# Mushroom and Herb Polysaccharides as Alternative for Antimicrobial Growth Promoters in Poultry

| | |
|---|---|
| Promotor: | **Prof. Dr. Ir. M. W. A. Verstegen**<br>Hoogleraar Diervoeding<br>Wageningen Universiteit |
| Co-promotor: | **Dr. Ir. R. P. Kwakkel**<br>Universitair docent<br>Departement Dierwetenschappen<br>Wageningen Universiteit |
| Co-promotor: | **Dr. B. A. Williams**<br>Universitair docent<br>Departement Dierwetenschappen<br>Wageningen Universiteit |
| Co-promotor: | **Prof. Z. Q. Yang**<br>Institute of Lanzhou Animal Science and Veterinary Pharmaceutics, Chinese Academy of Agricultural Sciences, P. R. China |

**Samenstelling Promotiecommissie:**

| | |
|---|---|
| Prof. Dr. H.F.J. Savelkoul | Wageningen Universiteit |
| Prof. Dr. Ir. S. Tamminga | Wageningen Universiteit |
| Prof. Dr. J. Bindels | Wageningen Universiteit |
| Prof. Dr. C. Wenk | Institute of Animal Sciences, ETH Zürich (Switzerland) |
| Prof. Dr. J.A. Stegeman | Universiteit Utrecht |

# MUSHROOM AND HERB POLYSACCHARIDES AS ALTERNATIVE FOR ANTIMICROBIAL GROWTH PROMOTERS IN POULTRY

Fucun Guo

**Proefschrift**

ter verkrijging van de graad van doctor

op gezag van de rector magnificus

van Wageningen Universiteit,

Prof. Dr. Ir. L. Speelman,

in het openbaar te verdedigen

op woensdag 18 juni 2003

des namiddags te vier uur in de Aula

Guo, F.C., 2003. Mushroom and herb polysaccharides as alternative for antimicrobial growth promoters in poultry.
Ph.D. Dissertation, Wageningen Institute of Animal Sciences, Department of Animal Nutrition, Wageningen University, P.O. Box 338, 6700 AH Wageningen, The Netherlands.
With ref.- with summary in Dutch and Chinese.

ISBN 90-5808-826-X

## 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 membranacea 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

First of all, I am grateful to my co-promotor, Dr. René Kwakkel, for bringing me into the classic and novel research field of Chinese herbal medicine use as antimicrobial growth promoters in poultry in the western world. He opened the door to my PhD study and is well known for his excellent supervision. Most important I learned from him is how to become thoughtful, logical and accurate as a scientist. I also appreciate very much the hospitality and the cordial atmosphere created by his wife Emmy and their daughters Hedi and Sanna, which made my stay in Wageningen a great pleasure. Words can never be sufficient to express my thanks to him.

Very special gratitude is given to my co-promotor Dr. Barbara Williams, for introducing me the new techniques, such as the gas production technique and the molecular technique based on 16S rDNA analysis, which certainly enriched my PhD study. I really appreciate all her instructions, suggestions and discussions. I also owe her very much for correcting the English language of this dissertation.

It is a great pleasure to thank my Promotor, Prof. Martin Verstegen, for his consistent support, encouragement and guidance. Despite his busy schedule, he always found time for discussions. I am very much appreciated for his caring, sense of humor and spending many weekends in reading and correcting my manuscripts even when his health was not always in good condition. I am also grateful to his wife Mariet for her great patience and hospitality.

I highly appreciate the people of the Animal Nutrition Group of Wageningen University for their help in all aspects. To Prof. Seerp Tamminga for all his support when I needed it most. I express my great gratitude to Dr. Huug Boer, Dick Bongers, Jane-Martine Muylaert and Truus Post for their guidance and assistance in chemical analysis. My special thanks to Betty Looijen and Anja Riksen for their kind help whenever I needed it. I also extend my thanks to the PhD candidates and the staff at Zodiac for their assistance in all ways.

I would like to express my sincere appreciation to my co-promotor, Prof. Yang Zhiqiang, and the employer of Institute of Animal Science and Veterinary Pharmaceutics, CAAS, for their supporting and helping me to fulfill the project. I also extend my thanks to my colleagues, Wei Yunxia, Li Xingpu, Li Hongsheng, Luo Jinying, Hu Tingjun, Yang Zuoting, and Hu Zhengying for their contribution to this dissertation. My special thanks to the followings in Gansu province for their either academic help or administrative support: Wang Yunlu, Liu Zaixing, Cheng Xiaohua, Liu Jiabiao, Cai Yingkui, and Bao Zhenggui.

I would like to express my thanks to Dr. Antoon Akkermans and his group, especially

Wilma Akkermans-van Vliet, Hans Heilig, Wen Yao and Sergey Konstantinov, Laboratory of Microbiology of Wageningen University, for their guidance with molecular biotechnology. I also owe thanks to Edwin Bakx, Laboratory of Food Chemistry of Wageningen University, for his sugar analysis.

I would express my thanks to Prof. Suo Xun, Laboratory of Parasitology and Parasitological Diseases, China Agricultural University, Li Wankun, Department of Parasitological Diseases, Gansu Animal Health Service, and their group for providing the experimental facilities, and for guiding and helping the experimental work.

I owe special thanks to Prof. Huub Savelkoul, Cell Biology and Immunology group, Dr. Henk Parmentier, Adaption Physiology Group, Wageningen University, Prof. John Patterson, Department of Animal Science, Purdue University, USA, and Enwu Liu, University of Saskatchewan, Canada, for critically reading the manuscripts.

I owe many thanks to Emmy Kwakkel for making the dissertation beautifully formatted. Special thanks go to Dr. Evert-Jan Bakker for his helping in statistics and for introducing Jesus Christ to me. I am very grateful to Dr. John Mattheij and his wife who have shown great concern on my study and provided me nice excursions and special dinners here.

During my stay in Wageningen, I also enjoyed all the helpful discussions and get-togethers with my Chinese friends who specialized in different research fields. My very special thanks go to those who brought me help and friendship.

At last, I would like to express my warmest gratitude to my parents, my husband Wankun and my daughter Tian for their love, understanding and support over years. I am indebted to them so much for long time separation. Their patience and encouragement are indispensable for the accomplishment of my study.

April 30, 2003
Wageningen

For Wankun and Tian

## CONTENTS

# 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 mannan oligosaccharides (MOS) (Lyons, 1994). It had been shown that non-digestible oligosacchaides 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 *Samonella* 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 Schaafsma, 1999), many beneficial effects have been reported from adding feed enzymes such as cellulase, amylase, arabinase, pectinase, phytase, protease, lipase, xylanase, ð-galactosidase and ß- 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 phytogenic 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 heterosides, 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, Codongopsis Radix, Astragalus membranacea Radix, Epimedii Herba, Ligustri 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 membranacea 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 enhanncers. 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 promotor 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.

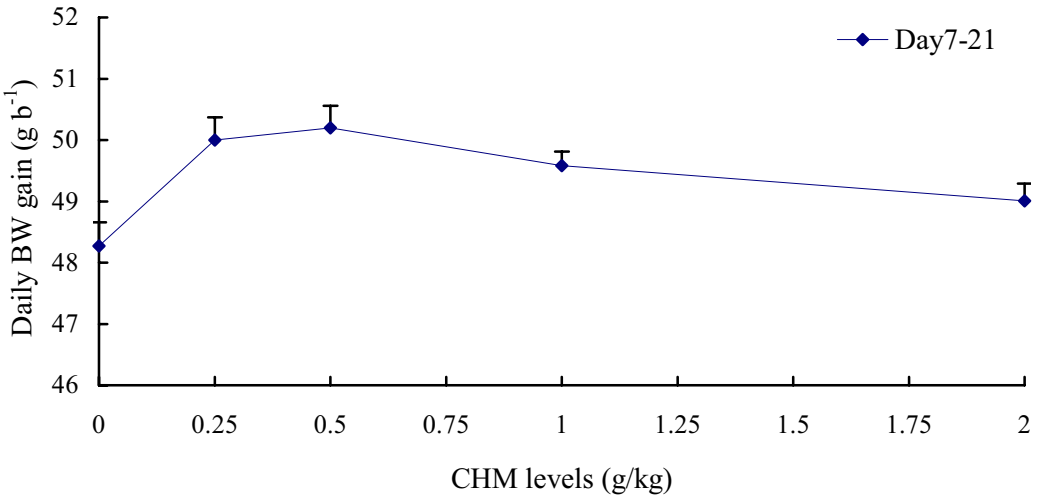



**Figure 1.** Effect of CHM supplemental levels on BW gain of female broiler chicks from day 7 to day 21, which was studied in the pilot experiment. Results are given as means (n=6) ± SE; CHM concentrations are 0, 0.25, 0.5, 1 and 2g/kg CHM. The quadratic regression function for BW gain from day 7 to 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 *Astragali membranacea* 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 membranacea 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 $NH_3$ 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 (cecal 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-r 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-lymphocye 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.

## REFERENCES

Andreason, M. 2000. Consequences of the ceased used of growth promoters presented at DVHS 2000, 4th and 5th May 2000, Denmark.

Bedford, M. R., and H., Schulze. 1998. Exogenous enzymes for pigs and poultry. Nutri. Res. Rev. 11: 91-114.

Brufau, J., M., Francesch, A. M., PèreZ-Vendrell. 2002. Exogenous enzymes in poultry feeding. Recent development. Arch. Geflügelk. 66 (Sonderheft II):46.

Buddington, P. K., and E., Weiher, 1999. The application of ecological principles and fermentable fibers to manage gastrointestinal tract ecosystem. J. Nutr. 129: 1446S.

Cheng, F. S., T. J., Hu, and J. L., Liang. 1998. Enhance of VMD vaccine by 8301 polysaccharide in chickens . J. of Traditional Chinese Vet. Medicine (in Chinese) 1: 8-9.

Damien, G., and B., Chantal. 1998. Synthesis of novel fructo-oligosaccharides (FOS) by enzymatic reaction. Comptes Rendus des Seances de la Societe de Biologie et de ses Filiales. 192 (4): 711-717.

Demir, E., and C., Eser. 2002. The comperative effects of the addition of enzyme, mannano-ligosaccharide, probiotic and antibiotic to wheat-based broiler chick diets on growth performance and some small intestine parameters. Arch. Geflügelk. 66 (Sonderheft II): 105-106.

Ensminger, M. E., J. E., Oldfield and W. W., Heinemann. 1990. Feeds and Nutrition, The Ensminger publishing company, Clovis, California, USA, 1544pp.

European Commision/scientific steering committee, DG XXIV. 1999. Opinion of the steering committee of antimicrobial resistance. 28 May.

Fan, B. T., Y. L., Yuan and J. L., Liang. 1993. Study of mechanism of enhanced avian cholera vaccine by 8301 polysaccharide. J. of Traditional Chinese Vet. Medicine (in Chinese) 1: 5-10.

Fernandez, F., M., Hinton, and B., Van Gils. 2000. Evaluation of mannan-oligosaccharides and the competitive exclusion of Samonella enteritidis colonization in broiler chicks. Avian Path. 29: 575.

Fuller, R. 1989. Probiotics in man and animals. J. Appl. Bacteriol. 66: 365-378.

Fulton, R. M., B. N., Nersessian, and W. M., Reed. 2002. Prevention of Salmonella enteritidis infection in commercial ducklings by oral chicken egg-derived antibody alone or in combination with probiotics. Poult. Sci. 81 (1): 34-40.

Gibson, G. R., and M. B., Roberfroid. 1995. Dietary modulation of the human colonic microflora: introducing the concept of prebiotics. J. Nutr., 125:1401.

Giesting, D. W., and R. A., Easter. 1985. Response of starter pigs to supplement of corn soybean meal diets with organic acids. Journal of Ani. Sci., 60: 1288-1294.

Gopal, K. 2002. Use of cud liquor cellulase-protease enzyme complex and gram negative bacilli as growth promoter in broiler-an information to enzyme manufacturing industries. Arch. Geflügelk.66 (Sonderheft II): 108.

Guo, F. C., R. P., Kwakkel, M. W. A., Verstegen. 2000. The use of Chinese herbs as alter-native for growth promoters in broiler diets (abs.) The XXI International Poultry Congress, Montreal, Canada.

Hayakawa, K., J., Mizutani, T., Wasai and I., Yoshihara. 1990. Effect of soybean oligosaccharides on human feacal flora. Microb. Ecol. Health Dis. 3: 293-303.

Hinton, M. H., 1988. Antibiotics, poultry production and public health. World's Poult. Sci. J. 44: 67-69.

Hu, T. J., J. L., Liang, and F. S., Cheng. 1998. The use of 8301 polysaccharide as an adjuvant in coccidial infected chickens. J. of Grass and Livestock (in Chinese), 6: 5-7.

Huyghebaert, G., G., de Groote, G., de Broek, and Velzeboer. 1999. Effect on technical results in broilers of several ways of addition organic acids versus addition of an antibiotic growth promoter. Proceedings of the 12th W. P. S. A. European Symposium on Poultry Nutrition, veldhoven, NL, pp136. Aug. 15-19.

Jamroz, D. J., Orda, C., Kamel, T., Wertelecki, J., Skorupiñska. 2002. Influence of phytogenic extracts on performance, digestibility and gut microbial status in broiler chickens. Arch. Geflügelk. 66 (Sonderheft II): 105.

Jensen, B. B., 1993. The possibility of manipulating the microbial acting in the digestive tract of monogastric animals. 44th Annual Meeting of the European Association for Animal Production Session IV. pp. 16-19.

Katta, Y. K., M., Ohkuma, R., Satouchi, and T., Yamamoto. 1993. Feed for livestock. Eur. Pat. Appl. 0549478A1: 1-14.

Korösi, L. 2002. Effects of natural products for broiler production parameters. Arch. Geflügelk. 66 (Sonderheft II):106.

Langhout, D. J., 1999. The role of the intestinal flora as affected by NSP in broilers. Proceedings of the 12th W.P.S.A. European Symposium on Poultry Nutrition, veldhoven, NL, pp 203-212. Aug. 15-19.

Langlois, B., E., Dawson, G. L., Cromwell, and T. S., Stahly. 1986. Antibiotic resistance following a 13 year ban. J. Anim. Sci. 62 (suppl.3): 18.

Li, C. M. 1998. Chinese Herb Feed Additives (in Chinese). Beijing: Chinese Agricultural University Press. No. ISBN 7-81002-360-8

Li, X. Y. 2000. Immuno-modulating components from Chinese medicines. Pharma. Biol. 38 (Supplement): 33-40.

Lizuka, N., K., Miyamoto, and S., Hazama. 2000. Anticachectic effects of Coptidis rhizoma, an anti-inflammatory herb, on esophageal cancer cells that produce interleukin 6. Cancer Letters, 158 (1): 35-41.

Lyons, T. P. 1994. A panorama of techniques, processes and products to address animal production problems today and tomorrow. In: Biotechnology in the Feed Industry: Proceedings of

Alltech's 10th Annual Symposium. Nottingham Unversity Press, Nottingham, UK pp 1-48.

Ma De Cesare A., G., Manfreda, V., Bondioli, F., Pasquali, and A., Franchini. 2002. Antibiotic resistance and ribotyping profiles of campylobacter isolates from a poultry meat processing plan. Arch. Geflügelk. 66 (Sonderheft II): 62.

MacManus, M. C. 1997. Mechanisms of bacterial resistance to anti-microbial agents. Am. J. Health Syst. Pharm. 54: 1420.

Mathlouthi, M, M., Larbier and M., Lessire. 1999. Effect xylanase supplementation on laying hen performance. Proceedings of the 12th W.P.S.A. European Symposium on Poultry Nutrition, veldhoven, NL, pp268. Aug. 15-19.

Miles, P. G., and S. T., Chang. 1997. Mushroom Biology – Concise Basics and Current Developments. World Scientific. Singapore. pp 194.

Nelson, F. E., L. S. Jenson and J. McGinnis. 1963. Studies on the stimulation of growth by dietary antibiotics. Poult. Sci. 42: 909-912.

Newman, M. G. 2002. Antibiotic resistance is a reality: novel techniques for overcoming antibiotic resistance when using new growth promoters. Pp98-106. Nutritional Biotechnology in the Feed and Food Industries. Proceedings of Alltech's 18th Annual Symposium. Nottingham University Press.

Pang, F. H., M. Q., Xie, and H. H., Ling. 2000. The investigation of Immunodulators tested for the results on the control of a coccidial infection. Chinese J. Vet. Parasit (in Chinese) 8(3): 1-3.

Peterson, R. A., G. H. Carpenter, W. T. Jones and J. A. kla. 1991. Effect of tetracycline hydrochloride and oxytetrocycline hydrochloride given via drinking water on early mortality of broiler chicks from twenty-eight-week-old dams. Poult. Sci. 70: 4, 1040-1042.

Policy and Laws, Ministry of Agriculture PRC. 2002. http://www.agri.gov.cn/zcfg/

Proudfoot, F. G., E. D., Jackson, and H. W., Hulan. 1990. The response of male chicken broilers to the dietary addition of virginiamycin. Poult. Sci. 69: 1713-1717.

Raddiffe, J. 2000. British Supermarkets: forging changes in poultry nutrition, Australian Poultry Science Symposium 12: 25-31.

Rolfe, R. D. 2000. The role of probiotic cultures in the control of gastrointestinal health. Symposium: Probiotic Bacteria: Implication for human Health. J. Nutr. 130:396S.

Rosen, G. D. 1995. Antibacterials in poultry and pig nutrition. In: Biotechnology In Animal Feeds And Feeding. VCH Publishers, New York NY, p. 143.

Rutkowski, A., A. J. M., Jansman, and R. P. M., Bremmers. 1999. Effect of enzyme supplementation to wheat and triticale based diets on digestibility and energetic value for chickens. Proceedings of the 12th W.P.S.A. European Symposium on Poultry Nutrition, veldhoven, NL, pp267. Aug. 15-19.

Samarasinghe, K., and C., Wenk. 2002. Turmeric (curcuma longa) and mannanoligosaccharides as antibiotic replacers in broiler diets. Arch. Geflügelk. 66 (Sonderheft II):107.

Schwarz, S., C., Kehrenberg, and E., Chaslus-Dancla. 2002. Antimicrobial resistance in bacteria from poultry: Resistance genes and their ways of spreading. Arch. Geflügelk. 66 (Sonderheft II): 57.

Shon, Y. H., J. H., Kim, and K. S., Nam. 2002. Effect of *Astragalus radix* extract on lipopolysaccharide-induced inflammation in human amnion. Biol. Pharma. Bull. 25 (1): 77-80.

Spring, P. C., Wenk, K. A., Dawson, and K. E., Newman. 2000. The effects of dietary mannoligosaccharide on cecal parameters and the samonella-challenged broiler chicks. Poult. Sci. 79: 205-211.

Stanley, V. G., Y. W., Park, and C., Gray. 1997. Effects of Mannan oligosaccharide (MOS) on aflatoxicosis, serum, liver, and egg cholesterol and egg production in chickens. Assoc. Res. Dir. 11th Biennial Research Symposium.

Subrata, S., L., Manda, G. C., Banerjee, and S., Sarkar. 1997. Effect of feeding yeasts and antibiotic on performance of broilers. Indian J. Poult. Sci. 32: 2, 126-131.

Tanaka, R., H., Takayama, and M., Morotomi. 1983. Effects of administration of Tos and Bifidobacterium breve 4006 on the fecal flora. Bifidobacteria Microflora 2: 17-24.

Thanga, T., M., Thiagarajan, V., Ramesh, T., Sivahumar, and T., Tamilvanan. 2001. Performance of broiler chicken under floor system of management fed with different processed feed and probiotics. Indian J. Ani. Res. 35: 2, 88-91.

U.S. Newswire. 2002. http://library.northernlight.com/FE20020226770000065.html?cb=0&dx=1006&sc=0

Verstegen, M. W. A., and G. J., Schaafsma. 1999. Some developments on alternatives for antibiotics as growth promotor. In: Nutrition and Gastrointestinal Physiology Today and Tomorrow. pp 65-73. Papers presented at the symposium held on the occasion of the retirement of J. Huisman.

Wang R. J., D. F., Li, and S., Bourne. 2002. Can 2000 years of herbal medicine history help us solve problems on the year 2000? Pp 273 – 291. in: Biotechnology in the feed industry. Proceedings of Alltech's 14th Anunual sysmposium. Nottingham University Press.

Wargovich, M. J., C., Woods, D. M., Hollis, and M. E., Zander. 2001. Herbals, cancer prevention and health. J. of Nutr., 131 (11Suppl): 3034-3036.

Wenk, C. 2002. Herbs, botanicals and other related substances. Arch. Geflügelk. 66 (Sonderheft II):46.

Wenk, C., and R., Messikommer. 2002. Turmeric (curcuma longa) as a feed additive in alying hens. Arch. Geflügelk.66 (Sonderheft II): 107

Wiese, A. C. and C. F. Petersen. 1959. Comparison of several antibiotics for chick growth. Poult. Sci. 38: 1259 (abstr.).

Wilson, S., and S., Kenyon. 2002. Avilamycin and mannanoligosaccharide as feed additives for broilers. Arch. Geflügelk. 66 (Sonderheft II): 115.

Wu, Y. H., and F., Wang. 1999. Research status on herbs' two-way immune regulating functions. J. of Traditional Chinese Vet. Medicine (in Chinese) 4: 19-22.

Xie, Z. Q. and S. Q., Niu. 1996. Comprehensive Book of Natural Resources and Chinese Herb Feed Additives (in Chinese). Xueyuan Science and Technology Press, Beijing, PRC.

Xue, M., and X. S., Meng. 1996. Review on research progress and prosperous of immune activities of bio-active polysaccharides. J. of Traditional Vet. Medicine (in Chinese) 3: 15-18.

Yang H. C., G., Lu and G., Lu 1998. Effect of pollen polysaccharide from corns on immunological afficacy of Newcastle disease vaccine and Egg Drop Syndrome vaccine in chickens. Chinese Vet. Medicine J. (in Chinese) 24 (6): 43.

Yang Y. and W. S., Feng. 1998. Brochures of isolation and extraction of chemical components from herbs (in Chinese). China Chinese Herb Medicine Press. Beijing.

Yu, J. G., and L.Y., Zhu. 2000. The use of AP against Infectious Bursa Disease in chickens. J. of Traditional Chinese Vet. Medicine (in Chinese) 6: 3-4.

Yuan, Y. L., B. T., Fang, and Y. X., Zhang. 1993. Efficacy of 8301 polysaccharides as an adjuvant against Avian Pasteurellosis in chickens. J. of Traditional Chinese Vet. Medicine (in Chinese) 3: 6-10.

Zhang, L. C., J. B., Wang, and Y. P., Sun. 1998. Effect of adding Chinese herb polysaccharide preparations to a Newcastle disease inactivated vaccine on the development of the immune organs in chickens. Chinese J. of Vet. Science (in Chinese) 18(4): 378-381.

Zhong, M., D. S., Zhang, J., Murata, I., Saiki, and Y., Itaru. 2001. Effects of *Astragalus* polysaccharide (APS) and *Astragalus* flavonoids (AF) on the cytotoxicity of LAK and NK cells in normal and tumor bearing mice. Sichuan Pharma. Bulletin (in Chinese) 38 (5): 718-722.

Zhou, R. H. 1993. Resource Science of Chinese Medicinal Materials (in Chinese), pp. 19-32. Bejing: China Medical & Pharmaceutical Science Press.

# Chapter 1

# IMMUNOACTIVE, MEDICINAL PROPERTIES OF MUSHROOM AND HERB POLYSACCHARIDES: ALTERNATIVE FOR ANTIMICROBIALS IN POULTRY DIETS

F.C. Guo

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands; Institute of Animal Science and Vet. Pharmaceutics, Chinese Academy of Agricultural Sciences, Lanzhou 730050, China*

H.F.J. Savelkoul

*Cell Biology and Immunology, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands;*

R.P. Kwakkel

*Animal Production Systems Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

B.A. Williams and M.W.A. Verstegen

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

World's Poultry Science Journal (2003) 59: 3 (in press)

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

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

**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 immunodulators 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 1.** Chinese herb medicine from plants (Zhou, 1993)

| Classification | Number of family | Number of species |
|---|---|---|
| Algae | 2 | 2 |
| Fungi | 5 | 6 |
| Pteridophyta | 6 | 7 |
| Spermatophyta | 110 | 398 |
| Gymnospermae | 5 | 8 |
| Angiospermae | 105 | 390 |
| Total | 123 | 413 |

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 membranacea Radix, Epimedii 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 ROPERTIES 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

| Polysaccharides | Origin | Glycoside linkages | Molecular weights (Daltons) | Reference |
|---|---|---|---|---|
| Lentinan | *Lentinus edodes* | ß-1, 3 glucan, | $5 \times 10^5$ | Liu *et al.*, 1999c |
| Glucan | *Saccharomyces cerevisiae* | ß-1, 6 glucan | $1\times 10^4$ | Blagoeva *et al.*, 1991 |
| Krestin | *Coriolus versicolor* | ß-1, 3; ß-1, 4, ß-1, 6 glucan | $1 \times 10^5$ | Zhang *et al.*, 2001 |
| Schizophyllan | *Schizophyllum commune* | ß-1, 3 and ß-1, 6 glucan | $1 \times 10^4$ - $1\times 10^6$ | Mol *et al.*, 1988 |
| Glucomannan | *Amorphophallus Konjak* | ß -1,4 D-mannose and D- glucose | 1- $2\times 10^6$ | Shimahara, 1975 |
| Fructan | *Gomphrena macrocephala* | ß-2, 6 fructan polymer | $5.5\times 10^3$- $1\times 10^4$ | Shiomi *et al.*, 1996 |

**Figure 1.** The roots of *Astragalus* (left), the fruit body of *Lentinus edodes* (middle) and the *Tremella ficuformis* (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 3** Characteristics of *Lentinus edodes* (*LenS*), *Tremella fuciformis* (TreS) and *Astragalus membranaceus Radix* (*AstS*)

| Parameters | Substrates | Contents | References |
|---|---|---|---|
| Family and common name | *AstS* | *Leguminosae* family; "Huang Qi" | Zhou, 1993; Xu, 1990 |
| | *LenS* | *Tricholomataceae* family; "Shiitake" | |
| | *TreS* | *Tremellaceae* family; "Silver Ear", "White Jerry Leaf", "Snow Ear", "Ying Er" | Chandra, 1989; Singer, 1961 |
| Cultivation | *AstS* | Seminal reproduction, stem grafting or branch separating | http://www.agri.ac.cn/agri_net/ |
| | *LenS* | Synthetic and natural logs | Royse *et al.*, 1985 |
| | *TreS* | Synthetic and natural logs | Huang, 1982 |
| Composition | *AstS* | Crude saponin (5.81%), astragaloside (0.14%), and polysaccharide (23%), flavonoids, triterpene glycosides, 22 kinds of amino acids and 14 kinds of micro-minerals. | Qi, 1987; Zheng *et al*., 1998<br>Ma *et al.*, 2000, 2002; Zhang *et al*., 200 |
| | *LenS* | Proteins, fats, carbohydrates, soluble fiber, vitamins, minerals and Lentinan, heteroglucan, Adenine derivative, guanosine 5'–mono- phosphate | Yang *et al.*, 1999 |
| | *TreS* | Dietary non-starch polysaccharides (70%) such as acidic polysaccharides and glucuronoxylomannan, and vitamin D. | Min, 1996 |
| Traditional use | *AstS* | White color, sweets and warm; Night sweats and deficiency of chi (fatigue, weakness, and loss of appetite), and diarrhea. | Zhou, 1993 |
| | *LenS* | Brown to black brown color, Steak-like with a pungent flavor; Asian cuisine as health maintaining food. | Zhou, 1993 |
| | *TreS* | White and translucent, sour, sweet and bland; Chinese cuisine, chronic tracheitis and other cough-related conditions such as asthma and dry cough. | Zhou, 1993 |
| Modern use | *AstS* | Cancers, common cold/sore throat, heart attack, hepatitis, infections, immune suppression and dysfunction. | Qun *et al.*, 1999 |
| | *LenS* | Immune modulation, anti-tumor, anti-viral, anti-HIV, liver protection, and lowering cholesterol and blood pressure. | Lin *et al.*, 1987; Min, 1996; Jones, 1998 |
| | *TreS* | Anti-tumor, lower cholesterol, anti-diabetes, calcium absorption, liver protection, and prevention of osteoporosis, anti-inflammation and slowing of the aging process. | Xia and Cheng, 1988<br>Min, 1996; |
| Production and price | *AstS* | 1.5 million tons (stock), 1.5 to 1.9 USD/kg | http://www.888888.org/rj.gif |
| | *LenS* | 80,000 tons (production), 1.9-2.5 USD/kg | http://www.888888.org/rj.gif |
| | *TreS* | 12,500 tons (production), 1.9-2.5 USD/kg | http://www.888888.org/rj.gif |

**Table 4.** Physico-chemical properties of mushroom and herb polysaccharides

| Parameters | Polysaccharides[1] | | Characteristics[2] | References |
|---|---|---|---|---|
| Physical properties | *AstP* | *AG*-1 | Water-soluble glucan | Huang *et al.*, 1982 |
| | | *AG*-2 | Water-insoluble glucan | |
| | | *AH*-1 | Acidic heterosaccharide | |
| | | *AH*-2 | Water-soluble heterosaccharide | |
| | *LenP* | $LenP_m$-A | Water-soluble polysaccharide-protein complex | Liu *et al.*, 1999c |
| | | $LenP_m$-B | Water-soluble polysaccharide-protein complex | Jin *et al.*, 1991 |
| | | $LenP_f$-A | Polysaccharides complex (GlcA, 24.1%; protein, 2.0%) | Yang *et al.*, 1999, 2001 |
| | | $LenP_f$-B | Polysaccharides complex (GlcA, 34.8%; protein, 7.4%) | |
| | | $LenP_f$-C | Polysaccharides complex (GlcA, 40.1%; protein, 25.3%) | |
| | *TreP* | $TreP_m$-A | Neutral heterosaccharide | Wu *et al.*, 1984 |
| | | $TreP_m$-B | Acidic heterosaccharide | |
| | | $TreP_m$-C | Acidic heterosaccharide | |
| | | $TreP_f$ | Water-soluble heterosaccharide | Xia and Cheng, 1988 |
| Molecular weights (Dalton) | *AstP* | *AG*-1 | $1.23 \times 10^4$ | Qi, 1987 |
| | | *AG*-2 | $3.46 \times 10^4$ | |
| | | *AH*-1 | $3.63 \times 10^4$ | |
| | *LenP* | $LenP_m$-A | $5.08 \times 10^5$ | Liu *et al.*, 1999c |
| | | $LenP_m$-B | $1.05 \times 10^5$ | Jin *et al.*, 1991 |
| | | $LenP_f$-A | $9.54 \times 10^5$ | Yang *et al.*, 1999, 2001 |
| | | $LenP_f$-B | $9.0 \times 10^4$ | |
| | | $LenP_f$-C | $1.4 \times 10^4$ | |
| | *TreP* | $TreP_m$-A | $7.6 \times 10^4$ | Wu *et al.*, 1984 |
| | | $TreP_m$-B | $7.6 \times 10^4$ | |
| | | $TreP_m$-C | $7.6 \times 10^4$ | |
| | | $TreP_f$ | $1.15 \times 10^5$ | Xia and Cheng, 1988 |

| | | | | |
|---|---|---|---|---|
| hSugar composition | *AstP* | *AG*-1 | Glu | Huang *et al.*, 1982 |
| | | *AG*-2 | Glu | |
| | | *AH*-1 | HexA: Glu: Rha: Ara = 1: 0.04: 0.02: 0.01 | |
| | | *AH*-2 | Glu: Ara = 1: 0.15 | |
| | *LenP* | $LenP_m$-A | 2,4-di-: 2,4,6-tri-: 2, 3, 4, 6-tetra-O-Me-glucose = 1: 2: 1 | Liu *et al.*, 1999c |
| | | $LenP_m$-B | Gal, Man, Xyl, Fru = 3.1: 4.8: 1.9: 1.8 | Jin *et al.*, 1991 |
| | | $LenP_f$-A | Ara: Xyl: Man: Gal: Glu = 0.39: 0.46: 1.0: 0.93: 14.1 | Yang *et al.*, 1999, 2001 |
| | | $LenP_f$-B | Ara: Xyl: Man: Gal: Glu = 0.19: 0.41: 1.0: 0.93: 10.7 | |
| | | $LenP_f$-C | Ara: Xyl: Man: Gal: Glu = 0.31: 0.47: 1.0: 1.15: 8.9 | |
| | *TreP* | $TreP_m$-A | L-Fru: L-Ara: D-Xyl: D-Man: D-Gal: D-Glu = 1.06: 1.0: 0.33: 0.29: 0.037: 0.75 | Wu *et al.*, 1984 |
| | | $TreP_m$-B | L-Fru: L-Ara: D-Xyl: D-Man: D-Glu: GlcA = 0.16: 1.0: 0.28: 0.73: 0.036: 0.19 | |
| | | $TreP_m$-C | L-Fru: L-Ara: D-Xyl: D-Man: D-Glu: GlcA = 0.086: 1.0: 0.37: 0.75: 0.058: 0.37 | |
| | | $TreP_f$ | L-Fru: L-Ara: D-Xyl: D-Man: D-Glu: GlcA = 0.92: 0.49: 0.18: 1.0: 1.15: 0.57 | Xia and Cheng, 1988 |
| Structure | *AstP* | *AG*-1 | α-1, 4 and α-1, 6 glucan linkage = 5: 2 | Huang *et al.*, 1982 |
| | | *AG*-2 | α-1, 4 glucan | |
| | *LenP* | $LenP_m$-A | β-1, 3 D-glucan backbone, β-1, 6 D-glucan side chain | Liu *et al.*, 1999c |
| | | $LenP_f$-A | α- the glycoside linkages | Yang *et al.*, 1999, 2001 |
| | | $LenP_f$-B | α- the glycoside linkages | |
| | | $LenP_f$-C | β- the glycoside linkages | |
| | *TreP* | $TreP_m$ | Repeat unit: D-GlcA-D-xyl-D-man; an α-1, 3 Mannan backbone with β- linked | De Baets and Vandamme, |
| | | $TreP_f$ | Xyl side chains and GlcA | 2001 |

[1] *AstP* = *Astragalus membranaceus* polysaccharides with four fractions *AG*-1, *AG*-2, *AH*-1 and *AH*-2; *LenP* = *Lentinus edodes* polysaccharides derived from mycelia ($LenP_m$-A and $LenP_m$-B) and body fruit ($LenP_f$-A, $LenP_f$-B and $LenP_f$-C) and *TreP* = *Tremella fuciformis* polysaccharides derived from mycelia ($TreP_m$-A, $TreP_m$-B, and $TreP_m$-C) and body fruit ($TreP_f$).

