasts</th>
<th>Probability level of the contrasts(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection vs. control</td>
<td>.037  .021  .056  .006  .003  .041</td>
</tr>
<tr>
<td>Extracts(^4) vs. Infection</td>
<td>.941  .566  .004  .012  .025  .158</td>
</tr>
<tr>
<td>LenE vs TreE</td>
<td>.473   .760   .733   .132   .341   .397</td>
</tr>
<tr>
<td>AstE vs LenE &amp; TreE</td>
<td>.251  .651   .748   .206   .638   .689</td>
</tr>
</tbody>
</table>

\(^1\) ERFC = erythrocyte rosette forming cells; EAC = erythrocyte-antibody-complemental cells.

\(^2\) Results are given as means (n = 3, 2 birds per replicate) for all treatments. *LenE* = *Lentinus edodes* extract; *TreE* = *Tremella fuciformis* extract; *AstE* = *Astragalus membranaceus* extract.

\(^3\) SEM = the standard error of the means.

\(^4\) Extracts = overall mean of the three extract groups (n = 9).

\(^5\) P < 0.05 are printed in bold.

The majority of IgA in the intestinal lumen is synthesized by plasma cells in the lamina propria and is transported across epithelial cells although a minor amount is derived from
blood via the hepatobility IgA transport system (Yu et al., 2000). Antigen-specific IgA can neutralize parasites intracellularly as it passes through epithelial cells during the secretion process. With regard to *Eimeria*, parasite-specific IgA can bind and damage sporozoites, causing loss in the ability of extracellular differentiation and thereby preventing parasite invasion and intracellular development (Yun et al., 2000). IgG is the major Ig in serum. IgG has also been found in the intestine of the infected animals although its origin is not well understood, Girard et al. (1997) detected IgG in culture supernatants of intestinal lymphocytes from *Eimeria*-infected chickens. IgM is the 3rd most common serum Ig produced by the fetus and virgin B cells when stimulated by antigen. IgM is a good complement fixing Ig and also a good agglutinating Ig because of its pentameric structure. IgM antibodies are very efficient in leading to the lysis of microorganisms or clumping microorganisms for eventual elimination from the body. Yun et al. (2000) reported that specific IgM antibody levels increased significantly in both the intestine and serum after *Eimeria* infection.

In the present study, *E. tenella* infected birds fed with polysaccharide extracts showed significantly higher specific antibody responses at 14 and 21 days p.i. Several studies in rats and chickens showed that the mushroom and herb polysaccharides enhanced antibody production. Mao (1988) reported that *AstE* significantly alleviated the reduction of splenocytes, plaque forming cells (PFC) and hemolysin antibody production as a result of using immune inhibitors through increasing plasmacytes and antibody protein metabolism in plasmacytes in rats. Cheng et al. (1998) reported that when used as an adjuvant of the vaccine, *TreE.m*, a polysaccharide isolated from mycelia of *T. fuciformis*, significantly enhanced serum antibody titers to Marek’s disease in chickens, and significantly enhanced antibody titers can be detected at 96 days after vaccination in chickens given *TreE*. Thus, the mushroom and herb polysaccharides might trickle specific humoral response of the infected animals. In the present study, *AstE*-fed birds showed faster immune responses than both *TreE* and *LenE* in terms of inducing *E. tenella* antigen-binding specific antibodies. An in vivo experiment conducted by Zhang et al. (1998b) showed that adding polysaccharide preparations, *AstE*, *LenE* or their mixture, to a Newcastle Disease vaccine significantly increased the weight of the immune organs, and the effect was more obvious in the groups which received the mixture than in those which received one of the single polysaccharide.
**Antigen-specific T-cell Proliferation**

Antigen-specific proliferation was evident, which can be concluded from the present study that the specific anti-parasitic proliferation of splenic lymphocytes following the infection with *Emieria* was significantly higher in infected birds than uninfected birds at day 14 and 21 p.i.. This is consistent with other studies that specific proliferation of lymphocytes in response to coccidial antigens is significantly increased after coccidial infections (Martin and Lillehoj, 1993; Girard *et al.*, 1997).

The present study showed that the mushroom and herb extracts significantly enhanced the specific proliferation of splenocytes at day 14 and 21 p.i.. A study by Liu *et al.* (1999) showed that T-lymphocyte proliferation of Marek’s disease infected chickens was significantly increased by *AstE* (85%) and by *LenE* (18%), and IL-2 production of lymphocytes was also significantly enhanced (37-52%). Fang *et al.* (1993) reported that *TreE*, which was used as an adjuvant of avian cholera vaccine, significantly enhanced the transformation rate of lymphocytes of the infected chickens. Tang *et al.* (1998) reported that functional activity of the T-lymphocytes as determined by the potential to proliferate upon stimulation with Con A was enhanced by *AstE*. The proliferation rate of T-lymphocytes of the chickens was increased with increased *AstE* doses.

Many studies in rats and humans demonstrated that *AstE*, *LenE* and *TreE* have a significant enhancing effect on T-cell mediated immune response by (1) increasing spleen and thymus index and lymphocyte transformation and proliferation (Xia and Cheng, 1988; Liang, 1994); (2) correcting immunosupression as result of immune inhibitors such as cyclophosphamide or deltacortisone (Mao, 1989; Wang, 1989; Wu and Wang, 1999); (3) inducing secretion and production of IL-2, TNF and IFN-γ (Hu *et al.*, 1995; Wang *et al.*, 1997 and Liang *et al.*, 1995); and (4) increasing mRNA expression level of IL-2, IL-1α, IL-1β, TNF-α, IFN-γ of T lymphocytes (Liang, 1994; Liu *et al.*, 1999a). The mechanism of enhanced cellular immune function using herb and mushroom polysaccharides was not well understood. It was suggested that mushroom and herb polysaccharides might increase DNA synthesis of lymphocytes by regulating DNA polymerase activity (Lei and Lin, 1993).
**ERFC and EAC**

The importance of immune cells in the immune response to coccidia has been well documented (Lillehoj, 1987; Rose and Hesketh, 1982). This was also found in the present study that both ERFC and EAC (%) were significantly higher in infected birds than in uninfected birds. Both ERFC and EAC (%) of the coccidial infected birds fed with the extract tended to be higher at 14 and 21 days p.i. A few studies showed the mushroom and herb extracts increased the number and activity of lymphocytes in rats and chickens. *TreEm*, which was used as adjuvant, significantly increased number of ERFC and EAC in chickens infected with avian cholera (Fang et al., 1993). Zhong et al. (1998) reported that injection of AstE and LenE significantly enhanced bursa, spleen and thymus lymphocytes’ reaction to mitogen, producing active oxygen-derived free radicals that promote lymphocyte differentiation and proliferation in chickens. Xia and Cheng (1988) reported that injection of TreE significantly (P<0.05) increased E-rosette production of T-lymphocytes, and also had antagonistic action to leukopenia caused by cyclophosphamide intoxication in rats.

In brief, the mushroom and herb polysaccharide extracts enhanced both systemic and cecal *E. tenella* antigen-binding antibody production in *E. tenella* infected chickens. The specific anti-parasitic proliferation of splenocytes, populations of peripheral ERFC and EAC of *E. tenella* infected chickens was also enhanced by feeding these extracts. It can be concluded from this study that the coccidial infected birds fed with AstE showed a higher cellular and humoral immune response at 7 and 14 days p.i., whereas those fed with LenE or TreE showed higher immune response at 21 days p.i.. Thus, the mushroom and herb extracts may have significant impacts on the inductive immune responses against *E. tenella* infection in chickens, by enhancing both cellular and humoral immunity. The lack of relatively less significant effects of the mushroom and herb extracts on immune response in this experiment might be related to the inclusion level of the extracts in the diets. The supplemental level of the mushroom and herb extracts in the present experiment may not be optimal for enhanced immune response in chickens. Further experiments are needed to carry out to investigate efficacy of the mushroom and herb extracts against coccidiosis, in terms of mediating host protective immune responses to coccidia in chickens.
ACKNOWLEDGEMENTS

This research is supported by International Foundation For Science (IFS, No. B/3175-1). The help of Depatment of Parasitic Disease, Gansu Animal Health Service, with providing the experimental facilities and carrying out most of the practical work for this experiment, of Wang Yonglu, Institute of Lanzhou Veterinary Medicine CAAS, with determining antibodies using ELISA, and of Li Hongsheng, Luo Jingying and Li Xipu Lab of Veterinary Clinic, CAAS, with determining the Antigen-specific T-cell proliferation and ERFC and EAC for this experiment is highly appreciated.

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GENERAL DISCUSSION
General Discussion

MOTIVATION OF THIS STUDY

Poultry meat is the second most important source of animal proteins in the world. According to the statistics of FAO, the global poultry meat production in 2002 was 72.2 million tons, which was 30% of the total meat production of the world (242.6 million tons). Poultry meat production in China in 2002 was around 13.5 million tons, which was 20% of the total meat production of China. The importance of chickens is further illustrated by the fact that overall poultry meat consumption in the developed world reached 23.7 kg per capita in 2000, representing 30% of total meat consumption, and rose by 8% in the EU (2001). In order to meet needs of world poultry meat, growth promoters are increasingly been used in poultry feed industry to increase poultry production. Until now, growth promoting antibiotics have been widely used as manipulators of the gastrointestinal microbiota, resulting in improvement of growth, production and feed efficiency (a.o., Miles et al., 1984; Ensminger, 1990; Peterson et al., 1991; Rosen, 1995). Antibiotics, however, may cause a disturbed gut eco-system and result in development of a resistant population of bacteria. The possibility of developing resistant populations of bacteria and the side-effects of using antibiotics as growth promoters in farm animals has led to the recent EU-ban on the use of several antibiotics in poultry diets. The restriction on antimicrobial growth promoters will have consequences for the health and production status of animals, especially broiler chickens. It has been reported that removal of antibiotic growth promoters has led to an increase in enteric conditions leading to increased mortality, bird and animal welfare problems, and an increase in prescription of therapeutic antibiotics (Andreason, 2000). Therefore, there is an intensive search for alternatives such as probiotics, prebiotics and other feed additives.

NATURAL MEDICINAL PRODUCTS AS FEED ADDITIVES IN CHINA

Natural medicinal products, such as herbs, botanicals and other related substances, have a wide range of bio-activities, and these products can be potential anti-microbial growth promoters. Natural medicinal products were popularly used as feed supplements, either as prevention or therapeutic agents, in farm animals such as poultry, pigs, sheep,
cattle, horses, rabbits, fish, and wild animals in China. According to Li (1998), about 200 medicinal feed additives, single products, combinations and extracts, have been developed and extensively used in the animal feed industry of China since 1970. It has been shown that some of these natural products have a role in enhancing productive performance and health of stressed animals, and others can improve quality of animal products.

AIMS OF THIS STUDY

The polysaccharide extracts from two medicinal mushrooms, *Lentinus edodes* and *Tremella fuciformis*, and an herb, *Astragalus membranaceus Radix*, are considered to play an important role in strengthening the animals’ defense system by improving the physical conditions of the gut ecosystem and enhancing functions of the immune system of chickens. These products have been used as immune enhancers in chickens and have shown anti-viral, anti-bacterial or anti-parasitic activity, as reviewed in Chapter 1.

The objectives of this research were:

- To isolate and identify polysaccharides from mushrooms and herbs and study their physico-chemical properties;
- To study the fermentation characteristics of mushroom and herb polysaccharides *in vitro*, using cecal microflora of chickens;
- To check the immune modulating effects of mushroom and herb polysaccharides against coccidiosis in chickens;
- To investigate effects of mushroom and herb polysaccharides on performance in chickens;
- To elucidate possible mode of action related to the gut ecosystem and to the immune system of chickens.

POLYSACCHARIDES FROM MEDICINAL MUSHROOMS AND HERBS

In an extensive review of the literature (Chapter 1), different natural species of mushrooms and herbs were detected with different physico-chemical properties and immune activities. Immune activities of the polysaccharides from two mushrooms, *Lentinus edodes* and *Tremella fuciformis*, and an herb, *Astragalus membranaceus Radix*, are reported, based on studies in rats, humans, and chickens. These immuno-active polysaccharides generally can stimulate growth of immune organs, enhance number and
activities of immunocytes, and induce both cellular and humoral immune responses in
these species. They have been used as either adjuvant of vaccines or feed supplements, and
have shown significant anti-viral, anti-bacterial and anti-parasitic activities in poultry.