[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 $C_3$ that enhances the animals' disease resistance; (4) increasing the number and activities of the phagocytes that release $H_2O_2$ 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 immunodulaters, 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 5.** The immune activities of mushroom and herb polysaccharides

| Parameters | Polysaccharides[1] | Dosage (mg/kg bw)[2] | Species | Response to treatments[3] | References |
|---|---|---|---|---|---|
| **Immune organs** | | | | | |
| Spleen weights | *AstP* | 200 × 3d | chickens | 166[a]; 125 | Zhang *et al.*, 1998a |
| | | 250 × 5d | rats | 146 | Liang *et al.*, 1994 |
| | | 1000 | mice | 123 | Mao *et al* , 1988 |
| | *LenP$_f$* | 200 × 3d | chickens | 162[a]; 123 | Zhang *et al.*, 1998a |
| | *TreP$_f$* | 100 × 7d | mice | 148 | Xia and Cheng, 1988 |
| | *TreP$_m$* | 100 × 7d | rats | 156 | Xia and Cheng, 1988 |
| Bursa weights | *AstP* | 200 × 3d | chickens | 183[a]; 122 | Zhang *et al.*, 1998a |
| | *LenP$_f$* | 200 × 3d | chickens | 162[a]; 108 | Zhang *et al.*, 1998a |
| Thymus weights | *AstP* | 250 × 5d | rats | 146 | Liang *et al.*, 1994 |
| | | 1000 | mice | 98 | Mao *et al* , 1988 |
| | *TreP$_f$* | 100 × 7d | rats | 108 | Xia and Cheng 1988 |
| **Immune cells** | | | | | |
| Number of NK cells | *LenP$_f$* | 2 × 5d | man | 91 to 131[c] | Shinya *et al.*, 1991 |
| Activity of NK cells | *AstP* | 20/b × 3d | chickens | 129 | Tang *et al.*, 1997 |
| | | 40/b × 3d | chickens | 119 | |
| | *LenP$_f$* | 1/m$^2$× 12d | man | 139[b] | Wu *et al.*, 1996 |
| Number of LAK cells | *LenP$_f$* | 2 × 5d | man | 110 to 122[c] | Shinya *et al.*, 1991 |
| Activity of LAK cells | *AstP* | 20/rat | rats | 300 | Wang 1992 |
| Phagocytosis of M$_Ø$ | *AstP* | 500 | rats | 188 | Li *et al.*, 1989 |
| | *LenP$_m$-B* | 20 × 1d | mice | 175 | Jin *et al.*, 1991 |
| | *TreP$_f$* | 100 × 7d | rats | 103 | Xia and Cheng, 1988 |
| | *TreP$_m$* | 100 × 7d | rats | 102 | Xia and Cheng, 1988 |
| | | 200 × 7d | rats | 254 | Fan *et al.*, 1993 |
| Activity of bursa lymphocytes | *AstP* | 40 × 7d | chickens | (P<0.05, P<0.01) | Zhong *et al.*, 1998 |
| Activity of spleen and thymus lymphocytes | *LenP$_f$* | 2 × 7d | chickens | (P<0.05, P<0.01) | Zhong *et al.*, 1998 |
| Number of CD4 cells | *LenP$_f$* | 2 × 5d | man | 115[c] | Shinya *et al.*, 1991 |
| Number of CD8 cells | *LenP$_f$* | 2 × 5d | man | 109[c] | Shinya *et al.*, 1991 |
| E-rosette production | *TreP$_f$* | 50ug/rat | rats | 159 | Xia and Cheng, 1988 |
| | *TreP$_m$* | 50ug/rat | rats | 208 | Fan *et al.*, 1993 |
| | | 100 × 3d | chickens | 136 | |
| **Cellular Immunity** | | | | | |
| Lymphocytes proliferation | *AstP* | 35 × 7d | chickens | 127 to 185[d] | Liu *et al.*, 1999b |
| | *LenP$_f$* | 8 × 7d | chickens | 111 to 118[d] | Liu *et al.*, 1999b |
| | *TreP$_f$* | 100ug/rat | rats | 211[e] | Xia and Cheng, 1988 |
| | *TreP$_m$* | 100ug/rat | rats | 220[e] | Xia and Cheng, 1988 |
| | | 50 × 4d | chickens | 170 | Fan *et al*., 1993 |
| Agglutination of red cell | *AstP* | 500 | mice | 113 | Mao *et al*., 1989 |
| | | 1000 | mice | 93 | |

| | | | | | |
|---|---|---|---|---|---|
| **Humoral immunity** | | | | | |
| Number of hemolytic | *$LenP_m$-B* | 20 | mice | 340 | Jin *et al.*, 1991 |
| plague forming cells | *TrePf* | 50 × 5d | mice | 178 | Xia and Lin, 1989 |
| | | 100 × 5d | mice | 182 | |
| Hemolysin production | *AstP* | 500 | mice | 104 | Mao *et al*., 1988 |
| | | 1000 | mice | 92 | |
| | *$TreP_f$* | 50 × 7d | rats | 130 | Xia and Cheng, 1988 |
| | *$TreP_m$* | 50 × 7d | rats | 321 | Xia and Cheng, 1988 |
| Number of spleen cells | *AstP* | 500 | rats | 127 | Mao *et al.*, 1988 |
| | | 1000 | rats | 96 | |
| Number of B-cells | *$TreP_m$* | 100 × 3d | chickens | 120 | Fan *et al.*, 1993 |
| Antibody titers | *$TreP_m$* | 200 | chickens | $P<0.05$[d] | Cheng *et al.*, 1998 |
| **Cytokines** | | | | | |
| IL-2 production | *AstP* | 35 × 7d | chickens | 112 to 137 | Liu *et al.*, 1999a |
| | *$LenP_f$* | 8 × 7d | chickens | 117 to 152 | Liu *et al.*, 1999a |
| | *$TreP_m$* | 100 × 4d | rats | 138 | Hu *et al.*, 1995 |
| | | 200 × 4d | rats | 148 | |
| TNF production | *AstP* | 50 × 9d | rats | 112[a] | Wang *et al.*, 1997 |
| IFN titers | *AstP* | 50 × 9d | rats | 139[a] | Wang *et al.*, 1997 |
| | *$TreP_m$* | 11/b × 7d | chickens | 118 to 155[f] | Hu *et al.*, 1996 |
| | | 200 × 3d | rats | 152 | Liang *et al.*, 1995 |
| | | 400 × 4d | rats | 129 | |
| mRNA levels of IL-1α, IL-1β, TNF-α, IFN-γ | *$LenP_m$-A* | 20 | mice | marked increase | Liu *et al.*, 1999a |
| **Complements** | *AstP* | 500 | rats | $P<0.05$ | Zhang, 1991 |
| | *$TreP_m$* | 100 × 3d | chickens | 107 | Fan *et al.*, 1993 |

[1] *AstP = Astragalus membranaceus Radix* polysaccharides; *LenP = Lentinus edodes* polysaccharides derived from mycelia (*$LenP_m$*, *$LenP_m$*-A) and body fruit (*$LenP_f$*) and *TreP = Tremella fuciformis* polysaccharides derived from mycelia (*$TreP_m$*) and body fruit (*$TreP_f$*).

[2] d = number of successive days of treatments executed.

[3] Response to the treatments was based on 100 of the negative control (without superscripts) or the positive control (with superscripts in which [a] = cyclophosphamide; [b] = chemical therapy; [c] = cancer patients; [d] = Marek's disease; [e] = phytahematoagglutinin; [f] = 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 *$TreP_m$* (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 6.** Efficacy of mushroom and herb polysaccharides against viral diseases in chickens

| Viral diseases | Substrates[1] | Dosage[2] (mg/kg bw) | Relative incidence[3] (%) | Relative mortality[3] (%) | References |
|---|---|---|---|---|---|
| Marek's disease | *AstP* | 35 × 7d | 52 | 47 | Liu *et al.*, 1999b |
| | | 40 × 7d | 65 | 38 | Wei *et al.*, 1997 |
| | *LenP* | 2 × 7d | 86 | 62 | Wei *et al.*, 1997 |
| | | 8 × 7d | 59 | 65 | Liu *et al.*, 1999b |
| Avian myelocytic | *AstP* | 40 × 7d | 74 | 73 | Qu *et al.*, 1998 |
| leukemia | *LenP* | 2 × 7d | 78 | 79 | Qu *et al.*, 1998 |
| Infectious bursal | *AstP* | 20 × 7d | - | 27 | Yu and Zhu, 2000 |
| disease | | 40 × 7d | - | 33 | Yu and Zhu, 2000 |

[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-γ secretion,

against coccidiosis in chickens (Hu *et al.*, 1996).

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

| Groups | Dose | BWG[1] | Mortality[1] | Lesion score[1] | Oocyst output[1] |
|---|---|---|---|---|---|
| Challenge | $5 \times 10^4$ oocyst $b^{-1}$ | 100 | 100 | 100 | 100 |
| Vaccine | 75 oocyst $b^{-1}$ | 87 | 90 | 67 | 25 |
| *TreP$_m$* | $200 \times 7$ mg $kg^{-1}$ d | 78 | 139 | 100 | 50 |
| *TreP$_m$*+Vaccine | | 135 | 0 | 67 | 50 |

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

### *Bacterial Infections*

The mushroom polysaccharide (*TreP$_m$*) 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$_m$* (80, 120, 150, 200 and 300 mg/kg bw) and the vaccine was 50, 37, 22, 37 and 0% respectively after avian *Pasteurellosis* (MLD $C48^{-1}$) 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$_m$* as an adjuvant. A further experiment conducted by Yuan *et al.*, (1993) showed that the mortality of the groups immunized with *TreP$_m$* (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$_m$*, indicating that *TreP$_m$* 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 immnuo-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.

## REFERENCES

Blagoeva, J., G., Stoev, and P., Venkov. 1991. Glucan structure in a fragile mutant of Saccharo- myces cerevisiae. Yeast, 7(5):455-462.

Buddington, P. K., and E., Weiher. 1999. The application of ecological principles and fermentable fibers to manage gastrointestinal tract ecosystem. J. Nutr., 129:1446S.

Chandra, A. 1989. Elsevier's Dictionary of Edible Mushrooms, Botanical and Common Names in Various Languages of the World. Elsevier Scientific Publishing Co. Amsterdam & New York.

De, Baets, S., and E. J., Vandamme. 2001. Extra-cellular Tremella polysaccharides: Structure, properties and applications. Biotechnology Letters, 23 (17):1361-1366.

Gibson, G. R., and M. B., Roberfroid. 1995. Dietary modulation of the human colonic microflora: introducing the concept of prebiotics. J. Nutr., 125:1401.

Hassan, H. 1988. Biosynthesis and regulation of superoxide dismutases, Free Radical Biol. Med. 5, 377.

Huisman, M. M. H., H. A., Schols and A. G. J., Voragen. 1998. Cell wall polysaccharides from soybean meal; isolation and characterization. Carbohydr. Polym. 37:87-95.

Jin, M. R., J. W., Bok, M. J., Shim, E. O., Choi, and B. K., Kim. 1991. Studies on constituents of the higher fungi of Korea (LXIX): Anti-tumor components of the cultured mycelia of Lentinus edodes. Seoul University Journal of Pharmaceutical Sciences, 16(0):66-96.

Jones, K. 1998. Shiitake: a major medicinal mushroom. Alternative and Complementary Therapies, 2:53-59.

Langhout, D. J. 1999. The role of the intestinal flora as affected by NSP in broilers. Proceedings of the 12th W.P.S.A. European Symposium on Poultry Nutrition, veldhoven, NL, pp 203-212. Aug. 15-19.

Lee, S. S., S. W., Hong, S. H., Kim and B. C., Kim. 2001. Several genes expressed during morphogenesis of Lentinus edodes (ImHyup-1). Microbology 29 (3):135-141.

Len, R., S., Wolda, E., Moon, J., Esselstyn, C., Hertel, and A., Lerner. 2002. PDE7A is expressed in human B-lymphocytes and is up-regulated by elevation of intracellular cAMP. Cellular Signalling, 14 (3):277-284.

Li, H. F., T., Waisman, Y., Maimon, K., Shakhar, E., Rosenne, and B. E., Shamgar. 2001. The effects of a Chinese herb formula, anti-cancer number one (ACNO), on NK cell activity and tumor metastasis in rats. International Immunopharmacology. 1(11):1947-1956.

Li, X. Y. 2000. Immuno-modulating components from Chinese medicines. Pharmaceutical Biology, 38 (Supplement):33-40.

Lien, E. J., and H., Gao. 1990. Higher plant polysaccharides and their pharmacological activities. Int. J. Oriental Med. 15:123.

Liu F., V. E. C., Ooi, and M. C., Fung. 1999a.. Analysis of immuno-modulating cytokine mRNAs in the mouse induced by mushroom polysaccharides. Life Sciences, 64 (12):1005-1011.

Lizuka, N., K., Miyamoto, and S., Hazama. 2000. Anticachectic effects of Coptidis rhizoma, an anti-inflammatory herb, on esophageal cancer cells that produce interleukin 6. Cancer Letters, 158 (1): 35-41.

Ma, X. Q., Q., Shi, J. A., Duan, T. T., Dong, and K. W., Tsim. 2002. Chemical analysis of Astragalus Radix (Huangqi) in China: a comparison with its adulterants and seasonal variations. J Agric Food Chem. 50(17):4861-6.

Miles, P. G., and S. T., Chang. 1997. Mushroom Biology – Concise Basics and Current Developments. World Scientific. Singapore. pp 194.

Min, S. D. 1996. Medicinal value of fungi. Acta Edulis Fungi. 3(4):55-64.

Mol, P. C., C. A., Vermeulen, and J. G. H., Wessels. 1988. Glucan-glucosaminoglycan linkages in

fungal walls. Acta Botanica Neerlandica, 37 (1):17-22.

Raddiffe, J. 2000. British Supermarkets: forging changes in poultry nutrition. Australian Poultry Science Symposium 12:25-31.

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

Royse, D. J., L. C. Schisler, and D. A. Diehle 1985. Shiitake mushrooms – consumption, production and cultivation. Interdisciplinary Science Reviews, 10 (4):329.

Salyers, V. G., Y. W., Park, and C., Gray. 1977. Effects of mannan oligosaccharide (MOS) on aflatoxicosis, serum, liver, and egg cholesterol and egg production in chickens. Assoc. Res. Dir. 11th Biennial Research Symposium.

Satonori, K., A., Yuki, and E., Fumio. 1999. Effects of Astragalus Radix extract on carcinogenesis, cytokine production, and cytotoxicity in mice treated with a carcinogen, N- butyl- N'- butanol-nitrosoamine. Cancer Investigation, 17 (1):30-35

Shimahara, H. 1975. Partial purification of ß-Mannanases from the konjac tubers and their substrate specificity in relation to the structure of konjac glucomannan. Agri. Biol. Chem. 39(2):301-312.

Shiomi, N., S., Onodera, and C. C. J., Vieira. 1996. Structure of fructan polymers from tuberous roots of Gomphrena macrocephala (Amaranthaceae) from the cerrado. New Phytologist 133 (4):643-650.

Shon, Y. H., J. H., Kim, and K. S., Nam. 2002. Effect of Astragalus radix extract on lipopolysaccharide-induced inflammation in human amnion. Biological & Pharmaceutical Bulletin, 25 (1):77-80.

Singer, R. 1961. Mushrooms and Truffles. Interscience Publishers, Inc., New York, NY.

Stamets, P. 2000. Growing gourmet and medicinal mushrooms. Ten Speed Press, Berkeley, CA.

Wang, R. J., D. F., Li and S., Bourne. 2002. Can 2000 years of herbal medicine history help us solve problems on the year 2000? pp 273 – 291. in: Biotechnology in the feed industry. Proceedings of Alltech's 14th Anunual sysmposium, Nottingham University Press.

Wargovich, M. J., C., Woods, D. M., Hollis, and M. E., Zander. 2001. Herbals, cancer prevention and health. J. of Nutr., 131 (11Suppl):3034-3036.

Xia, E. N. and Q. H., Cheng. 1988. Isolation, analysis and bioactivities of Tremella fuciformis fruit body polysaccharides. Acta Mycologica Sinica. 7(3):166-174.

Zhou, R. H. 1993. Resource science of Chinese medicinal materials (in Chinese), pp. 19-32. Bejing: China Medicinal & Pharmaceutical Science Press.

Zhou, Y. P. 1998. Guidelines for application of Chinese materia medica. pp. 27-653. Chinese Material Medica (chemistry, pharmacology and applications) Nedichin B. V. China- Netherlands. Medical and pharmaceutical center Groningen, The Netherlands.

References from Chinese Journals

Cheng, F. S., T. J., Hu, and J. L., Liang. 1998. Enhancement of Marek's disease vaccine by 8301 polysaccharide in chickens. J. of Traditional Chinese Vet. Medicine (in Chinese), 1:8-9.

Chi Y. J., Q. Z., Li, and H. X., Yu. 1998. Effect of Astragalus mongholicus Bga polysaccharide on superoxide dismutase (SOD) activity in avian myelocytic leukemia virus (VAMV) infected

chicks. Helongjiang Animal Science and Vet. Medicine (in Chinese), 2:3-5.

Fan, B. T., Y. L., Yuan, and J. L., Liang. 1993. Study of mechanism of enhanced efficacy of avian cholera vaccine by 8301 polysaccharide. J. of Traditional Chinese Vet. Medicine (in Chinese) 1:5-10.

Hu, T. J., B. T., Fan, and Q. Z., Huang. 1995. Effect of 8301 polysaccharide on spleen IL-2 production in rats. Chinese J of Vet. Science and Technology (in Chinese), 25(1):28.

Hu, T. J., J. L., Liang, and F. S., Cheng. 1998. The use of 8301 polysaccharide as an adjuvant in coccidial infected chickens. J. of Grass and Livestock (in Chinese), 6:5-7.

Hu, T. J., J. L., Liang, and M. X., Dong. 1996. Effect of 8301 polysaccharide on serum IFN-γ titers in chickens. Endemic Disease Bulletin (in Chinese), 11:6-7.

Huang, N. L. 1982. Cultivation of Tremella fuciformis in Fujian, China. Mushroom Newsl. Trop. (in Chinese), 2(3):2-5.

Huang, Q. S., G. B., Lu, Y. C., Li, and J. H., Guo. 1982. Study on the polysaccharides of "Huang Qi" (Astragalus mongholicus Bung). Acta Pharmaceutica Sinica (in Chinese), 17(3):200-206.

Li, C. M. 1998. Chinese Herb Medicine Feed Additives (in Chinese), pp. 11-235, Beijing: Chinese Agricultural University press.

Li, X.Y. 1989. Pharmacological study of Astragalus Radix polysaccharides 2: An immuno-pharmacology study. Chinese Medicine (in Chinese), 11(5):26-38.

Liang, H. P. 1996. Mechanism of the cellular immune function suppression and study on immune regulation by herbs in injured rats. Journal of Third Army Medical University (in Chinese), 18: 352.

Liang, H. P., Y., Zhang, and B., Geng. 1994. Effect of Astragalus membranacea Radix on cellular immunity in burn rats. Journal of China Plastic and Burn Surgery (in Chinese), 10(2):138-141.

Liang, J. L., T. J. Hu, and B. T., Fan. 1995. Experiment of 8301 polysaccharide inducing IFN production in rats. J. of Traditional Chinese Vet. Medicine (in Chinese), 4: 12-13.

Lin, Y. 1987. A double-blind treatment of 72 cases of chronic hepatitis with Lentinan injection. New Drugs and Clinical Remedies (in Chinese with English summary), 6:362-363

Liu, J. X., C. Y., Wang, and Y. M., Bai. 1999c.. Preparation of immuno-active Chinese herbal formulation and safe efficacy test. Chinese J. of Veterinary Science and Technology (in Chinese), 29(8):23-25.

Liu, Y. J., Q. Z., Li, and Y. H., Hao. 1999b. Effects of Lentinan and Astragalan on IL-2 inductive activity and lymphocyte proliferative reaction in chicks infected with Marek's disease. J. of China Vet. Medicine (in Chinese), 25 (7):3-5.

Ma, X. Q., J. A., Duan, D. Y., Zhu, T. X., Dong, and K. W. K., Tsim. 2000. Chemical comparison of Astragalus Radix (Huangqi) from different regions of China. Natural Medicines (in Chinese with English summary), 54 (5):213-218.

Mao, X. J. 1988. Effect of Astragalus membranacea Radix polysaccharide on humoral immunity in rats. J. of China Immunology (in Chinese), 4(3):158.

Mao, X. J. 1989. Immune opsonic action of Astragalus membranacea Radix polysaccharide. China Pharmacology Bulletin (in Chinese), 5:367.

Pang, F. H., M. Q., Xie, and H. H., Ling. 2000. The investigation of immuno-dulators tested for the results on the control of a coccidial infection. Chinese J. of Vet. Parasitology (in Chinese),

8(3):1-3.

Qi, Z. S. 1987. Review on chemical study of Astragalus membranacea Radix. Chinese Traditional and Herbal Drugs (in Chinese), 18(5):41-29.

Qu, Q. H., Y. J., Chi, Q. Z., Li, and B. R., Xu. 1997. Effect of Astragalus mongholicus Bga and Lentinus edodes polysaccharides on Se-GSHPX activity in chicks. Helongjiang Animal Science and Vet. Medicine (in Chinese), 5:8-9.

Qu, Q. H., Q. Z., Li, and B. R., Xu. 1998. Effect of Astragalus mongholicus Bga and Lentinus edodes polysaccharide on Se-GSHPX activity in avian myelocytic leukemia virus (VAMV) infected chicks. Helongjiang Animal Science and Vet. Medicine (in Chinese), 4:1-2.

Qun, L, Q., Luo, and Z. Y., Zhang. 1999. Effects of Astragalus membranacea Radix on IL-2/IL-2R system in patients with maintained hemodialysis. Clin Nephrol. (in Chinese), 52: 333–4.

Shinya, A., K., Nobuya, T., Kiyoshi, N., Shigern, I., Hiroshi, N., Masaaki U., Hiroaki, and A., Tsuyoshi. 1991. Reinforcement of inducing activity of lymphocyte activators by Lentinus polysaccharides in gastric carcinoma patient. Surgery (in Chinese), (1):874.

Tang, X. M., J. G., Liu, and D. L., Sun. 1997. Effect of Astragalus membranacea Radix polysaccharide on activity of NK cells in peripheral blood in chicks. J of China Vet. Medicine (in Chinese), 23(1): 44-45.

Wang, D. L. 1989. Effect of Astragalus membranacea Radix polysaccharide on immune function of blood lymphocytes in humans. J. of China Cancer (in Chinese), 11(3):180.

Wang, G. 1992. Effect of Astragalus membranacea Radix polysaccharide on activities of lymphocyte-activated killer (LAK) cells. China Hygiene Journal (in Chinese), 11:233.

Wang, L. R., Z. H., Liang, and W., Feng. 1997. Immunodulation of Astragalus membranacea Radix and Acanthopanax gracilistylus. Journal of China Experimental Clinic Immunology (in Chinese), 9(4):61-66.

Wei, P., Q. Z., Li, and Y. H., Hao. 1997. Effect of Astragalus mongholicus Bga and Lentinus edodes polysaccharide on lipid peroxide (LPO) content in Marek's disease infected chicks. J. of Traditional Chinese Vet. Medicine (in Chinese), 4:3-4.

Wei, X.Y. 1991. Research on effects of Astragalus membranacea Radix and Polygoni multiflori Radix thymus in old rats. J. of China Pharmaceutical University (in Chinese), 22:359.

Wu, S., Z. Y., Zhou, and L. X., Pan. 1996. Effect of China Lentinus edodes polysaccharide on activity of NK cells in cancerous patients. Jiangsu Chinese Medicine (in Chinese), 17(9):41.

Wu, W. T., P. H., Yu, E. N., Xia, W. X., Shi, B. R., Xu, and Q. H., Cheng. 1984. Isolation and purification of polysaccharides TF-A, TF-B and TF-C from the spores of Tremella fuciformis Berk and determination of their molar ratios. Acta Biochimica et Biophysica Sinica (in Chinese with English summary), 16(4):393-398.

Wu, Y. H. and F., Wang. 1999. Research status on two-way immune regulating functions of herbs. J. of Traditional Chinese Vet. Medicine (in Chinese), 4:19-22.

Xia, D. and Z. B., Lin. 1989. Effects of Tremella polysaccharides on immune function in mice. Acta Pharmacologica Sinica (in Chinese with English summary), 10 (5):453-456.

Xu, G. J. 1990. Radix Astragalus. pp:176-177 in Coloured Illustrations of Chinese Traditional and Herbal Drugs (reversion) (in Chinese). Fujian Science and Technology Press.

Xue, M. and X. S., Meng 1996. Review on research progress and prosperous of immune activities of

bio-active polysaccharides. J. of Traditional Chinese Vet. Medicine (in Chinese), 3:15-18.

Yang, H. C., G., Lu, and G. Lu, 1998. Effect of pollen polysaccharide from corns on immunological efficacy of Newcastle disease vaccine and egg drop syndrome vaccine in chickens. J. of Chinese Vet. Medicine (in Chinese), 24 (6):43.

Yang, J., M. C., Wu, and S. H., Zhang. 1999. Study on isolation, purification and characteristics of polysaccharide Le-III from the fruit body of Lentinus edodes. J. of Huazhong Agricultural University (in Chinese with English summary), 18(2):188-190.

Yang, J., M. C., Wu, and S. H., Zhang. 2001. Isolation, purification and characterization of polysaccharides from the fruit body of Lentinus edodes (Berk.) Journal of Plant resources and Environment (in Chinese with English summary), 10 (2):4-6.

Yang, Y., and W. S., Feng. 1998. Brochures of isolation and extraction of chemical components from herbs (in Chinese), China Chinese Herb Medicine Press. Beijing.

Yu, J. G., and L.Y., Zhu. 2000. The use of Astragalus membranacea Radi polysaccharide extract against infectious bursa disease in chickens. J. of Traditional Chinese Vet. Medicine (in Chinese with English summary), 6:3-4.

Yuan, W. S., and S. M., Shi. 2000. Pharmacological functions of Chinese medicinal fungus Cordyceps sinensis and related species. J. of Food and Drug Analysis (in Chinese and English summary), 8 (4):248-257.

Yuan, Y. L., B. T., Fan, and Y. X., Zhang. 1993. Efficacy of 8301 polysaccharides as an adjuvant against avian Pasteurellosis in chickens. J. of Traditional Chinese Vet. Medicine (in Chinese) 3:6-10.

Zhang, F. Y. 1991. Effect of Astragalus membranacea Radix on CMSC in elders. Journal of Immunology (in Chinese), 8(2):3.

Zhang, J. S., W. W., Han, and Y. J., Pan. 2001. Studies on chemical structure of polysaccharide from fruit body of Coriolus versicolor. Acta Pharmaceutica Sinica (in Chinese with English summary), 36(9):664-667.

Zhang, L. C., J. B., Wang, Y. P., Sun, D. Z., Ma, M. L., Shi, and Z. H., Wang. 1998a. Effect of a herb polysaccharide complex on immune organs morphology in chickens. Chinese J. of Vet. Science and Technology (in Chinese), 28(8):26-28.

Zhang, L. C., J. B., Wang, Y. P., Sun, D. Z., Ma, M. L., Shi, and Z. H., Wang. 1998b. Effect of adding Chinese herb polysaccharide preparations to a Newcastle disease inactivated vaccine on the development of the immune organs in chickens. Chinese Journal of Vet. Science and Technology (in Chinese), 18(4):378-381.

Zhang, Z. Z., X. M., Liang, Q., Zhang, Qing, and P. Z., Lu. 2001. Characterization and recognition key components in Astragalus membranaceus. Acta Pharmaceutica Sinica (in Chinese with English summary), 36 (7):523-527.

Zhao, K. S. 1993. Study on Astragalus membranacea Radix polysaccharide enhancement of peripheral blood monocyte producing tumour necrosis factors in human. J. of China Traditional and Western Medicine (in Chinese), 13:263.

Zhao, R. H., H. Y., Zhang, and T., Tiao. 1995. The resources and stock of the common use Chinese herb medicine in China. J. of Chinese Medicine (in Chinese), 20(12):712.

Zheng, Z., D., Liu, C., Song, C., Cheng, and Z., Hu. 1998. Studies on chemical constituents and

immunological function activity of hairy root of Astragalus membranaceus Radix. Chin J Biotechnol. (in Chinese with English summary), 14(2):93-7.

Zhong, M., D. S., Zhang, J., Murata, I., Saiki, and Y., Itaru. 2001. Effects of Astragalus polysaccharide (APS) and Astragalus flavonoids (AF) on the cytotoxicity of LAK and NK cells in normal and tumor bearing mice. Sichuan Pharmaceutical Bulletin (in Chinese with English summary), 38 (5):718-722.

Zhong, P., Q. Z., Li, M., Ge, X. J., Gao, and F. J., Mao. 1998. Effect of Lentinus edodes and Astragalus membranacea Radix polysaccharides on lymphocytes' chemiluminescence. J. of Traditional Chinese Vet. Medicine (in Chinese), 3:5-7.

Zhou, R. H. 1993. Resource science of Chinese medicinal materials (in Chinese), pp. 19-32. Bejing: China Medicinal & Pharmaceutical Science Press.