Polysaccharides from different species of the mushroom and herbal species and even
from the same species differ in their physico-chemical properties such as the molecular
weights, sugar composition and structure. *Lentinus edodes* (*LenS*) contains proteins, fats,
carbohydrates, fiber, vitamins, and minerals and a variety of bioactive constituents such as
*Lentinan*, a heteroglucon, guanosine 5’-monophosphate (Yang et al., 1999). Three
polysaccharides have been isolated and purified from the fruit body of *LenS*, and these are
polysaccharide-protein complexes (Yang et al., 2001). *Tremella fuciformis* (*TreS*) contains
more than 70% dietary fibers such as acid polysaccharides and glucurono-xylomannan
(Min, 1996). Xia and Cheng (1988) obtained a polysaccharide fraction from the fruit body
of *TreS* by hot water extraction. The hairy roots of the large-scale cultured *Astragalus
membranaceus Radix* (*AstS*) contained 5.81% crude saponin, 0.14% astragaloside, 23%
total polysaccharide (water-soluble polysaccharide 14.88%) (Zheng et al., 1998). Active
constituents of *AstS* include saponins, flavonoids, polysaccharides, triterpene glycosides,
22 different amino acids and 14 micro-minerals (Qi, 1987). Pang et al. (1995) reported that
two kinds of glucans and two kinds of complex polysaccharides were isolated from *AstS.*
Thus, there probably are a great variety of the complex carbohydrates in the mushroom and
herb species. The physico-chemical properties of the polysaccharide fractions from *LenS*,
*TreS* and *AstS* were summarized in Table 1, according to Chapters 1 and 2.

Table 1 showed that sugar composition of the polysaccharide extracts was different.
*AstE* contains only glucose; *TreE*, however, was composed of fructose, galactose, glucose,
mannose, xylose, and glucuronic acid with a molar ratio of 0.8: 0.2: 3.0: 1.6: 4.5: 0.2; *LenE*
contains galactose, glucose, and xylose with a molar ratio of 0.8: 8.8: 0.5. Thus, there were
probably a variety of complex carbohydrates in the crude extracts obtained in this study.
However, the polysaccharides from these mushroom and herb species were not purified in
this study. *AstE* may be glucans, which was consistent with a study of Huang et al. (1982)
that two kinds of water-soluble glucans were isolated from *AstS*. However, most probably a
water-soluble heterosaccharide from *AstS*, composed of glucose and arabinose, was not
obtained in this extraction. According to a study of Xia and Cheng (1988), a
polysaccharide derived from *TreS* was composed of fructose, arabinose, xylose, mannose,
glucose and glucuronic acid, which fits well the sugar composition of *TreE* obtained in this
study, except galactose that was also found in *TreE.*
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Poly-saccharides/ extracts</th>
<th>Characteristics</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical properties</td>
<td><em>AstP</em></td>
<td><em>AstP</em>-1, water-soluble glucan; <em>AstP</em>-2, water-insoluble glucan; <em>AstP</em>-3, acidic heterosaccharide; <em>AstP</em>-4, water-soluble heterosaccharide</td>
<td>Huang et al., 1982</td>
</tr>
<tr>
<td></td>
<td><em>LenP</em></td>
<td>Polysaccharide-protein complex: <em>LenP</em>-1, GlcA, 24.1%; protein, 2.0%; <em>LenP</em>-2, GlcA, 34.8%; protein, 7.4%; <em>LenP</em>-3, GlcA, 40.1%; protein, 25.3%</td>
<td>Yang et al., 1999, 2001</td>
</tr>
<tr>
<td></td>
<td><em>TreP</em></td>
<td>water-soluble heterosaccharide</td>
<td>Xia and Cheng, 1988</td>
</tr>
<tr>
<td>Molecular weights</td>
<td><em>AstP</em></td>
<td><em>AstP</em>-1, 1.23 × 10^4; <em>AstP</em>-2, 3.46 × 10^4; <em>AstP</em>-3, 3.63 × 10^4;</td>
<td>Qi, 1987</td>
</tr>
<tr>
<td>(Dalton)</td>
<td><em>LenP</em></td>
<td><em>LenP</em>-1, 9.54 × 10^5; <em>LenP</em>-2, 9.0 × 10^5; <em>LenP</em>-3, 1.4 × 10^4</td>
<td>Yang et al., 1999, 2001</td>
</tr>
<tr>
<td></td>
<td><em>TreP</em></td>
<td>1.15 × 10^5</td>
<td>Xia and Cheng, 1988</td>
</tr>
<tr>
<td>Sugar composition</td>
<td><em>AstP</em></td>
<td><em>AstP</em>-1, Glu; <em>AstP</em>-2, Glu; <em>AstP</em>-3, HexA: Glu: Rha: Ara = 1: 0.04: 0.02: 0.01; <em>AstP</em>-4, Glu: Ara = 1: 0.15</td>
<td>Huang et al., 1982</td>
</tr>
<tr>
<td></td>
<td><em>AstE</em></td>
<td>Glu</td>
<td>Chapter 2</td>
</tr>
<tr>
<td></td>
<td><em>LenP</em></td>
<td><em>LenP</em>-1, Ara: Xyl: Man: Gal: Glu = 0.39: 0.46: 1.0: 0.93: 14.1; <em>LenP</em>-2, Ara: Xyl: Man: Gal: Glu = 0.19: 0.41: 1.0: 0.93: 10.7; <em>LenP</em>-3, Ara: Xyl: Man: Gal: Glu = 0.31: 0.47: 1.0: 1.15: 8.9</td>
<td>Yang et al., 1999, 2001</td>
</tr>
<tr>
<td></td>
<td><em>LenE</em></td>
<td>Gal: Glu: Xyl = 0.8: 8.8: 0.5.</td>
<td>Chapter 2</td>
</tr>
<tr>
<td></td>
<td><em>TreP</em></td>
<td>L-Fru: L-Ara: D-Xyl: D-Man: D-Glu: GlcA = 0.92: 0.49: 0.18: 1.0: 1.15: 0.57</td>
<td>Xia and Cheng, 1988</td>
</tr>
<tr>
<td></td>
<td><em>TreE</em></td>
<td>Fru: Gal: Glu: Man: Xyl: GlcA = 0.8: 0.2: 3.0: 1.6: 4.5: 0.2</td>
<td>Chapter 2</td>
</tr>
</tbody>
</table>

1 *AstP* = *Astragalus membranaceus* polysaccharides with four fractions (*AstP*-1, *AstP*-2, *AstP*-3 and *AstP*-4); *LenP* = *Lentinus edodes* polysaccharides derived from body fruit with three fractions (*LenP*-1, *LenP*-2 and *LenP*-3) and *TreP* = *Tremella fuciformis* polysaccharides derived from body fruit. *LenE* = *Lentinus edodes* polysaccharide extract; *TreE* = *Tremella fuciformis* polysaccharide extract; *AstE* = *Astragalus membranaceus* polysaccharide extract.

2 Fru = fructose; Ara = arabinose; Xyl = xylene; Man = mannose; Gal = galactose; Glu = glucose; Rha = rhamnose; GlcA = glucuronic acid; HexA = Hexuronic acid.
There are three kinds of polysaccharide-protein complexes, composed of arabinose, xylose, mannose, galactose and glucose, isolated from the fruit body of LenS (Yang et al., 2001). In present study, both arabinose and mannose presented in polysaccharides of LenS were not found in LenE. It may be because the composition of arabinose and mannose in these products is quite low (the intact material of LenS contains only 1 mol % mannose).

The yield of polysaccharide fractions varied in different batches of the substrates. The two mushrooms gave lower yields (8-10%) of polysaccharides than that of the herb (31%) (Chapter 2). It was found in Chapter 5 and 6, however, LenS and AstS gave lower yield (7-10%) than TreS (16%), when the polysaccharide fractions were extracted from second batches of the intact materials. Factors that could affect the constituents of these products may range from the natural source, the time of collection, and the place of production, to artificial conditions such as the growing medium, drying and storage (Li, 1998). The water-soluble polysaccharides made up over 60% of the extracted dry matter in all these products. As indicated in Chapter 2, proteins and other unidentified compounds were also present in the water extracts. Uncharacterized materials were extracted along with the polysaccharides. On the basis of the review (Chapter 1), we hypothesized that the water-soluble polysaccharides are major bio-active component. It would be interesting however to identify the remaining compounds to determine whether they also have bio-activities.

IN VITRO TRIALS

The intestinal environment can be influenced by diet composition and by dietary additives such as prebiotics, and may thus “change” the composition and activity of the microbiota (Gardiner et al., 1993, Jensen, 1993). A food ingredient can be classified as a prebiotic according to the following criteria (Gibson and Roberfroid, 1995). It must:

- be neither hydrolyzed nor absorbed in the upper part of the gastro-intestinal tract;
- be a selective substrate for one or a limited number of beneficial bacteria, which are stimulated to grow and/or are metabolically activated;
- be able to alter the hindgut flora in favor of a healthier composition;
- induce systemic effects that are beneficial to host health.

In poultry, fermentation occurs mainly in the ceca. The ceca provide a stable environment for microorganisms and, as a result, contain the largest and most complex
ecosystem. In order to evaluate some prebiotic effects of the mushroom and herb materials and their polysaccharide extracts, *in vitro* fermentation trials (Chapters 3 and 4) were conducted using cecal microflora of chickens.

These medicinal mushrooms and the herb, and more particularly their polysaccharide extracts, influenced the activity and composition of the microbial population after fermentation, in terms of fermentation kinetics, and changes in both fermentation end-products and cecal microbial community (Figure 1).

The increased volatile fatty acids (VFA) and decreased ammonia (NH₃) are perceived as being beneficial for long-term host health (Williams *et al.*, 2000). Fermentation of intact mushroom and herb materials resulted in less gas production, higher pH values, more branched-chain fatty acids and more NH₃ compared with their extracts (Figure 1). The fermentation of these polysaccharide extracts, however, resulted in the production of more gas, low pH values, mainly straight-chain fatty acids and decreased NH₃. The difference of the fermentation end products between the intact materials and their extracts was larger in Ast (*Asts* versus *AstE*) than in Len (*Lens* versus *LenE*) and Tre (*Tres* versus *Tred*). Denaturing gradient gel electrophoresis (DGGE) band patterns of chicken cecal microflora *in vitro* as determined by 16S rDNA analysis, demonstrated that both intact mushroom and herb materials and their polysaccharide extracts led to significant shifts in the bacterial community when fermented *in vitro*, suggesting that mushroom and herbal supplements had stimulated certain bacterial species. Of the substrates, AstE associated with the highest total gas and VFA productions and lowest pH value and NH₃ overall. However, DGGE profiles of the *Tres* cultures in intact materials and the *LenE* cultures in the extracts were more diverse. This suggests that these two materials had led to the greatest shifts in the microbial community. The use of different substrates may result in different fermentation kinetics and enrichment of certain microbial species.

Generally, these medicinal mushroom and herb materials, particularly their polysaccharide extracts, show that they are capable of stimulating microbial activities and altering composition in chicken ceca, based on the *in vitro* studies (Chapters 3 and 4). However, whether the different fermentation patterns and shifts in the microbial community by the extracts also affect performance of the birds *in vivo* was the subject of the following investigations.
Figure 1. Fermentation characteristics of mushroom and herb materials (LenS = Lentinus edodes; TreS = Tremella fuciformis; AstS = Astragalus membranaceus) and their polysaccharide extracts (LenE = Lentinus edodes extract; TreE = Tremella fuciformis extract; AstE = Astragalus membranaceus extract). BCR = (AIBU + AIVAL + AVAL)/(AAC + APRO + ABUT). AAE = Acetic acid equivalents. abc: Means within different substrates with no common superscript differ significantly (P<0.05).
IN VIVO TRIALS

Growth Performance

Usually feed additives are supplemented to a diet for the whole period of production. In the present trial, the polysaccharide extracts were supplemented for only one week (from 7 to 14 days of age), which was considered to be the critical period of broilers with a fast growth rate and adequate development of the immune system, and bacterial populations appears to be already established in the gut ecosystem (Smith, 1965). A study of Hu et al. (1998) showed the immune response of chickens was significantly increased by a mushroom polysaccharide supplement from 7 to 14 days of age, and its efficacy could be observed in the third week after feed supplementation. In this study, a significant effect of the antibiotic and the extracts on performance was found only in the first two weeks and especially during their supplemental period (from day 7 to 14). This effect however didn’t carry on into the third week (Chapter 5).

It appears that growth performance of the birds fed with the extracts had no significant difference from those fed the antibiotic in normal broilers (Chapter 5). However, growth of the infected birds fed with the extracts was lower compared with those fed the antibiotics (Chapter 6). This may suggest that antibiotic growth promoters could only be partially replaced by the extracts. When compare the growth response of the birds to LenE in Chapter 5 and Chapter 6, it was found that the extract showed more clearly a significant growth promoting effect in the infected birds than in non-infected birds (Figure 2).

Growth response of the birds under non-infected and infected conditions showed that hardly any difference in BW gain between different levels of LenE was detected in non-infected birds as increased LenE dose (A), however, a significant growth response was found in AMG infected birds as increased LenE dose (B).