# Chapter 2

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

F.C. Guo

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands; Institute of Animal Science and Vet. Pharmaceutics, Chinese Academy of Agricultural Sciences, Lanzhou 730050, China*

E.J. Bakx

*Laboratory of Food Chemistry, Departement of Agrotechnology and Food Sciences, Wageningen University P.O. Box 8129, 6700 EV Wageningen, Netherlands*

R.P. Kwakkel

*Animal Production Systems Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

B.A. Williams, H. Boer and M.W.A. Verstegen

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

To be submitted

# Extraction and Identification of Water-Soluble Polysaccharides from Two Mushrooms and An Herb

F.C. Guo, E.J. Bakx, B.A. Williams, R.P. Kwakkel, H. Boer and M.W.A. Verstegen

**Abstract**

The aim of the present study was to isolate and characterize water-soluble polysaccharide fractions from two mushrooms, *Letinus edodes* (*LenS*) and *Tremella fuciformis* (*TreS*), and an herb, *Astragalus membranaceus Radix* (*AstS*), so as to study their bio-activities *in vitro* and *in vivo*. The dried and grounded intact mushroom and herb materials (*LenS*, *TreS* and *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 *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 only 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. 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 amperometeric 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, *Letinus 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$^{o}$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 $^{o}$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) $H_2SO_4$. The tubes were put in a heating block at 30 $^oC$ 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 $^oC$ 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$^oC$ 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

| Compositions | Intact mushroom and herb materials[2] | | |
|---|---|---|---|
| | *LenS* | *TreS* | *AstS* |
| Dry matter (DM) (g/kg) | 957 | 958 | 967 |
| Crude protein (g/kg DM) | 208 | 103 | 157 |
| Yields of polysaccharides (%)[1] | 8.0 | 10.0 | 31.1 |
| | Polysaccharide extracts[3] | | |
| | *LenE* | *TreE* | *AstE* |
| DM (g/kg) | 952 | 955 | 940 |
| Crude protein (g/kg DM) | 114 | 47 | 67 |
| Total sugar content (g/kg DM) | 632 | 687 | 647 |

[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.

### *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 glucoses, 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

| Substrates[1] | Sugar composition[2] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Fru | Rha | Ara | Gal | Glu | Man | Xyl | GalA | GlcA |
| | w/w %[3] (mol %)[4] | | | | | | | | |
| *LenS* | 0 (1) | | | 2 (2) | 80 (91) | 1 (1) | 3 (5) | | 1 (1) |
| *TreS* | 4 (9) | | | 0 (1) | 19 (35) | 8 (15) | 19 (41) | | 3 (5) |
| *AstS* | | 3 (6) | | 1 (2) | 44 (83) | 2 (4) | 1 (1) | 2 (3) | |
| *LenE* | | | | 7 (8) | 74 (88) | | 3 (5) | | |
| *TreE* | 3 (8) | | | 1 (2) | 13 (30) | 7 (16) | 15 (45) | | 2 (2) |
| *AstE* | | | | | 100 (100) | | | | |
| *LenR* | | | | 0 (2) | 11 (82) | | 1 (6) | 1 (10) | |
| *TreR* | 6 (8) | | | 0 (0) | 23 (27) | 24 (28) | 26 (37) | | 3 (4) |
| *AstR* | | 9 (8) | | 4 (3) | 90 (75) | 10 (9) | 1 (1) | 5 (4) | |

[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 glucoses, 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

| Parameters | Polysaccharides[1] | Characteristics[2] | References |
|---|---|---|---|
| Physical properties | *AstP* | *AstP*-1, water-soluble glucan; *AstP*-2, water-insoluble glucan; *AstP*-3, acidic heterosaccharide; *AstE*-4, water-soluble heterosaccharide | Huang *et al.*, 1982 |
| | *LenP* | Polysaccharide-protein complex: *LenP*-1, GlcA, 24.1%; protein, 2.0%; *LenP*-2, GlcA, 34.8%; protein, 7.4%; *LenP*-3, GlcA, 40.1%; protein, 25.3% | Yang *et al.*, 1999, 2001 |
| | *TreP* | water-soluble heterosaccharide | Xia and Cheng, 1988 |
| Molecular weights (Dalton) | *AstP* | *AstP*-1, $1.23 \times 10^4$ ; *AstP*-2, $3.46 \times 10^4$; *AstP*-3, $3.63 \times 10^4$; | Qi, 1987 |
| | *LenP* | *LenP*-1, $9.54 \times 10^5$; *LenP*-2, $9.0 \times 10^4$; *LenP*-3, $1.4 \times 10^4$ | Yang *et al.*, 1999, 2001 |
| | *TreP* | $1.15 \times 10^5$ | Xia and Cheng, 1988 |
| Sugar composition | *AstP* | *AstP*-1, Glu; *AstP*-2, Glu; *AstP*-3, HexA: Glu: Rha: Ara = 1: 0.04: 0.02: 0.01; *AstP*-4, Glu: Ara = 1: 0.15 | Huang *et al.*, 1982 |
| | *LenP* | *LenP*-1, Ara: Xyl: Man: Gal: Glu = 0.39: 0.46: 1.0: 0.93: 14.1; *LenP*-2, Ara: Xyl: Man: Gal: Glu = 0.19: 0.41: 1.0: 0.93: 10.7; *LenP*-3, Ara: Xyl: Man: Gal: Glu = 0.31: 0.47: 1.0: 1.15: 8.9 | Yang *et al.*, 1999, 2001 |
| | *TreP* | L-Fru: L-Ara: D-Xyl: D-Man: D-Glu: GlcA = 0.92: 0.49: 0.18: 1.0: 1.15: 0.57 | Xia and Cheng, 1988 |

[1] *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.

[2] 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.

## ACKNOWLEDGEMENTS

This work was founded in part by Alltech Inc., USA. The authors wish to thank Dick Bongers, Jane Martine Muijlaert and Truus Post of Animal Nutrition Group, Wageningen University for their assistance in chemical analyses.

## REFERENCES

Dubois M, K. A., Gilles J. K., Hamilton D. A., Rebers, and F., Smith. 1956. Analytical Chemistry 28, 350-356 (Cardbox GR CD11, FK method).

Guo, F. C., H. F. J., Savelkoul, R. P., Kwakkel, B. A., Williams, and M. W. A., Verstegen. 2003.

Immunoactive, medicinal properties of mushroom and herb polysaccharides: alternative for antimicrobials in poultry diets. World's Poultry Science Journal (in press).

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

Huang, Q. S., G. B., Lu, Y. C., Li, and J. H., Guo. 1982. Study on the polysaccharides of "Huang Qi" (*Astragalus mongholicus Bung*. Acta Pharmaceutica Sinica (in Chinese), 17(3): 200-206.

Huisman, M. M. H., H. A., Schols, and A. G. J., Voragen. 1998. Cell wall polysaccharides from soybean meal; isolation and characterization. Carbohydr. Polym. 37: 87-95.

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

Jin, M. R., J. W., Bok, M. J., Shim, E. O., Choi, B. K., Kim. 1991. Studies on constituents of the higher fungi of Korea (LXIX): Antitumor components of the cultured mycelia of *Lentinus edodes*. Seoul University Journal of Pharmaceutical Sciences, 16(0): 66-96.

Li, X. Y. 2000. Immuno-modulating components from Chinese medicines. Pharmaceutical Biology, 38 (Supplement): 33-40.

Liu J. X., C. Y., Wang and Y. M., Bai. 1999. Preparation of immune active Chinese herbal formulation and safe efficacy test. Chinese J. of Veterinary Science and Technology (in Chinese). 29(8): 23-25.

Min, S. D. 1996. Medicinal value of fungi. Acta Edulis Fungi, 3(4): 55-64.

Qi, Z. S. 1987. Review on chemical study of *Astragalus membranacea Radix*. Chinese Traditional and Herbal Drugs (in Chinese), 18(5): 41-29.

Stroop, C. J. M., C. A., Bush, R. L., Marple, and W. R., LaCourse. 2002. Carbohydrate analysis of bacterial polysaccharides by high-pH anion-exchange chromatography and online polarimetric determination of absolute configuration. Analytical Biochemistry, 303 (2): 176-185.

Wu, W. T., P. H., Yu, and E. N., Xia. 1984. Isolation and purification of polysaccharides TF-A, TF-B and TF-C from the spores of *Tremella fuciformis* Berk and determination of their molar ratios. Acta Biochimica et Biophysica Sinica, 16(4): 393-398.

Xia, E. N., and Q. H., Cheng. 1988. Isolation, analysis and bioactivities of *Tremella fuciformis* fruit body polysaccharides. Acta Mycologica Sinica (in Chinese), 7(3): 166-174.

XU, G. J. 1990. *Radix Astragalus*. pp:176-177 in Coloured Illustrations of Chinese Traditional and Herbal Drugs (reversion) (in Chinese). Fujian Science and Technology Press.

Xue, M., and X. S., Meng. 1996. Review on research progress and prosperous of immune

activities of bio-active polysaccharides. J. of Traditional Vet. Medicine (in Chinese), 3: 15-18.

Yang, J., M. C., Wu, and S. H., Zhang. 1999. Study on isolation, purification and characteristics of polysaccharide Le-III from the fruit body of *Lentinus Edodes.* J. of Huazhong Agricultural University (in Chinese), 18(2): 188-190.

Yang, J., M. C., Wu, and S. H., Zhang. 2001. Isolation, purification and characterization of polysaccharides from the fruitbody of *Lentinus edodes* (Berk.) Journal of Plant Resources and Environment (in Chinese), 10 (2): 4-6.

Yang, Y., and W. S., Feng. 1998. Brochures of isolation and extraction of chemical components from herbs (in Chinese), China Chinese Herb Medicine Press. Beijing.

Zheng, Z., D., Liu, C., Song, C., Cheng, and Z., Hu. 1998. Studies on chemical constituents and immunological function activity of hairy root of *Astragalus membranaceus Radix*. Chin J Biotechnol. (in Chinese), 14(2): 93-7.

# Chapter 3

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

F.C. Guo

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands; Institute of Animal Science and Vet. Pharmaceutics, Chinese Academy of Agricultural Sciences, Lanzhou 730050, China*

R.P. Kwakkel

*Animal Production Systems Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

B.A. Williams and M.W.A. Verstegen

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

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 diphasic 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 Schaafsma, 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$^{o}$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$^{o}$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 $^{o}$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 $CO_2$. 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 $CO_2$.

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$^{o}$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 $N_2$ saturated with methanoic acid, 190 $^{o}$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 (Motulsky 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 ($R_{max}$) and the time at which it occurs ($T_{Rmax}$) were calculated according to the following equations (W.J.J., Gerrits, Animal Nutrition Group, Wageningen University- personal communication):

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

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

***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

| Compositions | Intact mushroom and herb materials[2] | | |
|---|---|---|---|
| | *LenS* | *TreS* | *AstS* |
| Dry matter (DM) (g/kg) | 957 | 958 | 967 |
| Crude protein (g/kg DM) | 208 | 103 | 157 |
| Yields of polysaccharides (%)[1] | 8.0 | 10.0 | 31.1 |
| | Polysaccharide extracts[3] | | |
| | *LenE* | *TreE* | *AstE* |
| DM (g/kg) | 952 | 955 | 940 |
| Crude protein (g/kg DM) | 114 | 47 | 67 |
| Total sugar content (g/kg DM) | 632 | 687 | 647 |

[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.

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 ($T_{Rmax}$). 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 ($R_{max)}$ and tended to produce more gas compared with their intact substrates. *AstE* had the fastest $R_{max}$ 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[1]

| | Gas production kinetics[5] | | | | |
|---|---|---|---|---|---|
| Substrates[2] | $T_{Rmax}$ (h) | C (h) | $R_{max}$ (ml/h) | DMCV (ml/g DM) | pH |
| *LenS* | 1.5$^{b}$ | 7.0$^{c}$ | 20.8$^{cd}$ | 235.5$^{bc}$ | 6.3$^{ab}$ |
| *TreS* | ND[6] | ND[6] | ND[6] | 184.7$^{c}$ | 6.1$^{ab}$ |
| *AstS* | 0.9$^{b}$ | 9.9$^{c}$ | 15.2$^{cd}$ | 188.0$^{c}$ | 6.5$^{a}$ |
| *LenE* | 3.4$^{b}$ | 5.3$^{c}$ | 33.8$^{b}$ | 273.3$^{ab}$ | 6.1$^{bc}$ |
| *TreE* | 13.8$^{a}$ | 34.0$^{b}$ | 11.4$^{d}$ | 214.7$^{c}$ | 6.2$^{ab}$ |
| *AstE* | 2.7$^{b}$ | 3.6$^{c}$ | 65.7$^{a}$ | 332.1$^{a}$ | 5.4$^{d}$ |
| MSD[3] | 3.0 | 8.3 | 4.3 | 24.2 | 0.1 |
| *P*-values[4] | 0.0001 | 0.0008 | 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] C = the half-time to the asymptotic gas production; $T_{Rmax}$ = the time when there is the maximum rate of gas production; $R_{max}$ = the maximum rate of gas production; DMCV = total gas production.

[6] ND indicates poor data fit using both mono- and diphasic models.

$^{abc}$: 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 144.3mg/l ammonia after incubation. At the end of fermentation, ammonia concentrations were higher for the intact materials than for the extracts (Table 3).

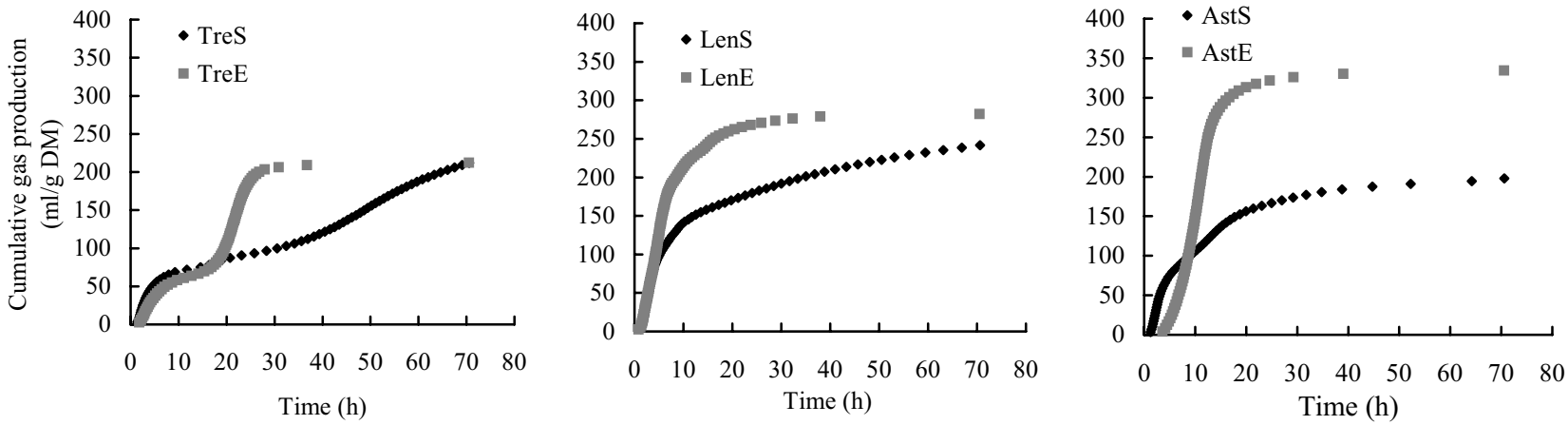



**Figure 1.** Gas production profiles from fermentation of the intact mushroom and herb materials and their polysaccharide extracts. [1]Values are means of 3 determinations for the intact materials (◆), *LenS = Lentinus edodes; TreS = Tremella fuciformis; AstS = Astragalus membranaceus,* and 4 determinations 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 ($NH_3$) production measured at the end of fermentation of intact mushroom and herb materials and their extracts[1]

| Substrates[2] | AAC[5] | APR[5] | ABU[5] | AIBU[5] | AIVAL[5] | AVAL[5] | MMTOT[5] | ATOT[5] | BCR[6] | $NH_3$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | mg AAE[7] /g DM | | | | | | mmol/g DM | mg AAE[7]/g DM | | mg/L |
| *LenS* | 251.4$^{ab}$ | 178.8$^{c}$ | 114.9$^{ab}$ | 7.8$^{ab}$ | 15.6$^{a}$ | 21.4$^{bc}$ | 7.5$^{bc}$ | 590$^{b}$ | 0.082$^{b}$ | 419.1$^{a}$ |
| *TreS* | 236.3$^{ab}$ | 173.5$^{c}$ | 60.5$^{bd}$ | 7.0$^{b}$ | 9.0$^{bc}$ | 16.7$^{bc}$ | 6.6$^{cd}$ | 503$^{c}$ | 0.069$^{b}$ | 322.0$^{b}$ |
| *AstS* | 217.3$^{b}$ | 93.1$^{d}$ | 120.1$^{ab}$ | 8.1$^{ab}$ | 16.3$^{a}$ | 20.5$^{bc}$ | 6.0$^{d}$ | 475$^{c}$ | 0.104$^{a}$ | 399.2$^{a}$ |
| *LenE* | 263.4$^{ab}$ | 213.4$^{b}$ | 90.9$^{c}$ | 3.2$^{cd}$ | 5.6$^{cd}$ | 12.3$^{c}$ | 7.7$^{ab}$ | 589$^{b}$ | 0.037$^{c}$ | 245.6$^{c}$ |
| *TreE* | 275.0$^{a}$ | 237.8$^{a}$ | 57.3$^{d}$ | 5.9$^{bc}$ | 4.2$^{de}$ | 15.3$^{c}$ | 7.9$^{ab}$ | 596$^{b}$ | 0.045$^{c}$ | 253.0$^{c}$ |
| *AstE* | 254.4$^{ab}$ | 232.0$^{ab}$ | 165.4$^{a}$ | 1.2$^{d}$ | 1.9$^{e}$ | 23.0$^{ab}$ | 8.4$^{a}$ | 678$^{a}$ | 0.040$^{c}$ | 144.7$^{d}$ |
| MSD[3] | 16.7 | 8.1 | 8.0 | 1.2 | 1.3 | 3.3 | 0.3 | 25 | 0.021 | 23.5 |
| *P*-values[4] | 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 + APRO + 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 $NH_3$ for growth of bacteria, while the fermentation of protein as a source of energy will lead to net production of $NH_3$ which then diffuses across the intestinal wall into the bloodstream. In terms of animal health, increase of VFA and decreased $NH_3$ are perceived as being beneficial for long-term host health, while the opposite is true for low VFA and increasing $NH_3$ (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.

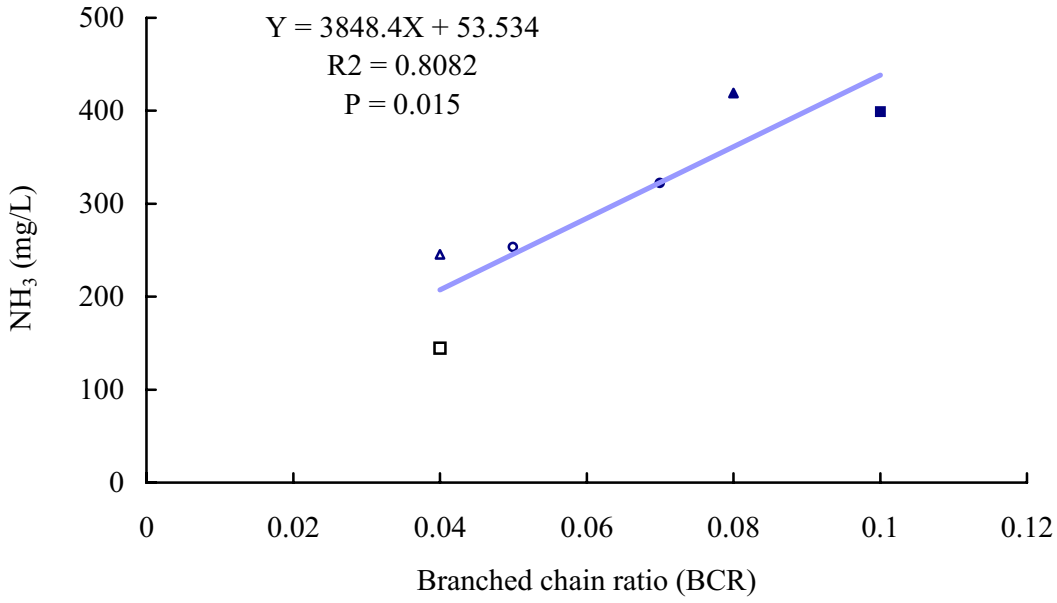



**Figure 2.** Correlation between the ammonia concentration and the branch chain ratio (BCR = (AIBU + AIVAL + AVAL)/(AAC + APRO + ABUT)) measured at the end of fermentation of intact mushroom and herb materials and their extracts. Values are means of 3 determinations for the intact materials, *LenS*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

## ACKNOWLEDGEMENTS

This work was founded in part by Alltech Inc., USA. The authors wish to thank Dick Bongers of the Department of Human and Animal Physiology, Wageningen University for his assistance in analysis of VFA and Huug Boer, Jane Martine Muijlaert and Truus Post of Animal Nutrition Group for their assistance in preparing the fermentation trial and chemical analyses.

## REFERENCES

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

Burrit, E. A., A. S. Bittner, J. C. Street, and M. J. Anderson. 1984. Correlations of phenolic acids and xylose content of cell wall with *in vitro* dry matter digestibility of three maturing grasses. J. Dairy Sci. 67:1209-1213.

Canibe, N., K. E. Bach-Kundsen, and B. O. Eggum. 1997. Apparent digestibility of non-starch polysaccharides and short chain fatty acids production in large intestine in pigs fed dried or toasted peas. Acta Agric. Scand (Animal Science) 47:106-116.

Chesson, A. 1981. Effects of sodium hydroxide on cereal straws in relation to the enhanced degradation of structural polysaccharides by rumen microorganisms. J. Sci. Food Agric. 32:745-758.

Choct, M. 2001. Carbohydrate and fibre digestion in monogastric animals. ASA Technical Bulletin

AN34-2001. http://www.asasea.com/technical/AN34-Choct.pdf MITA(P).No. 070/10/2001.

Cummings, J. H., and G. T. Macfarlane. 2002. Gastrointestinal effects of prebiotics. Br. J. Ntri. 87(Suppl. 2):S145-S151.

Davies, Z. S., D. Mason, A.E. Brooks, G. W. Griffith, R. J. Merry, and M. K. Theodorou. 2000. An automated system for measuring gas production from forages inoculated with rumen fluid and its use in determining the effect of enzymes. Anim. Feed Sci. Technol. 83(3-4):205-221.

Dubois, M. K., J. K. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Pfizer flocon 4800 procedure-phenol/sulfuric acid method. Analytical Chemistry. 28: 350-352.

Engels, F. M. 1996. Developments in application of light and scanning electron microscopy techniques for cell wall degradation studies. Neth. J. Agric. Sci. 44:357-373.

France, F. and R. C. Siddons. 1993. Volatile fatty acid production. Pages 107-121 in: Quantitative Aspects of Ruminant Digestion and Metabolism. J. M. Forbes ed. J. France, CAB International, Wallingford.

Gardiner, K. R., P. J. Erwin, N. H. Anderson, J. G. Barr, M. I. Halliday, and B. J. Rowlands. 1993. Colonic bacteria and bacterial translocation in experimental colitis. Br. J. Surg. 80:512-516.

Getachew, G., M. Bluemmel, H. P. S. Makkar, and K. Becker. 1998. *In vitro* gas measuring techniques for assessment of nutritional quality of feed: A review. Anim. Feed Sci. Technol. 72(3-4):261-281.

Gibson, G. R., and M. B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. J. of Nutr. 125(6):1401-1412.

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

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

Houdijk, J. G. M. 1998. Effects of non-digestible oligosaccharides in young pig diets. PhD thesis, Wageninge Agricultural University.

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

Liu J. X., C. Y. Wang, and Y. M. Bai. 1999. Preparation of immunologically active Chinese herbal formulation and safe efficacy test. Chinese J. of Veterinary Science and Technology. 29(8): 23-25.

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

131:2225-2229.

Macfarlane, G. T., G. R. Gibson, E. Beatty, and J. H. Cummings. 1992. Estimation of short-chain fatty acid production from protein by human intestinal bacterial based on branched-chain fatty acid measurements. FEMS Microbial Ecology. 101: 81-88.

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

Pang, F., Y. X. Feng, and Y. Zhang. 1995. Review on current research of *Astragalus mongholicus Bga*. World Phytomedicines (CN). 10(3):110-115.

Raibaud, P. 1992. Bacterial interactions in the gut. Pages 9-28 in: Probiotics-The Scientific Basis. R. Fuller. Chapman Hall, Londen.

SAS Institute Inc. 1995. SAS/STAT User's Guide, Version 6.11., Cary, NC: The SAS Institute.

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

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

Sunvold, G. D., H. S. Hussein, G. C. Fahey Jr, N. R. Merchen, and G. A. Reinhart. 1995. *In vitro* fermentation of cellulose, beet pulp, citrus pulp, and citrus pectin using fecal inocula from cats, dogs, horses, humans, and pigs and fuminal fluid from cattle. J. Anim. Sci. 73:3639-3648.

Theodorou, M. K., B. A. Williams, M. S. Dhanoa, A. B. McAllan, and J. France. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Technol. 48:185-197.

Titgemeyer, E. C., M. G. Cameron, L. D. Bourquin, and G. C. Fahey Jr. 1991. Digestion of cell wall components by dairy heifers fed diets based on alfalfa and chemically treated oat hulls. J. Dairy Sci. 74: 1026-1037.

Van Houtert, M. F. J. 1993. The production and metabolism of volatile fatty acids by ruminants fed roughages: A review. Anim. Feed Sci. Technol. 43:189-225.

Van Laar, H., S. Tamminga, B. A. Williams, M. W. A. Verstegen, and F. M. Engels. 1999. Fermentation characteristics of cell-wall sugars from soya bean meal, and from separated endosperm and hulls of soya beans. Anim. Feed Sci. Technol. 79:179-193.

Verstegen, M. W. A. and G. J. Schaafsma. 1999. Some developments on alternatives for antibiotics as growth promotor. Pages 65-73 in: Nutrition and Gastrointestinal Physiology Today and Tomorrow. A. J. M. Jansman ed. J. Huisman, Wageningen, The Netherlands.

Williams, B. A., S. Tamminga, and M. W. A. Verstegen. 2000. Fermentation kinetics to assess microbial activity of gastro-intestinal microflora. Pages 97-100 in An EAAP Satellite

Symposium on Gas Production: Fermentation Kinetics for Feed Evaluation and to Assess Microbial Activity. British Society of Animal Science. Aug. 18-19. Wageningen, Netherlands.

Xie, Z. Q., and S. Q. Niu. 1996. Comprehensive Book of Natural Resources and Chinese Herb Feed Additives. Xueyuan Science and Technology Press, Beijing, China.

Xue, M., and X. S. Meng. 1996. Review on research progress and prosperous of immune activities of bioactive polysaccharides. J. of Traditional Vet. Medicine. 3:15-18.

Yang Y. and W. S. Feng. 1998. Brochures of isolation and extraction of chemical components from herbs. China Chinese Herb Medicine Press. Beijing. China.

## 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

F.C. Guo

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands; Institute of Animal Science and Vet. Pharmaceutics, Chinese Academy of Agricultural Sciences, Lanzhou 730050, China*

A.D.L. Akkermans

*Laboratory of Microbiology, Department of Agrotechnology and Food Sciences, 6703 CT Wageningen, Wageningen University, Netherlands*

R.P. Kwakkel

*Animal Production Systems Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

B.A. Williams and M.W.A. Verstegen

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

Submitted to: Poultry Science

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

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

---

## 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 ($10^{8}$-$10^{9}$ 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 $^{o}$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$^{o}$C.

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

| Compositions | Intact mushroom and herb materials[2] | | |
|---|---|---|---|
| | *LenS* | *TreS* | *AstS* |
| Dry matter (DM) (g/kg) | 957 | 958 | 967 |
| Crude protein (g/kg DM) | 208 | 103 | 157 |
| Yields of polysaccharides (%)[1] | 8.0 | 10.0 | 31.1 |
| | Polysaccharide extracts[3] | | |
| | *LenE* | *TreE* | *AstE* |
| DM (g/kg) | 952 | 955 | 940 |
| Crude protein (g/kg DM) | 114 | 47 | 67 |
| Total sugar content (g/kg DM) | 632 | 687 | 647 |

[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 $CO_2$. 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 $CO_2$.

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 $AgNO_3$ 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:

$$Cs = [2j/(a + b)] \times 100$$

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[1]

| | Similarity indices (%) | | |
|---|---|---|---|
| Enrichment comparisons | *LenS* | *TreS* | *AstS* |
| Inoculum | 46.2±2.6$^{a}$ | 39.9±3.5$^{a}$ | 51.5±5.3$^{a}$ |
| Blanks before incubation | 57.7±1.9$^{b}$ | 41.5±3.1$^{a}$ | 56.6±7.3$^{b}$ |
| Blanks after incubation | 44.8±2.7$^{a}$ | 43.5±3.7$^{a}$ | 51.7±7.4$^{a}$ |
| | *LenE* | *TreE* | *AstE* |
| Inoculum | 23.3±1.9$^{a,***}$ | 57.1±8.7$^{b}$ | 76.8±1.2$^{c,**}$ |
| Blanks before incubation | 31.1±2.3$^{a,***}$ | 58.3±9.9$^{b}$ | 79.8±0.7$^{c,*}$ |
| Blanks after incubation | 36.2±2.1$^{a,*}$ | 54.3±8.2$^{b}$ | 79.6±1.2$^{c,**}$ |

[1] Results are given as means (n=6) ± SE; 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).

$^{abc}$: Means within rows with no common superscript differ significantly (P<0.05).

$^{*, **, ***}$: Means differ significantly between each intact material and its polysaccharide extract (per inoculum or blank (*P<0.05, **P<0.01, ***P<0.001).

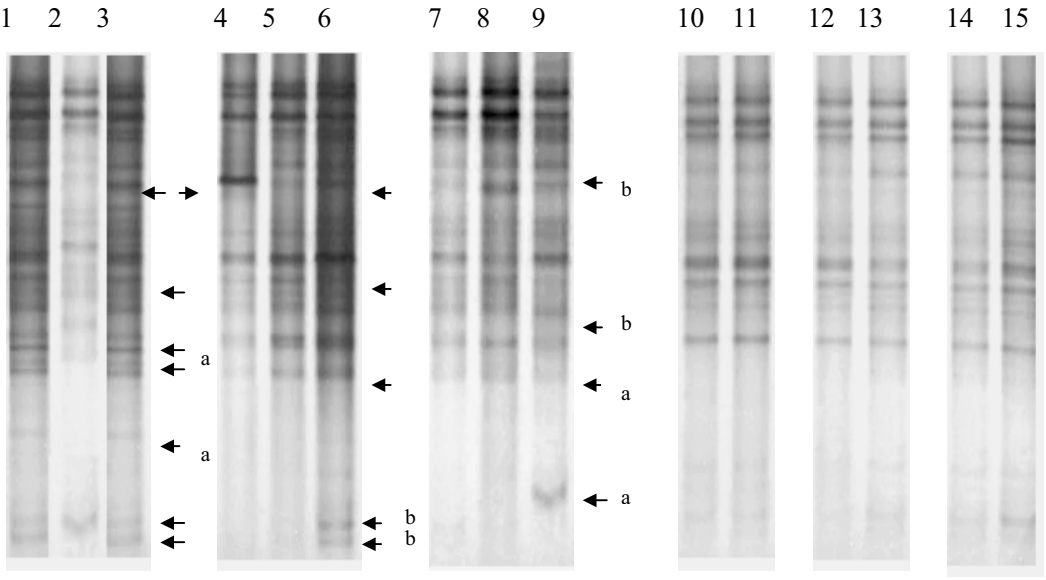



**Figure 1.** DGGE band pattern of bacterial community profiles of the samples containing the intact materials. DGGE profile lanes: 1, 2, 3: *Lentinus edodes* (*LenS*); 4, 5, 6: *Tremella fuciformis* (*TreS*); 7, 8, 9: *Astragalus membranaceus* (*AstS*) 10, 11: the inoculum; 12, 13: blank before incubation; 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.

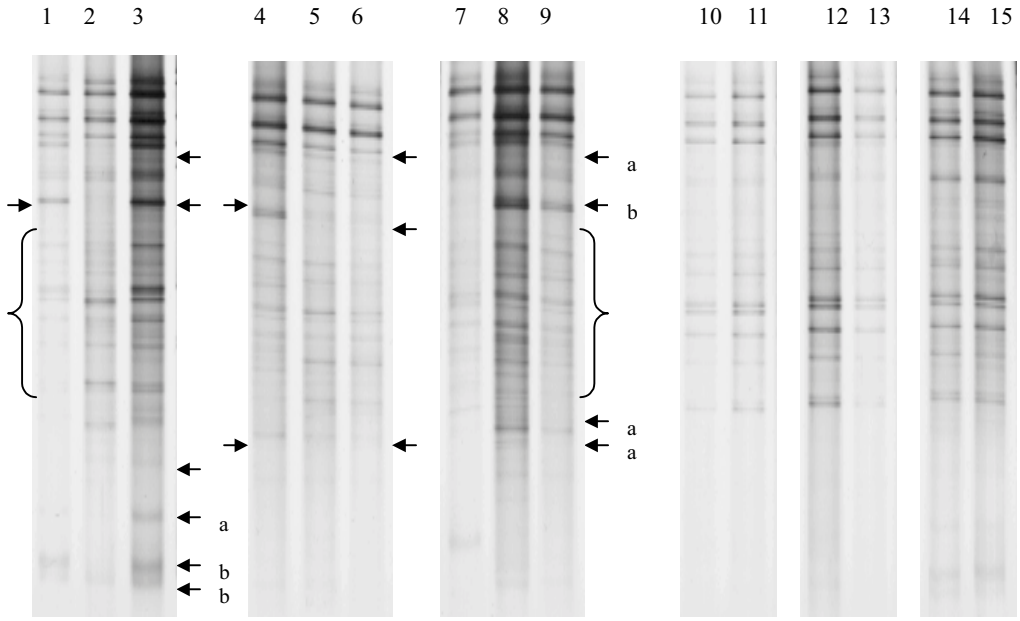



**Figure 2.** DGGE band pattern of bacterial community profiles of the samples containing the extracts. DGGE profile lanes: 1, 2, 3: *Lentinus edodes* extract (*LenE*); 4, 5, 6: *Tremella fuciformis* extract (*TreE*); 7, 8, 9: *Astragalus membranaceus* extract (*AstE*); 10, 11: inoculum; 12, 13: blank 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.

## ACKNOWLEDGEMENTS

This project was supported in part by Alltech Inc. USA. The authors would like to thank Wilma Akkermans-van Vliet, Hans Heilig, Wen Yao, and Sergey Konstantinov from the Laboratory of Microbiology, Agrotechnology and Food Sciences, Wageningen University for their assistance with the DGGE and other laboratory analyses. John Patterson of the Department of Animal Science, Purdue University, USA, is also thanked for his critical reading of the manuscript.

## REFERENCES

Amann, R. L., W., Ludwig, and K. H., Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbial. Rev. 59:143-169.

Barnes, E. M, G. C., Mead, D. A., Barnum, and E. G., Harry. 1972. The instestinal flora of the chicken in the period 2 to 6 weeks of age, with particular reference to the anaerobic bacteria. Br. Poult. Sci.; 13:311-326.

Cummings, J. H., and G. T., Macfarlane. 2002. Gastrointestinal effects of prebiotics. Br. J. Ntri. 87(Suppl.:2) 1S45-S51.

Davies, Z. S., D., Mason, A. E., Brooks, G. W., Griffith, R. J., Merry, M. K., Theodorou. 2000. An automated system for measuring gas production from forages inoculated with rumen fluid and its use in determining the effect of enzymes. Anim. Feed Sci. Technol.; 83(3-4):205-221.

Dubois, M. K., J. K., Gilles, J. K., Hamilton, P. A., Rebers, and F., Smith. 1956. Pfizer. flocon 4800 procedure-phenol/sulfuric acid method. Analytical Chemistry 28:350-352.

Gardiner, K. R., P. J., Erwin, N. H., Anderson, J. G., Barr, M. I., Halliday, and B. J., Rowlands. 1993. Colonic bacteria and bacterial translocation in experimental colitis. Br. J. Surg. 80:512-516.

Gibson, G. R., and M. B., Roberfroid. 1995. Dietary modulation of the human colonic microflora: introducing the concept of prebiotics. J. Nutr. 125:1401.

Gillan, D., A., Speksnijder, G., Zwart, and C., de Ridder. 1998. Genetic diversity in the biofilm covering Montacuta ferruginosa (Malluska, Bolivia) as evaluated by denaturing gradient gel electrophoresis and cloning of PCR-amplified gene fragment coding for 16S rRNA. Appl. Environ. Microb. 64:3464-3472.

Heilig, G. H. J., E. G., Zoetendal, E. E., Vaughan, P., Marteau, and A. D. L., Akkermans. 2002. Molecular diversity of *Lactobacillus spp*. and other lactic acid bacteria in the human intestine as determined by pecific amplification of 16S ribosomal DNA. Appl. Environ. Microb. 68 (1): 114-123.

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

Jensen, B. B. 1993. The possibility of manipulating the microbial activity in the digestive tract of monogastric animals. 44th Animal Meeting of the European Association for Animal Production. Århus, Denmark, pp. 20.

Jensen, B. B. 1999. Impact of feed composition and feed processing on the gastrointestinal ecosystem in pigs. in: A.J.M. Jansman and J.Huisman (Eds). Nutrition and gastrointestinal physiology-today and tomorrow, Wageningen, The Netherlands, pp. 43-56.

Langhout, D. J. 1998. The role of the intestinal flora as affected by non-starch polysaccharides in broiler chicks. PhD thesis, Wageningen Agricultural University, The Netherlands.

Liu, J. X., C. Y., Wang, and Y. M., Bai. 1999. Preparation of immune active Chinese herbal formulation and safe efficacy test. Chinese J. of Vet. Science and Technology 29(8):23-25.

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

Magurran, A. 1988. Density indices and species abundance models. in: Ecological Density and Its Measurement, Princeton, NJ, Princeton University Press, pp. 8-45.

Min, S. D. 1996. Medicinal value of fungi. Acta Edulis Fungi. 3(4):55-64.

Muyzer, A., J., Hollibaugh, and C., Orrego. 1996. Phylogenetic compositions of bacterioplankton

from two California estuaries compared by denaturing gradient gel eletrophoresis of 16S rDNA fragments. Appl. Environ. Microb. 62:2676-2680.

Muyzer, G., and K., Smalla. 1998. Application of the DGGE and TGGE in microbial ecology. Antonie van Leeuwenhoec. 73:127-141.

Muyzer, G. E., E. C., de Waal and G. A., Uitterlinden. 1993. Profiling of complex populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes for 16S rDNA. Appl. Environ. Microb. 59:695-700.

Pang, F., Y. X., Feng, and Y., Zhang. 1995. Review on current research of *Astragalus mongholicus Bga*. World Phytomedicines 10(3):110-115.

Qi, Z. S. 1987. Review on chemical study of *Astragalus*. Chinese Traditional and Herbal Drugs 18(5):41-29.

Raskin, L., W., Capman, R., Sharp, L., Poulsen, and D., Stahl. 1997. Molecular ecology of gastrointestinal ecosystems. in: Mackie, R.I. White, B.A., Isaacson, R.E. (Eds.), Gastrointestinal Microbes and Host Interactions, Gastrointestinal Microbiology, Vol. 2, Chapman and Hall, New York, pp. 243-298.

Sambrook, J., E. F., Fritsch, and T., 1989. Maniatis. Molecular cloning, a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Sanguinetti, C. J., E., Dias Neto, and A. J. G., Simpson. 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. BioTechniques 17:915-919.

Satokari, R. M., E. E., Vaughan, A. D. L., Akkermans, M., Saarela, and W. M., de Vos. 2001. Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. Appl. Environ. Microb. 67(2):504-513.

Simpson, J. M., V. J., McCracken, H. R., Gaskins, and R. I., Mackie. 2000. Denaturing gradient gel electrophoresis analysis of 16s ribosomal DNA amplicons to monitor changes in fecal bacterial populations of weaning pigs after introduction of Lactobacillus reuteri strain MM53. Appl. Environ. Microb. 66 (11):4705-4714.

Simpson, J. M., V. J., McCracken, B. A., White, H. R., Gaskins, and R. I., Mackie. 1999. Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointectinal microbiota. J. of Microb. Methods 36:167-179.

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

Sunvold, G. D., H. S., Hussein, G. C., Fahey Jr, N. R., Merchen, and G. A., Reinhart. 1995. *In vitro* fermentation of cellulose, beet pulp, citrus pulp, and citrus pectin using fecal inoculum from cats, dogs, horses, humans, and pigs and fuminal fluid from cattle. J. Anim. Sci. 73:3639-3648.

Theodorou, M. K., B. A., Williams, M. S., Dhanoa, A. B., McAllan, J., France. 1994. A simple ruminant feeds. Anim. Feed Sci. Technol. 48:185-197.

Van der Wielen, D. A., J. J., Keuzenkamp, L. J. A., Lipman, F., van Knapen, and S., Biesterveld. 2002. Spatial and Temperal Variation of the intestinal bacterial community rasied broiler chicks during growth. Microb. Ecol. 44: 286-293.

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

Verstegen, M. W. A., and G. J., Schaafsma. 1999. Some developments on alternatives for antibiotics as growth promotor. In Nutrition and Gastrointestinal Physiology Today and Tomorrow. A. J. M. Jansman and J. Huisman (Eds), Wageningen The Netherlands. p. 65-73.

Williams, B. A., S., Tamminga, and M. W. A., Verstegen. 2000. Fermentation kinetics to assess microbial activity of gastro-intestinal microflora. An EAAP Satellite Symposium on Gas Production: Fermentation kinetics for feed evaluation and to assess microbial activity. Wageningen, Netherlands. p. 97-100.

Xia, E. N., and Q. H., Cheng. 1988. Isolation, analysis and bioactivities of *Tremella fuciformis* fruit body polysaccharides. Acta Mycologica Sinica, 7(3):166-174.

Xie, Z. Q., and S. Q., Niu. 1996. Comprehensive Book of Natural Researces and Chinese Herb Feed Additives (in Chinese). Beijing, China, Xueyuan Science and Technology Press.

Xue, M., and X. S., Meng. 1996. Review on research progress and prosperous of immune activities of bioactive polysaccharides. J. of Traditional Vet. Medicine 3:15-18.

Yang, J, M. C., Wu, and S. H., Zhang. 1999. Study on isolation, purification and characteristics of polysaccharide Le-III from the fruit body of *Lentinus Edodes.* J. of Huazhong Agricultural University 18(2):188-190.

Zheng, Z., D., Liu, C., Song, C., Cheng, and Z., Hu. 1998. Studies on chemical constituents and immunological function activity of hairy root of *Astragalus membranaceus*. Chin J Biotechnol. 14(2):93-7.

Zhu, X. Y., T., Zhong, Y., Pandya, and R. D., Joerger. 2002. 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. Appl. Environ. Microb., 68(1):124-137.

Zoetendal, E. G., A. D. L., Akkermans, and W. M., de Vos. 1998. Temperature Gradient Gel Electrophoresis Analysis of 16S rRNA from Huamn Fecal Samples and Host-Specific Communities of Active Bacteria. Appl. Environ. Microb. 58:2292-2295.

Zoetendal, E. G., A. D. L., Akkermans, W. M., Akkermans, J. A., van Vliet, G. M., de Visser, and W. M., de Vos. 2001. The host genotype affects the bacterial community in the human gastrointestinal tract. Microbial Ecology in Health and Disease 13(3):129-134.

Zoetendal, E. G., A., von Wright, S. T., Vilpponen, K., Ben Amor, A. D. L., Akkermans, and W. M., de Vos. 2002. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. Appl. Environ. Microb. 68(7):3401-3407.

# Chapter 5

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

F.C. Guo

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands; Institute of Animal Science and Vet. Pharmaceutics, Chinese Academy of Agricultural Sciences, Lanzhou 730050, China*

R.P. Kwakkel

*Animal Production Systems Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

B.A. Williams and M.W.A. Verstegen

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

H.S. Li, J.Y. Luo, X.P. Li, Y.X. Wei, and Z.T. Yan

*Laboratory of Veterinary Clinic, Institute of Lanzhou Animal Science and Veterinary Pharmaceutics, CAAS,* Lanzhou *730050, P. R. China*

W.K. Li

*Department of Parasitological Diseases, Gansu Animal Health Service, 730046, Lanzhou, P. R. China*

Submitted to: British Poultry Science

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

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

## 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 immunodulators 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 $cm^2$ 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 $m^2$ during the first two weeks and 15 watt /20 $m^2$ 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[1]

| Compositions | *LenE*[1] | *TreE*[1] | *AstE*[1] |
|---|---|---|---|
| Yields of polysaccharides (%)[2] | 6.8 | 16.0 | 10.1 |
| Dry matter (g/kg)[3] | 958 | 965 | 967 |
| Total sugar content (g/kg DM)[4] | 655 | 613 | 687 |

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

[2] Yields of polysaccharides were expressed on basis of air-dry matter of the intact mushroom and herb materials.

[3] The dry matter contents of the extracts were determined by freeze-drying.

[4] 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)

| Ingredients | Starter diet | Grower diet |
|---|---|---|
| Maize | 570 | 635.3 |
| Soybeans | 320 | 270 |
| Fish meal | 40 | 30 |
| Animal fat | 20 | 20 |
| Limestone | 14 | 12 |
| Salt (NaCl) | 3.5 | 3.5 |
| Calcium hydrophosphate ($CaHPO_4$) | 17 | 16 |
| DL-Methionine | 2.6 | 2.1 |
| Lysine | 2.9 | 1.1 |
| Premix[1] | 10 | 10 |
| Calculated analysis (g/kg) | | |
| Crude protein | 210 | 190 |
| Metabolisable energy (kcal/kg) | 3,000 | 3,050 |
| Calcium | 10 | 9 |
| *Available phosphate* | 4.5 | 4.3 |
| Lysine | 11.7 | 10.5 |
| Methionine | 6.0 | 5.1 |

[1] Supplied for per kg of diet: vitamin A (retinal acetate) 8500 IU, cholechalciferol 1800 IU, vitamin E (DL-α-topherol acetate) 13 mg, vitamin $K_3$ 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, $Na_2SeO_3 \cdot 5H_2O$ 0.15 mg, $FeSO_4 \cdot 7H_2O$ 80 mg, $MnO_2$ 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:

$$Y = b0 + b1\ X \qquad [1]$$

$$Y = b0 + b1X + b2\ X^2 \qquad [2]$$

Where Y = predicted response; X = the dose of the extract; b0 = intercept (i.e., BW gain, feed intake or FCR on the basal diet), b1 and b2 = 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

| Treatments[1] | Day 7-14 | | | Day 15-21 | | | Day 22-28 | | | Day 7-28 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BW gain ($g\ b^{-1}d^{-1}$) | FI ($g\ b^{-1}d^{-1}$) | FCR | BW gain ($g\ b^{-1}d^{-1}$) | FI ($g\ b^{-1}d^{-1}$) | FCR | BW gain ($g\ b^{-1}d^{-1}$) | FI ($g\ b^{-1}d^{-1}$) | FCR | BW gain ($g\ b^{-1}d^{-1}$) | FI ($g\ b^{-1}d^{-1}$) | FCR |
| Non-suppl. | $31.8^{b}$ | $47.1^{b}$ | $1.51^{ab}$ | $32.2^{c}$ | 81.0 | $2.52^{a}$ | 44.3 | 80.0 | 1.83 | 36.1 | 69.8 | 1.93 |
| VRG | $32.8^{a}$ | $46.8^{b}$ | $1.43^{c}$ | $35.0^{ab}$ | 80.5 | $2.32^{ab}$ | 44.6 | 81.3 | 1.82 | 37.3 | 69.1 | 1.87 |
| *LenE* | $32.3^{a}$ | $48.4^{ab}$ | $1.52^{ab}$ | $36.3^{a}$ | 78.9 | $2.17^{b}$ | 44.2 | 79.9 | 1.83 | 37.5 | 68.9 | 1.83 |
| *TreE* | $32.6^{a}$ | $52.7^{a}$ | $1.62^{a}$ | $32.9^{bc}$ | 79.7 | $2.44^{a}$ | 45.2 | 80.7 | 1.80 | 36.9 | 71.0 | 1.92 |
| *AstE* | $31.9^{ab}$ | $51.1^{ab}$ | $1.60^{a}$ | $32.8^{bc}$ | 78.8 | $2.42^{a}$ | 45.1 | 80.7 | 1.79 | 36.7 | 70.2 | 1.92 |
| SEM[2] | 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 means (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 4.** BW gain of broiler chicks supplemented with mushroom and herb extracts at different dietary levels[1]

| | Extracts[2] | Dietary level of extracts (g/kg) | | | | | | Sources of variance | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.5 | 1 | 2 | 3 | 4 | SEM[3] | Extract | Dose | Dose[2] | Extract*Dose | Extract*Dose[2] |
| Day 7-14 | *LenE* | 31.7 | 32.1 | 33.1 | 32.6 | 32.1 | .19 | **.051** | .591 | .544 | .107 | .154 |
| | *TreE* | 33.2 | 32.0 | 32.7 | 32.5 | 32.5 | .21 | | | | | |
| | *AstE* | 32.0 | 32.1 | 32.1 | 31.9 | 31.6 | .18 | | | | | |
| | SEM | .34 | .25 | .22 | .23 | .24 | | | | | | |
| Day 15-21 | *LenE* | 34.8 | 37.8 | 37.4 | 35.8 | 35.7 | .33 | .726 | **.035** | **.042** | .376 | .316 |
| | *TreE* | 32.3 | 33.7 | 32.7 | 32.9 | 32.7 | .47 | | | | | |
| | *AstE* | 32.3 | 31.7 | 33.8 | 33.6 | 32.7 | .34 | | | | | |
| | SEM | .52 | .79 | .59 | .59 | .63 | | | | | | |
| Day 22-28 | *LenE* | 44.4 | 45.0 | 46.6 | 42.2 | 42.6 | .61 | .670 | .879 | .495 | .748 | .671 |
| | *TreE* | 46.1 | 45.8 | 45.0 | 45.2 | 44.2 | .44 | | | | | |
| | *AstE* | 45.1 | 46.3 | 44.6 | 45.2 | 44.3 | .45 | | | | | |
| | SEM | .76 | .64 | .54 | .72 | .51 | | | | | | |
| Day 7-28 | *LenE* | 37.0 | 37.8 | 39.0 | 36.8 | 36.8 | .25 | .512 | .140 | **.073** | .292 | .290 |
| | *TreE* | 37.1 | 37.4 | 36.8 | 36.9 | 36.6 | .26 | | | | | |
| | *AstE* | 36.5 | 36.7 | 37.3 | 36.6 | 36.2 | .19 | | | | | |
| | SEM | .30 | .27 | .36 | .30 | .26 | | | | | | |

[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.

**Table 5.** Parameter estimates and asymptotic SE (in parenthesis) of BW gain, described by linear and quadratic functions in broilers

| Type of model[1] | | Extracts[2] | b0 | b1 | b2 | SE | P-value[3] | $R^2$ | F (1, 27)[4] |
|---|---|---|---|---|---|---|---|---|---|
| | Day 7-14 | *LenE* | 32.05 (0.37) | 0.13 (0.15) | | 1.06 | .414 | .02 | |
| | | *TreE* | 32.72 (0.41) | -0.07 (0.17) | | 1.16 | .683 | .01 | |
| | | *AstE* | 32.16 (0.35) | -0.11 (0.14) | | 1.01 | .452 | .02 | |
| | Day 15-21 | *LenE* | 36.54 (0.65) | -0.11 (0.26) | | 1.85 | .670 | .01 | |
| Linear | | *TreE* | 32.92 (0.92) | -0.03 (0.37) | | 2.63 | .935 | .002 | |
| | | *AstE* | 32.18 (0.66) | 0.31 (0.27) | | 1.88 | .263 | .04 | |
| | Day 22-28 | *LenE* | 45.76 (1.13) | -0.77 (0.46) | | 3.22. | .105 | .09 | |
| | | *TreE* | 46.27 (0.82) | -0.49 (0.33) | | 2.34 | .154 | .07 | |
| | | *AstE* | 45.78 (0.87) | -0.33 (0.35) | | 2.47 | .354 | .03 | |
| | Day 7-28 | *LenE* | 37.85 (0.48) | -0.18 (0.20) | | 1.37 | .361 | .03 | |
| | | *TreE* | 37.33 (0.49) | -0.18 (0.20) | | 1.41 | .369 | .03 | |
| | | *AstE* | 36.82 (0.37) | -0.08 (0.15) | | 1.06 | .606 | .01 | |
| | Day 7-14 | *LenE* | 30.92 (0.59) | 1.63 (0.66) | -0.34 (0.14) | 0.99 | **.061** | .19 | **5.41** |
| | | *TreE* | 33.02 (0.71) | -0.47(0.79) | 0.09 (0.17) | 1.18 | .806 | .02 | 0.27 |
| | | *AstE* | 31.90 (0.62) | 0.24 (0.69) | -0.08 (0.15) | 1.02 | .663 | .03 | 0.27 |
| | Day 15-21 | *LenE* | 34.67 (1.05) | 2.40 (1.17) | -0.56 (0.26) | 1.74 | **.099** | .16 | **4.83** |
| Quadratic | | *TreE* | 32.61 (1.61) | 0.39 (1.80) | -0.09 (0.39) | 2.67 | .969 | .002 | 0.06 |
| | | *AstE* | 30.85 (1.11) | 2.09 (1.24) | -0.40 (0.27) | 1.84 | .189 | .12 | 2.19 |
| | Day 22-28 | *LenE* | 44.06 (1.93) | 1.52 (2.16) | -0.51 (0.47) | 3.21 | .155 | .13 | 1.18 |
| | | *TreE* | 46.27 (1.44) | -0.49 (1.60) | 0.01 (0.35) | 2.39 | .369 | .07 | 0.00 |
| | | *AstE* | 45.46 (1.51) | 0.11 (1.69) | -0.10 (0.37) | 2.52 | .633 | .03 | 0.07 |
| | Day 7-28 | *LenE* | 36.34 (0.76) | 1.85 (0.85) | -0.45 (0.19) | 1.27 | **.045** | .21 | **5.95** |
| | | *TreE* | 37.37 (0.86) | -0.24 (0.97) | 0.01 (0.21) | 1.44 | .672 | .03 | 0.00 |
| | | *AstE* | 35.98 (0.62) | 1.04 (0.69) | -0.25 (0.15) | 1.03 | .239 | .10 | 2.73 |

[1] Models: Linear: $Y = b0 + b1\ X$; Quadratic: $Y = b0 + b1\ X + b2\ X^2$.

[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 significancy 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 6.** Feed intake of broiler chicks supplemented with mushroom and herb extracts at the different dietary levels[1]

| | Extracts[2] | Dietary level of extracts (g/kg) | | | | | | Sources of variance | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.5 | 1 | 2 | 3 | 4 | SEM[3] | Extract | Dose | Dose[2] | Extract*Dose | Extract*Dose[2] |
| Day 7-14 | *LenE* | 49.3 | 46.7 | 47.3 | 46.9 | 51.9 | .85 | **.073** | .721 | .381 | **.024** | **.031** |
| | *TreE* | 52.7 | 53.2 | 53.5 | 52.1 | 51.9 | .38 | | | | | |
| | *AstE* | 46.2 | 53.8 | 47.5 | 54.3 | 53.9 | .79 | | | | | |
| | SEM | 1.0 | 1.1 | 1.1 | .95 | .71 | | | | | | |
| Day 15-21 | *LenE* | 75.1 | 78.8 | 77.1 | 80.6 | 83.8 | .85 | .890 | **.035** | .106 | .780 | .443 |
| | *TreE* | 76.1 | 81.6 | 80.2 | 80.2 | 80.3 | .66 | | | | | |
| | *AstE* | 77.8 | 80.8 | 78.3 | 81.0 | 76.2 | .85 | | | | | |
| | SEM | 1.1 | .54 | .77 | .66 | 1.4 | | | | | | |
| Day 22-28 | *LenE* | 80.1 | 78.0 | 79.3 | 79.5 | 82.5 | .68 | .570 | .413 | .206 | .309 | .275 |
| | *TreE* | 79.4 | 80.3 | 80.0 | 82.0 | 81.8 | .42 | | | | | |
| | *AstE* | 82.5 | 80.1 | 81.5 | 80.2 | 80.5 | .65 | | | | | |
| | SEM | .87 | .86 | .73 | .68 | .63 | | | | | | |
| Day 7-28 | *LenE* | 68.2 | 67.8 | 67.9 | 68.3 | 72.5 | .50 | .476 | .560 | .843 | **.023** | **.009** |
| | *TreE* | 69.4 | 71.7 | 71.2 | 71.4 | 71.3 | .31 | | | | | |
| | *AstE* | 68.0 | 72.0 | 68.7 | 71.9 | 70.5 | .50 | | | | | |
| | SEM | .58 | .65 | .56 | .48 | .53 | | | | | | |

[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.

**Table 7.** Parameter estimates and asymptotic SE (in parenthesis) of feed intake, described by linear and quadratic functions in broilers

| Type of model[1] | Periods | Extracts[2] | b0 | b1 | b2 | SE | P-value[3] | $R^2$ | F (1, 27)[4] |
|---|---|---|---|---|---|---|---|---|---|
| Linear | Day 7-14 | *LenE* | 46.96 (1.63) | 0.69 (0.66) | | 4.66 | .305 | .04 | |
| | | *TreE* | 53.36 (0.72) | -0.32 (0.29) | | 2.06 | .280 | .04 | |
| | | *AstE* | 47.71 (1.36) | 1.63 (0.55) | | 3.87 | **.006** | .24 | |
| | Day 15-21 | *LenE* | 74.88 (1.39) | 1.92 (0.57) | | 4.00 | **.002** | .29 | |
| | | *TreE* | 78.33 (1.26) | 0.63 (0.51) | | 3.58 | .227 | .05 | |
| | | *AstE* | 79.70 (1.65) | -0.42 (0.67) | | 4.69 | .537 | .01 | |
| | Day 22-28 | *LenE* | 78.28 (1.28) | 0.76 (0.52) | | .3.66 | .156 | .07 | |
| | | *TreE* | 79.17 (0.76) | 0.73 (0.31) | | 2.16 | **.025** | .17 | |
| | | *AstE* | 80.42 (1.26) | 0.14 (0.51) | | 3.60 | .783 | .003 | |
| | Day 7-28 | *LenE* | 66.74 (0.85) | 1.05 (0.34) | | 2.41 | **.005** | .25 | |
| | | *TreE* | 70.26 (0.58) | 0.35 (0.23) | | 1.65 | .144 | .07 | |
| | | *AstE* | 69.27 (0.95) | 0.45 (0.39) | | 2.70 | .250 | .05 | |
| Quadratic | Day 7-14 | *LenE* | 51.30 (2.66) | -5.13 (2.98) | 1.30 (0.65) | 4.42 | **.092** | .16 | 4.01 |
| | | *TreE* | 52.55 (1.25) | 0.77 (1.39) | -0.24 (0.31) | 2.07 | .411 | .06 | 0.64 |
| | | *AstE* | 47.46 (2.37) | 1.97 (2.65) | -0.08 (0.58) | .3.94 | **.026** | .24 | 0.02 |
| | Day 15-21 | *LenE* | 75.76 (2.43) | 0.75 (2.71) | 0.26 (0.59) | .4.04 | **.009** | .30 | 0.20 |
| | | *TreE* | 75.90 (2.12) | 3.90 (2.37) | -0.73 (0.52) | 3.52 | .186 | .12 | 2.00 |
| | | *AstE* | 76.68 (2.79) | 3.63 (3.11) | -0.91 (0.68) | 4.63 | .352 | .07 | 1.77 |
| | Day 22-28 | *LenE* | 80.76 (2.17) | -2.57 (2.42) | 0.75 (0.53) | 3.60 | .143 | .13 | 1.99 |
| | | *TreE* | 79.04 (1.32) | 0.90 (1.48) | -0.04 (0.32) | 2.20 | **.084** | .17 | 0.01 |
| | | *AstE* | 80.85 (2.21) | -0.44 (2.47) | 0.13 (0.54) | 3.67 | .936 | .01 | 0.06 |
| | Day 7-28 | *LenE* | 69.64 (1.31) | -2.84 (1.47) | 0.87 (0.32) | 2.18 | **.008** | .41 | **7.38** |
| | | *TreE* | 69.13 (0.97) | 1.86 (1.09) | -0.34 (0.24) | 1.62 | .132 | .14 | 2.02 |
| | | *AstE* | 68.31 (1.64) | 1.74 (1.83) | -0.29 (0.40) | 2.72 | .404 | .06 | 0.52 |

[1] Models: Linear: $Y = b0 + b1\ X$; Quadratic: $Y = b0 + b1\ X + b2\ X^2$.

[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 significancy 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 8.** Feed conversion ratio of broiler chicks supplemented with mushroom and herb extracts at the different dietary levels[1]

| | Extracts[2] | Dietary level of the extracts (g/kg) | | | | | | Sources of variance | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.5 | 1 | 2 | 3 | 4 | SEM[3] | Extract | Dose | Dose[2] | Extract*Dose | Extract*Dose[2] |
| Day 7-14 | *LenE* | 1.57 | 1.47 | 1.43 | 1.45 | 1.62 | .028 | .189 | .581 | .322 | **.016** | **.015** |
| | *TreE* | 1.58 | 1.67 | 1.67 | 1.60 | 1.60 | .018 | | | | | |
| | *AstE* | 1.47 | 1.67 | 1.47 | 1.68 | 1.70 | .028 | | | | | |
| | SEM | .035 | .035 | .04 | .03 | 02 | | | | | | |
| Day 15-21 | *LenE* | 2.13 | 2.07 | 2.08 | 2.27 | 2.30 | .030 | .475 | .660 | .855 | .533 | .324 |
| | *TreE* | 2.35 | 2.47 | 2.48 | 2.48 | 2.47 | .043 | | | | | |
| | *AstE* | 2.42 | 2.57 | 2.33 | 2.43 | 2.33 | .037 | | | | | |
| | SEM | .04 | .07 | .05 | .06 | .06 | | | | | | |
| Day 22-28 | *LenE* | 1.83 | 1.75 | 1.72 | 1.88 | 1.95 | .028 | .224 | .380 | **.091** | .211 | .182 |
| | *TreE* | 1.73 | 1.75 | 1.78 | 1.85 | 1.87 | .020 | | | | | |
| | *AstE* | 1.77 | 1.75 | 1.78 | 1.80 | 1.85 | .027 | | | | | |
| | SEM | .04 | .03 | .02 | .03 | .03 | | | | | | |
| Day 7-28 | *LenE* | 1.83 | 1.80 | 1.73 | 1.85 | 1.95 | .021 | .829 | .715 | .340 | **.072** | **.038** |
| | *TreE* | 1.88 | 1.92 | 1.93 | 1.93 | 1.95 | .016 | | | | | |
| | *AstE* | 1.87 | 2.0 | 1.83 | 1.97 | 1.93 | .019 | | | | | |
| | SEM | .02 | .03 | .03 | .02 | .02 | | | | | | |

[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

| Type of the model[1] | Periods | Extracts[2] | b0 | b1 | b2 | SE | P-value[3] | $R^2$ | F (1, 27)[4] |
|---|---|---|---|---|---|---|---|---|---|
| Linear | Day 7-14 | *LenE* | 1.48 (0.05) | 0.01 (0.02) | | .15 | .536 | .01 | |
| | | *TreE* | 1.64 (0.04) | -0.01 (0.01) | | .10 | .647 | .01 | |
| | | *AstE* | 1.49 (0.05) | 0.05 (0.02) | | .14 | **.016** | .19 | |
| | Day 15-21 | *LenE* | 2.04 (0.05) | 0.06 (0.02) | | .23 | **.004** | .25 | |
| | | *TreE* | 2.38 (0.08) | 0.03 (0.03) | | .20 | .394 | .03 | |
| | | *AstE* | 2.49 (0.07) | -0.04 (0.03) | | .14 | .217 | .05 | |
| | Day 22-28 | *LenE* | 1.73 (0.05) | 0.05 (0.02) | | .10 | **.033** | .15 | |
| | | *TreE* | 1.71 (0.03) | 0.04 (0.01) | | .12 | **.007** | .23 | |
| | | *AstE* | 1.74 (0.04) | 0.03 (0.02) | | .11 | .162 | .07 | |
| | Day 7-28 | *LenE* | 1.76 (0.04) | 0.03 (0.02) | | .11 | **.028** | .16 | |
| | | *TreE* | 1.89 (0.03) | 0.02 (0.01) | | .09 | .200 | .06 | |
| | | *AstE* | 1.90 (0.04) | 0.01 (0.02) | | .10 | .552 | .01 | |
| Quadratic | Day 7-14 | *LenE* | 1.66 (0.08) | -0.23 (0.09) | 0.055 (0.021) | .14 | **.034** | .22 | **7.24** |
| | | *TreE* | 1.58 (0.06) | 0.07 (0.07) | -0.018 (0.014) | .10 | .419 | .06 | 1.53 |
| | | *AstE* | 1.53 (0.08) | 0.01 (0.10) | 0.010 (0.020) | .14 | **.049** | .20 | 0.30 |
| | Day 15-21 | *LenE* | 2.12 (0.09) | -0.05 (0.10) | 0.025 (0.021) | .14 | **.009** | .29 | 1.44 |
| | | *TreE* | 2.30 (0.14) | 0.14 (0.16) | -0.026 (0.035) | .24 | .536 | .04 | 0.54 |
| | | *AstE* | 2.48 (0.12) | -0.02 (0.14) | -0.004 (0.030) | .21 | .470 | .05 | 0.01 |
| | Day 22-28 | *LenE* | 1.87 (0.08) | -0.14 (0.09) | 0.040 (0.020) | .13 | **.014** | .27 | **4.38** |
| | | *TreE* | 1.71 (0.06) | 0.05 (0.07) | -0.001 (0.015) | .10 | **.027** | .24 | 0.01 |
| | | *AstE* | 1.76 (0.07) | -0.01 (0.08) | 0.008 (0.018) | .12 | .346 | .08 | 0.20 |
| | Day 7-28 | *LenE* | 1.90 (0.06) | -0.15 (0.06) | 0.041 (0.014) | .09 | **.002** | .38 | **9.35** |
| | | *TreE* | 1.87 (0.05) | 0.04 (0.06) | -0.005 (0.013) | .09 | .411 | .06 | 0.12 |
| | | *AstE* | 1.91 (0.06) | -0.01 (0.07) | 0.003 (0.016) | .11 | .821 | .01 | 0.04 |

[1] Models: Linear: $Y = b0 + b1\ X$; Quadratic: $Y = b0 + b1\ X + b2\ X^2$.