Growth of Immune Organs

The relative weight of immune organs of the birds fed with the extracts is shown in Figure 3. The extracts did not have a significant effect on the relative weight of immune organs in normal broilers, although the relative weights of thymus and bursa were larger in a numerical sense for the three extracts as compared with the VRG and non-supplemented group (Figure 3, A; Chapter 5). The extracts-fed birds showed a
more significant development of immune organs in infected birds (Figures 5, B; Chapter 7) than the non-infected birds.

**A (Chapter 5)**

![Graph for non-infected birds]

**B (Chapter 6)**

![Graph for infected birds]

**Figure 2.** Growth response of non-infected (A) and infected (*Avian mycoplasma Gallisepticum*) (B) chickens fed with different levels of *Lentinus edodes* polysaccharide extract (*LenE*). *abc*: Means between levels with no common superscript differ significantly (*P*<0.05).
**Development of GIT**

The relative weight of the GIT fragments of the birds fed with the extracts is shown in Figure 4. The extracts did not have a significant effect on the relative weight of GIT organs in normal broilers, although the relative weights of the GIT organs (proventriculus+gizzard and duodenum) were somewhat larger in a numerical sense for the three extracts as compared with the VRG and non-supplemented group (Figures 4, A; Chapter 5). The extracts-fed birds showed a more significant development of GIT in infected birds (Figures 4, B; Chapter 7), as compared with the non-infected birds.

A (Chapter 5)

![Non-infected GIT organs](image)

B (Chapter 7)

![Infected GIT organs](image)

**Figure 3.** Growth of immune organs in non-infected (A) or *E. tenella* infected (B) chickens fed with the polysaccharide extracts (*LenE* = *Lentinus edodes* extract; *TreE* = *Tremella fuciformis* extract; *AstE* = *Astragalus membranaceus* extract). \( a, b, c \). Means within organs between different treatments with no common superscripts differ significantly (\( P<0.05 \)).
A (Chapter 5)

![Graph showing weights of GIT organs in non-infected chickens fed with polysaccharide extracts](image)

B (Chapter 7)

![Graph showing weights of GIT organs in infected chickens fed with polysaccharide extracts](image)

**Figure 4.** Growth of immune organs in non-infected (A) or *E. tenella* infected (B) chickens fed with the polysaccharide extracts (*LenE* = *Lentinus edodes* extract; *TreE* = *Tremella fuciformis* extract; *AstE* = *Astragalus membranaceus* extract). *abc*: Means within organs between different treatments with no common superscripts differ significantly (P<0.05).

In conclusion, of the three extracts, *LenE* was proved a better growth promoter than *TreE* and *AstE* in non-infected birds (Chapter 5). *LenE* and *TreE* showed a better growth promoting effect than *AstE* in coccidial infected birds (Chapter 7). However, *AstE* fed birds gained more weight in AMG infected birds than in those fed with *LenE* and *TreE* (Chapter 6). The hypothesis of this study was that the polysaccharide extracts may have some prebiotic or immunomodulating effects. Therefore, it is not surprising that the extract-fed birds under different conditions showed different growth responses.
**Intestinal Microbial Ecosystem**

In order to evaluate whether the polysaccharide extracts which led to microbial shifts *in vitro* (Chapters 3 and 4) also have prebiotic properties *in vivo*, an *in vivo* experiment was conducted in broilers, which were naturally infected with *Avian mycoplasma Gallisepticum* (AMG) prior to the experiment (Chapter 6). It was expected that the extracts have a positive response *in vivo*. BW gain of the infected birds was enhanced and the extracts had a great influence on the cecal microbial ecosystem, such as increased cecal viscosity and microbial populations.

The intestinal microflora is known to play an important role in the health status of host animals. In general, intestinal bacteria may be divided into species that exert either harmful (pathogenic) or beneficial effects on host (Macfarlane and Cummings, 1991). Therefore, a common approach to maintain host health is to increase the number of desirable bacteria in order to inhibit colonization by invading pathogens (Rolfe, 1991; Brown, 1996). The composition and activity of intestinal microbiota can be altered by diet composition and dietary manipulations such as feed additives (Jensen, 1993). Carbohydrates, especially oligo- and poly-saccharides, have been used as prebiotics to influence the composition of the bacterial populations in the large intestine of a number of animal species (a.o., Newman, 1995; Grizard and Barthomeuf, 1999; Hughes et al., 2000; Breves et al., 2001; Korakli et al., 2002). In this work, the extracts stimulated the number of potentially beneficial bacteria such as *Bifidobacteria* and *Lactobacilli*, and reduced the number of potentially harmful bacteria such as *Bacteroides* and *E.coli* (Chapter 6). Of the three extracts (at level of 2g/kg), LenE was associated with the highest numbers of cecal *Bifidobacteria* and *Lactobacilli*. Thus, the mushroom and herb polysaccharide extracts, LenE, TreE and AstE, may have had some prebiotic effects leading to shifts in the intestinal microbial populations of chickens.

In this experiment, with increased LenE levels, birds tended to have higher BW gain and microbial counts. The number of predominant cecal bacteria, in particular, *Bifidobacteria* and *Lactobacilli*, was significantly increased with increased LenE dose (Figure 5). It would seem that the use of these specific mushroom and herb polysaccharide extracts holds great promise as potential modifiers of intestinal microbiota in diseased chickens.
**Figure 5.** Cecal microbial counts in chickens one-week after feeding different levels of *Lentinus edodes* polysaccharide extract (LenE).

**Immune Responses**

The immune status of the host is known to play an important role in its ability to resist infections. The cell-mediated immune response plays a major role in resistance against various pathogens in poultry (Lillehoj, 1998). Cell-mediated immunity has been investigated in chickens suffering from a coccidial infection (Lillehoj *et al.*, 1989; Martin *et al.*, 1993, 1994; Lillehoj, 1998). Cytokines, particularly IFN-γ and IL-2, are involved in the induction of protective immunity against coccidial infection (a.o., Breed *et al.*, 1997; Yun *et al.*, 2000; Li *et al.*, 2002; Miyamoto *et al.*, 2002), and have been used as indicators of cell-mediated immunity in infected hosts (Martin *et al.*, 1993, 1994; Ottenhoff and Mutis, 1995).

The fast development of drug-resistant coccidia species has stimulated searches for alternative control methods, one of which is vaccination. In recent years, there is an increasing interest to develop vaccines for the control of poultry diseases. The live, attenuated oocyst vaccine showed better results with significantly increased immune response and growth performance in coccidial infected birds (Figure 6; Chapter 7).
Figure 6. Growth and immune response of *E. tenella* infected chickens fed with the polysaccharide extracts (A) (*LenE* = *Lentinus edodes* extract; *TreE* = *Tremella fuciformis* extract; *AstE* = *Astragalus membranaceus* extract) or the extract plus vaccine treatments (B) (*LenE+V* = *Lentinus edodes* extract plus vaccine; *TreE+V* = *Tremella fuciformis* extract plus vaccine; *AstE+V* = *Astragalus membranaceus* extract plus vaccine). \( ^{abc} \): Means within different treatments with no common superscript differ significantly (P<0.05).
The results (Figure 6; Chapter 7) demonstrated that at 7 days post infection, birds fed the extracts (LenE and TreE) without vaccination had slightly higher BW gain than those infected birds. However, the extracts in conjunction with the vaccine significantly enhanced BW gain of the infected chickens compared with the vaccine only. Of the three extracts, LenE and TreE showed a better growth promoting effect. The cecal peak oocyst output were higher in groups fed AstE and LenE than in the infected group (Figure 6, A), whereas those of the extract plus vaccine groups (LenE+V and TreE+V) were lower than the vaccine group (Figure 6, B). Of the three extracts, both LenE and AstE-fed groups showed lower cecal oocyst output. Lesion scores of the three extract groups was slightly lower than the infected group (Figure 6, A), but there was no significant difference between the extracts plus vaccine groups and the vaccine only (Figure 6, B). A study of Hu et al. (1998) showed that the chickens fed with TreEm, a polysaccharide derived from mycelia of Tremella fuciformis, when used as a adjuvant, significantly (P<0.001) enhanced BW gain and reduced the mortality compared with the vaccine only, and the oocyst output in coccidial infected chickens was largely reduced by feeding TreEm.

Intensive use of chemotherapy, anti-coccidial drugs, has led to the rapid appearance of resistant strains of the disease (Rana and Tikaram, 2001a, 2001b). The mushroom and herb polysaccharides may not cause the drug-resistance in chickens. It was reported that polysaccharides enter the large intestine and may serve as substrates for endogenous colonic bacteria, and indirectly provide the host with energy, metabolic substrates and essential micronutrients (Gibson and Roberfroid, 1995).

Based on the results of Chapter 8, it was suggested that the polysaccharide extracts, LenE, TreE and AstE, could enhance T-cell immune responses against coccidia, as characterized by cytokine IFN-γ and IL-2 secretion. Serum IFN-γ titers and IL-2 production of splenocytes determined at 7 and 14 days post-infection (p.i.) is presented in Figure 7. The serum IFN-γ of the groups fed with the extracts and immunized with vaccine was significantly higher compared with the vaccine group at 7 and 14 days p.i.. Of the three extracts, AstE showed the highest IFN-γ titers at 7 days p.i. (Figure 7, A). The IL-2 production of splenocytes showed a similar pattern to the serum IFN-γ titers. Seven days p.i., the IL-2 level of the groups fed with extracts and immunized with vaccine was higher compared with the group immunized with the oocyst vaccine only. At 14 days p.i., the IL-2 production of the groups fed with the extracts and immunized with vaccine was not significantly different from the vaccine group (Figure 7, B).
Figure 7. Cytokines IFN-γ and IL-2 secretion of *E. tenella* infected chickens fed with the polysaccharide extracts (*LenE* = *Lentinus edodes* extract; *TreE* = *Tremella fuciformis* extract; *AstE* = *Astragalus membranaceus* extract) (A) or the extract plus vaccine treatments (B) (*LenE+V* = *Lentinus edodes* extract plus vaccine; *TreE+V* = *Tremella fuciformis* extract plus vaccine; *AstE+V* = *Astragalus membranaceus* extract plus vaccine). *abce*: Means within different treatments with no common superscript differ significantly (P<0.05).
Figure 8. Immuno-modulation of the polysaccharide extracts (LenE = Lentinus edodes extract; TreE = Tremella fuciformis extract; AstE = Astragalus membranaceus extract) in chickens infected with *E. tenella*. Cecum samples were diluted for specific IgA (1:2), IgM (1:1) and IgG (1:8). A 450 = absorbance (A) at 450 nm; ERFC = erythrocyte rosette forming cells; EAC = erythrocyte-antibody-complemetal cells; cpm = counts per min.
This indicates that the extracts have promise in immune protection against experimental infections when they are used as an adjuvant for a vaccine. Both in vitro and in vivo studies in mammals provide evidence that certain polysaccharides derived from mushrooms and herbs show cytokine-stimulating activities and could mediate protective immunity against infections and cancers (a.o., Hu et al., 1995; Liang et al., 1995; Liu et al., 1998; Gao et al., 1997).

Therefore, the extracts used as vaccine adjuvant were proved to be most promising against the experimental coccidial infection, in terms of stimulating growth and mediating host protective immune responses to coccidia in chickens.

Following a coccidial infection, both cell-mediated and humoral immunity may play a role in resistance of birds to Eimeria spp. T-cells induce a positive immune response that limits oocyst production in both primary and subsequent infections (Lillehoj et al., 1989; Martin et al., 1993, 1994; Lillehoj, 1998). Also, antibodies produced systematically in serum, and locally in the intestine play a part in resistance against coccidial infections (Wallach et al., 1994). A considerable amount of specific immunoglobulins, IgA, IgM and IgG, was measured in intestinal mucosa of chickens with an E. tenella infection (Girard et al., 1997).

Supplementation of diets with mushroom and herb extracts resulted in an enhanced resistance to E. tenella, by enhancing both cellular and humoral immune responses against E. tenella in chickens (Chapter 9). The mushroom and herb polysaccharide extracts enhanced both systemic and cecal E. tenella antigen-binding antibody production, splenocyte proliferation, and the populations of peripheral erythrocyte rosette forming cells (ERFC %) and erythrocyte-antibody-complemental cells (EAC %) of E. tenella infected chickens, as measured at 7, 14 and 21 days post-infection (Figure 8).

CONCLUSIONS

In brief, the results presented and discussed in this dissertation have demonstrated that:

- Intact mushroom and herb materials and their polysaccharide extracts showed differences in their physico-chemical properties in terms of protein contents, and sugar contents and sugar compositions.
- Intact mushroom and herb materials and their polysaccharide extracts showed differences in fermentability, fermentation end products as well as the bacterial community when fermented in vitro. These mushroom and herb materials, particularly their polysaccharide extracts, showed some prebiotic effects in terms of altering the
microbial activities and composition in chicken ceca.