[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 significancy 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

| Treatments[1] | Heart | Liver | Thymus | Bursa | Spleen |
|---|---|---|---|---|---|
| | | | (per 100g BW ) | | |
| Non-suppl. | 0.55 | 2.21 | 0.39 | 0.13 | 0.11 |
| VRG | 0.55 | 2.07 | 0.37 | 0.14 | 0.10 |
| *LenE* (2 g/kg) | 0.60 | 2.19 | 0.44 | 0.16 | 0.11 |
| *TreE* (1g/kg) | 0.55 | 2.10 | 0.41 | 0.18 | 0.10 |
| *AstE* (2 g/kg) | 0.62 | 2.17 | 0.48 | 0.12 | 0.12 |
| SEM[2] | 0.013 | 0.058 | 0.088 | 0.008 | 0.004 |

[1] 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

| Treatments[1] | Prov +giz[3] | Crop + Eso[3] | Duodenum | Jejunum | Ileum | Ceca |
|---|---|---|---|---|---|---|
| | | | (per 100g BW) | | | |
| Non-suppl. | 2.84 | 0.62 | 0.56 | 1.16 | 0.93 | 0.21 |
| VRG | 2.85 | 0.57 | 0.57 | 1.17 | 1.05 | 0.19 |
| *LenE* (2 g/kg) | 3.16 | 0.67 | 0.63 | 1.16 | 1.05 | 0.19 |
| *TreE* (1g/kg) | 3.10 | 0.61 | 0.61 | 1.19 | 0.96 | 0.20 |
| *AstE* (2 g/kg) | 3.19 | 0.73 | 0.62 | 1.29 | 1.00 | 0.20 |
| SEM[2] | .075 | 0.03 | 0.02 | 0.03 | 0.03 | 0.01 |

[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 Weiderman, 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; Buresh *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 $NH_3$ (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

This research was supported by the International Foundation For Science (IFS), No. B/3175-1. The help of Wei Liping, with providing the experimental facilities, and of Bao Zhenggui, Lanzhou transporting the feed and animals, of Liu Jiabiao, Lanzhou Shunda Veterinary Pharmacy and Cai Yingkui, the Poultry Farm of People's Government of Gansu Province with guiding most of the practical work for this experiment, and of Evert-Jan Bakker, Biometrics, WUR with helping for statistics is highly appreciated.

## REFERENCES

Aarestrup, F. M., H., Kruse, E., Tast, A. M., Hammerum, and L. B., Jensen. 2000. Associations between the use of antimicrobial agents for growth promotion and the occurrence of resistance among Enterococcus faecium from broilers and pigs in Denmark, Finland, and Norway. Microbial Drug Resistance. 6 (1): 63-70.

Armstrong, D. G. 1986. Gut-active growth promoters in Buttery at al. (eds.). Control and Metabolism of Animal Growth, 21-37.

Bird, H. R. 1980. Chick growth response to dietary antibiotics remains undiminished after 30 years. Feedstuffs, 52 (40): 16-24.

Buresh, R. E., R. H., Harms, and R. D., Miles. 1986. A differential response in turkey poults to various antibiotics in diets designed to be deficient or adequate in certain essential nutrients. Poultry Science, 65: 2314-2317.

Dubois, M. K., J. K., Gilles, J. K., Hamilton, P. A., Rebers, and F., Smith. 1956. Pfizer flocon 4800 procedure-phenol/sulfuric acid method. Analytical Chemistry, 28: 350-352.

Ensminger, M. E., J. E., Oldfield, and W. W., Heinemann. 1990. Feeds and Nutrition, The Ensminger publishing company, Clovis, California, USA, pp.1544

European Commision/Scientific Steering Committee, DG XXIV. 1999. Opinion of the steering committee of antimicrobial resistance. 28 May.

Eyssen, H. V., and P., De Somer. 1963. The mode of action of antibiotic number 899 in stimulating growth in chicks. Journal of Experimental Medicine, 117: 127-138.

Fukuyasu, T., and T., Oshida. 1986. Use of Neosugar in piglets. Proc. Third Neosugar Res. Conf. pp. 1-18.

Fulton, R. M., B. N., Nersessian, and W. M., Reed. 2002. Prevention of Salmonella enteritidis infection in commercial ducklings by oral chicken egg-derived antibody alone or in combination with probiotics. Poultry Science, 81 (1): 34-40.

Gordon, H. A., and E., Bruckner-Kardoss. 1961a. Effects of the normal microbial flora on various tissue elements of the small intestine. Acta Anat, 44: 210-225.

Gordon, H. A., and E., Bruckner-Kardoss. 1961b. Effects of the normal microbial flora on various tissue elements of the small intestine. Acta Anat, 201: 175-178.

Guo, F. C., B. A., Williams, R. P., Kwakkel, and M. W. A., Verstegen, 2003. *In vitro* fermentation characteristics of two mushrooms, an herb, and their polysaccharide fractions, using chicken cecal contents as inoculum. Poultry Science (accepted), Chapter 3.

Guo, F. C., B. A., Williams, R. P., Kwakkel, and M. W. A., Verstegen. 2003. The effect of mushroom and herb polysaccharide fractions on DGGE band patterns of chicken cecal microflora *in vitro* as determined by 16s rdna analysis. Poultry Science (submitted), Chapter 4.

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

Hinton, M. H. 1988. Antibiotics, poultry production and public health. World's Poultry Science Journal, 44: 67-69.

Hu, T. J., J. L., Liang, and F. S., Cheng. 1998. The use of 8301 polysaccharide as an adjuvant in coccidial infected chickens. Journal of Grass and Livestock (in Chinese), 6: 5-7.

Huang, Q. S., G. B., Lu, Y. C., Li, and J. H., Guo. 1982. Study on the polysaccharides of "Huang Qi" (*Astragalus mongholicus Bung*). Acta Pharmaceutica Sinica (in Chinese), 17(3): 200-206.

Jensen, B. B. 1999. Impact of feed composition and feed processing on the gastrointestinal ecosystem in pigs. in: A.J.M. Jansman and J.Huisman (Eds). Nutrition and gastrointestinal Physiology-Today and Tomorrow, Wageningen, The Netherlands. pp. 43-56.

Katta, Y., K., M., Ohkuma, R., Satouchi, and T., Yamamoto. 1993. Feed for livestock. Eur. Pat. Appl. 0549478A1: 1-14.

Li, C. M. 1998. Chinese herb medicine feed additives, Beijing: Chinese Agricultural University press.

Liang, H. P., Y., Zhang, and B., Geng. 1994. Effect of *Astragalus membranacea Radix* on cellular immunity in burn rats. Journal of China Plastic and Burn Surgery (in Chinese), 10(2): 138-141.

Liu, J. X., C. Y., Wang, and Y. M., Bai. 1999. Preparation of immune active Chinese herbal formulation and safe efficacy test. Chinese Journal of Veterinary Science and Technology (in Chinese). 29(8): 23-25.

Mao, X. J. 1988. Effect of *Astragalus membranacea Radix* polysaccharide on humoral immunity in rats. Journal of China Immunology (in Chinese), 4(3):158.

March, B. E., R., Soong, and C., Macmillian. 1978. Growth rate, feed conversion, and dietary

metabolizable energy in response to VRG supplementation of different diets. Poultry Science, 57: 1346-1350.

Miles, R. O., C. R., Douglas, and R. H., Harms. 1984a. Influence of VRG in pullets and broilers fed diets containing sub-optional protein and sulfur amino acid levels. Nutr. Rep. Int., 30: 983-989.

Miles, R. O., D. M., Janky, and R. H., Harms. 1984. VRG and broiler performance. Poultry Science, 63: 1218-1221.

Narayanankutty, K., A., Ramakrishnan, and A., Viswannath. 1992. Efficacy of virginiamycin as growth promoter in commercial broiler chicks. Journal of Veterinary and Animal Sciences. 23 (1) 96-97.

Nelson, F. E., L. S., Jenson, and J., Mcginnis. 1963. Studies on the stimulation of growth by dietary antibiotics. Poultry Science, 42: 909-912.

Newman, K. E. 2002. Antibiotic resistance is a reality: novel techniques for overcoming antibiotic resistance when using new growth promoters. Pp98-106. Nutritional Biotechnology in the Feed and Food Industries. Proceedings of Alltech's 18th Annual Symposium. Nottingham University Press.

Odunsi, A. A., A. A., Onifade, and G. M., Babatunde. 1999. Response of broiler chicks to VRG and dietary protein concentrations in the humid tropics. Archivos de Zootecnia, 48 (183): 317-325.

Pang, F. H., M. Q., Xie, and H. H.. Ling. 2000. The investigation of immuno-dulators tested for the results on the control of a coccidial infection. Chinese Journal of Veterinary Parasitology (in Chinese), 8(3): 1-3.

Pang, F., Y. X., Feng, and Y., Zhang. 1995. Review on *Astragalus membranaceus(Fisch.) Bge* research. Oversea Medicine-Plants 10(3): 110-111.

Peterson, R. A., G. H., Carpenter, W. T., Jones, and J. A., Kla. 1991. Effect of tetracycline hydrochloride and oxytetrocycline hydrochloride given via drinking water on early mortality of broiler chicks from twenty-eight-week-old dams. Poultry Science, 70: 4, 1040-1042.

Proudfoot, F. G., E. D., Jackson, and H. W., Hulan, 1990. The response of male chicken broilers to the dietary addition of VRG. Poultry Science, 69: 1713-1717.

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

Sherman, W. C., G. A., Donovan, W. M., Reynolds, and H. G., Luther. 1959. Studies of nutritional level of oleandomycin in broiler rations. Poultry Science, 38: 869-873.

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

Spring, P., C., Wenk, K. A., Dawson, and K. E., Newman. 2000. The effects of dietary

mannoligosaccharide on cecal parameters and the samonella-challenged broiler chicks. Poultry Science, 79: 205-211.

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

Stanley, V. G., Y. W., Park, and C., Gray. 1997. Effects of Mannan oligosaccharide (MOS) on aflatoxicosis, serum, liver, and egg cholesterol and egg production in chickens. Assoc. Res. Dir. 11th Biennial Research Symposium.

Thanga, T., M., Thiagarajan, V., Ramesh, T., Sivahumar, and T., Tamilvanan. 2001. Performance of broiler chicken under floor system of management fed with different processed feed and probiotics. Indian Journal of Animal Research. 35: 2, 88-91.

Veltmann, J. R. Jr., and C. A., Weiderman. 1987. Comparison of pullet performance fed diets formulated to different specifications with and without VRG supplementation. Nutr. Rep. Int., 35: 179-190.

Waible, P. E., O. J., Abbott, C. A., Baumann, and H. I., Bird. 1954. Disappearance of the growth response of chicks to dietary antibiotics in an “old” environment. Poultry Science, 33: 1141-1146.

Wiese, A. C., and C. F., Petersen. 1959. Comparison of several antibiotics for chick growth. *Poultry Science*, 38: 1259 (abstr.).

Xia, E. N., and Q. H., Cheng. 1988. Isolation, analysis and bioactivities of *Tremella fuciformis* fruit body polysaccharides. Acta Mycologica Sinica (in Chinese). 7(3): 166-174.

Xue, M., and X. S., Meng. 1996. Review on research progress and prosperous of immune activities of bio-active polysaccharides. Journal of Traditional Chinese Veterinary. Medicine (in Chinese), 3: 15-18.

Yang, H. C., G., Lu, and G., Lu. 1998. Effect of pollen polysaccharide from corns on immunological afficacy of Newcastle disease vaccine and Egg Drop Syndrome vaccine in chickens. Journal of Chinese Veterinary Medicine (in Chinese). 24 (6): 43.

Yang, J., M. C., Wu, and S. H., Zhang. 1999. Study on isolation, purification and characteristics of polysaccharide Le-III from the fruit body of *Lentinus Edodes.* Journal of Huazhong Agricultural University (in Chinese), 18(2): 188-190.

Yuan, Y. L., B. T., Fang, and Y. X., Zhang. 1993. Efficacy of 8301 polysaccharides as an adjuvant against avian *Pasteurellosis* in chickens. Journal of Traditional Chinese Veterinary Medicine (in Chinese), 3: 6-10.

Zhang, L. C., J. B., Wang, and Y. P., Sun. 1998. Effect of adding Chinese herb polysaccharide preparations to a Newcastle disease inactivated vaccine on the development of the immune organs in chickens. Chinese Journal of Veterinary Science (in Chinese) 18(4): 378-381.

# Chapter 6

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

F.C. Guo

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands. Laboratory of Veterinary Clinic, Institute of Lanzhou Animal Science and Veterinary Pharmaceutics, CAAS,* Lanzhou *730050, P. R. China*

B.A. Williams and M.W.A. Verstegen

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

R.P. Kwakkel

*Animal Production Systems Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

H.S. Li, X.P. Li, and J.Y. Luo

*Laboratory of Veterinary Clinic, Institute of Lanzhou Animal Science and Veterinary Pharmaceutics, CAAS,* Lanzhou *730050, P. R. China*

W.K. Li

*Department of Parasitological Diseases, Gansu Animal Health Service, 730046, Lanzhou, P. R. China*

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 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, 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 membranacea 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 /$m^2$. The brooder temperature was set at 30 $^oC$ to 33 $^oC$ during the first week and gradually decreased by 2 $^oC$ per week, until 28 $^oC$ 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 $^{o}$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 ($C_{21}H_{41}N_5O_{11}$) 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)

| Ingredients | Starter diet | Grower diet |
|---|---|---|
| Maize | 570 | 635.3 |
| Soybeans (d) | 320 | 270 |
| Fish meal | 40 | 30 |
| Animal fat | 20 | 20 |
| Limestone | 14 | 12 |
| Salt (NaCl) | 3.5 | 3.5 |
| Calcium hydrophosphate(CaHPO4) | 17 | 16 |
| DL-Methionine | 2.6 | 2.1 |
| Lysine | 2.9 | 1.1 |
| Premix[1] | 10 | 10 |
| Calculated analysis (g/kg) | | |
| Crude protein | 210 | 190 |
| Metabolisable energy (kcal/kg) | 3,000 | 3,050 |
| Calcium | 10 | 9 |
| Available phosphate (P) | 4.5 | 4.3 |
| Lysine | 11.7 | 10.5 |
| Methionine | 6.0 | 5.1 |

[1] Supplied for per kg of diet: vitamin A (retinal acetate), 8,500 IU; cholechalciferol, 1,800 IU; DL-α-topherol acetate, 13.0 IU; vitamin $K_3$, 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 $B_{12}$, 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]

| Compositions | LenE | *TreE* | *AstE* |
|---|---|---|---|
| Yields of polysaccharides (%)[2] | 6.8 | 16.0 | 10.1 |
| Dry matter (g/kg) [3] | 958 | 965 | 967 |
| Total sugar content (g/kg DM) [4] | 655 | 613 | 687 |

[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$^{o}$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:

$$Y = b0 + b1\ x \quad [1]$$

$$Y = b0 + b1\ x + b2\ x^2 \quad [2]$$

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[1]

| Treatments[2] | BW gain | pH | Viscosity | Aerobes | Anaerobes | *Bacteroides* | *E. coli* | *Enterococci* | *Bifidobacteria* | *Lactobacilli* |
|---|---|---|---|---|---|---|---|---|---|---|
| | ($g\ b^{-1}d^{-1}$) | | (cPm)[6] | | | log10 (cfu)[7] | | | | |
| Control | 5.7 | 7.0 | 0.88 | 5.93 | 9.52 | 6.70 | 6.93 | 7.36 | 8.55 | 7.54 |
| APR | 25.6 | 7.2 | 1.29 | 9.08 | 9.39 | 8.72 | 9.69 | 7.25 | 8.12 | 6.58 |
| *LenE* – 2 g/kg | 17.6 | 6.8 | 1.17 | 6.19 | 9.53 | 6.38 | 6.58 | 7.29 | 9.09 | 8.33 |
| *TreE* – 2 g/kg | 18.9 | 7.0 | 1.22 | 5.99 | 9.09 | 5.74 | 6.14 | 7.16 | 8.69 | 8.16 |
| *AstE* – 2 g/kg | 21.6 | 7.0 | 1.17 | 6.18 | 9.26 | 6.45 | 6.73 | 7.23 | 9.03 | 8.21 |
| SEM[3] | 1.84 | 0.13 | 0.03 | 0.32 | 0.05 | 0.27 | 0.03 | 0.02 | 0.10 | 0.096 |
| Contrast statements[4] | | | | | | Probability levels of contrasts | | | | |
| Control vs APR | < .000 | .725 | .008 | .001 | .071 | < .000 | < .000 | .029 | .015 | .005 |
| Extracts[5] vs APR | .001 | .346 | .081 | < .000 | .062 | < .000 | < .000 | .496 | < .000 | < .000 |
| *LenE* vs *TreE* | .558 | .706 | .039 | .109 | .002 | .002 | .029 | .226 | .001 | .008 |
| *AstE* vs *LenE* & *TreE* | .066 | .359 | .141 | .220 | .413 | .003 | .005 | .960 | .032 | .203 |

[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 $\log_{10}$ colonies per g cecal contents.

**Table 4.** BW gain, cecal pH, viscosity and microbial counts of chicken fed with different levels of *LenE*[1]

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

[1] Results are given as means (n=3) ± SE.

[2] *LenE* = *Lentinus edodes* polysaccharide extract;

[3] SEM = Mean of the standard error

[4] cPm = cycles per minutes.

[5] The colony-forming unit (cfu) is expressed as $\log_{10}$ colonies per g cecal contents.

[abcd] Means with different superscripts within the column are significantly different ($P<0.05$).

**Table 5.** Dose response of *LenE*[1], Parameter estimates and asymptotic SE (in parenthesis), described by linear and quadratic functions in broilers

| Type of model[2] | Observed traits | b0 | b1 | b2 | SE | P-value[3] | $R^2$ | F (1, 12)[4] |
|---|---|---|---|---|---|---|---|---|
| Linear | BW gain | 17.92 (1.57) | 0.69 (0.30) | | 3.68 | **.037** | .29 | |
| | PH | 6.79 (0.23) | -0.03 (0.04) | | 0.53 | .478 | .04 | |
| | Viscosity | 1.14 (0.02) | 0.002 (0.003) | | 0.04 | .433 | .05 | |
| | Aerobes | 5.90 (0.12) | 0.02 (0.02) | | 0.28 | .318 | .08 | |
| | Anaerobes | 8.99 (0.07) | 0.02 (0.01) | | 0.17 | .120 | .18 | |
| | *Bacteroides* | 6.41 (0.10) | 0.02 (0.02) | | 0.24 | .348 | .07 | |
| | *Enterococci* | 7.36 (0.09) | 0.06 (0.03) | | 0.20 | .717 | .01 | |
| | *E. coli* | 6.47 (0.07) | 0.06 (0.01) | | 0.17 | **.001** | .59 | |
| | *Bifidobacteria* | 8.71 (0.09) | 0.02 (0.02) | | 0.20 | .187 | .13 | |
| | *Lactobacilli* | 8.14 (0.08) | 0.03 (0.02) | | 0.19 | .113 | .18 | |
| Quadratic | BW gain | 10.98 (1.85) | 4.65 (0.91) | -0.35 (0.08) | 2.35 | **< .001** | .73 | 19.81 |
| | PH | 7.46 (0.37) | -0.42 (0.18) | 0.034 (0.016) | 0.47 | .107 | .31 | 4.74 |
| | Viscosity | 1.09 (0.02) | 0.03 (0.01) | -0.003 (0.001) | 0.03 | **.048** | .40 | 7.64 |
| | Aerobes | 5.42 (0.16) | 0.30 (0.08) | -0.002 (0.007) | 0.21 | **.009** | .54 | 12.14 |
| | Anaerobes | 8.71 (0.10) | 0.18 (0.05) | -0.014 (0.004) | 0.13 | **.006** | .57 | 10.98 |
| | *Bacteroides* | 6.26 (0.19) | 0.10 (0.09) | -0.007 (0.008) | 0.24 | .450 | .12 | 0.78 |
| | *Enterococci* | 7.83 (0.04) | -0.26 (0.02) | 0.024 (0.002) | 0.05 | **< .001** | .94 | 200.80 |
| | *E. coli* | 6.13 (0.07) | 0.25 (0.03) | -0.017 (0.003) | 0.09 | **< .001** | .89 | 32.56 |
| | *Bifidobacteria* | 8.65 (0.16) | 0.06 (0.08) | -0.003 (0.007) | 0.21 | .388 | .15 | 0.23 |
| | *Lactobacilli* | 7.98 (0.15) | 0.12 (0.07) | -0.008 (0.006) | 0.19 | .135 | .28 | 1.70 |

[1] *LenE =Lentinus edodes* polysaccharide extract.
[2] Models: Linear: $Y = b0 + b1\ X$; Quadratic: $Y = b0 + b1\ X + b2\ X^2$.
[3] P <0.05 are printed in bold.
[4] F (1, 12) represents the significancy 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 membranacea 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 $TreP_m$ 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 $TreP_m$ 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

This research was supported by a grant from International Foundation For Science (IFS) No. B/3175-1. We thank Fu Siwu, Institute of Biological Products, China Hygiene Ministry for providing the medium for culture of *Bifidobacteria* and Hu Zhengying, of the Antibiotic Lab Institute of Animal Science and Vet. Pharmaceutics CAAS for providing APR. John Patterson, of the Department of Animal Sciences, Purdue University is thanked for his critical reading of the manuscript.

## REFERENCES

Armstrong, D. G. 1986. Gut-active growth promoters. In: Control and Metabolism of Animal Growth. Buttery at al. (eds.). 21-37.

Bailey, J. S., L. C., Blankenship, and N. A., Cox. 1991. Effect of fructooligosaccharide on *Salmonella* colonization of the chicken intestine. Poult. Sci. 70:2433-2438.

Barnes, E. M., and C. S., Impey. 1970. The isolation and properties of the predomiant anaerobic bacteria in the caeca of chickens and turkeys. Br. Poult. Sci. 11:467-481.

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

Bird, H. R. 1980. Chick growth response to dietary antibiotics remains undiminished after 30 years. Feedstuffs 52(40):16-24.

Breves G., L., Szentkuti, and B., Schroeder. 2001. Effects of oligosaccharides on functional parameters of the intestinal tract of growing pigs. DTW Deutsche Tieraerztliche Wochenschrift 108(6):246-248.

Brown, R. H. 1996. The living gut must be considered when trying to eliminate pathogens. Feedstuffs 68:14.

Cheng F. S., T. J., Hu, and J. L., Liang. 1998. Enhance of VMD vaccine by 8301 polysaccharide in chickens . J. of Traditional Chinese Vet. Medicine (in Chinese) 1:8-9.

Coates, M. E., C. B., Cole, R., Fuller, S. B., Houghton, and H., Yokota. 1981. The gut microflora and the uptake of glucose from the small intestine of the chick. Br. Poult. Sci. 22:289-294.

Cummings, J. H. 1981. Short chain fatty acids in the human colon. Gut 22:763-779.

Cummings, J. H., and G. T., Macfarlane. 1991. A review: the control and consequences of bacterial fermentation in the human colon. J. Appl. Bacteriol. 70:443-459.

Cummings, J. H., and G. T., Macfarlane. 2002. Gastrointestinal effects of prebiotics. Br. J. Ntri. 87(Suppl.2):S145-S151.

Dubois, M. K., J. K., Gilles, J. K., Hamilton, P. A., Rebers, and F., Smith. 1956. Pfizer flocon 4800 procedure-phenol/sulfuric acid method. Analytical Chemistry. 28:350-352.

Eastwood, M. A. 1992. The physiological effect of dietary fibre: an update. Annual Reviews in the Nutrition. 12:19-35

Ensminger, M. E., J. E. Oldfield, and W. W., Heinemann. 1990. Feeds and Nutrition, The Ensminger publishing company, Clovis, California, USA, 1544 pp.

Eyssen, H. V., and P., De Somer. 1963. The mode of action of antibiotic number 899 in stimulating growth in chicks. J. Expt. Med., 117:127-138.

Gardiner, K. R., P. J., Erwin, N. H., Anderson, J. G., Barr, M. I., Halliday, and B. J., Rowlands. 1993. Colonic bacteria and bacterial translocation in experimental colitis. Br. J. Surg. 80:512-516.

Gibson, R. G., and M. B., Roberfroid. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Appl. Bacteriol. 1401-1412.

Grizard, D., and C., Barthomeuf. 1999. Non-digestible oligosaccharides used as prebiotic agents: Mode of production and beneficial effects on animal and human health. Reprod. Nutri. Develop. 39(5-6):563-588.

Guo, F. C., B. A., Williams, R. P. Kwakkel, and M. W. A., Verstrgen. 2003. *In vitro* fermentation characteristics of two mushrooms, an herb, and their polysaccharide fractions, using chicken cecal contents as inoculum. Poult. Sci. (accepted).

Guo, F. C., B. A., Williams, R. P. Kwakkel, and M. W. A., Verstrgen. 2003. The effect of mushroom and herb polysaccharide fractions on DGGE band patterns of chicken cecal microflora *in vitro* as determined by 16s rdna analysis. Poult. Sci. (submitted).

Hesselman, K. and P., Aman. 1986. The effect of B-glucanase on the utilization of starch and nitrogen by broiler chickens fed on barley of low- or high-viscosity. Ani. Feed Sci. Technol. 15:83-93.

Hu, T. J., J. L., Liang, and F. S., Cheng. 1998. The use of 8301 polysaccharide as an adjuvant in coccidial infected chickens. J. of Grass and Livestock (in Chinese), 6:5-7.

Hughes, R. J., M., Choct, A., Kocher, and R. J., Van Barneveld. 2000. Effect of food enzymes on AME and composition of digesta from broiler chickens fed on diets containing non-starch polysaccharides isolated from lupin kernel. Br. Poult. Sci. 41(3):318-323.

Iji, P. A., Saki, A. A., and D. R., Tivey. 2001. Intestinal development and body growth of broiler chicks on diets supplemented with non-starch polysaccharides. Ani. Feed Sci. Technol. 89 (3-4):175-188.

Jensen, B. B. 1993. The possibility of manipulating the microbial activity in the digestive tract of monogastric animals. 44th Animal Meeting of the European Association for Animal Production. Århus, Denmark. pp. 20

Jensen, B. B. 1999. Impact of feed composition and feed processing on the gastrointestinal ecosystem in pigs. in: A.J.M. Jansman and J.Huisman (Eds). Nutrition and Gastrointestinal Physiology-

Today and Tomorrow, Wageningen, The Netherlands. pp. 43-56.

Korakli, M., M. G., Gaenzle, and R. F., Vogel. 2002. Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye, and exopolysaccharides produced by *Lactobacillus sanfranciscensis*. J. Appl. Microbiol. 92(5):958-965.

Langhout, D. J. 1999. The role of the intestinal flora as affected by NSP in broiler chicks. in: proceedings of 12th European Symposium on Poultry Nutrition. Pp 203-212. WPSA Dutch Branch.

Liu, J. X., C. Y., Wang, and Y. M., Bai. 1999a. Preparation of immune active Chinese herbal formulation and safe efficacy test. Chinese J. of Veterinary Science and Technology (in Chinese). 29(8):23-25.

Liu, Y. J., Q. Z., Li, and Y. H., Hao. 1999b Effects of Lentinan and Astragalan on IL-2 inductive activity and lymphocyte proliferative reaction in chicks infected with Marek's disease. J. of China Vet. Medicine (in Chinese) 25(7):3-5.

Macfarlane, G. T., G. R. Gibson, E. Beatty, and J. H. Cummings. 1992. Estimation of short-chain fatty acid production from protein by human intestinal bacterial based on branched-chain fatty acid measurements. FEMS Microbial Ecology. 101: 81-88.

Macfarlane, G. T., and J. H., Cummings. 1991. The colonic flora, fermentation and large bowel digestive function. In: The Large Intestine: physiology, Pathophysiology and Disease (Phillips, S. F., Pemberton J. H. and Shorter R.G., eds.), pp. 51-92. Raven Press, New York, NY.

Macy, J. M. and I., Probst. 1979. The biology of gastrointestinal bacteroides. Annu. Rev. Microbiol. 33:561-594.

Miles, R. O., D. M., Janky, and R. H., Harms. 1984. Virginiamycin and broiler performance. Poult. Sci. 63:1218-1221.

Newman, K. E. 1995. Mannan-oligosaccharides: natural polymers with siginificant impact on the gastrointestinal microflora and immune system. In: Biotechnology in the feed industry. Proc. Alltech's Tenth Annual Symposium. T.P. Lyons and K. A. Jaques(Eds):pp. 167-174. Nottingham University Press, Nottingham.

Pang, F. H., M. Q., Xie, and H. H., Ling. 2000. The investigation of Immunodulators tested for the results on the control of a coccidial infection. Chinese J. of Vet. Parasitology (in Chinese) 8(3):1-3.

Pang, F., Y. X., Feng, and Y., Zhang. 1995. Review on current research of *Astragalus mongholicus Bga*. World Phytomedicines (in Chinese). 10(3):110-115.

Peterson, R. A., G. H., Carpenter, W. T., Jones, and J. A., kla, 1991. Effect of tetracycline hydrochloride and oxytetrocycline hydrochloride given via drinking water on early mortality of broiler chicks from twenty-eight-week-old dams. Poult. Sci. 70(4):1040-1042.

Rolfe, R. D. 1991 population dynamics of the intestinal tract. In: Colonization control of human enteropathogens in poultry (Blankenship, L.C., ed.) Academic Press; Inc. San Diego, CA, pp. 59-75.

Rosen, G. D. 1995. Antibacterals in poultry and pig nutrition. In: Boitechnology in animal feeds and

animal feeding. R. J. Wallance and A. Chesson (Eds): 143-172. VCH, Weinhiem.

Rycroft, C. E., M. R., Jones, G.R., Gibson and R. A., Rastall. 2001. A comparative *in vitro* evaluation of the fermentation properties of prebiotic oligosaccharides. J. Appl. Microbiol. 91(5):878-887.

Salyers, A. A. 1979. Energy sources of major intestinal termentative anaerobes. Am. J. Clin. Nutr. 32: 158-163.

Solomons, N. W. 1993. Pathways to the impairment of human nutritional status by gastrointestinal pathogens. Parasitology 107:S19-S35.

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

Sunvold, G. D., H. S., Hussein, G. C., Fahey Jr, N. R., Merchen, and G. A., Reinhart. 1995. *In vitro* fermentation of cellulose, beet pulp, citrus pulp, and citrus pectin using fecal inoculum from cats, dogs, horses, humans, and pigs and fuminal fluid from cattle. J. Anim. Sci. 73:3639-3648.

Verstegen, M. W. A., and G. J., Schaafsma. 1999. Some developments on alternatives for antibiotics as growth promotor. Pages 65-73 in: Nutrition and Gastrointestinal Physiology Today and Tomorrow. A. J. M. Jansman ed. J. Huisman, Wageningen, The Netherlands.

Wang R. J., D. F., Li, and S., Bourne. 2002. Can 2000 years of herbal medicine history help us solve problems on the year 2000? pp 273 – 291. in: Biotechnology in the feed industry. Proceedings of Alltech's 14th Anunual sysmposium, Nottingham University Press.

Wei, P., Q. Z., Li, and Y. H., Hao. 1997. Effect of *Astragalus mongholicus Bga* and *Lentinus edodes* polysaccharide on lipid peroxide (LPO) content in Marek's disease infected chicks. J. of Traditional Chinese Vet. Medicine (in Chinese) 4:3-4.

Xia, E. N., and Q. H., Cheng. 1988. Isolation, purification of polysaccharides of artificial cultivated fruit body of *Tremella fuciformis Berk*. Acta Mycologica Sinica (CN). 7(3):166-174.

Xie, Z. Q., and S. Q., Niu. 1996. Comprehensive Book of Natural Resources and Chinese Herb Feed Additives (in Chinese). Xueyuan Science and Technology Press, Beijing, China.

Xue, M., and X. S., Meng. 1996. Review on research progress and prosperous of immune activities of bio-active polysaccharides. J. of Traditional Vet. Medicine (in Chinese) 3:15-18.

Yang, Y., and W. S., Feng. 1998. Brochures of isolation and extraction of chemical components from herbs (in Chinese). China Chinese Herb Medicine Press. Beijing. China.

Yang, J., Wu, M. C. and S. H., Zhang. 1999. Study on isolation, purification and characteristics of polysaccharide Le-III from the fruit body of *Lentinus edodes.* J. of Huazhong Agricultural University (in Chinese) 18(2):188-190.

Yang, J., M. C., Wu and S. H., Zhang. 2001. Isolation, purification and characterization of polysaccharides from the fruit body of *Lentinus edodes* (Berk.) Journal of Plant resources and Environment (in Chinese) 10(2):4-6.

Yu, J. G., and L.Y., Zhu. 2000. The use of *Astragalus* polysaccharide against Infectious Bursa Disease in chickens. J. of Traditional Chinese Vet. Medicine (in Chinese). 6:3-4.

Yuan, Y. L., B. T., Fang, and Y. X., Zhang. 1993. Efficacy of 8301 polysaccharides as an adjuvant against Avian *Pasteurellosis* in chickens. J. of Traditional Chinese Vet. Medicine (in Chinese). 3:6-10.

Zimmermann, B., E., Bauer, and R., Mosenthin. 2001. Pro- and prebiotics in pig nutrition: Potential modulators of gut health? J. Ani. Feed Sci. 10(1):47-56.

# Chapter 7

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

F.C. Guo

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands. Laboratory of Veterinary Clinic, Institute of Animal Science and Veterinary Pharmaceutics, Chinese Academy of Agricultural Sciences, 730050 Gansu, China*

B.A. Williams and M.W.A. Verstegen

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

R.P. Kwakkel

*Animal Production Systems Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

X. Suo

*Laboratory of Parasitology and Parasitological Diseases, College of Veterinary Medicine, China Agricultural University, P.R. China*

Z.Q. Yang

*Laboratory of Veterinary Clinic, Institute of Animal Science and Veterinary Pharmaceutics, Chinese Academy of Agricultural Sciences, 730050 Gansu, China*

Submitted to: British Poultry Science

# Efficacy of Mushroom and Herb Polysaccharides Against Avian Coccidiosis: Immunization with or without Live Oocyst

F.C. Guo, R.P. Kwakkel, B.A.Williams, X. Suo, Z.Q. Yang and M.W.A. Verstegen

## 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 $9 \times 10^4$ 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 coccdial 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 Bedrnik (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 $^{o}$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 /m$^2$. The formal experiment was conducted from 4 to 31 days of age (a total of 28 days). The temperature was set at 30 $^{o}$C to 33 $^{o}$C during the first week and gradually declined by 2 $^{o}$C per week until 20 $^{o}$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

| Ingredients | Basal diet (g/kg) |
|---|---|
| Maize | 555 |
| Soybean meal | 326.6 |
| Fish meal | 40 |
| Soya oil | 40 |
| Monocalciumphosphate | 13 |
| Limestone | 11.3 |
| Salt (NaCl) | 3 |
| DL-Methionine | 1 |
| Lysine | 0.12 |
| Vitamin and mineral mix[1] | 10 |
| 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, cholechalciferol 1800 IU, vitamin E (DL-α-topherol acetate) 20 mg, vitamin $K_3$ 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, $FeSO_4$•7 $H_2O$ 80 mg, $ZnSO_4$•4 $H_2O$ 75 mg, $MnO_2$ 100 mg, $Na_2SeO_3$•5$H_2O$ 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% $K_2Cr_2O_7$ 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, 0.2ml 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 $9\times10^4$ 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

| Treatment groups | | BWG ($g\ b^{-1}d^{-1}$)[1] | | |
|---|---|---|---|---|
| | | Day 7-17 | Day 18-24 | Day 25-30 |
| Non-infected | | 10.7 ± 0.3 | 22.4 ± 0.5 | 15.6 ± 0.5 |
| Infected | | 11.3 ± 0.2 | 17.0 ± 0.7 | 17.4 ± 1.2 |
| Vaccinated | | 11.8 ± 0.2 | 20.3 ± 0.6 | 18.6 ± 1.4 |
| Mean extracts (E)[2] | | 11.1 ± 0.1 | 17.6 ± 0.7 | 20.2 ± 0.7 |
| | *AstE* | 11.5 ± 0.2 | 14.6 ± $0.8^{b}$ | 19.2 ± 1.1 |
| | *LenE* | 10.9 ± 0.2 | 19.5 ± $1.2^{a}$ | 21.1 ± 0.8 |
| | *TreE* | 11.2 ± 0.3 | 18.8 ± $1.1^{a}$ | 20.5 ± 1.6 |
| Mean (E+V)[3] | | 11.9 ± 0.2 | 22.8 ± 0.4 | 17.1 ± 0.8 |
| | *AstE*+V | 12.2 ± $0.3^{A}$ | 21.5 ± $0.8^{B}$ | 17.6 ± 2.1 |
| | *LenE*+V | 12.3 ± $0.2^{A}$ | 24.0 ± $0.5^{A}$ | 16.6 ± 1.0 |
| | *TreE*+V | 11.2 ± $0.2^{B}$ | 22.8 ± $0.5^{AB}$ | 17.2 ± 0.9 |
| Contrast statements | | Probability level of contrasts[4] | | |
| Infected vs Non-infected | | .098 | **< .001** | .167 |
| Vaccinated vs Infected | | **.050** | **.001** | .514 |
| E vs Vaccinated | | **.007** | **.002** | .315 |
| E+V vs Vaccinated | | .639 | **.001** | .376 |

[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 occured 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 occured 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 oocured 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).

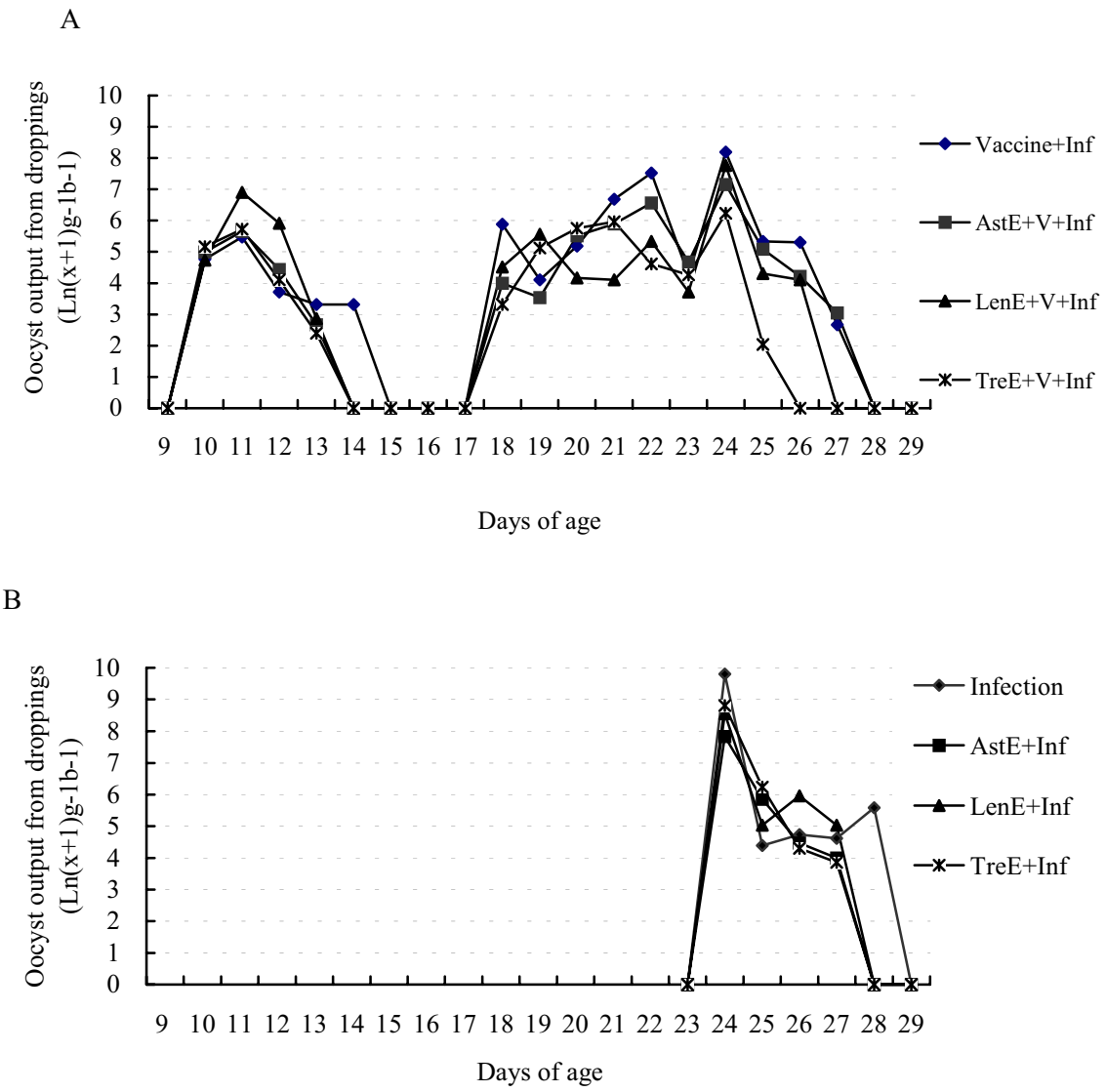



**Figure 1.** The daily logged oocyst output from droppings post-vaccination and infection for the extract plus vaccine groups (*LenE*+V = *Lentinus edodes* extract plus vaccine; *TreE*+V = *Tremella fuciformis* extract plus vaccine; *AstE*+V = *Astragalus membranaceus* extract plus vaccine) (A) and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

| Treatment groups | | Cecal oocyst output Ln (x+1) | Lesion score (mean rank[4]) |
|---|---|---|---|
| Infected | | 14.93 ± 0.15 | 3.4 ± 0.2 (73.7) |
| Vaccinated | | 13.29 ± 0.41 | 1.5 ± 0.3 (35.8) |
| Mean extracts (E)[2] | | 14.55 ± 0.10 | 2.7 ± 0.1 (60.1) |
| | *AstE* | 14.42 ± 0.12[b] | 2.7 ± 0.3 (59.1) |
| | *LenE* | 14.25 ± 0.15[b] | 2.8 ± 0.3 (62.0) |
| | *TreE* | 14.99 ± 0.11[a] | 2.7 ± 0.3 (59.1) |
| Mean (E+V)[3] | | 12.51 ± 0.34 | 1.6 ± 0.2 (37.3) |
| | *AstE*+V | 13.46 ± 0.58[A] | 1.7 ± 0.4 (39.3) |
| | *LenE*+V | 12.69 ± 0.53[AB] | 1.6 ± 0.3 (37.5) |
| | *TreE*+V | 11.38 ± 0.48[B] | 1.5 ± 0.4 (35.2) |
| Contrasts | | Probability level of contrasts[5] | |
| Vaccinated vs. Infected | | **.003** | **< .001** |
| E vs. Vaccinated | | **.014** | **.006** |
| E+V vs. Vaccinated | | .145 | .807 |

[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.

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

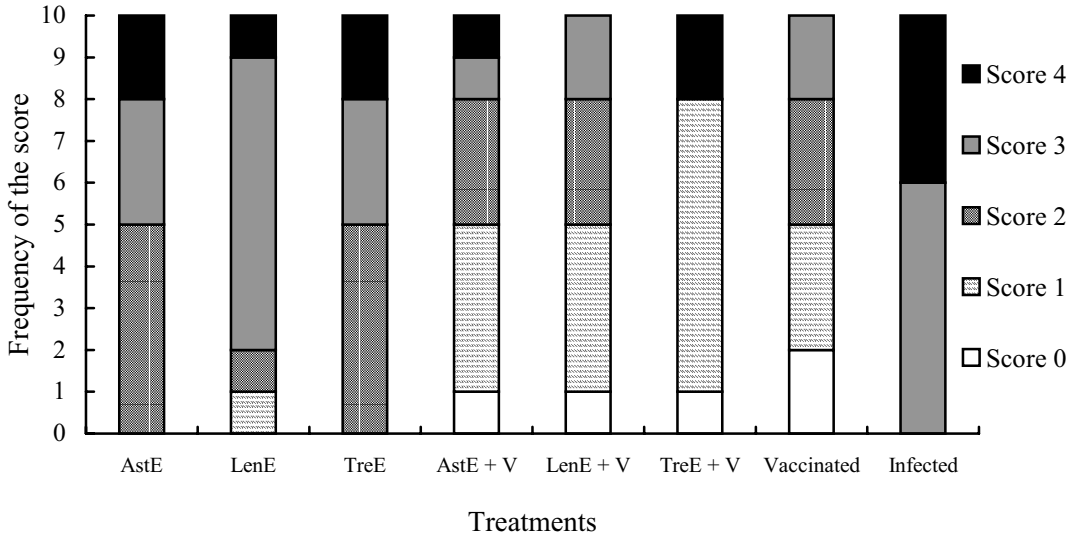



**Figure 2.** Lesion score of ceca in chickens at 7 days post *Eimeria* infection. The infected ceca (n=10) were examined and scored on a scale of lesion from Score 0 (no obvious lesion) to 4 (most serious lesion). Score 1: Very few scattered petechiae on the caecal wall, no thickening of the caecal 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-oesphagus, 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)

| Treatment groups | Thymus | | Liver | | Spleen | | Bursa | |
|---|---|---|---|---|---|---|---|---|
| | 7 d p.i. | 14 d p.i. | 7 d p.i. | 14 d p.i. | 7 d p.i. | 14 d p.i. | 7 d p.i. | 14 d p.i. |
| Non-infected | 0.83 | 0.69 | 3.01 | 2.92 | 0.19 | 0.31 | 0.44 | 0.30 |
| Infected | 0.43 | 0.53 | 3.70 | 3.26 | 0.20 | 0.33 | 0.32 | 0.35 |
| Vaccinated | 0.71 | 0.88 | 2.85 | 2.42 | 0.14 | 0.18 | 0.44 | 0.42 |
| Mean extracts (E)[2] | 0.59 | 0.62 | 3.48 | 2.72 | 0.20 | 0.27 | 0.36 | 0.30 |
| *AstE* | 0.55 | 0.67 | 3.44 | 2.73 | 0.19 | 0.31 | 0.34 | 0.31 |
| *LenE* | 0.55 | 0.60 | 3.45 | 2.47 | 0.20 | 0.21 | 0.36 | 0.27 |
| *TreE* | 0.66 | 0.59 | 3.56 | 2.95 | 0.22 | 0.28 | 0.37 | 0.31 |
| Mean (E+V)[3] | 0.48 | 0.59 | 3.06 | 2.72 | 0.17 | 0.24 | 0.36 | 0.30 |
| *AstE*+V | $0.72^{A}$ | 0.57 | $3.35^{A}$ | 2.75 | 0.19 | 0.26 | 0.34 | 0.26 |
| *LenE*+V | $0.39^{B}$ | 0.58 | $3.10^{AB}$ | 2.77 | 0.17 | 0.22 | 0.39 | 0.32 |
| *TreE*+V | $0.35^{B}$ | 0.63 | $2.73^{B}$ | 2.63 | 0.16 | 0.24 | 0.35 | 0.33 |
| SEM[4] | 0.02 | 0.03 | 0.05 | 0.05 | 0.01 | 0.01 | 0.01 | 0.01 |
| Contrast statements | Probability level of contrasts[5] | | | | | | | |
| Infected vs Non-infected | **< .001** | .119 | **.001** | .153 | .562 | .657 | **.024** | .327 |
| Vaccinated vs Infected | **.001** | **.004** | **< .001** | **.003** | **.009** | **.024** | **.004** | .301 |
| E vs Vaccinated | .065 | **.007** | **< .001** | **.044** | **<.001** | **.010** | **.031** | .085 |
| E+V vs Vaccinated | **.003** | **.004** | **.038** | **.036** | **.024** | **.038** | **.041** | .096 |

[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 standred error mean of the treatments.

[5] P <0.05 are printed in bold.

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

**Table 6.** The relative weight of gastro-intestinal tracts in *Eimeria* infected chickens at 7 and 14 days post infection (%BW)[1]

| Treatment groups | Crop + oeso. | | Prov. + gizzard | | Duodenum | | Jejunum | | Ileum | | Ceca | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 7d p.i. | 14d p.i. | 7d p.i. | 14d p.i. | 7d p.i. | 14d p.i. | 7d p.i. | 14d p.i. | 7d p.i. | 14d p.i. | 7d p.i. | 14d p.i. |
| Non-infected | 0.95 | 0.99 | 3.9 | 3.9 | 0.87 | 0.84 | 1.20 | 1.39 | 1.32 | 1.05 | 1.00 | 0.83 |
| Infected | 1.08 | 0.89 | 3.8 | 3.7 | 0.61 | 0.96 | 1.09 | 1.74 | 0.62 | 1.15 | 0.99 | 0.81 |
| Vaccinated | 1.09 | 1.01 | 4.1 | 2.4 | 0.75 | 0.91 | 1.37 | 1.39 | 0.99 | 1.07 | 0.73 | 0.65 |
| Mean extracts (E)[2] | 1.03 | 0.86 | 4.3 | 4.0 | 0.90 | 0.86 | 1.44 | 1.53 | 1.14 | 1.17 | 0.97 | 0.66 |
| *AstE* | 1.12 | 0.84 | 4.9[a] | 3.7 | 0.92 | 0.85 | 1.38 | 1.45 | 1.27 | 1.12 | 0.89 | 0.68 |
| *LenE* | 1.01 | 0.81 | 3.8[b] | 3.9 | 0.92 | 0.84 | 1.40 | 1.70 | 1.09 | 1.21 | 1.01 | 0.62 |
| *TreE* | 0.96 | 0.93 | 4.2[b] | 4.4 | 0.87 | 0.89 | 1.54 | 1.45 | 1.06 | 1.18 | 1.02 | 0.67 |
| Mean (E+V)[3] | 0.87 | 0.87 | 3.6 | 3.6 | 0.72 | 0.84 | 1.14 | 1.35 | 0.74 | 1.05 | 0.76 | 0.61 |
| *AstE*+V | 0.85 | 1.00 | 3.6[AB] | 3.6 | 0.72 | 0.87 | 1.24[A] | 1.36 | 0.81 | 1.10 | 0.71 | 0.63 |
| *LenE*+V | 0.88 | 0.77 | 3.9[A] | 3.6 | 0.63 | 0.82 | 1.19[AB] | 1.46 | 0.69 | 1.03 | 0.83 | 0.64 |
| *TreE*+V | 0.87 | 0.85 | 3.2[B] | 3.7 | 0.80 | 0.84 | 1.01[B] | 1.23 | 0.71 | 1.01 | 0.75 | 0.55 |
| SEM[4] | 0.02 | 0.02 | 0.01 | 0.09 | 0.02 | 0.02 | 0.03 | 0.04 | 0.03 | 0.02 | 0.03 | 0.03 |
| Contrast statements | Probability level of contrasts[5] | | | | | | | | | | | |
| Infected vs Non-infected | .083 | .252 | .554 | .318 | **.011** | .167 | .426 | **.029** | **<.001** | .200 | .952 | .905 |
| Vaccinated vs Infected | .920 | .064 | .265 | **< .001** | .101 | .575 | **.018** | **.015** | **.001** | .338 | **.015** | .341 |
| E vs Vaccinated | .298 | **.013** | .205 | **< .001** | **.003** | .526 | .398 | .140 | **.038** | .202 | **.001** | .953 |
| E+V vs Vaccinated | **.001** | **.021** | **.018** | **< .001** | .535 | .384 | **.003** | .654 | **.002** | .802 | .574 | .456 |

[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 standred error mean of the treatments.

[5] $P < 0.05$ are printed in bold.

[abc, ABC] 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 Bedrnik (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 ($TreE_m$) against *Eimeria tenella* infection in chickens (Hu *et al*., 1998)

| Groups | Dose | BWG[1] | Mortality[1] | Lesion score[1] | Oocyst output[1] |
|---|---|---|---|---|---|
| Challenge | $5 \times 10^4$ oocyst $b^{-1}$ | 100 | 100 | 100 | 100 |
| Vaccine | 75 oocyst $b^{-1}$ | 87 | 90 | 67 | 25 |
| $TreP_m$ | $200 \times 7$ mg $kg^{-1}$ d | 78 | 139 | 100 | 50 |
| $TreP_m$+Vaccine | | 135 | 0 | 67 | 50 |

[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* ($5 \times 10^4$) infection. In this study, the birds were infected with $9 \times 10^4$ 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 coccidal infection in this study was most probably a result from the coccdial 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 cocciodial 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 cyclophosphamide 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 cacum, 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.

## REFERENCES

Banfield, M. J., R. P., Kwakkel, and J. M., Forbes. 2002. Effect of wheat structure and viscosity on coccidiosis in broiler chickens. Animal Feed Science and Technology, 98: 37-48.

Banfield, M. J., R. P., Kwakkel, M., Groeneveld, R. A., Ten Doeschate, and J. M., Forbes, 1999. Effects of whole wheat substitution in broiler diets and viscosity on a coccidial infection in broilers. British Poultry Science, 40 (Suppl): S58-9.

Breed, D. G. J., J., Dorresten, T. P. M., Shetters, L.V. D., Waart E., Rijke, and A. N., Vermeulen. 1997. Peripheral blood lymphocytes from *Eimeria tenella* infected chickens produce gamma-interferon after stimulation *in vitro*. Parasite Immunology Oxford, 19(3): 127-135.

Chapman, H. D. 1997. Biochemical, genetic and applied aspects of drug resistance in *Eimeria* parasites of the fowl. Avian Pathology, 26(2): 221-244.

Chapman, H. D. 2001. Use of anticoccidial drugs in broiler chickens in the USA: Analysis for the years 1995 to 1999. Plutry Science, 80(5): 572-580.

Chapman, H. D., T. E., Cherry, H. D., Danforth, G., Richards, M. W., Shirley, and R. B., Williams. 2002. Sustainable coccidiosis control in poultry production: The role of live vaccines. International Journal of Parasitology, 32(5): 617-629.

Choi, S. H., H. J., Youn, K. S., Kim, and S., Namgoong. 1988. Studies on anticoccidial drug resistance against chicken coccidia: I. Effectiveness of therapeutic anticoccidial drugs against field isolates of *Eimeria acervulina* and *Eimeria tenella*. Research Reports of the Rural Development Administration (Suweon), 30(3VET.): 66-76.

Conway, D. P., G. F., Mathis, and M., Lang. 2002. The use of diclazuril in extended withdrawal anticoccidial programs: 2. Immunity to *Eimeria tenella* challenge after drug withdrawal. Poultry Science, 81(3): 353-355.

Dubois, M. K., J. K., Gilles, J. K., Hamilton, P. A., Rebers, and F., Smith. 1956. Pfizer flocon 4800 procedure-phenol/sulfuric acid method. Analytical Chemistry, 28: 350-352.

Eastwood, M. A. 1992. The physiological effect of dietary fibre: an update. Annual Reviews in the

Nutrition, 12:19-35

Gibson, G. R., and M. B., Roberfroid. 1995 Dietary modulation of the human colonic microflora: introducing the concept of prebiotics. Jouranl of Nutrition, 125:1401.

Girard, F., G., Fort, P., Yvoré , and P., Qué ré . 1997. Kinetics of specific IgA, IgM and IgG production in the duodenal and caecal mucosa of chickens infected with *E. Acervulina* and *E.tenella*. International Journal for Parasitology, 27(7): 803-809.

Hesselman, K., and P., Aman. 1986. The effect of β-glucanase on the utilization of starch and nitrogen by broiler chickens fed on barley of low- or high-viscosity. Animal Feed Science Technology, 15: 83-93.

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

Hu, T. J., J. L., Liang, and F. S., Cheng. 1998. The use of 8301 polysaccharide as an adjuvant in coccidial infected chickens. Journal of Grass and Livestock (in Chinese), 6: 5-7.

Hughes, R. J., M., Choct, A., Kocher, and R. J., Van Barneveld. 2000. Effect of food enzymes on AME and composition of digesta from broiler chickens fed on diets containing non-starch polysaccharides isolated from lupin kernel. British Poultry Science, 41(3): 318-323.

Iji, P. A., A. A., Saki, and D. R., Tivey. 2001. Intestinal development and body growth of broiler chicks on diets supplemented with non-starch polysaccharides. Animal Feed Science Technology, 89(3-4): 175-188.

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

Li, C. M. 1998. Chinese Herb Medicine Feed Additives (in Chinese). Beijing: Chinese Agricultural University press.

Li, G. X., E. P., Lillehoj, and H. S., Lillehoj, 2002. Interleukin-2 production in SC and TK chickens infected with *Eimeria tenella*. Avian Diseases, 46 (1): 2-9.

Liang, H. P., Y., Zhang, and B., Geng. 1994. Effect of *Astragalus membranacea Radix* on cellular immunity in burn rats. Journal of China Plastic and Burn Surgery (in Chinese), 10(2): 138-141.

Lien, E. J., and H., Gao. 1990. Higher plant polysaccharides and their pharmacological activities. Int. J. Oriental Med. 15:123.

Lillehoj, H. S., and E. P., Lillehoj, 2000. Avian coccidiosis. A review of acquired intestinal immunity and vaccination strategies. Avian Disease, 44: 408-425.

Lillehoj, H. S. 1998. Role of T lymphocytes and cytokines in coccidiosis. International Journal of

Parasitology, 28: 1071-1081.

Lillehoj, H. S. 2000. Review on vaccine development against enteric parasites *Eimeria* and Cryptosporidium. Japanese Poultry Science, 37(3): 117-141.

Lillehoj, H. S., S. Y., Kang, L., Keller, and M., Sevoian. 1989. *Eimeria tenella* and *E. Acervulina:* lymphokines secreted by an avian T cell lymphoma or by sporozoite-stimulated lymphocytes protect chickens against avian coccidiosis. Experimental Parasitolology, 69: 54-64.

Liu, J. X., C. Y.. Wang, and Y. M., Bai. 1999. Preparation of immune active Chinese herbal formulation and safe efficacy test. Chinese Journal of Veterinary Science and Technology (in Chinese), 29(8): 23-25.

Long, P. L. 1982. The Biology of the Coccidia. University Park Press. Baltimore, M.D.

Long, P. L. 1984. Coccidiosis Control: Past, Present and Future. British Poultry Science, 25: 3-18.

Long, P. L., J., Johnson, M., Mckenzie, E., Perry, M., Crane, and P., Murray. 1986. Immunization of young broiler chickens with low-level infections of *Eimeria tenella, E. acervulina* or *E. maxima.* Avian Pathology, 15: 271-278.

Mao, X. J. 1988. Effect of *Astragalus membranacea Radix* polysaccharide on humoral immunity in rats. Journal of China Immunology (in Chinese), 4(3):158.

Martin, A., H. S., Lillehoj, B., Kaspers, and D., Bacon. 1994. Mitogen-induced lymphocyte proliferation and interferon production induced by *coccdia* infection. *Avian Disease*, 38: 262-268.

Martin, A., H. S., Lillehoj, B., Kaspers, and L. D., Bacon. 1993. Antigen-specific T cell proliferation following *coccdia* infection. Poultry Science, 72: 2084-2094.

Matsuzawa, T. 1979. Effect of carbohydrate in the diet on *Eimeria tenella* infection. Japnaese Poultry Science, 16(3): 137-140.

Miyamoto, T., W., Min, and H. S., Lillehoj. 2002. Kinetics of interleukin-2 production in chickens infected with *Eimeria tenella*. Comparative Immunology Microbiology and Infectious Diseases, 25(3): 149-158.

Newman, K. E. 2002. Antibiotic resistance is a reality: novel techniques for overcoming antibiotic resistance when using new growth promoters. Pp98-106. Nutritional Biotechnology in the Feed and Food Industries. Proceedings of Alltech's 18th Annual Symposium. Nottingham University Press.

Ovington, K. S., L. M., Alleva, and E. A., Kerr. 1995. Cytokines and immunological control of *Eimeria spp*. International Journal for Parasitology, 25(11): 1331-1351.

Pang, F. H., M. Q., Xie, and H. H., Ling, 2000. The investigation of immuno-dulators tested for the results on the control of a coccidial infection. Chinese Journal of Veterinary Parasitology (in

Chinese), 8(3): 1-3.

Rai, R. B., S., Senani, S. P. S., Ahlawat, and A., Gupta. 1996. Studies on development of vaccine against fowl coccidiosis. Indian Veterinary Journal, 73(2): 181-183.

Rana, N., and S. M., Tikaram. 2001a. In-vivo assessment of drug resistance in field isolates of *Eimeria tenella*. Indian Journal of Animal Science, 71(7): 627-630.

Rana, N., and S. M., Tikaram. 2001b. Performance index based evaluation of anticoccidials against field strains of *Eimeria tenella* in broiler chickens. Indian Journal of Animal Science, 71(7): 623-626.

Rodriguez, J. C., J. C., Segura, A., Alzina, and M. A., Gutierrez. 1997. Factors affecting mortality of crossbred and exotic chickens kept under backyard systems in Yucatan, Mexico. Tropical Animal Health and Production, 29(3): 151-157.

Salyers, A. A. 1979. Energy sources of major intestinal termentative anaerobes. The American Joural of Clinical Nutrition, 32: 158-163.