- An in vivo test further demonstrated that the polysaccharide extracts significantly enhanced growth of AMG infected chickens. The cecal microbial ecosystem in terms of cecal viscosity and microbial species present in the infected chickens was also changed, which, in turn, selectively stimulate growth of potentially beneficial bacteria in ceca of chickens. Chicken growth and cecal microbial populations were enhanced with increased LenE dose.

- The polysaccharide extracts showed a more pronounced effect on growth performance, development of the immune and GIT organs in AMG infected birds compared to non-infected birds.

- The polysaccharide extracts showed significant effects on body growth, immune responses as well as growth of immune organs and development of GIT fragments in coccidial-infected chickens, and particularly when they were used in conjunction with vaccine.

- The use of the mushroom and herb polysaccharide extracts enhance T-cell immune responses, characterized by IFN-γ and IL-2 secretion, against coccidiosis in chickens.

- Supplementation of mushroom and herb extracts resulted in an enhancement of resistance to *E. tenella*, probably by enhancing both cellular and humoral immune responses against *E. tenella* in chickens.

**PRACTICAL IMPLICATIONS**

Based on the results of Chapter 5 of this dissertation, it seems that supplementation of the extracts in commercial broilers’ diets led to enhanced growth rate and slightly increased weights of immune and GIT organs. LenE demonstrated to be the most potential growth promoter of the three extracts, with higher BW gain and lower FCR. An increased quadratic response was found in BW gain of the birds fed LenE. Therefore, it may suggest supplementing LenE in birds’ diets to achieve optimum growth performance in normal broiler practice.

The three extracts showed significantly enhanced growth rate when supplemented to the diet of the infected birds. However, the three extracts showed different effects on growth rate and immune response of the infected birds. Higher dose of the extracts (5 and 10g/kg LenE) showed a better growth-stimulating effect in AMG infected birds (Chapter 6). Therefore, it may suggest that a higher dose of the extracts is to be recommend in infected birds.
In the present study, the polysaccharide extracts were supplemented for one week (from 7 to 14 days of age) only, which was considered to be the most critical period for broilers. In this study, a significant effect of the extracts on performance was found in the first two weeks only. Thus, it may suggest that the supplementation period of the extracts is prolonged in order to achieve desirable growth performance and immune responses in those areas where the extracts are available and not too expensive.

The polysaccharide extracts showed significant effects on body growth and immune responses in coccidial infected chickens, and particularly when they are used as adjuvant of the live oocyst vaccine. In practice, these products should be used in conjunction with vaccine to enhance the immune responses of chickens against the invasion of pathogens.

ECONOMIC EFFECTS

China is the world’s second largest producer of poultry meat with an annual output of 13.52 million tones (Table 2). With the popularity of ‘green products’, natural herbal medicine acquires a better prospect, sharing 30% of the world medicine market (totally 20 billion in the year 2001), and it is estimated to keep growing by 20% annually within 20 years. China, as the origin, the largest producer of natural herbal medicine. According to incomplete statistics, there are more than 5,000 herb medicine pharmaceutical companies in China with a total industrial production output of around 7.5 billion USD (China International Fair on Traditional Chinese Medicine and Natural herbal medicine, 2003).

The advances in cultivation technology may account for the increased availability of herbs and mushrooms and the reduction in price in recent years. There are over 2,000 types of Astragalus which exist worldwide, of which around 130 Chinese varieties have been extensively used medicinally (Ma et al, 2002). The annual production of Astragalus from 1950’s to 1970’s was around 3,000 tons. However, in 1983 the production of Astragalus reached around 21,000 tons (Zhao et al., 1995). China is the major producer of edible mushrooms according to an investigation of FAO (Table 2). The popularity of the culinary characteristics and medicinal purpose offered by Lentinus and Tremella mushrooms will stimulate for continued growth and development of the industry worldwide.

The restriction on the use of antimicrobial growth promoters in feeds will result in increased mortality and animal welfare problems (Andreason, 2000). Avian diseases adversely affect the development of the poultry industry. Only coccidiosis is responsible for 6-10% of all broiler mortality, and global economic losses, as a result of the reduction in
growth rate, feed intake and feed conversion efficiency, are estimated at 1 billion USD annually (Banfield et al., 1999). It was estimated that annual cost of anticoccidial drugs were about 720 million US dollars in China (Suo and Li, 1998) and 60 million USD in USA (Long, 1984). In the United Kingdom, the total costs of coccidiosis in chickens was estimated to have been at least 54.9 million USD in 1995, of which 98% involved broilers (Williams, 1999).

Table 2. Poultry and mushroom production in China (Source: FAOSTAT, 2003)

<table>
<thead>
<tr>
<th></th>
<th>China</th>
<th>World</th>
<th>China contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poultry production in 2002</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of chickens (x10^8)</td>
<td>3.92</td>
<td>15.42</td>
<td>25</td>
</tr>
<tr>
<td>Total meat production (mt)</td>
<td>67.32</td>
<td>242.63</td>
<td>28</td>
</tr>
<tr>
<td>Poultry meat production (mt)</td>
<td>13.52</td>
<td>72.24</td>
<td>19</td>
</tr>
<tr>
<td>Poultry meat/total meat (%)</td>
<td>20</td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>

| Mushroom production (mt)     |       |       |                        |
| 2002                         | 1.24  | 2.96  | 42                     |
| 2000                         | 0.81  | 2.54  | 32                     |
| 1990                         | 0.36  | 1.76  | 21                     |
| 1980                         | 0.27  | 1.10  | 25                     |
| 1970                         | 0.21  | 0.59  | 35                     |
| 1961                         | 0.10  | 0.30  | 35                     |

Here are some basic calculations for broiler production, based on a small poultry enterprise in Gansu, China (Chinese currency: 1 RMB = 0.12 USD):

Broiler slaughter weight: 2kg; FCR: 2; Consumed feed per bird: 4 kg

Broiler market price: 20 RMB/bird (10 RMB/kg)

*Chicken costs: 3 RMB/bird*

*Feed costs: 8.8 RMB/bird (2.2 RMB/kg)*

*Extract supplements: 0.28g/bird (2 g/kg of feed (20g x 7 days) from 8 to 14 days of age)*

*Extract cost: 0.056 RMB/bird (200 RMB/kg for extracts; 10-20 RMB/kg for intact materials)*

Flock depletion/mortality: 5%

Vaccine costs: 0.25 RMB/bird

Costs of labor and electricity, etc.: 0.25 RMB/bird

The estimated costs and benefits for a small poultry enterprise in Gansu, China (Table 3):
### Table 3. Costs and benefits based on a small poultry enterprise with a total of 1000 broilers

<table>
<thead>
<tr>
<th>Items</th>
<th>RMB (1 RMB ≈ 0.12 USD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Benefits</strong></td>
<td></td>
</tr>
<tr>
<td>Broilers</td>
<td>20,000</td>
</tr>
<tr>
<td><strong>Costs</strong></td>
<td></td>
</tr>
<tr>
<td>Chickens</td>
<td>3,000</td>
</tr>
<tr>
<td>Feed</td>
<td>8,800</td>
</tr>
<tr>
<td>Mortality (5%)</td>
<td>1,000</td>
</tr>
<tr>
<td>Vaccine</td>
<td>250</td>
</tr>
<tr>
<td>Labor and electricity, etc.</td>
<td>250</td>
</tr>
<tr>
<td><strong>Sub-total of the costs</strong></td>
<td>13,300</td>
</tr>
<tr>
<td>Extract supplements</td>
<td>56</td>
</tr>
<tr>
<td>Margin (without extract supplementation)</td>
<td>6,700</td>
</tr>
<tr>
<td>Margin (with extract supplementation)</td>
<td>6,644</td>
</tr>
</tbody>
</table>

Assume that a retarded growth will cost 0.20 RMB per bird due to diseases and withdraw antibiotic growth promoters. It will cost a farmer 200 RMB per flock for a commercial broiler farm with 1,000 broilers. With five flocks per year, it will cost 1,000 RMB per year. Therefore it is may be possible to invest 280 RMB extracts to achieve the optimum growth performance in broilers.

### SUGGESTIONS FOR FUTURE RESEARCH

- Further study is needed to purify, where possible, the bio-active polysaccharide components from the extracts and to study their physico-chemical properties related to the bio-activities.
- The combination of two or more polysaccharide extracts may be more effective in stimulating growth and immune responses in animals than a single extract. It would be worthwhile to conduct experiments to investigate the effects of a mixture of extracts on growth and health in poultry.
- The polysaccharides extracts used as an adjuvant for the live oocyst vaccine increased immune responses of *E. tenella* infected birds. However, it was not clear whether the polysaccharides could be used as an adjuvant for other vaccines, or, whether it should be mixed with vaccine or given as feed supplements. Researchers should find the optimal way of supplementation according to different vaccines.
- Polysaccharides can be isolated and purified from the cell culture of *Astragalus* and from
mycelia of *Lentinus* and *Tremella*. It might be more economical to extract polysaccharide from the cultures instead of using fruit bodies of mushrooms or collecting the roots from two or three year-old plants. Further studies are needed to compare the efficacy of the polysaccharides from the cultures and from the fruit bodies/roots.

- The effective components from medical products are quite complex. Other bio-active components from these mushroom and herb materials might also be good candidates as potential anti-microbial growth promotors in animals. Researchers should identify these components and study their efficacy.

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SUMMARY
Summary

Antibiotics are widely used as therapeutics agents and also as growth promoters in poultry production. However, use of antibiotics in animals’ diets will cause a disturbed gut eco-system and may develop a resistant population of bacteria in birds. The possibility of developing resistant populations of bacteria and the side effects of using antibiotics as growth promoters in the farm animals has led to the recent EU-ban on the use of several antibiotics in poultry diets. The restriction on antimicrobial growth promoters will have consequences for the health and production status of animals especially young pigs and broiler chickens. Therefore, there is an intensive search for alternatives such as probiotics, prebiotics and other feed additives.

In the first experiment, Chinese herbal medicine (CHM) was considered to be a potential antimicrobial growth promoter. Four dietary levels (0.25, 0.5, 1 and 2 g/kg) of a CHM complex were used as alternatives to virginiamycin (VRG, 20 mg/kg). A total of 720 female broiler chicks were used to test the effects of VRG and CHM on BW gain, feed intake, feed conversion ratio and viability at 7 to 28 days of age. At the end of the experiment, a total of 72 birds, with 4 birds from each replicate of three treatments (control, VRG and 0.5 g/kg CHM), were killed for the dissection analysis. Liver color and the relative weight of liver and intestinal tract organs including duodenum-pancreas-jejunum, ileum and ceca were determined to study their mechanisms.

In this experiment, CHM dietary treatments did significantly increase BW gain at 7 to 21 days of age but not at 21 to 28 days of age, and the birds fed with 0.5 g/kg CHM showed higher BW gain at the first two weeks of the experiments. Feed intake was increased by the CHM treatments at 21 to 28 days of age, as a result, FCR in those groups was increased. The dissection results showed that dietary CHM and VRG had no effects on liver and intestinal tract weights. However liver color of the birds fed with CHM was more uniform than those fed with VRG and the non-supplemented birds. Thus, CHM showed a promising effect on performance of chickens.

However, the effective components from CHM were unknown and the mechanisms for enhanced growth performance are not well understood. Therefore, the following experiments were carried out to further investigate the bio-active components (polysaccharides) from natural medicines and to study their mode of action related to the gut ecosystem and immune stimulation for enhanced growth performance in poultry.

On the basis of Chinese literature, immuno-active polysaccharides derived from two
mushrooms, *Tremella fuciformis* (*TreS*) and *Lentinus edodes* (*LenS*), and the herb *Astragalus membranacea* Radix (*AstS*), seem to be potential alternatives for antimicrobial growth and health promoters. However, the effects of these products on growth performance of animals and their mode of actions related to gut health and immuno-modulation in farm animals, like poultry, are not yet well studied. It is hypothesized in this project that mushroom and herb polysaccharides (as a prebiotic) may selectively stimulate growth and/or activity of one or a limited number of favorite bacterial species of large intestine, and as such beneficially affect gut health of the bird. In addition, mushroom and herb polysaccharides may enhance immune response of chickens. Therefore these products could play an important role in strengthening the animals’ defense system by improving the physical conditions of gut ecosystem and enhancing functions of the immune system of chickens. The objectives of this research are:

1) To isolate and identify polysaccharides from mushrooms and herbs and study their physico-chemical properties;

2) To study the fermentation characteristics of mushroom and herb polysaccharides *in vitro*;

3) To check immune modulating effects of mushroom and herb polysaccharides against coccidiosis in chickens;

4) To investigate effects of mushroom and herb polysaccharides on performance in chickens;

5) To elucidate possible modes of action related to the gut ecosystem and immune system of chickens.

Chapter 1 gives a review on immuno-active polysaccharides from medicinal plants and fungus. Moreover, two mushrooms, *LenS* and *TreS*, and an herb, *AstS*, are discussed in more detail. In an extensive review of the literature, different natural species of mushrooms and herbs were detected with different physico-chemical properties and immune activities. Immune activities of the polysaccharides from two mushrooms, *LenS* and *TreS*, and an herb, *AstS*, are reported, based on studies in rats, humans, and chickens. These immuno-active polysaccharides generally can stimulate growth of immune organs, enhance number and activities of immunocytes, and induce both cellular and humoral immune response in these species. They have been used as either adjuvant of vaccines, therapeutic agents or feed supplements, and have shown anti-bacterial, anti-viral and anti-parasitic activities in chickens.

In the second experiment (Chapter 2), water-soluble polysaccharide fractions from two
medicinal mushrooms (LenS and TreS) and an herb (AstS) were isolated by hot water extraction, and protein was partially removed with tri-chloroacetic acid. Finally 96% ethanol was used to precipitate polysaccharides containing material. Polysaccharide extracts, LenE, TreE and AstE, were obtained from this study. Total sugar content of the extracts was determined using the phenol-sulphuric method and qualitative chemical analyses such as HPAEC were used for physico-chemical property analysis, such as polysaccharide contents and sugar composition of these extracts. The two mushrooms gave lower yields (8-10%) of polysaccharides than that of the herb (31%) with polysaccharides making up over 60% of the DM of the extract. The sugar composition analysis showed that LenS contains fructose, galactose, glucose, mannose, xylose and glucuronic acid with molar ratio of 0.1: 0.2: 9.1: 0.1: 0.5: 0.1; TreS contains fructose, galactose, glucose, mannose, xylose and glucuronic acid with molar ratio of 0.9: 0.1: 3.5: 1.5: 4.1: 0.5; and AstS contains rhamnose, galactose, glucose, mannose, xylose and galacturonic acid with molar ratio of 0.6: 0.2: 8.3: 0.4: 0.1: 0.3. Of the three extracts, AstE contains considerable amounts of glucose, and TreE, however, contains fructose, galactose, glucose, mannose, xylose, and glucuronic acid with a molar ratio of 0.8: 0.2: 3.0: 1.6: 4.5: 0.2; LenE contains galactose, glucose, and xylose with a molar ratio of 0.8: 8.8: 0.5. Thus, the intact mushroom and herb materials and their polysaccharide extracts showed differences in their physico-chemical properties in terms of protein and sugar contents as well as the sugar compositions.

In order to evaluate the prebiotic effects of these mushroom and herb materials and their polysaccharide extracts, in vitro experiments (Chapter 3 and 4) were carried out to investigate in vitro fermentabilities of the intact materials (LenS, TreS and AstS) and their polysaccharide extracts (LenE, TreE and AstE), and their effects on bacterial community, using microflora from chicken ceca. Fermentation kinetics was determined using the in vitro cumulative gas production technique. End-products such as gas, volatile fatty acids (VFA) and ammonia were also determined. In addition, specific PCR amplification of 16S rDNA gene fragments in combination with denaturing gradient gel electrophoresis (DGGE) was used to analyze the microbial community before and after in vitro fermentation.

The gas profiles of intact materials were similar for AstS and LenS, which showed mono-phasic kinetics, however, TreS had a diphasic fermentation pattern. The extracts had similar profiles to the intact materials, though gas production rates were faster. AstE was rapidly degraded and highly fermentable, whereas TreE was less readily fermented. The gap of total gas production between the extracts and the intact materials were greater for Ast than for both Len and Tre. Fermentation of AstE and LenE resulted in the lowest final pH values.
Intact materials tended to produce less VFA than the extracts, though *LenS* and *AstE* had the highest total VFA production overall. Fermentation of the intact materials resulted in more branched-chain fatty acids and ammonia.

The intact mushroom and herb materials and the extracts led to a shift in the bacterial community fingerprint after *in vitro* fermentation. The similarity indices of the DGGE band pattern of the intact substrates compared with the inoculum and blanks ranged from 40 to 58%, of these, *TreS* showed the lowest similarity, whereas *AstS* showed the highest. The similarity indices of the band pattern of the extracts compared with the inoculum and blanks ranged from 23 to 80%, of which *LenE* showed the lowest similarity, whereas *AstE* showed the highest. The similarity indices of DGGE were significantly lower in the *LenE* samples than in the *LenS* samples compared with the inoculum and blanks, whereas it was higher in the *AstE* samples than in the *AstS* samples. However, there were no significant difference between *TreS* and *TreE*. Fermentation kinetics and end-point products demonstrated differences in availability of substrates for the fermentative microflora between the mushrooms and herb.

In general, both intact mushroom and herb materials and their polysaccharide extracts led to significant shifts in the bacterial community when fermented *in vitro*. These medicinal mushroom and herb materials, particularly their polysaccharide extracts, show promise in altering microbial activities and composition in chicken ceca. It will be important to establish whether this selection may also occur *in vivo*, if the same substrates are included in poultry diets. *In vivo* experiments are necessary for confirmation of this hypothesis.

In order to assess the potential growth-promoting polysaccharide extracts *in vivo*, a pilot study was conducted to investigate the effects of these products on growth performance and development of immune and GIT organs in broilers (Chapter 5). Three extracts (*LenE, TreE* and *AstE*) were supplemented at levels of 0.5, 1, 2, 3 and 4 g/kg from 7 to 14 days of age, which was compared with an antibiotic treatment group (20 mg/kg, VRG) as well as a group of non-supplemented birds.

In this experiment, BW gain, feed intake and feed conversion ratio of the extract-supplemented groups were not significantly different from those of the antibiotic group. Significant effects of type of extract and level on growth performance were found from 7 to 28 days of age. Generally, the birds fed with *LenE* showed higher BW gain and lower feed conversion ratio than those fed with *TreE* and *AstE* from 7 to 28 days of age, and 1g/kg in *TreE* group and 2 g/kg in *LenE* and *AstE* groups were considered the optimum levels of supplementation for enhanced broiler growth. However, the extracts did not show a
significant effect on the relative weight of both immune and GIT organs compared with the antibiotic group. Further experiments are needed to find out which mode of action is responsible for different responses of the birds to the extracts as observed in this experiment.

In order to elucidate the mode of action for enhanced growth performance of chickens by these products, the second in vivo trial was conducted in broilers, which were naturally infected with *Avian mycoplasma Gallisepticum* (AMG) prior to the experiment. A total of 135 birds were randomly assigned to nine dietary treatments: addition of *LenE* at level of 1, 2, 3, 5 and 10 g/kg of the diet, *TreE* and *AstE* both at level of 2 g/kg, antibiotic (Apramycin-APR, 20 mg/kg), and the control. Polysaccharide extracts and APR were supplemented from 15 to 21 days of age. BW gain, cecal pH, viscosity and predominant microbial populations were measured one week after treatments. The data were analyzed to test significance between the extracts (*LenE*, *TreE* and *AstE* at level of 0.2%), the antibiotic and control groups. In addition, a dose response of *LenE* (1, 2, 3, 5 and 10 g/kg) was assessed.

In this experiment, both the extracts and APR significantly stimulated growth of the chickens infected with AMG compared to the infected-control group. However, overall mean BW gain of the groups fed with the extracts was significantly lower compared with that of the antibiotic, and the extracts had no significant effect on cecal pH. However, cecal viscosity and microbial populations were significantly increased by the extracts and APR. In contrast to APR, the extracts stimulated the number of the potentially beneficial bacteria (*Bifidobacteria* and *Lactobacilli*), while reducing number of the potential harmful bacteria (*Bacteroides* and *E.coli*). Of the three extracts, *LenE* was associated with the highest numbers of cecal Bifidobacteria and Lactobacilli. The dose response results showed that with increases in the *LenE* level, birds tended to have higher BW gain and higher total aerobic and anaerobic counts. The number of predominant cecal bacteria, in particular, *Bifidobacteria* and *Lactobacilli*, was significantly increased with increased *LenE* dose. It would seem that the use of these specific mushroom and herb polysaccharide extracts holds great promise as potential modifiers of intestinal microbiota in diseased chickens.

To further study the mode of action of these products in terms of immuno-modulation in chickens, two in vivo trials were conducted to assess the immune response again *E.tenella* infection in broilers. BW growth and development of immune and GIT organs, oocyst output, cecal lesions, and immune parameters, such as cytokines (IFN-γ and IL-2), systemic and cecum antibodies, splenocyte proliferation, and E-rosette production of T, B lymphocytes were measured before or after infection.
In the first anti-coccidial trial, a total of 180 broiler chicks were assigned to nine groups: three groups fed with extracts (LenE, TreE and AstE), three groups fed with extracts and immunized with live oocyst vaccine (LenE+V, TreE+V and AstE+V), the group immunized with oocyst vaccine, and two controls (E. tenella infected and uninfected groups). The oocyst vaccine was given at 4 days of age and the extracts (0.1% of the diet) were supplemented from 8 to 14 days age. At 18 days of age, all the birds except those of the negative control group were infected with 9 x 10^4 sporulated oocysts.

In this experiment, the infected birds fed with the extracts had lower BW gain than those which received the vaccine only at 7 days post infection. However, the extracts, when used as an adjuvant of a live oocyst vaccine, significantly enhanced BW gain of the infected chickens. Of the three extracts, LenE and TreE showed a better growth promoting effect. The extracts largely increased oocyst excretion of droppings during the primary response post vaccination. The cecal peak oocyst output and lesion score measured at 7 days post infection were higher in the groups fed the extracts than in the group immunized with the vaccine only, whereas those of the groups fed with the extracts and immunized with the vaccine were not significantly different from the vaccine group. Of the three extracts, both LenE and AstE groups showed lower cecal oocyst output. The infected birds fed with the extracts gained slightly more weights of thymus and bursa but less liver weights. The birds immunized with vaccine showed significantly higher thymus and bursa weights but lower liver and spleen weights compared with the birds fed with the extracts at 7 days p.i. A significant difference was found in relative weights of the GIT organs between the different treatments. As compared with the vaccine group, the birds fed with the extracts gained significantly more weights of the GIT organs (duodenum, ileum and cecum), however, the birds fed with the extracts and immunized with vaccine showed significantly lower weights of the GIT organs (crop-oesphagus, proventriculus+gizzard, jejunum and ileum) at day 7 p.i. Of the three extracts, the AstE fed birds gained more weights of proventriculus+ gizzard and jejunum at day 7 p.i.. Thus as compared with the extracts, the live, attenuated vaccine showed better results with significantly increased immune response and growth performance in coccidial infected birds. The polysaccharide extracts may prove useful against avian coccidiosis, and particularly when they used in conjunction with vaccine have shown preliminary promise against the experimental coccidial infection.

In this experiment, serum IFN-γ titers and IL-2 production of splenocytes were determined at 7 and 14 days post-infection. The overall mean serum IFN-γ of the groups fed
with the extracts and immunized with vaccine was significantly (P<0.01) higher compared with the vaccine group at 7 and 14 days p.i., whereas that of the groups fed with the extracts was not significantly different from the vaccine group. Of the three extracts, AstE showed the highest IFN-γ titers at 7 days p.i., and the TreE+V group had significantly higher IFN-γ titers compared with both LenE +V and AstE+V groups. The IL-2 production of splenocytes showed a similar pattern to the serum IFN-γ titers. Seven days p.i., the overall mean IL-2 level of the groups fed with extracts and immunized with vaccine was significantly (P<0.01) higher compared with the group immunized with the oocyst vaccine only, but that of the groups fed with the extracts were not significantly different from the vaccine group. At 14 days p.i., the overall mean IL-2 production of both the groups fed with the extracts and the groups fed with the extracts and immunized with vaccine was not significantly different from the vaccine group. A significant difference in IL-2 production of splenocytes between different extracts was not found. The use of the mushroom and herb polysaccharide extracts might enhance T-cell immune responses, characterized by IFN-γ and IL-2 secretion, against coccidiosis in chickens, and particularly when they are used in conjunction with vaccine.