Shirley, M. W., and M. J., Bedrnik. 1977. Studies on the growth, chemotherapy and enryme variation of *Eimeria acervulina* var. dimi.nuted and *E. acervulina var.* mivati. Parasitology, 75: 165-182.

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

Stiff, I., and K. W., Bafundo. 1993. Development of immunity in broilers continuously exposed to *Eimeria Sp.* Avian Disease, 37(2): 295-301.

Suo, X., and G. Q., Li. 1998. Coccidia and Coccidiosis of Domestic Fowl. Chinese Agriculture University Press. Beijing.

Vermeulen, A. N. 1998. Progress in recombinant vaccine development against coccidiosis: A review and prospects into the next millennium. International Journal of Parasitology, 28(7): 1121-1130.

Vermeulen, A. N., D. C., Schaap, and Th. P. M., Schetters. 2001. Control of coccidiosis in chickens by vaccination. Veterinary Parasitology, 100(1-2): 13-20.

Wallach, M., N. C., Smith, C. M. D., Miller, J., Eckeri, and M. E., Rose. 1994. *Eimeria Maxima:* Elisa and western blot analyses of protective sera. Parasite Immuniolology, 16: 377-383.

Wang, R. J., D. F., Li, and S., Bourne. 2002. Can 2000 years of herbal medicine history help us solve problems on the year 2000? pp 273 – 291. in: Biotechnology in the feed industry. Proceedings of Alltech's 14th Anunual sysmposium, Nottingham University Press.

Williams, R. B. 1999. A compartmentalised model for the estimation of the cost of coccidiosis to the world's chicken production industry. International Journal of Parasitology, 29(8):

1209-1229.

Williams, R. B. 2002. Anticoccidial vaccines for broiler chickens: Pathways to success. Avian Pathology, 31(4): 317-353.

Williams, R. B., and L., Gobbi. 2002. Comparison of an attenuated anticoccidial vaccine and an anticoccidial drug programme in commercial broiler chickens in Italy. Avian Pathology, 31(3): 253-265.

Williams, R. B., W. W. H., Carlyle, D. R., Bond, and I. A. G., Brown. 1999. The efficacy and economic benefits of Paracox (R), a live attenuated anticoccidial vaccine, in commercial trials with standard broiler chickens in the United Kingdom. International Journal of Parasitology, 29(2): 341-355.

Xia, E.N., and Q.H., Cheng. 1988. Isolation, analysis and bioactivities of *Tremella fuciformis* fruit body polysaccharides. Acta Mycologica Sinica (in Chinese). 7(3): 166-174.

Xue, M., and X. S., Meng. 1996. Review on research progress and prosperous of immune activities of bio-active polysaccharides. Journal of Traditional Chinese Veterinary. Medicine (in Chinese), 3: 15-18.

Yun, C. H. 2000. Coccidiosis and recent progress in development of vaccines. Asian Australasian Journal of Animal Sciences, 13 (Special Issue): 88-112.

Zhang, L. C., J. B., Wang, Y. P., Sun, D. Z., Ma, M. L., Shi, and Z. H., Wang. 1998. Effect of adding Chinese herb polysaccharide preparations to a Newcastle disease inactivated vaccine on the development of the immune organs in chickens. Chinese Journal of Veterinary Science (in Chinese). 18(4): 378-381.

## Chapter 8

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

F.C. Guo

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands; Institute of Animal Science and Vet. Pharmaceutics, Chinese Academy of Agricultural Sciences, Lanzhou 730050, China*

R.P. Kwakkel

*Animal Production Systems Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

H.K. Parmentier

*Adaptation Physiology Group, 6700 AH Wageningen, Wageningen University, The Netherlands*

B.A. Williams and M.W.A. Verstegen

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

Z.Q. Yang

*Laboratory of Veterinary Clinic, Institute of Animal Science and Veterinary Pharmaceutics, Chinese Academy of Agricultural Sciences, 730050 Gansu, China.*

Submitted to: British Poultry Science

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

F.C. Guo, R.P. Kwakkel, B.A. Williams, H.K. Parmentier, Z.Q. Yang, and M.W.A.Verstegen

---

**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 (Lillehoj, 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; Lillehoj 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 (Lillehoj, 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 (Selleri *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 /$m^2$. 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

| Ingredients | Basal diet (g/kg) |
|---|---|
| Maize | 555 |
| Soybean meal | 326.6 |
| Fish meal | 40 |
| Soya oil | 40 |
| Monocalciumphosphate | 13 |
| Limestone | 11.3 |
| Salt (NaCl) | 3 |
| DL-Methionine | 1 |
| Lysine | 0.12 |
| Vitamin and mineral mix[1] | 10 |
| 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, cholechalciferol 1800 IU, vitamin E (DL-α-topherol acetate) 20 mg, vitamin $K_3$ 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[1]

| Composition[2] | *LenS* | *TreS* | *AstS* |
|---|---|---|---|
| Dry matter (DM) (g/kg) | 957 | 958 | 967 |
| Crude protein (g/kg DM) | 208 | 103 | 157 |
| Yields of polysaccharides (%) | 8.0 | 10.0 | 31.1 |
| | *LenE* | *TreE* | *AstE* |
| DM (g/kg) | 952 | 955 | 940 |
| Crude protein (g/kg DM) | 114 | 47 | 67 |
| Total sugar content (g/kg DM) | 632 | 687 | 647 |

[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% $K_2Cr_2O_7$ 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, 0.2ml 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 $9\times10^4$ 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-γ 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-γ titers.

IFN-γ 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, 4×104 cells/50μl 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 μl (1×102) 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-γ 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-γ 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 μg/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-γ Titers*

Serum IFN-γ 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-γ 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-γ 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-γ 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-γ 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-γ 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-γ 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

| Treatments[1] | IFN-γ (IU/ml) | | IL-2 ($\times 10^3$ cpm)[2] | |
|---|---|---|---|---|
| | Day 7 | Day 14 | Day 7 | Day 14 |
| Non-infection | 158.7±7.5 | 188.9±7.9 | 0.97±.09 | 1.03±.06 |
| Infection | 274.5±8.3 | 360.5±4.6 | 1.34±.06 | 1.25±.07 |
| Vaccine | 329.7±7.0 | 443.4±13.8 | 1.43±.07 | 1.41±.09 |
| Extracts | 312.8±5.6 | 478.7±3.3 | 1.34±.04 | 1.43±.05 |
| Extracts+Vaccine | 356.2±5.6 | 488.0±3.4 | 1.71±.05 | 1.45±.05 |
| Contrast statements | Probability levels of the contrasts[3] | | | |
| Infection vs. Non-infection | **.000** | **.000** | **.007** | **.043** |
| Vaccine vs. Infection | **.001** | **.001** | .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.

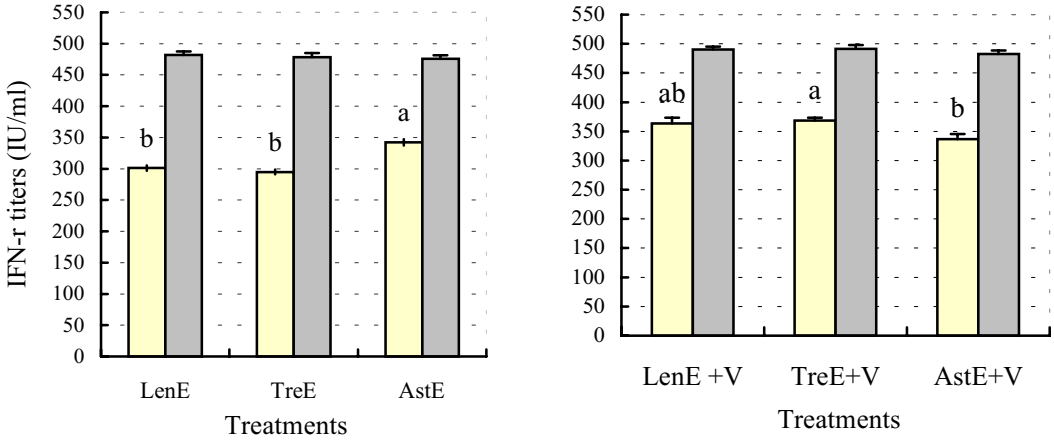



**Figure 1.** The effect of mushroom and herb polysaccharides on serum IFN-γ titers of chickens at 7 (gray-colored columns) and 14 days (dark-colored columns) after *Eimeria tenella* infection. Results are given as means ± SE (n=6) for *LenE* = *Lentinus edodes* extract, *TreE* = *Tremella fuciformis* 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; [abc] Means between two treatments with different superscripts differ significantly (P<0.05).

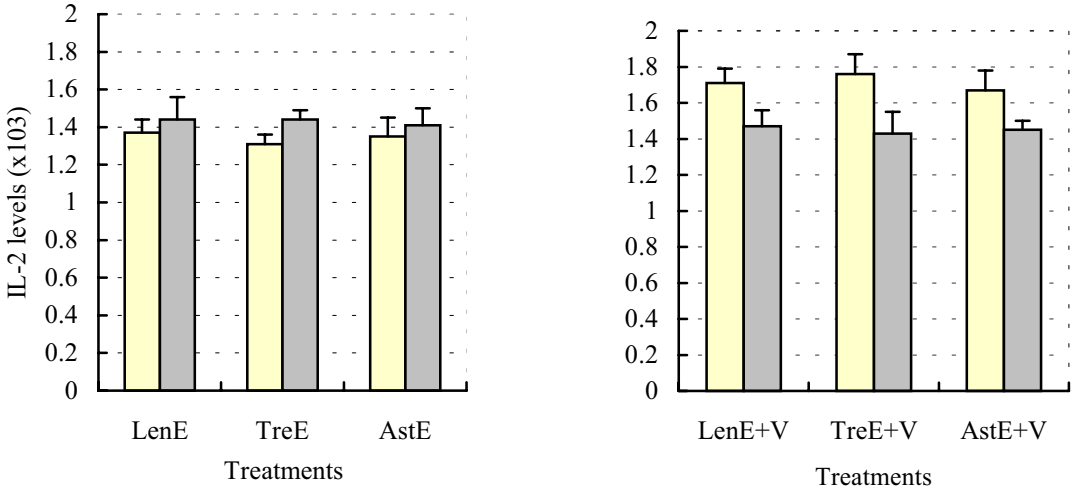



**Figure 2.** Effect of mushroom and herb polysaccharides on IL-2 production of splenocytes induced by phytahematoagglutinin at 7(gray-colored columns) and 14 days (dark-colored columns) after *Eimeria tenella* infection in chickens. Results are given as means ± SE (n = 6) for *LenE = Lentinus edodes* 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-r 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-α and TNF-β 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-γ against invasion of organisms in humans and animals.

It was found from the present experiment that IFN-γ 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 $TreE_m$ 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 $TreE_m$, 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.

## ACKNOWLEDGEMENTS

This research was partially supported by International Foundation For Science (No. B/3175-1). The help of Suo Xun, Laboratory of Parasitology and Parasitological Diseases,

China Agricultural University with providing the experimental facilities, reagents and guiding the practical work for this experiment and Zhao Hui with helping grouping and taking samples is highly appreciated.

## REFERENCES

Banfield, M. J., R. P., Kwakkel, M., Groeneveld, R. A., Ten Doeschate, and J. M., Forbes. 1999. Effects of whole wheat substitution in broiler diets and viscosity on a coccidial infection in broilers. British Poultry Science, 40 (Suppl):S58-9.

Breed, D. G. J., J., Dorresten, T. P. M., Shetters, L.V. D., Waart, E., Rijke, and A. N., Vermeulen. 1997. Peripheral blood lymphocytes from *Eimeria tenella* infected chickens produce gamma-interferon after stimulation *in vitro*. Parasite Immunology Oxford, 19(3):127-135.

Cavallon, J. M. 2001. Pro- versus anti-inflammatory cytokines: Myth or reality. Cellular and Molecular Biology Noisy Le Grand, 47(4):695-702.

Digby, M. R., and J. W., Lowenthal. 1995. Cloning and expression of the chicken interferon-gamma gene. Journal of Interferon and Cytokine Research, 15:939-945.

Dubois, M. K., J. K., Gilles, J. K., Hamilton, P. A., Rebers, and F., Smith. 1956. Pfizer flocon 4800 procedure-phenol/sulfuric acid method. Analytical Chemistry, 28: 350-352.

Fan, B. T., Y. L., Yuan, and J. L., Liang. 1993. Study of mechanism of enhanced avian cholera vaccine by 8301 polysaccharide. Journal of Traditional Chinese Veterinary Medicine, 1:5-10.

Gao, Q. P., M. K., Killie, H. C., Chen, R. Z., Jiang, R., Seljelid, Q. P., Gao, H. C., Chen, and R Z., Jiang. 1997. Characterization and cytokine-stimulating activities of acidic heteroglycans from *Tremella fuciformis*. Planta Medica, 63(5):457-460.

Guo, Y. P., and J., Lei. 1999. The comparison of two methods in detecting the activity of IL-2 in humanperipheral blood. Journal of Chuan Bei Medical College, 1(14):10-12.

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

Hu, T. J., B. T., Fang, and Q. Z., Huang. 1995a. Effect of 8301 polysaccharide on activities of spleen NK cells in rats. China Veterinary Science and Technology, 25:10.

Hu, T. J., M. X., Dong, and J. L., Liang. 1996. Study on 8301 polysaccharide induces immune protection in chicks infected with *Eimeria tenella*. Gansu Animal Husbandry and Veterinary Medicine, (5):8-9.

Hu, T. J., J. L., Liang, and F. S., Cheng. 1998. The use of 8301 polysaccharide as an adjuvant in

coccidial infected chickens. Journal of Grass and Livestock (in Chinese), 6:5-7.

Hu, T. J., B. T., Fang, and Q. Z., Huang. 1995b. Effect of 8301 polysaccharide on spleen IL-2 production in rats. Chinese Veterinary Science and Technology, 25(1):28.

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

Jing, H. 1989. Study on immune function of *Astragalus Mongholicus Bga* polysaccharide (APS-G). Journal of Chinese Immunology, 5(5):40.

Kaiser, P., D., Sonnemans, and L. M., Smith. 1998. Avian IFN-γ genes: sequence analysis suggests proble cross-species reactivity among galliforms. Journalof Interferon and Cytokines Research, 18:711-719.

Katsikis, P. D., S. B. A., Cohen M., Londei, and M., Feldmann. 2001. Are CD4+ T-h1 cells pro-inflammatory or anti- inflammatory? The ratio of IL-10 to IFN-GAMMA or IL-2 determines their function. International Immunology, 7(8):1287-1294.

Klasing, K. C. 1994. Avian leukocytic cytokines. Poultry Science, 73(7):1035-1043.

Lange, A., L., Karabon, A., Klimczak, D., Dlubek, K., Bogunia-Kubik, C., Swider, and K., Suchnicki. 1996. Serum interferon-gamma and C-reative protein levels as predictors of acute graft-vs.-host disease in allogeneic hematopoietic precursor cell (marrow or peripheral blood progenitor cells) recipients. Transplant Proceedings, 28:3522-3525.

Li, G. X., E. P., Lillehoj, and H. S., Lillehoj. 2002. Interleukin-2 production in SC and TK chickens infected with *Eimeria tenella*. Avian Diseases, 46(1):2-9.

Liang, H. P. 1996. Mechanism of cellular immune function suppression and study on immune regulation by herbs in injured rats. Journal of China Third Army Medical University, 18:352.

Liang, J. L., T. J., Hu, and B. T., Fan. 1995. Experiment of 8301 polysaccharide inducing IFN production in rats. Journal of Traditional Chinese Veterinary Medicine, 4:12-13.

Lien, E. J., and H., Gao. 1990. Higher plant polysaccharides and their pharmacological activities. International Journal of Oriental Medicine, 15:123.

Lillehoj, H. S., and K. D., Choi. 1998. Recombinant chicken interferon-gamma-mediated inhibition of *Eimeria tenella* development *in vitro* and reduction of oocyst production and body weight loss following *Eimeria acervulina* challenge infection. Avian Disease, 42:307-314.

Lillehoj, H. S. and E. P., Lillehoj. 2000. Avian coccidiosis. A review of acquired intestinal immunity and vaccination strategies. Avian Disease, 44:408-425.

Lillehoj, H. S., S. Y., Kang, L., Keller, and M., Sevoian. 1989. *Eimeria tenella* and *E. Acervulina:* lymphokines secreted by an avian T cell lymphoma or by sporozoite-stimulated lymphocytes

protect chickens against avian coccidiosis. Experimental Parasitolology, 69:54-64.

Lillehoj, H. S. 1998. Role of T lymphocytes and cytokines in coccidiosis. International Journal of Parasitology, 28:1071-1081.

Liu, J. X., C. Y., Wang, and Y. M., Bai. 1999. Preparation of immune active Chinese herbal formulation and safe efficacy test. Chinese Journal of Veterinary Science and Technology, 29(8): 23-25.

Liu, M., J. Z., Li, F. Z., Kong, J. Y., Lin, and Y., Gao. 1998. Induction of immunomodulating cytokines by a new polysaccharide-peptide complex from culture mycelia of *Lentinus edodes*. Immunopharmacology, 40(3):187-198.

Lowenthal, J.W., B., Lambrecht, T. P., Van Den Berg, M. E., Andrew, A. D. G., Strom, and A. G. D., Bean. 2000. Avian cytokines: The natural approach to therapeutics. Development and Comparative Immunology, 24 (2-3):355-365.

Lowenthal, J. W., J. J., York, T. E., O'neil, S., Rhodes, D. G., Prowse, D. G., Strom, and M. R., Digby. 1997. *In vivo* effects of chicken interferon-gamma during infection with *Eimeria.* Journal of Interferon and Cytokines Research, 17:551-558.

Lowenthal, J. W., J. J., York, T. E., O'neil, R. A., Steven, D. G., Strom, and M. R., Digby. 1998. Potential use of cytokine therapy in poultry. Veterinary Immunology and Immunopathology, 63: 191-198.

Martin, A., H. S., Lillehoj, B., Kaspers, and D., Bacon. 1994. Mitogen-induced lymphocyte proliferation and interferon production induced by *coccdia* infection. Avian Disease, 38:262-268.

Martin, A., H. S., Lillehoj, B., Kaspers, and L. D., Bacon. 1993. Antigen-specific T cell proliferation following *coccdia* infection. Poultry Science, 72:2084-2094.

Min, W. G., H. S., Lillehoj, J., Burnside, K. C., Weiming, P., Staeheli, and J. J., Zhu. 2001. Adjuvant effects of IL-1beta, IL-2, IL-8, IL-15, IFN-alpha, IFN-gamma, TGF-beta4 and lymphotactin on DNA vaccination against *Eimeria acervulina*. Vaccine, 20(1-2):267-274.

Miyamoto, T., W., Min, and H. S., Lillehoj. 2002. Kinetics of interleukin-2 production in chickens infected with *Eimeria tenella*. Comparative Immunology Microbiology and Infectious Diseases, 25(3):149-158.

Murray, P. J., R. A., Young, and G. Q., Daley. 1998. Hematopoietic remodeling in interferon-gamma-deficient mice infected with mycobacteria. Blood, 91:2914-2924.

Ottenhoff, T. H. and Mutis, T. (1995) Role of cytoxic cells in the protective immunity against and immunopathology of intracellular infections. European Journal of Clinic Investigation, 25:371-377.

Ovington, K. S., L. M., Alleva, and E. A., Kerr. 1995. Cytokines and immunological control of *Eimeria spp*. International Journal for Parasitology, 25(11):1331-1351.

Pang, F., Y. X., Feng, and D. Y., Zhang. 1995. Review on current research of *Astragalus mongholicus Bga*. World Phytomedicines, 10(3):110-115.

Rong, K. T. 1987. General Introduction of Immunophamaceutics. pp: 21-25 (Zhejing Science and Technology Press).

Rose, M. E., D., Wakelin, and P., Hesketh. 1991. Interferon-gamma-mediated effects upon immunity to coccidial infections in the mouse. Parasite Immunology, 13:63-74.

Selleri, C., T., Sato, S., Anderson, N. S., Young, and J. P., Maciejewski. 1995. Interferon-gamma and tumor necrosis factor-alpha suppress both early and late stages of haemotopoiesis and induce programmed cell death. Journal of Cellular Physiology, 165:538-546.

Shinji, W., T., Takahiro, K., Satoshi, K., Tetsuya, H., Hiroshi, and T., Kunio. 2002. Involvement of phosphodiesterase isozymes in osteoblastic differentiation. Journal of Bone and Mineral Research, 17(2):249-256.

Song, K.D., H. S., Lillehoj, K.D., Choi, D., Zalenga, and J. Y., Han. 1997. Expression and functional characterization of recombinant chicken interferon-gamma. Veterinary Immunology and Immunopathology, 58:321-333.

Suo, X. and G. Q., Li. 1998. Coccidia and Coccidiosis of Domestic Fowl. Chinese Agriculture University Press. Beijing.

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

Tang, X. M., Y. L., He, and B. K., Zong. 1998. Effect of Astragalus polysaccharides on T lymphocyte transformation in the peripheral blood of chickens. Chinese Journal of Veterinary Science, 18(3): 269-271.

Thakur, A., M., Xue, F., Stapleton, A. R., Lioyd, D., Wakefield, and M. D. P., Willcox. 2002. Balance of pro- and anti-inflammatory cytokines correlates with outcome of acute experimental Pseudomonas aeruginosa keratitis. Infection and Immunity, 70(4):2187-2197.

Wang, D. L. 1989. Effect of *Astragali Membranacea Radix* polysaccharide on immune function of blood lymphocytes in human. Journal of China Cancer, 11(3):180.

Wei, X.Y. 1991. Research on effects of *Astragali Membranacea Radix* and *Polygoni Multiflori Radix* thymus in old rats. Journal of China Pharmaceutical University, 22:359.

Wu, Y. H. and F., Wang, 1999. Research status on herbs' two-way immune regulating functions. Journal of Traditional Chinese Veterinary Medicine, 4:19-22.

Xia, E. 1986. Effect of *Lentinus edodes* polysaccharide against humors in rats. Jounal of Nanjing

Pharmacy College, 17(2):129.

Xia, E. N., and Q. H., Cheng. 1988. Isolation, analysis and bioactivities of *Tremella fuciformis* fruit body polysaccharides. Acta Mycologica Sinica, 7(3):166-174.

Xue, M., and X. S., Meng. 1996. Review on research progress and prosperous of immune activities of bio-active polysaccharides. J. of Traditional Vet. Medicine. 3:15-18.

Yang, J., M. C., Wu, and S. H., Zhang. 1999. Study on isolation, purification and characteristics of polysaccharide Le-III from the fruit body of *Lentinus Edodes.* Journal of Huazhong Agricultural University, 18(2):188-190.

Yang, T. S. and Y. F., Wang. 1998. Cytokine analysis techniques. in: Immunology and Immunological Techniques, pp188-193 (China People's Hygiene press).

Yun, C. H., H. S., Lillehoj, and K. D., Choi. 2000a. Chicken IFN-γ monoclonal antibodies and their application in enzyme-linked immunosorbent assay. Veterinary Immunology and Immunopathology, 73:297-308.

Yun, C. H., H. S., Lillehoj, J. T., Zhu, and W. G., Min. 2000b. Kinetic differences in intestinal and systemic interferon-γ and antigen-specific antibodies in chickens experimentally infected with *Eimeria maxima*. Avian Disease, 44 (2):305-512.

Zhao, K. S. 1993. Study on *Astragali membranacea Radix* polysaccharide enhancement of peripheral blood monocytes producing tumour necrosis factors in human. Journal of Chinese Traditional and Western Medicine, 13:263.

Zhao, K. S., C., Mancini, and G., Doria. 1990. Enhancement of immune response in mice by *Astragalus memebranaceus* extracts. Immunopharmacology, 20:225-341.

## Chapter 9

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

F.C. Guo

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands; Institute of Animal Science and Vet. Pharmaceutics, Chinese Academy of Agricultural Sciences, Lanzhou 730050, China*

R.P. Kwakkel

*Animal Production Systems Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

H.K. Parmentier

*Adaptation Physiology Group, 6700 AH Wageningen, Wageningen University, The Netherlands*

B.A. Williams and M.W.A. Verstegen

*Animal Nutrition Group, Wageningen University, P.O. Box 338, 6700 AH Wageningen, Netherlands*

W.K. Li

*Department of Parasitological Diseases, Gansu Animal Health Service, 730046 Gansu, China.*

Submitted to: Poultry Science

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

F.C. Guo, R.P. Kwakkel, B.A. Williams, H.K. Parmentier, W.K. Li, and M.W.A.Verstegen

## 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 cocciodial 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 $^{o}$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 /m$^2$. The formal experiment was conducted from 8 to 34 days of age. The temperature was set at 30 $^{o}$C to 33 $^{o}$C during the first week and gradually declined by 2 $^{o}$C per week until 20 $^{o}$C was reached. Relative humidity was about 60-80%. The lighting program was 24h light.

**Table 1.** Composition of the experimental diets (g/kg)

| Ingredients | Starter | Grower |
|---|---|---|
| Maize | 579 | 624 |
| Soybean meal (50%) | 240 | 200 |
| Fish meal | 40 | 30 |
| Rape seed meal | 30 | 30 |
| Peanut meal | 50 | 50 |
| Monocalciumphosphate | 10 | 13 |
| Limestone (Ca 38%) | 15 | 12 |
| Salt (NaCl) | 3 | 3 |
| DL-Methionine | 2.5 | 2.0 |
| Lysine | 0.5 | 1.0 |
| Soybean oil | 20 | 25 |
| Premix[2] | 10 | 10 |
| Caculated analysis (g/kg) | | |
| Crude protein | 210.0 | 200.0 |
| Metabolisable energy (kcal/kg) | 2,900 | 3,000 |
| Calcium | 10.0 | 9.0 |
| Available Phosphors | 5.0 | 4.0 |
| Lysine | 11.0 | 10.5 |
| Methionine | 5.0 | 5.1 |
| Met + Cys. | 9.0 | 8.0 |

[1] Supplied for per kg of diet: vitamin A (retinal acetate), 10,000 IU; cholechalciferol, 2,000 IU; DL-α-topherol 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[1]

| Composition[2] | *LenS* | *TreS* | *AstS* |
|---|---|---|---|
| Dry matter (DM) (g/kg) | 957 | 958 | 967 |
| Crude protein (g/kg DM) | 208 | 103 | 157 |
| Yields of polysaccharides (%) | 8.0 | 10.0 | 31.1 |
| | *LenE* | *TreE* | *AstE* |
| DM (g/kg) | 952 | 955 | 940 |
| Crude protein (g/kg DM) | 114 | 47 | 67 |
| Total sugar content (g/kg DM) | 632 | 687 | 647 |

[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 $^{o}$C in PBS (pH 7.2), and centrifuged at 10,000g for 10min at 4 $^{o}$C. The protein content in the supernatant was measured with a Bicinchoninic acid protein assay (Smith *et al*., 1985) and stored at -20 $^{o}$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 $10^4$ 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 $ml^{-1}$, Zaozhuang 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 $^{o}$C for serum antibody measurements. Chickens were then killed by cervical dislocation. Spleen samples were removed and stored at -20 $^{o}$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 ug $ml^{-1}$ 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 2-3mm), washed with HBSS penicillin-streptomycin (Lab of Vet. Clinic, CAAS, China ), and resuspended in 5ml RPMI 1640 Dutch modified (GIBCO) supplemented with 100 ug 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 oC, 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 μg/ml soluble E. tenella antigen in 0.05 mol/l carbonate buffer (pH 9.6)(100 μl/well) by incubation overnight at 37 oC. Two percent bovine serum albumin (Lanzhou Bio-products Company, China) (200 μl/well) was then added for 1h at 37 oC. The wells were washed 3 times with 300 μl PBS-Tween 20 buffer (0.05%, pH 7.2, Lab of Vet. Clinic, CAAS, China), and sera or cultured cecal samples (100 μl/well) diluted with the same PBS-Tween 20 serially, and incubated for one hour at 37 oC. One of the following polyclonal antibodies (100 μl) 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 μl per well): 0.1 μg/ml solution of 2-2'-azino-bis (3-ethylbenzthiazoline 6-sulfonic acid) diammonium in citrate buffer (pH 4) for IgA and IgM, or a l μg/ml solution of p-nitrophenylphosphate in diethanolamine (pH 9.8) for IgG. The reaction was stopped by addition of 50 μl 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 μm 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 effecter 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

μl/well in RPMI 1640 (GIBCO) supplemented with L-glutamine (300 μg/ml), penicillin (100 UI/ml), streptomycin (100 μg/ml), bovine insuline (5 μg/ml), 2-β-mercaptoethanol (5x10-5 M) and 2.5% chicken serum (Lab of Vet. Clinic, CAAS, China) with 100μl soluble E. tenella antigen at several concentrations (0, 25, 12.5, 6.25, or 3.12 μg/ml with 3 replicates each) at 41 oC in a humidified atmosphere of 5% CO2 for 96 hrs. Tritiated thymidine (50μl) (3H-TdR, 3.7 kBq, Institute of Ganzhou Medical Industry) was added to each well for the final 20 hrs. Frozen cells (at -20 oC) 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 (pH7.4, 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 (100μl) were incubated with a suspension of 20% bovine serum in Hank's solution (pH: 7.4, Lanzhou Bio-products, China) (100μl) and 2% SRBC Hank's solution (Lab of Vet. Clinic, CAAS China) (100μl) at 37 oC for 15 min, and then centrifuged for 5min at 100g. The cell pellets were harvested and stored at 4 oC 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 μl) were incubated with a sensitized erythrocyte-antibody-complemental suspension (100μl) 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.** Seral specific antibody production of *Eimeria tenella* infected chickens fed with mushroom and herb extracts

| Treatments[1] | Seral specific antibody production ($A_{450}$ nm)[4] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Day 7 | | | Day 14 | | | Day 21 | | |
| | IgA | IgM | IgG | IgA | IgM | IgG | IgA | IgM | IgG |
| Control | 0.027 | 0.07 | 0.33 | 0.03 | 0.09 | 0.33 | 0.03 | 0.03 | 0.22 |
| Infection | 0.065 | 0.20 | 0.75 | 0.15 | 0.25 | 2.9 | 0.04 | 0.04 | 2.1 |
| *LenE* + Infected | 0.066 | 0.22 | 0.76 | 0.18 | 0.26 | 3.0 | 0.12 | 0.21 | 2.3 |
| *TreE* + Infected | 0.065 | 0.20 | 0.76 | 0.17 | 0.26 | 3.0 | 0.15 | 0.25 | 2.9 |
| *AstE* + Infected | 0.068 | 0.22 | 0.78 | 0.21 | 0.27 | 3.0 | 0.11 | 0.21 | 2.2 |
| SEM[2] | 0.004 | 0.02 | 0.05 | 0.02 | 0.02 | 0.28 | 0.01 | 0.03 | 0.2 |
| Contrasts | Probability level of the contrasts[5] | | | | | | | | |
| Infection vs. control | **<.001** | **<.001** | **<.001** | **<.001** | **<.001** | **<.001** | .557 | .525 | **<.001** |
| Extracts[3] vs. Infection | .658 | .398 | .202 | **.003** | **.020** | .166 | **<.001** | **<.001** | **<.001** |
| *LenE* vs *TreE* | .803 | .294 | .766 | .647 | .574 | .944 | .214 | .052 | **<.001** |
| *AstE* vs *LenE* & *TreE* | .365 | .417 | .115 | .111 | .361 | .081 | .061 | .152 | **<.001** |

[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

| Treatments[1] | Cecal specific antibody production ($A_{450}$ nm)[4] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Day 7 | | | Day 14 | | | Day 21 | | |
| | IgA | IgM | IgG | IgA | IgM | IgG | IgA | IgM | IgG |
| Control | 0.13 | 0.05 | 0.04 | 0.15 | 0.11 | 0.07 | 0.10 | 0.04 | 0.02 |
| Infection | 0.15 | 0.16 | 0.43 | 0.16 | 0.21 | 2.4 | 0.14 | 0.20 | 2.0 |
| *LenE* + Infected | 0.16 | 0.18 | 0.44 | 0.21 | 0.26 | 2.8 | 0.19 | 0.24 | 2.6 |
| *TreE* + Infected | 0.14 | 0.17 | 0.40 | 0.18 | 0.24 | 2.6 | 0.23 | 0.27 | 3.0 |
| *AstE* + Infected | 0.17 | 0.20 | 0.78 | 0.25 | 0.29 | 3.0 | 0.15 | 0.21 | 2.2 |
| SEM[2] | 0.005 | 0.02 | 0.06 | 0.01 | 0.02 | 0.3 | 0.01 | 0.02 | 0.3 |
| Contrasts | Probability level of the contrasts[5] | | | | | | | | |
| Infection vs. control | .286 | **.001** | **.015** | .340 | **.025** | **.001** | **.044** | .096 | **<.001** |
| Extracts[3] vs. Infection | .316 | .220 | .147 | **.010** | .120 | **.021** | **.006** | .564 | **<.001** |
| *LenE* vs *TreE* | .191 | .690 | .620 | .404 | .405 | .389 | .213 | .097 | **.017** |
| *AstE* vs *LenE* & *TreE* | .470 | .095 | **<.001** | .061 | .058 | .147 | .053 | **.006** | **<.001** |

[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

| Treatments[1] | Proliferation of splenocytes (cpm x$10^3$)[3] | | |
|---|---|---|---|
| | Day 7 | Day 14 | Day 21 |
| Control | 2.5±0.14 | 2.1±0.3 | 2.4±0.11 |
| Infection | 9.5±2.5 | 14.8±1.2 | 12.7±1.9 |
| *LenE* + Infected | 13.5±3.7 | 26.5±4.4 | 25.0±3.1 |
| *TreE* + Infected | 10.3±2.8 | 22.8±3.2 | 25.7±2.9 |
| *AstE* + Infected | 13.3±4.9 | 28.4±2.4 | 22.2±2.9 |
| Contrasts | Probability level of the contrasts[4] | | |
| Infection vs Control | .108 | **.007** | **.033** |
| Extracts[2] vs. Infection | .430 | **.002** | **.005** |
| *LenE* vs *TreE* | .538 | .543 | .884 |
| *AstE* vs *LenE* & *TreE* | .811 | .345 | .428 |

[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

| Treatments[2] | Day 7 | | Day 14 | | Day 21 | |
|---|---|---|---|---|---|---|
| | ERFC (%) | EAC (%) | ERFC (%) | EAC (%) | ERFC (%) | EAC (%) |
| Control | [25.9] | 13.6 | 22.4 | 11.8 | 23.2 | 11.7 |
| Infection | 29.4 | 17.6 | 25.8 | 14.9 | 27.6 | 15.5 |
| *LenE* + Infected | 29.5 | 16.9 | 30.1 | 18.5 | 29.5 | 16.6 |
| *TreE* + Infected | 28.4 | 16.4 | 30.3 | 16.8 | 30.7 | 18.1 |
| *AstE* + Infected | 30.5 | 17.2 | 31.2 | 16.5 | 29.6 | 18.0 |
| SEM[3] | 0.57 | .0.55 | 1.02 | 0.66 | 0.77 | 0.78 |
| Contrasts | Probability level of the contrasts[5] | | | | | |
| Infection vs. control | **.037** | **.021** | .056 | **.006** | **.003** | **.041** |
| Extracts[4] vs. Infection | .941 | .566 | **.004** | **.012** | **.025** | .158 |
| *LenE* vs *TreE* | .473 | .760 | .733 | .132 | .341 | .397 |
| *AstE* vs *LenE* & *TreE* | .251 | .651 | .748 | .206 | .638 | .689 |

[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 proporia 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 $TreE_m$, 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. $TreE_m$, 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.