In the second anti-coccidial trial, a total of 150 broiler chicks were assigned to 5 groups: three groups infected with *E. tenella* and fed with extracts (LenE, TreE and AstE), and two control groups infected with or without *E. tenella*. The three extracts were given at the level of 1 g/kg of the diet from 8 to 14 days of age. Specific systemic and cecum mucosal antibody production, proliferation of splenocytes, and peripheral T and B lymphocytes were measured during three weeks following the *Eimeria* infection in order to study cellular and humoral immune responses of *Eimeria tenella* infected chickens. A significant higher production of specific IgA, IgM (at day 14 and 21 p.i.) and IgG (at day 21 p.i.) were detected in the *Eimeria* infected groups fed with the extracts than in the infected group not fed with the extracts. Of the three extracts, *TreE* stimulated a slightly high production of specific IgM, and a high IgG production at day 21 p.i.. The cecal antibody production showed a similar trend to that of serum antibodies. The overall mean levels of cecal specific IgA and IgG of the groups fed with extracts was significantly higher compared with the group not fed with extracts at 14 and 21 days p.i. Of the three extracts, AstE-fed group showed the highest IgG production at day 7 p.i. Both TreE and LenE groups had significantly higher IgM and IgG levels compared with the AstE group at day 21 p.i.. The extract-fed groups also showed a higher antigen-specific proliferation of splenocytes compared with the group not fed with the extracts at 14 and 21 days p.i. The overall mean of both ERFC (at day 14 and 21) and EAC (%) (at day 14) of the groups fed with the extracts was significantly higher compared with the
group not fed with the extracts. It is concluded from this study that supplementation of mushroom and herb extracts resulted in enhancement of resistance to *E. tenella*, probably by enhancing both cellular and humoral immune responses against *E. tenella* in chickens.

Finally, an overview of effects of the mushroom and herb polysaccharide extracts, as alternatives for antimicrobials, on growth and health of chickens, and their mode of actions related to the gut ecosystem and immune system of chickens has been made. Some scientific and practical implications are also discussed in the general discussion.

In summary, the results presented and discussed in this dissertation demonstrated several clear points as follows:

1) Intact mushroom and herb materials and their polysaccharide extracts showed differences in their physico-chemical properties in terms of protein contents, sugar contents and sugar compositions.

2) The intact mushroom and herb materials and their polysaccharide fractions demonstrated differences in fermentability and led to significant shifts in the bacterial community when fermented *in vitro*. These medicinal mushroom and herb materials, particularly their polysaccharide extracts, show promise in altering microbial activities and composition in chicken ceca.

3) The polysaccharide extracts showed a slightly significant effect on growth performance during the first two weeks only after supplementation. Almost no significant effects on weights of immune and GIT organs were found in normal broilers.

4) The polysaccharide extracts significantly enhanced body growth and manipulated cecal microbial ecosystem such as viscosity and microbial species in infected chickens (AMG). Potential beneficial bacteria were significantly increased by the extracts. Chicken growth and microbial populations were increased with increased *LenE* dose.

5) The polysaccharide extracts showed significant effects on growth, immune responses as well as growth of immune organs and development of GIT fragments in coccidial infected chickens, particularly when they were used in conjunction with vaccine.

6) The use of the mushroom and herb polysaccharide extracts might enhance T-cell immune responses, characterized by IFN-γ and IL-2 secretion, against coccidiosis in chickens.

7) Supplementation of mushroom and herb extracts resulted in enhancement of resistance to *E. tenella*, probably by enhancing both cellular and humoral immune responses against *E. tenella* in chickens.
SAMENVATTING
**Samenvatting**

In de pluimveehouderij worden antibiotica gebruikt voor medicinale doeleinden, maar vooral als voerbespaaider (‘groeibevorderaar’). Het gebruik van deze middelen veroorzaakt echter een imbalans in de maagdarmflora en er kunnen resistentie bacteriestammen ontstaan. Dit laatste is vanuit het oogpunt van de humane geneeskunde uiterst onwenselijk. Vanwege deze nadelen heeft de EU het gebruik van bepaalde antibiotica als groeibevorderaar recent verboden. Zo’n verbod binnen de intensieve veehouderij heeft verstrekkelijke gevolgen voor de gezondheidsstatus en het groeivermogen van jonge landbouwhuisdieren, zoals biggen en vleeskuikens. Vanaf het moment dat het verbod van kracht werd, is men in de veehouderij intensief op zoek gegaan naar alternatieven (probiotica, prebiotica of andere voederadditieven). In dit proefschrift worden chinese medicinale kruiden als mogelijk alternatief bestudeerd.

In een eerste studie werden vier doseringen (0.25, 0.5, 1 en 2 g/kg) van een Chinees kruidencomplex (CKC) vergeleken met een antibioticum (Virginiamycine (VRG); 20 mg/kg). Groei, voeropname, voederconversie en uitval werden bestudeerd over een periode van 7 tot 28 dagen leeftijd bij 720 vrouwelijke vleeskuikens, verdeeld over de verschillende proefgroepen. Na 28 dagen werden kuikens van drie groepen (geen dosering, VRG en 0.5 g/kg CKC) gedissecteerden ten behoeve van het bestuderen van levergewicht en leverkleur, alsook gewichten van delen van het maagdarmkanaal. Uit dit experiment kwam naar voren dat de CKC-groepen in een aantal gevallen een positieve respons liet zien in vergelijking tot de controle groep (= geen dosering). De ontwikkeling van het maagdarmkanaal (in termen van gewichtstoename) bleek echter niet verbeterd. De CKC gevoerde groepen vertoonden, in vergelijking tot de VRG- en de controlegroep, een meer uniforme leverkleur, hetgeen in de chinese geneeskunde duidt op een betere gezondheidsstatus. Ondanks het feit dat de resultaten wat wisselend waren, bleek dat het Chinees kruidencomplex over het algemeen een licht positief effect liet zien op de bestudeerde parameters.

Er is nog maar weinig bekend over de bio-actieve componenten (met name suikers) in verschillende kruiden en ook het werkingsmechanisme is nog grotendeels onopgehelderd. In het vervolg van de studie werd daarom een aantal
experimenten uitgevoerd met als doel het werkingsmechanisme van bio-actieve componenten van een aantal bekende kruiden te bestuderen, alsook het verkrijgen van inzicht in het effect van toevoeging van deze componenten in een vleeskuikenvoer op de immuunrespons, de maagdarmflora en de groei van vleeskuikens.

Uit de Chinese literatuur blijkt dat de koolhydraatfractie van twee paddestoel-species, te weten *Tremella fuciformis* (*TreS*) en *Lentinus edodes* (*LenS*), en een kruid, *Astragalus Membranacea Radix* (*AstS*), immuun-actieve eigenschappen bezitten en dus in potentie als vervanger zouden kunnen dienen voor antibiotica bij pluimvee. De mogelijk positieve effecten op groei en immuunmodulatie van deze producten, specifiek bij pluimvee, zijn echter nog nauwelijks bestudeerd. Vandaar dat specifiek voor deze drie producten is gekozen in het vervolg van deze studie, uitgaande van de hypothese dat de koolhydraatfractie van deze producten selectief de groei van bepaalde bacteriestammen kan bevorderen (met name in de blinde darmzakken) en op die manier darmgezondheid verbetert en het afweersysteem versterkt.

Het doel van dit onderzoek was:

1. De koolhydraatfractie van deze 3 producten te isoleren en te identificeren, alsook de fysisch-chemische eigenschappen van de extracten te bestuderen.
2. *De in vitro* fermentatie-activiteit van de extracten te bestuderen.
3. De immuunmodulerende werking van de extracten te onderzoeken bij kuikens met een coccidiose infectie.
4. De effecten op groei en voeropname van de extracten te bepalen.
5. Enige opheldering te verkrijgen over het werkingsmechanisme op maagdarmniveau, gerelateerd aan het afweersysteem bij kuikens.

In hoofdstuk 1 wordt een uitgebreid overzicht van de beschikbare literatuur gegeven die deels, vanwege de chinese taal, voor niet-chinezen moeilijk toegankelijk is. Er wordt een overzicht gegeven van de producten die medicinale activiteit vertonen en in detail wordt ingegaan op de drie producten uit dit proefschrift. De meeste publikaties, beschreven in dit overzicht, baseren hun conclusies op studies met ratten, mensen en enkele met pluimvee. In het algemeen blijkt uit de literatuur dat de betreffende extracten de groei van immuun-actieve organen stimuleren, het aantal en de activiteit van immuun-actieve cellen vergroten en de cellulaire en humorale immuunrespons verbeteren. De meeste producten worden
gebruikt als adjuvant van een vaccin, in therapeutische doseringen of als voedingssupplement.

Bij aanvang van de studie werd de koolhydraatfractie uit de drie producten geïsoleerd met behulp van tri-chloorazijnzuur (verwijdering eiwitfractie) en een 96%-ethanoloplossing (hoofdstuk 2). Beide paddestoelen hadden een duidelijk lagere koolhydraatfractie (8-10 %) dan het kruid (31 %). Een volledige suikeranalyse (fenol-zwavelzuur methode) van de drie producten (S), alsook van de drie extracten (E), leverde de volgende suikersamenstelling op: LenS bevatte fructose, galactose, glucose, mannose, xylose en glucoronzuur in een molaire verhouding van 0.1: 0.2: 9.1: 0.1: 0.5: 0.1; TreS bevatte fructose, galactose, glucose, mannose, xylose en glucoronzuur in een molaire verhouding van 0.9: 0.1: 1.5: 4.1: 0.5; en AstS bevatte rhamnose, galactose, glucose, mannose, xylose en glucoronzuur in een molaire verhouding van 0.6: 0.2: 8.3: 0.4: 0.1: 0.3. Van de drie extracten bevatte AstE aanzienlijke hoeveelheden glucose. TreE bevatte daarentegen fructose, galactose, glucose, mannose, xylose en glucoronzuur in een molaire verhouding van 0.8: 0.2: 3.0: 1.6: 4.5: 0.2; LenE bevatte galactose, glucose, en xylose in een molaire verhouding van 0.8: 8.8: 0.5. Zowel de suikerhoeveelheid als de suikersamenstelling verschillen nogal tussen de producten en de respectievelijke extracten, maar ook tussen de extracten onderling.

In de hoofdstukken 3 en 4 is de in vitro fermentatie-activiteit van de producten en de respectievelijke extracten bestudeerd, alsook de veranderingen in de microbieëse populatie, daarbij gebruikmakend van microflora afkomstig uit de blinde darmen van kuijens op een standaardvoer. De fermentatiekinetiek werd bestudeerd met behulp van de cumulatieve gasproductie-techniek, waarbij fermentatiegassen, vluchtige vetzuren (VVZ) en ammoniak werden gemeten. Met behulp van PCR-technieken in combinatie met gel electroforese (DGGE) werden veranderingen in de microbieëse populatie vóór en na fermentatie bestudeerd. Gasproductiekinetiek vertoonde een monofasisch patroon voor AstS en LenS en een difasisch patroon voor TreS. Gasproductiecurves waren vergelijkbaar tussen producten en extracten, ofschoon de extracten een duidelijk hogere fermentatiesnelheid lieten zien. AstE werd snel afgebroken en gefermenteerd, terwijl TreE duidelijk langzamer werd afgebroken. Verschillen in totale gasproductie tussen product en extract bleken groter voor Ast dan voor Len en Tre. AstE en LenE hadden de laagste pH-waarde. De intacte producten produceerden minder VVZ dan de extracten. Daarentegen was er wel
een duidelijker vertakking aanwezig.

Zowel de producten alsook de extracten veroorzaakten een verandering in de bacteriepopulatie na \textit{in vitro} fermentatie. De ‘similarity index’ van de DGGE bandpatronen van de producten, vergeleken met het inoculum en de blanco’s, varieerde tussen de 40 en 58%. Binnen deze range vertoonde \textit{TreS} de laagste indexwaarde en Ast\textit{S} de hoogste. Voor de extracten gold een variatie tussen de 23 en 80%. Hierbinnen vertoonde \textit{LenE} de laagste indexwaarde en Ast\textit{E} de hoogste. De ‘similarity index’ van de DGGE bandpatronen was duidelijk lager bij \textit{LenE} dan bij \textit{LenS}, vergeleken met het inoculum en de blanco’s, en hoger bij Ast\textit{E} dan bij Ast\textit{S}. Er was geen verschil in index tussen \textit{TreS} en \textit{TreE}. De resultaten van deze studie toonden aan dat er verschillen waren in beschikbaarheid van substraat voor de microflora tussen de verschillende producten. Of de hierbovengenoemde \textit{in vitro} verschillen ook aantoonbaar zouden zijn in een \textit{in vivo} experiment werd in een groepstrof bestudeerd.

Een experiment werd uitgevoerd, waarbij het effect van de drie extracten in verschillende doseringen (0.5, 1, 2, 3 en 4 g/kg) werd onderzocht op de dagelijkse groei en de ontwikkeling van het maagdarmkanaal en de immuunorganen in vleeskuikens (hoofdstuk 5). De extracten werden aan het voer toegevoegd gedurende de periode van 7 tot 14 dagen leeftijd en vergeleken met een ongesupplementeerde groep en een antibioticum groep (Virginiamycine (VRG), 20 mg/kg). Er bleek geen verschil te zijn tussen de extract- en de VRG-groepen ten aanzien van groei, voeropname en voederconversie. Tussen de extracten bleek er wel verschil te bestaan, vooral in de groei over de gehele periode (7 tot 28 dagen). Met name \textit{LenE}-gesupplementeerde kuikens lieten een betere groei en vc zien dan \textit{TreE}- en Ast\textit{E}-kuikens. Er was geen verschil tussen de extracten en VRG ten aanzien van de ontwikkeling van organen van het verteringsapparaat en de immuunorganen.

De verwachting was dat de kuikens wellicht meer profijt van de extracten zouden ondervinden als er een zogenaamde ‘challenge’ zou plaatsvinden. Een volgend experiment (hoofdstuk 6) werd uitgevoerd, waarbij 135 kuikens werden geïnfecteerd met \textit{Aviaire Mycoplasma Gallisepticum} (AMG) en verdeeld over 9 proefgroepen: Len\textit{E} supplementatie in doseringen van 1, 2, 3, 5 en 10 g/kg, Tre\textit{E} en Ast\textit{E} beide in een dosering van 2 g/kg, een antibioticum (Apramycine (APR), 20 mg/kg) en een controlegroep (=zonder toevoegingen). De toevoegingen werden verstrekt op een
leeftijd van 15 tot 21 dagen. Groei, pH van de ceca-inhoud, viscositeit en bepaalde bacteriepopulaties werden gemeten op 28 dagen leeftijd. De groei was hoger in de APR-groep ten opzichte van de ongesupplementeerde groep. De extract-groepen namen een tussenpositie in. De toevoeging van extract had geen effect op de pH van de ceca-inhoud. Daarentegen was de viscositeit wel verhoogd ten gevolge van de extract-toevoeging. In tegenstelling tot het antibioticum, stimuleerden de extracten het aantal *Bifidobacteriën* en *Lactobacilli* en verlaagden het aantal *Bacteriodes* en *E.coli* bacteriën. Binnen de extracten gaf *LenE* het grootste effect te zien. Ook de respons bleek significant toe te nemen bij een hogere dosering.

Om het werkingsmechanisme van een extract-supplementatie goed te kunnen bestuderen, werden twee experimenten uitgevoerd (hoofdstukken 7, 8 en 9). In deze experimenten werd de immuunrespons bestudeerd bij vleeskuikens die geïnfecteerd werden met *Eimeria tenella*. In het eerste experiment (hoofdstukken 7 en 8) werd voornamelijk gekeken naar de lichaamsgroei en de ontwikkeling van immuun- en maagdarmorganen, oocyst output, lesie score en enkele immunologische parameters zoals cytokinen (IFN-γ en IL-2), ‘splenocyt proliferatie’ en E-rosetvorming van T en B-lymfocyten, gemeten vóór en na infectie. In totaal 180 vleeskuikens werden verdeeld over negen proefgroepen: drie groepen kregen elk één van de drie extracten (*LenE*, *TreE* en *AstE*), drie andere groepen kregen eveneens de drie extracten maar werden ook gevaccineerd met levend (oocyst) vaccin (*LenE +V*, *TreE+V* en *AstE+V*), één gevaccineerde groep en twee controlegroepen (een geïnfecteerde en een niet-geïnfecteerde groep). Het vaccin werd verstrekt op vier dagen leeftijd en de extracten (1 g/kg in het voer) van 8 tot 14 dagen leeftijd. Op 18 dagen leeftijd werden de kuikens van acht groepen geïnfecteerd met $9 \times 10^4$ gesporuleerde oocysten.

Kuikens die gevoerd werden met één van de extracten hadden een lager lichaamsgewicht, meer oocyst output en een hogere lesie score op 7 dagen na de infectie dan de kuikens die waren gevaccineerd. Er bleek echter een additioneel effect te bestaan van extract toevoeging én immunisatie ten aanzien van lichaamsgroei (met name *LenE* en *TreE* verhoogden het lichaamsgewicht nog meer dan alleen immunisatie) en, in mindere mate, oocyst output (met name *LenE* en *AstE* verlaagden de output meer dan alleen immunisatie). Thymus en bursa werden duidelijk zwaarder door vaccinatie; extractsupplementatie leverde iets zwaardere immuunorganen op. De ontwikkeling van het maagdarmkanaal werd niet beïnvloed.
door de vaccinatie *per se*, licht positief beïnvloed door de extractbehandelingen en significant negatief beïnvloed door de extract- + vaccinatiebehandeling. Het lijkt er dus op dat er soms een additioneel effect van extractsupplementatie optreedt naast een vaccinatie.

In hetzelfde experiment (hoofdstuk 8) werden serum IFN-γ titers en IL-2 productie bepaald op 7 en 14 dagen na de infectie (7 en 14 dagen p.i.), als maat voor de T-cel immuunrespons. Extracttoevoeging gaf dezelfde respons als een vaccinatie, echter een combinatie van extract + vaccinatie gaf een duidelijk hogere respons voor IFN-γ (7 en 14 dagen p.i.; met name *TreE* en *LenE*) en IL-2 (7 dagen p.i.).

In een volgend infectie-experiment (hoofdstuk 9) werden 150 kuikens toegewezen aan vijf proefgroepen: vier groepen werden geïnfecteerd met *E. tenella*, waarvan drie groepen elk één van de extracten kreeg en één groep niet werd gesupplementeerd. Groep vijf was de niet-geïnfecteerde controlegroep. De extracten (1 g/kg in het voer) werden gesupplementeerd aan het voer in de periode van 8 tot 14 dagen leeftijd. De cellulaire en humorale immuunrespons werd gemeten aan een aantal parameters gedurende drie weken, volgend op de infectie. Groepen die de extracten gevoerd kregen, hadden een significant hogere productie aan IgA, IgM (14 en 21 dagen p.i.) en IgG (21 dagen p.i.) dan de geïnfecteerde groep zonder extractsupplementatie. Cecale antilichaamproductie gaf een identiek beeld te zien. De extract gesupplementeerde groepen lieten ook een hoger antigeen-specifieke proliferatie van splenocyten zien, alsook hogere percentages aan ERFC en EAC, ten opzichte van de niet-gesupplementeerde groep.

In een afsluitende discussie worden de bevindingen uitgebreid bediscussieerd, waarbij geconcludeerd wordt dat deze extracten met name werken onder ‘challenge’ omstandigheden bij vleeskuikens. Enkele praktische implicaties worden eveneens behandeld.

Samenvattend hebben de resultaten, zoals beschreven in dit proefschrift, het volgende aangetoond:

1. Intact natuurlijk materiaal, afkomstig van bepaalde paddestoel- en kruiden-species, alsook de extracten daarvan, vertoonden duidelijke verschillen
in fysisch-chemische eigenschappen, zoals eiwit- en suikergehalte, alsook suikersamenstelling.

2. De intacte producten en extracten vertoonden verschillen in in vitro fermentatiekinetiek die leidden tot verschillen in bacteriepopulaties gemeten via DGGE.

3. Bij gezonde kuikens werd slechts een gering effect op groei en voederconversie waargenomen, indien extracten waren toegevoegd aan het voer. Er was geen effect waarneembaar op de gewichten van immuun- en maagdarmorganen.

4. De extracten (met name LenE) verhoogden de groei en beïnvloedden de microflora en de viscositeit in de blinde darmzakken van kuikens besmet met Aviaire Mycoplasma Gallisepticum. Bacteriestammen zoals Bifido en Lactobacilli werden verhoogd door extracttoevoeging.

5. De extracten verhoogden in een aantal experimentele situaties de groei, de cellulaire en humorale immuunrespons en de ontwikkeling van immuun- en maagdarmorganen in E. tenella geïnfecteerde kuikens, met name als de extracten werden gecombineerd met een immunisatie.
食用菌和中药多糖作为抗生素饲料添加剂替代品对鸡抗病促生长作用的研究

摘要
食用菌和中药多糖作为抗生素饲料添加剂替代品对鸡抗病促生长作用的研究

摘要

抗生素作为疾病防治药物和生长促进剂广泛应用于家禽养殖中。然而，饲喂抗生素饲料会扰乱动物胃肠道微生态系统，长期使用抗生素饲料添加剂还可能产生耐药性菌株和引起毒副作用及药物残留，因此，欧盟现已禁止使用抗生素作为鸡的生长促进剂。但是，由于抗生素饲料添加剂的使用对病原动物的健康生长，特别是对家猪与雏鸡的生长具有重要作用，因此，广泛寻找抗生素饲料添加剂替代品如益生菌、益生素和其他饲料添加剂已成为该领域的研究热点。

在初试中，我们选用了一中草药复方（CHM）（按 0.25、0.5、1 和 2 g/kg 四个水平添加）作为抗生素饲料添加剂 - 维及尼亚霉素（VRG，20 mg/kg）的替代品来饲喂肉鸡。试验采用 720 只肉雏鸡，观察 VRG 和 CHM 对 7 至 28 日龄肉鸡的生长发育的影响。试验结束后，分别从对照、VRG 和 0.5 g/kg CHM 三组中取 72 只鸡（每个重复 4 只）进行剖杀，检测肝脏颜色、肝脏及肠道各段（十二直肠+胰腺+空肠、回肠和盲肠）重量来探讨中药复方的作用机理。本次试验证明中药复方 CHM 能显著提高 7 至 21 日龄肉鸡的增重，但它对 21 至 28 日龄阶段的肉鸡没有明显作用。在添加的最初两周，0.5 g/kg 的 CHM 能显著提高鸡的日增重。同时，CHM 显著提高 21 至 28 日龄肉鸡的采食量，CHM 添组的饲料转化率在此阶段也较高。剖检结果表明，添加 CHM 或 VRG 对肝脏和肠道段的相对重量没有明显影响。但 CHM 组鸡的肝脏颜色较 VRG 和对照组均衡。本试验表明，复方 CHM 对提高鸡的生长发育有效。

在此试验基础上，我们开展了以下试验研究，来进一步探索天然药物的生物活性成分（多糖）及其对鸡生产性能及其肠道微生态系统和免疫系统的影响。

根据文献，来自食用菌 - 香菇 Tremella fuciformis (TreS) 和银耳 Lentinus edodes (LenS)，以及植物药 - 黄芪 Astragalus membranaceae Radix (AstS) 的免疫活性多糖似乎是非常有潜力的抗生素促生长效应替代品。食用菌和植物药类多糖（作为益生菌）可能选择性地刺激大肠中一种或少数几种有益菌的生长和/或活性，从而对动物肠道健康起重要作用。另外，食用菌和植物药类多糖可能提高鸡的免疫应答，具有抗病促生长作用。因此，中药多糖可能通过改善动物肠道生态环境和提高免疫功能，对动物的防御系统起着重要作用。

本项目的研究目的：
1. 分离和鉴定食用菌和植物药类多糖并研究其理化性质。
2. 研究食用菌和植物药类多糖的离体发酵特性。
3. 研究食用菌和植物药类多糖的抗病性（鸡球虫病）及其免疫调节作用。
4. 探索食用菌和植物药类多糖对鸡的促生长作用。
5. 探讨食用菌和植物药类多糖与动物肠道生态和免疫系统的相关性，阐明可能的作用机制。

第一章是有关真菌和植物药类（重点探讨香菇、银耳和黄芪）免疫活性多糖的研究现状。大量的文献报道，来自不同种类的真菌类药物和植物类药的多糖具有不同的理化性质和免疫活性作用。本章介绍了香菇（LenS）、银耳（TreS）和黄芪（AstS）多糖对鼠、人和鸡的免疫活性作用。一般来说，这些免疫活性多糖能刺激动物免疫器官的生长发育，增加免疫细胞的数量和活性，并能诱导细胞和体液免疫应答。中药多糖作为疫苗佐剂、治疗药物或饲料添加剂对鸡具有抗病作用，如抗菌、抗病毒或抗寄生虫活性。

在第二章中，重点介绍了分离提取香菇、银耳和黄芪水溶性多糖的方法。先用热水进行浸提，用三氯醋酸除去部分蛋白，再用 96%的乙醇沉淀多糖。最后得到了三种多糖提取物（LenE, TreE 和 AstE）。用酯-硫酸法测定多糖提取物的总糖含量。用 HPAEC 测定多糖的单糖组成。两种食用菌的多糖产量（8-10%）低于植物药的多糖产量（31%），其中多糖含量占干物质提取物的60%以上。单糖组成为：LenS 含有果糖、半乳糖、葡萄糖、甘露糖、木聚糖和葡萄糖醛酸，其摩尔比为 0.1: 0.2: 9.1: 0.1: 0.5: 0.1, TreS 含有果糖、半乳糖、葡萄糖、甘露糖、木聚糖和葡萄糖醛酸，其摩尔比为 0.9: 0.1: 3.5: 1.5: 4.1: 0.5; AstS 含有鼠李糖、半乳糖、葡萄糖、甘露糖、木聚糖 和半乳糖醛酸，其摩尔比为 0.6: 0.2: 8.3: 0.4: 0.1: 0.3。三种提取物中，AstE 含有大量的葡萄糖；然而，TreE 含有果糖、半乳糖、葡萄糖、甘露糖、木聚糖和葡萄糖醛酸，其摩尔比为 0.8: 0.2: 3.0: 1.6: 4.5: 0.2; LenE 含有半乳糖、葡萄糖和木聚糖，其摩尔比为 0.8: 8.8: 0.5。由此可见，原药及其多糖提取物具有不同的理化性质。

为了检测上述这些食用菌和植物药类及其多糖提取物的益生作用，我们开展了体外试验（见第三章和第四章）。采用鸡盲肠微生物作为接种物，研究原药（LenS、TreS 和 AstS）及其多糖提取物（LenE, TreE 和 AstE）的发酵特性和对肠道微生物菌群的影响。发酵动力学的检测，采用离体累积发酵产气技术。并测定发酵终产物的含量，如产气量、挥发性脂肪酸（VFA）和氨（NH₃）的含量。另外，通过对微生物 16S rDNA 基因片段的 PCR 扩增，结合变性梯度电泳（DGGE），分析发酵前后微生物菌群的变化。

试验结果表明，原药 AstS 和 LenS 的气体分布模式相近，呈单项分布模式。而 TreS 呈两阶段发酵模式。虽然多糖提取物的产气速率较原药快，但两者具有相似的发酵模式。AstE 发酵速度较快，而 TreE 发酵较慢。比较原药及其多糖提取物的总产气量，Ast 的总产气量差异较 Len 和 Tre 要大。AstE 和 LenE 发酵后，pH 值降得最低。虽然 LenS 和 AstE 发酵后的 VFA 产量最高，但原药发酵后所产生的 VFA 量较多糖提取物要少。发酵后的原药样品中含有较多的支链脂肪酸和较高的 NH₃浓度。
发酵后，原药及其多糖提取物引起盲肠微生物菌群的变化。原药样品中的微生物
DGGE 条带与原接种物和空白对照样相比，相似指数在 40%至 58%之间。其中，TreS 样
的相似指数最低，而 AstS 样的相似指数最高。多糖提取物样品中微生物 DGGE 条带与
原接种物和空白对照样相比，相似指数在 23%至 80%之间。其中，LenE 样的相似指数
最低，而 AstE 样的相似指数最高。与原接种物和空白对照样相比，LenE 样的 DGGE
相似指数较 LenS 样显著低，而 AstE 样的 DGGE 相似指数较 AstS 样显著高，而 TreS
样和 TreE 样的 DGGE 相似指数没有显著差异。发酵动力学及其终产物含量分析结果表
明，不同的原药（食用菌和植物药）及其多糖提取物具有不同的体外发酵特性，因而对肠道微生物区系及其活性有不同的影响。一般来说，原药及其多糖提取物，特别是
多糖提取物，能显著提高肠道微生物活性及其菌群变化。

为了评价多糖提取物对鸡的促生长作用，我们开展了田间试验（第五章）：三种
多糖提取物（LenE、TreE 和 AstE）分别以 0.5、1、2、3 和 4 g/kg 的水平添加，添
加期为一周（7至 14 日龄）；设两个对照，抗生素（20 mg/kg VRG）和非添加组。该试
验结果表明，多糖提取物添加组的平均日增重、采食量和饲料转化率与抗生素组相比
没有显著差异；不同的提取物及其水平对 7至 28 日龄鸡的生产性能有显著影响。在 7
至 28 日龄期间，与 TreE 和 AstE 添加组相比，LenE 组日增重较高，饲料转化率
较低。1g/kg TreE 和 2 g/kg LenE 和 AstE 有较好的促生长作用。然而，与抗生素
相比，多糖提取物对免疫器官重量和胃肠道重量没有显著影响。

为了阐明多糖提取物的促生长作用机理，我们进行了第二个田间试验。正式试验
前，试验鸡自然感染了禽败血性支原体病（AMG）。135 只病鸡随机分成 9 个试验组：
LenE 添加组（以 1、2、3、5 和 10 g/kg 添加）；TreE 和 AstE 添加组（以 2 g/kg
添加）；安慰剂组（20 mg/kg APR）和非添加组。多糖和 APR 添加时间为一周（15 至
21 日龄）。添加饲喂一周后，测定鸡的日增重，和宰杀后盲肠内容物的 pH 值、粘度和
主要微生物菌群的含量。检验多糖提取物添加组（0.2%的 LenE、TreE 和 AstE）、
APR 和对照组间的显著性差异。另外，检测不同剂量的 LenE (1、2、3、5 和 10 g/kg)
对鸡增重和盲肠微生物菌群的影响。

本次试验表明，多糖提取物和 APR 能显著促进 AMG 感染鸡的生长，但多糖提取物
添加组的总体平均日增重显著地低于抗生素组。多糖提取物对盲肠内容物 pH 值没有
显著影响，然而，提取物和抗生素均显著提高盲肠内容物的粘度和微生物菌群的数量。
与 APR 相反，多糖提取物提高有益菌的数量，如双歧和乳酸菌的数量，而减少有害菌
的数量，如大肠杆菌和肠杆菌的数量。三种多糖提取物中，LenE 可明显增加鸡盲
肠双歧和乳酸菌的数量。试验结果表明，随着 LenE 剂量的增加，鸡的日增重及总需
氧菌和总厌氧菌的数量也增加，特别是双歧和乳酸菌显著增加。本试验证明，中药多
糖提取物能选择性促进 AMG 感染鸡肠道主要微生物的生长。
为了进一步探讨这些多糖提取物对动物的免疫调节作用，我们进行了两个抗球虫
试验，来观察它们对鸡免疫力的影响。在第一个抗球虫试验中，180只雏鸡被随机分
为9组：3个多糖提取物添加组（LenE、TreE 和 AstE），3个组添加提取物并免疫接
种活卵囊（LenE+V、TreE+V 和 AstE+V），球虫免疫组和两个对照组（球虫感染组和非
感染组）。鸡4日龄时，给活卵囊疫苗，8至14日龄给多糖提取物（0.1% 添加于饲料
中）。18日龄时，除了阴性对照组外，所有鸡感染 9×10^8个孢子化卵囊。

在此试验中，感染球虫7天后，添加多糖提取物的鸡的日增重明显低于疫苗组。
但是，多糖提取物作为卵囊疫苗佐剂可显著提高感染鸡的日增重。三种提取物中，LenE
和 TreE 具有较好的促生长作用。卵囊免疫后的初次免疫应答中，多糖提取物大大增加
粪便卵囊的产量。感染7天后，添加提取物组的盲肠卵囊峰值和病变分高于疫苗组，
但提取物加疫苗组与单纯疫苗组间没有显著差异。三种多糖中，LenE 和 AstE 组的盲
肠卵囊峰值较低。添加提取物组鸡的胸腺和腔上囊相对重量较单纯感染组稍高，但肝
脏重量较低。感染7天后，与多糖提取物添加组相比，单纯疫苗组鸡的胸腺和腔上囊
相对重量较高，肝脏和脾脏相对重量较低。试验发现，各组鸡的GIT相对重量也有显
著差异。感染7天后，与单纯疫苗组相比，多糖提取物添加组的GIT（十二指肠、回肠
和盲肠）重量显著提高，然而，添加提取物并加疫苗组的GIT（食道+胃囊、腺胃+肌胃、
空肠和回肠）显著降低。感染7天后，三种多糖提取物中，AstE 组的腺胃+肌胃和空
肠较重。因此，与多糖提取物相比，卵囊活疫苗能显著提高感染鸡的生长和免疫应答。
多糖提取物与疫苗一起使用时效果更佳。

本试验还测定了球虫感染后第 7 和 14 天血清 IFN-γ 效价和脾脏细胞 IL-2 的产
量。添加多糖提取物和疫苗组的血清 IFN-γ 效价显著高于单纯疫苗组。然而，单纯
提取物添加组与单纯疫苗组之间没有显著差异。三种多糖提取物中，感染第 7 天后，
AstE 组的 IFN-γ 效价最高，而 TreE+V 组的 IFN-γ 的效价显著高于 LenE+V 组和 AstE
+V 组。脾脏细胞 IL-2 产量与血清 IFN-γ 效价基本一致。感染第 7 天后，提取物且加
疫苗组的平均 IL-2 水平显著（P<0.01）高于单纯疫苗组，提取物添加组的平均 IL-2 水
平与单纯疫苗组之间没有显著差异。感染第十四天后，提取物添加组及多糖加疫苗组
的平均 IL-2 水平都与单纯疫苗组没有显著差异。IL-2 产量在不同提取物添加组间没
有显著差异。多糖提取物抗球虫作用可能与提高 T- 细胞免疫应答（刺激细胞因子
IFN-γ 和 IL-2 的分泌）有关。特别是当提取物与疫苗一起使用时，效果更为明显。

在第二个抗球虫试验中，150 只鸡分为 5 个组：3 个组感染 E. tenella 并添加多
糖提取物（LenE、TreE 和 AstE），2 个对照组（E. tenella 感染组和非感染组）。三种
多糖提取物以 1 g/kg 剂量添加于饲料中。添加时间为一周（8 至 14 日龄）。感染 Eimeria
第 7、14 和 21 天后，分别测定外周和盲肠特异性抗体、脾细胞增殖量和外周 T、B 淋
巴细胞百分数，来观察 E. tenella 感染后，鸡的细胞和体液免疫应答。多糖添加组的
特异性抗体 IgA、IgM（感染后第 14 和 21 天）和 IgG（感染后第 21 天）水平高于单纯感染组。三种多糖提取物中，感染第 21 天后，TreE 组的特异性 IgM 和 IgG 都较高。

盲肠与血清抗体水平基本一致。感染第 14 和 21 天后，多糖提取组的特异性 IgA 和 IgG 平均水平显著高于单纯感染组。三种多糖提取物中，感染第 7 天后，AstE 组的 IgG 水平最高。感染第 21 天后，与 AstE 组相比，TreE 和 LenE 组的 IgM 和 IgG 水平显著提高。感染第 14 和 21 天后，多糖提取物添加组的抗原特异性淋巴细胞增殖量显著高于单纯感染组。多糖提取物添加组的平均 ERFC（第 14 和 21 天）和 EAC (%)（第 14 天）显著高于单纯感染组。由此可见，添加中药多糖可提高鸡的细胞和体液免疫，因而提高鸡对球虫病的免疫力。

在最后一章中，我们总结和讨论了食用菌（香菇和银耳）和植物药（黄芪）及其多糖提取物作为抗生素替代品对鸡的抗病促生长作用，及它们对肠道微生态系和免疫系统的作用机制。另外，阐述了中药多糖在禽养殖业中的实际应用，展望了今后对中药多糖的研究前景。

综上所述，本项目研究阐明了以下几点；

1. 食用菌（香菇和银耳）和植物药（黄芪）及其多糖提取物具有不同化学性质如蛋白含量、总糖含量和单糖组份。

2. 离体发酵实验结果表明，食用菌（香菇和银耳）和植物药（黄芪）及其多糖提取物具有不同的发酵特性，且能显著改变盲肠微生物的活性及其组份，特别是多糖提取物，其效果更为显著。

3. 添加香菇、银耳和黄芪多糖提取物只在前两周对正常鸡的生产性能稍有提高，但对正常鸡的免疫器官和胃肠道各段的生长发育没有明显影响。

4. 添加香菇、银耳和黄芪多糖提取物能显著提高感染鸡（AMG）的日增重和改变盲肠微生态环境如粘度，以及盲肠微生物的数量，特别是增加有益菌的数量。而且随着 LenE 剂量的增加，鸡的日增重和肠道微生物数量也逐渐增加。

5. 添加香菇、银耳和黄芪多糖提取物能显著提高鸡对球虫病的抵抗力，表现在感染鸡的体重、免疫应答和免疫器官和胃肠道各段的重量，特别是当多糖与球虫疫苗一起使用时。

6. 添加香菇、银耳和黄芪多糖提取物能提高鸡的 T-细胞免疫应答和诱生细胞因子（IFN-γ 和 IL-2）的分泌，从而提高鸡对球虫病的免疫力。

7. 添加香菇、银耳和黄芪多糖提取物可提高球虫感染鸡的细胞和体液免疫应答，从而提高鸡对疾病的抵抗力。

本中文摘要是特别为我亲爱的父亲，母亲和家人而作。
CURRICULUM VITAE

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