## REFERENCES

Banfield, M. J., R. P. Kwakkel, M. Groeneveld, R. A. Ten Doeschate, and J. M. Forbes. 1999. Effects of whole wheat substitution in broiler diets and viscosity on a coccidial infection in broilers. Br. Poult. Sci. 40 (Suppl): S58-9.

Brain, P., Gordon, J., and W. A. Willetts. 1970. Rosette formation by peripheral lymphocytes. Clin. Exp. Immunol. 6:681-688.

Cheng, F. S., T. J., Hu, and J. L., Liang. 1998. Effect of 8301-polysaccharide on MD vaccine efficacy in chickens. Journal of Traditional Chinese Veterinary Medicine (in Chinese) 17 (1): 8-9.

Cheng, Y. L. 1988. Polysaccharide from fruit body of *Tremella Fuciformis*. Journal of Medical Academy of Martial Sciences (in Chinese) 12(1): 70.

Chi, Y. J., Q. Z., Li, and H. X., Yu. 1998. Effect of *Astragalus mongholicus Bga* polysaccharide on SOD activity in avian myelocytic leukemia virus infected chicks. Helongjiang Animal Science and Veterinary Medicine (in Chinese) 2:3-5.

Dang, P., B. T., Fan, J. L., Liang, and T. J., Hu. 1994. Effects of the *Tremella* mycelia polysaccharide on cellular immune function in chickens. Journal of Traditional Chinese Veterinary Medicine (in Chinese) 3: 4-6.

Dubois, M. K, Gilles, J. K., Hamilton, J. K., Rebers, P. A., Smith, F. 1956. Pfizer flocon 4800 procedure-phenol/sulfuric acid method. Analytical Chemistry 28: 350-352.

Fang, B. T., Y. L., Yuan, and J. L. Liang. 1993. Study of mechanism of enhanced avian cholera vaccine by 8301 polysaccharide. Journal of Traditional Chinese Veterinary Medicine (in Chinese) 1:5-10.

Froebel, K. S., N. G., Pakker, F., Aiuti, M., Bofill, H., Choremi-Papadopoulou, J., Economidou,

C., Rabian, M. T. L., Roos, L. P., Ryder, and F., Miedema. 1999. Standardization and quality assurance of lymphocyte proliferation assays for use in the assessment of immune function. J. Immunol. Methods. 227: 85-97.

Gao, F., J. L., Liang, and J. C., Meng. 1995. Polysaccharides content determination. Journal of Chinese Traditional Veterinary Medicine (in Chinese) 4:12-13.

Girard, F., G. Fort, P. Yvoré , and P. Qué ré . 1997. Kinetics of specific IgA, IgM and IgG production in the duodenal and caecal mucosa of chickens infected with *E. Acervulina* and *E.tenella*. International Journal for Parasitology 27(7): 803-809.

Girard, F., P., Pé ry, M., Naciri, and P., Qué ré . 1999. Adjuvant effect of cholera toxin on systemic and mucosal immune responses in chickens infected with *E.tenella* or given recombinant parasitic antigen per os. Vaccine 17:1516-1524.

Hu, T. J., M. X., Dong, and J. L., Liang. 1996. Study on 8301 polysaccharide induces immune protection in chicks infected with *E. tenella*. Gansu Animal Husbandry and Veterinary Medicine (in Chinese) (5):8-9.

Huisman, M. M. H., H. A., Schols, and A. G. J., Voragen. 1998. Cell wall polysaccharides from soybean meal; isolation and characterization. Carbohydr. Polym. 37: 87-95.

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

Jing, H. 1989. Study on Immune function of *Astragalus Mongholicus Bga* polysaccharide (APS-G). Journal of Chinese Immunology (in Chinese) 5(5): 40.

Kajimura, K, Y, Takagi, K, Miyano, Y, Sawabe, M., Mimura, Y., Sakagami, H., Yokoyama, and K., Yoneda. 1997. Polysaccharide of *Astragalus radix* enhances IgM antibody production in aged mice. Biological & Pharmaceutical Bulletin 20: 1178-82.

Lei, L. S., and Z. B., Lin. 1993. Effects of Ganoderma polysaccharides on the activity of DNA polymerase alpha of splenocytes and immune function in aged mice. Acta Pharmaceutica Sinica (in Chinese) 28: 577-582.

Li, C. M. 1998. Chinese herb medicine feed additives (Chinese) 2nd rev. ed. Chinese Agriculture University Press. Beijing.

Liang, H. P. 1994. Effect of *Astragali Membranacea Radix* polysaccharide on cellular immunity of burn rats. Journal of China Burn Plastic Surgery (in Chinese) 10:138.

Lien, E. J., and H., Gao. 1990. Higher plant polysaccharides and their pharmacological activities. Int. J. Oriental Med. 15:123.

Lillehoj, H. S. 1987. Effect of immunosupression on avian coccidiosis: cyclosporin A but not

hormonal bursectomy abrogates host protect immunity. Infection and Immunity 55: 1616-1621.

Liu, J. X., C. Y., Wang and Y. M., Bai. 1999. Preparation of immune active Chinese herbal formulation and safe efficacy test. Chinese Journal of Veterinary Science and Technology (Chinese) 29(8): 23-25.

Mao, X. J. 1989. Immune opsonic action of *Astragali Membranacea Radix* polysaccharide. China Pharmacology Bulletin (in Chinese). 5:367.

Martin, A., and H. S., Lillehoj. 1993. Antigen-specific T cell proliferation following coccidian infection. Poultry Science. 72 (2): 2085-2094.

Miao, X. J. 1988. Effect of *Astragali Membranacea Radix* polysaccharide on humoral immunity in rats. Journal of China Immunology (in Chinese) 4(3): 158.

Pang, F. H., M. Q., Xie, and H. H., Ling. 2000. The investigation of Immunodulators tested for the results on the control of a coccidial infection. Chinese Journal of Veterinary Parasitology (in Chinese) 8(3):1-3.

Qu, Q. H., Q. Z., Li, and B. R., Xu. 1998. Effect of *Astragalus mongholicus Bga* and *Lentinus edodes* polysaccharide on Se-GSHPX activity in avian myelocytic leukemia virus infected chicks. Helongjiang Animal Science and Veterinary Medicine (in Chinese) 4:1-2.

Ranjit, C. 1984. Measuring impact using immunologic techniques. In: Methods for the Evaluation of the Impact of Food and Nutrition Programmes. David E. Sahn, Richard Lockwood, ed. Nevin S. Scrimshaw United Nations University Press

Rose, M. E., and P., Hesketh. 1982. Immunity to coccidian in chickens: adaptive transfer with peripherial blood lymphocytes and splenocytes. Parasit. Immunol. 54:171-185.

Shi, X. M., X. M., Wang, and H. F., Gao. 1996. Tests on the antiviral efficacy of a water-soluble preparation of a compound polysaccharide of *Astragalus* root. Chinese Journal of Vet. Science and Technology (in Chinese) 26(12):39-41.

Smith, P. K., R. I., Krohn, G. T., Hermanson, A. K., Mallia, F. H., Gartner, M. D., Provnzano, E. K., Fujimoto, N. M., Goeke, B. J., Olson, and D. C., Klenk. 1985. Measurement of protein using bicinchoninic acid. Analytical Biochemistry 150:76-85.

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

Stiff, M. I., and K.W., Bafundo, 1993. Development of immunity in broilers continuously exposed to *Eimeria Sp*. Avi. Dis. 37(2): 295-301.

Suo, X. and G. Q., Li. 1998. Coccidia and Coccidiosis of Domestic Fowl (in Chinese). Chinese Agriculture University Press. Beijing.

Tang, X. M., Y. L., He, and B. K., Zong. 1998. Effect of *Astragalus membranacea Radix* polysaccharides on T lymphocyte transformation in the peripheral blood of chickens. Chinese Journal of Veterinary Science (in Chinese) 18(3): 269-271.

Wallach, M., N. C., Smith, C. M. D., Miller, J., Eckeri, and M. E., Rose. 1994. *Eimeria Maxima:* Elisa and western blot analyses of protective sera. Parasit. Immuniol. 16: 377-383.

Wang, D. L. 1989. Effect of *Astragali Membranacea Radix* polysaccharide on immune function of blood lymphocytes in human. Journal of China Cancer (in Chinese) 11(3):180.

Wang, J. H. 1991. Isolation of water-soluble, acid-soluble and alkali-soluble polysaccharide from fruit of *Lentinus edodes*. Edible Fungi (in Chinese) 13(3):14.

Wang, J. W. 1991. Review on immune function of Chinese herb medicine. Journal of Chinese Traditional Veterinary Medicine (in Chinese) 6: 19-21.

Wei, P., Q. Z., Li, and Y. H., Hao. 1997. Effect of *Astragalus mongholicus Bga* and *Lentinus edodes* polysaccharide on lipid peroxide (LPO) content in VMDV infected chicks. Journal of Traditional Chinese Veterinary Medicine (in Chinese) 4:3-4.

Wu, Y. H., and F., Wang. 1999. Research status on herbs' two-way immune regulating functions. Journal of Traditional Chinese Veterinary Medicine (in Chinese) 4:19-22.

Xia, E. N., and Q. H., Cheng. 1988. Isolation, analysis and bioactivities of *Tremella fuciformis* fruit body polysaccharides. Acta Mycologica Sinica (in Chinese), 7(3): 166-174.

Xie, Z. Q., and S. Q., Niu. 1996. Comprehensive Book of Natural Researces and Chinese Herrb Feed Additives. Xueyuan Science and Technology Press, Beijing.

Xue, M., and X. S., Meng. 1996. Review on research progress and prosperous of immune activities of bio-active polysaccharides. Journal of Traditional Chinese Veterinary Medicine (in Chinese) 3:15-18.

Yang, H. C., G., Lu, and G., Lu. 1998. Effect of pollen polysaccharide from corns on immunological afficacy of Newcastle disease vaccine and Egg Drop Syndrome vaccine in chickens. Journal of Chinese Veterinary Medicine (in Chinese) 24 (6): 43.

Yang, T. S., and Y. F., Wang. 1998. Cytokine technology. Pages 188-193. Immunology and Immunological Techniques (in Chinese), People's Hygiene Press. PRC

Yu, J. G. and L. Y., Zhu. 2000. The use of *Astragalus Radix* polysaccharides against Infectious Bursa Disease in chickens. Journal of Traditional Chinese Veterinary Medicine (in Chinese) 6: 3-4.

Yun, C. H., H. S., Lillehoj, J. T., Zhu, and W. G., Min. 2000. Kinetic differences in intestinal and systemic interferon-γ and antigen-specific antibodies in chickens experimentally infected with *Eimeria maxima*. Avi. Dis. 44 (2): 305-512.

Zhang, L. C., J. B., Wang, and Y. P., Sun, D. Z., Ma, M. L., Shi, and Z. H., Wang. 1998. Effect of adding Chinese herb polysaccharide preparations to a Newcastle disease inactivated vaccine on the development of the immune organs in chickens. Chinese Journal of Veterinary Science (in Chinese) 18(4): 378-381.

# 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 meant 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 heteroglucan, 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 1.** Physico-chemical properties of mushroom and herb polysaccharides (Chapters 1 and 2)

| Parameters | Poly-saccharides/ extracts[1] | Characteristics[2] | References |
|---|---|---|---|
| Physical properties | *AstP* | *AstP*-1, water-soluble glucan; *AstP*-2, water-insoluble glucan; *AstP*-3, acidic heterosaccharide; *AstP*-4, water-soluble heterosaccharide | Huang *et al.*, 1982 |
| | *LenP* | Polysaccharide-protein complex: *LenP*-1, GlcA, 24.1%; protein, 2.0%; *LenP*-2, GlcA, 34.8%; protein, 7.4%; *LenP*-3, GlcA, 40.1%; protein, 25.3% | Yang *et al.*, 1999, 2001 |
| | *TreP* | water-soluble heterosaccharide | Xia and Cheng, 1988 |
| Molecular weights (Dalton) | *AstP* | *AstP*-1, $1.23 \times 10^4$ ; *AstP*-2, $3.46 \times 10^4$; *AstP*-3, $3.63 \times 10^4$; | Qi, 1987 |
| | *LenP* | *LenP*-1, $9.54 \times 10^5$; *LenP*-2, $9.0 \times 10^4$; *LenP*-3, $1.4 \times 10^4$ | Yang *et al.*, 1999, 2001 |
| | *TreP* | $1.15 \times 10^5$ | Xia and Cheng, 1988 |
| Sugar composition | *AstP* | *AstP*-1, Glu; *AstP*-2, Glu; *AstP*-3, HexA: Glu: Rha: Ara = 1: 0.04: 0.02: 0.01; *AstP*-4, Glu: Ara = 1: 0.15 | Huang *et al.*, 1982 |
| | *AstE* | Glu | Chapter 2 |
| | *LenP* | *LenP*-1, Ara: Xyl: Man: Gal: Glu = 0.39: 0.46: 1.0: 0.93: 14.1; *LenP*-2, Ara: Xyl: Man: Gal: Glu = 0.19: 0.41: 1.0: 0.93: 10.7; *LenP*-3, Ara: Xyl: Man: Gal: Glu = 0.31: 0.47: 1.0: 1.15: 8.9 | Yang *et al.*, 1999, 2001 |
| | *LenE* | Gal: Glu: Xyl = 0.8: 8.8: 0.5. | Chapter 2 |
| | *TreP* | L-Fru: L-Ara: D-Xyl: D-Man: D-Glu: GlcA = 0.92: 0.49: 0.18: 1.0: 1.15: 0.57 | Xia and Cheng, 1988 |
| | *TreE* | Fru: Gal: Glu: Man: Xyl: GlcA = 0.8: 0.2: 3.0: 1.6: 4.5: 0.2 | Chapter 2 |

[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 = xylose; 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 ($NH_3$) 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 $NH_3$ 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 $NH_3$. 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 *TreE*). 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 $NH_3$ 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.

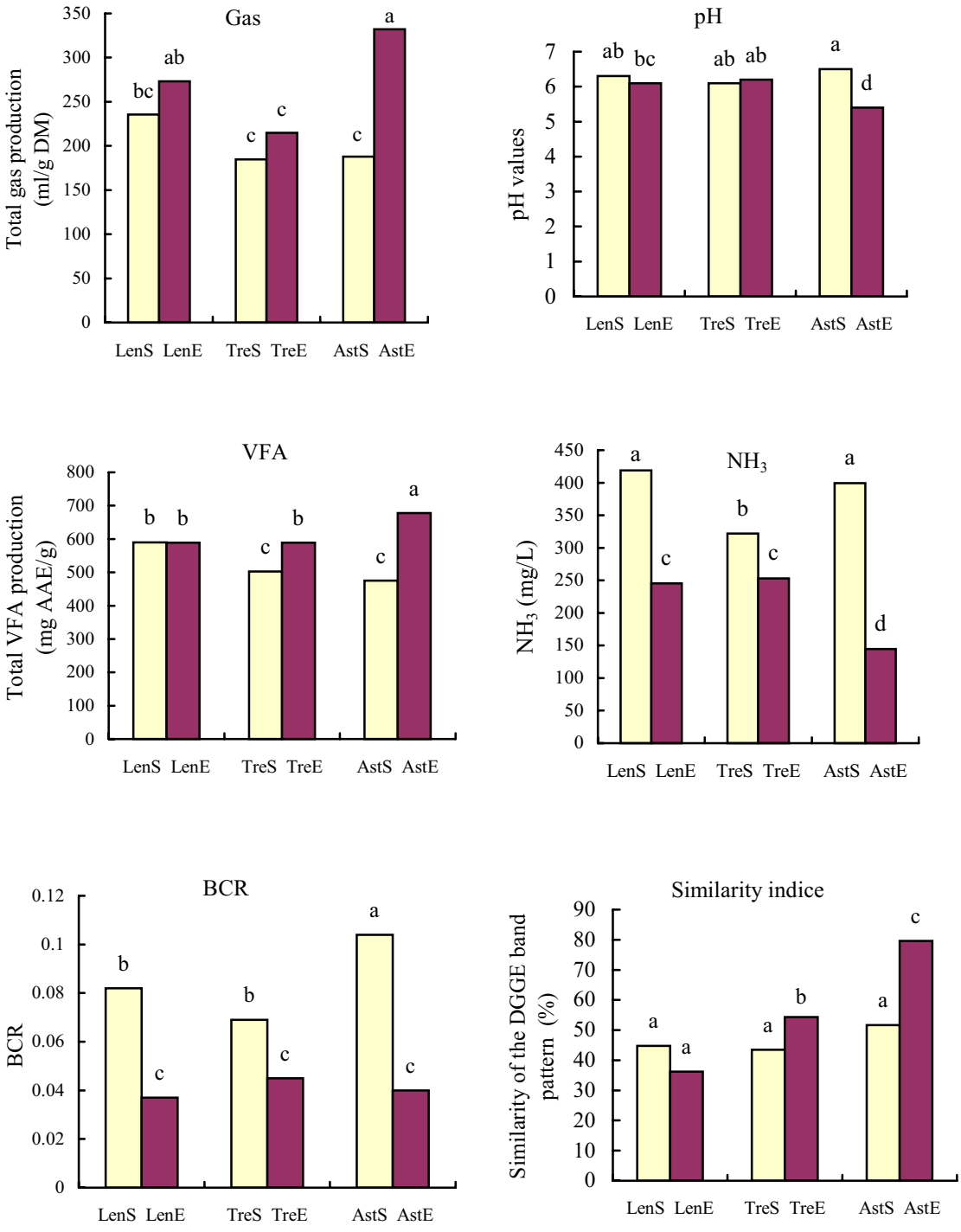



**Figure 1.** Fermentation characteristics of mushroom and herb materials (*LenS = Lentinus edodes; TreS = Tremella fuciformis; AstS = Astragalus membranaceus*) and their polysaccharide extracts (*LenE = Lentinus edodes* extract*; TreE = Tremella fuciformis* extract*; AstE= Astragalus membranaceus* extract).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

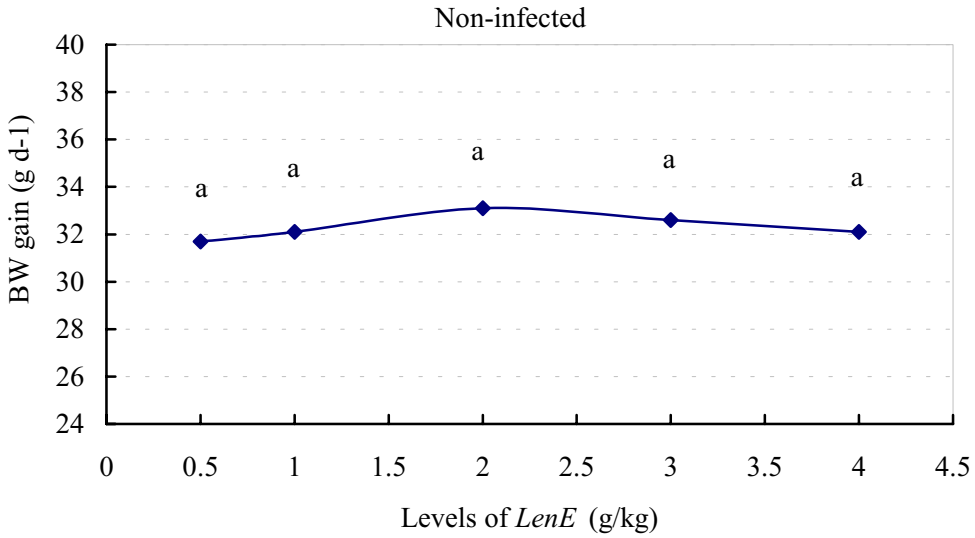
Growth response of the birds under non-infected and infected conditions showed that hardly any difference in BW gain between different levels of *LenE* was detected in non-infected birds as increased *LenE* dose (A), however, a significant growth response was found in AMG infected birds as increased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




B (Chapter 6)

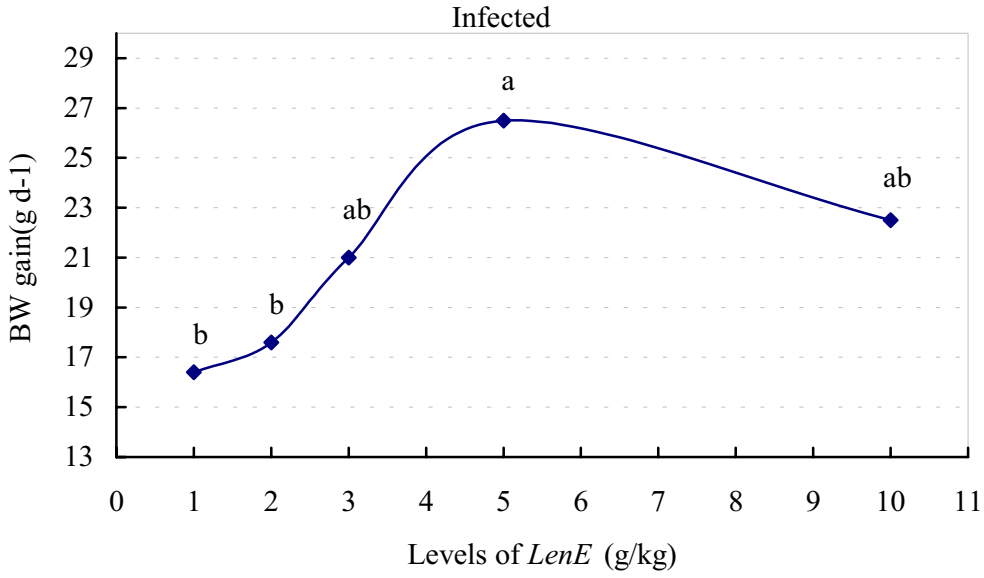
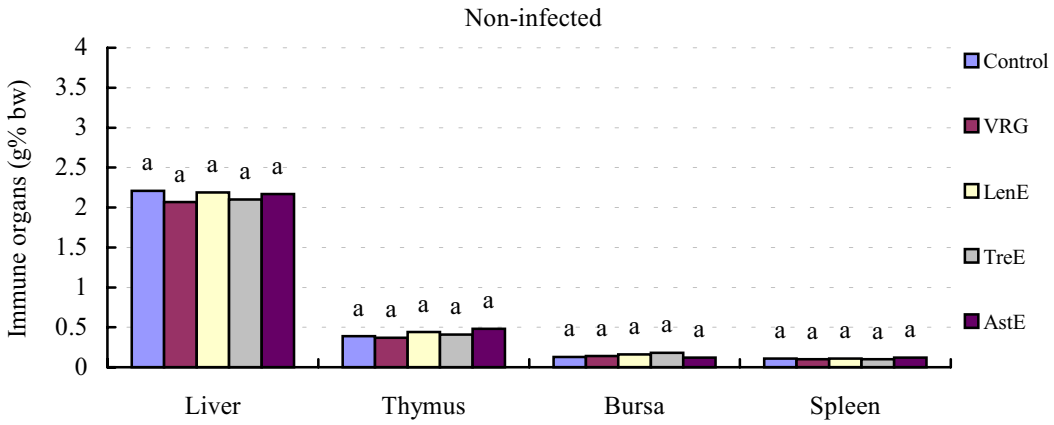



**Figure 2.** Growth response of non-infected (A) and infected (*Avian mycoplasma Gallisepticum*) (B) chickens fed with different levels of *Lentinus edodes* polysaccharide extract (*LenE*). [abc]: Means between levels with no common superscript differ significantly (P<0.05).

### *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)




B (Chapter 7)

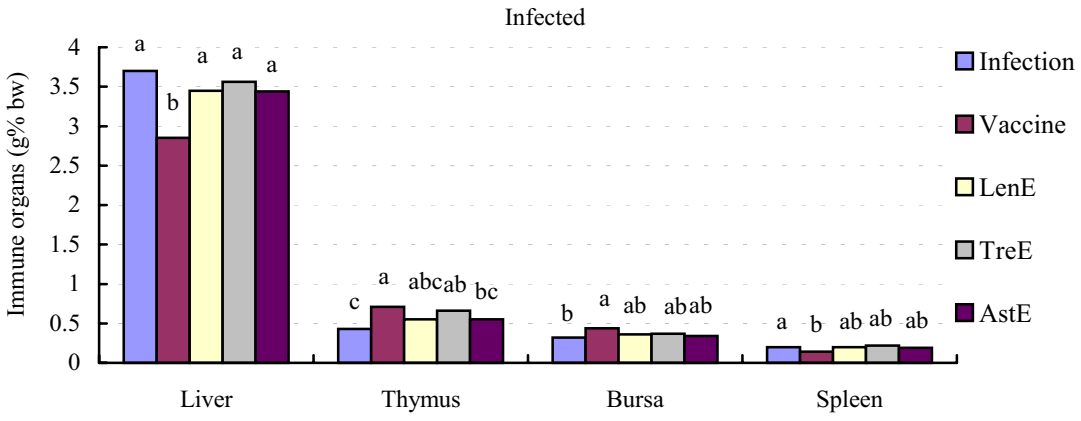
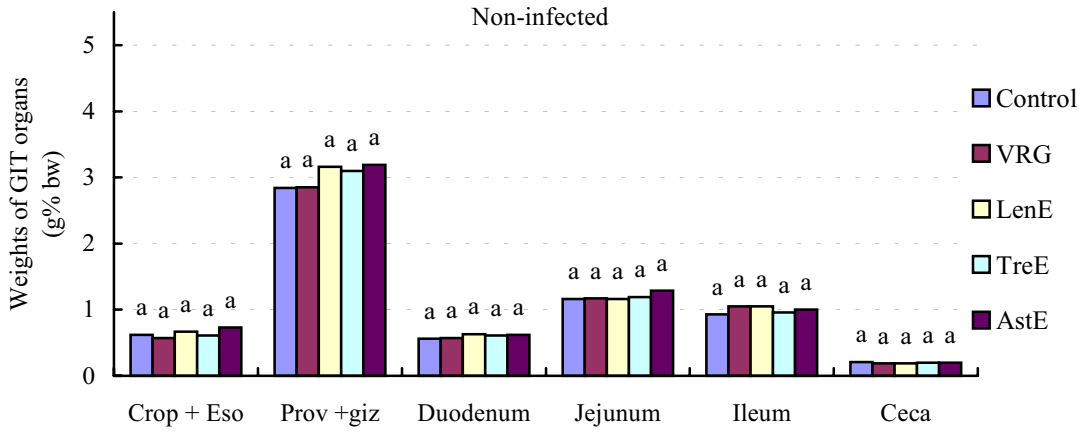



**Figure 3.** Growth of immune organs in non-infected (A) or *E. tenella* infected (B) chickens fed with the polysaccharide extracts (*LenE = Lentinus edodes* extract*; TreE = Tremella fuciformis* extract*; AstE= Astragalus membranaceus* extract). [abc]: Means within organs between different treatments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

A (Chapter 5)




B (Chapter 7)

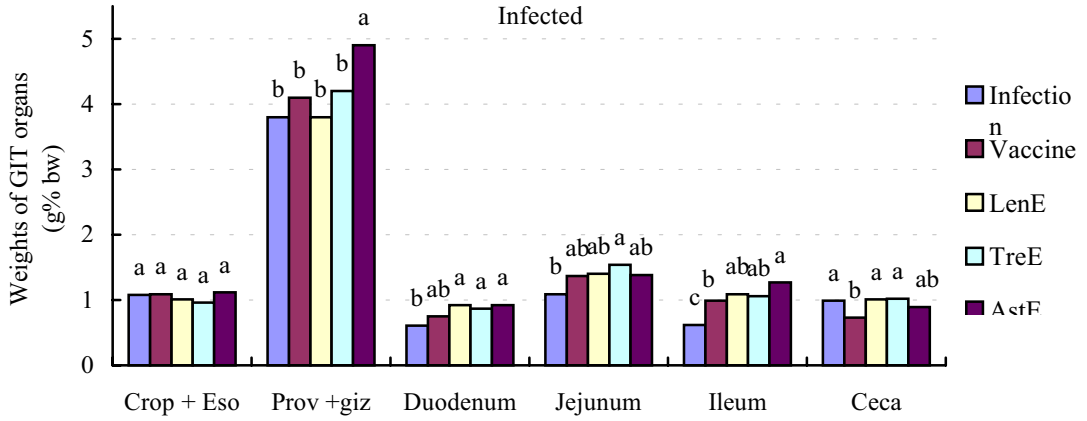



**Figure 4.** Growth of immune organs in non-infected (A) or *E. tenella* infected (B) chickens fed with the polysaccharide extracts (*LenE = Lentinus edodes* extract*; TreE = Tremella fuciformis* extract*; AstE= Astragalus membranaceus* extract). [abc]: Means within organs between different treatments 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.

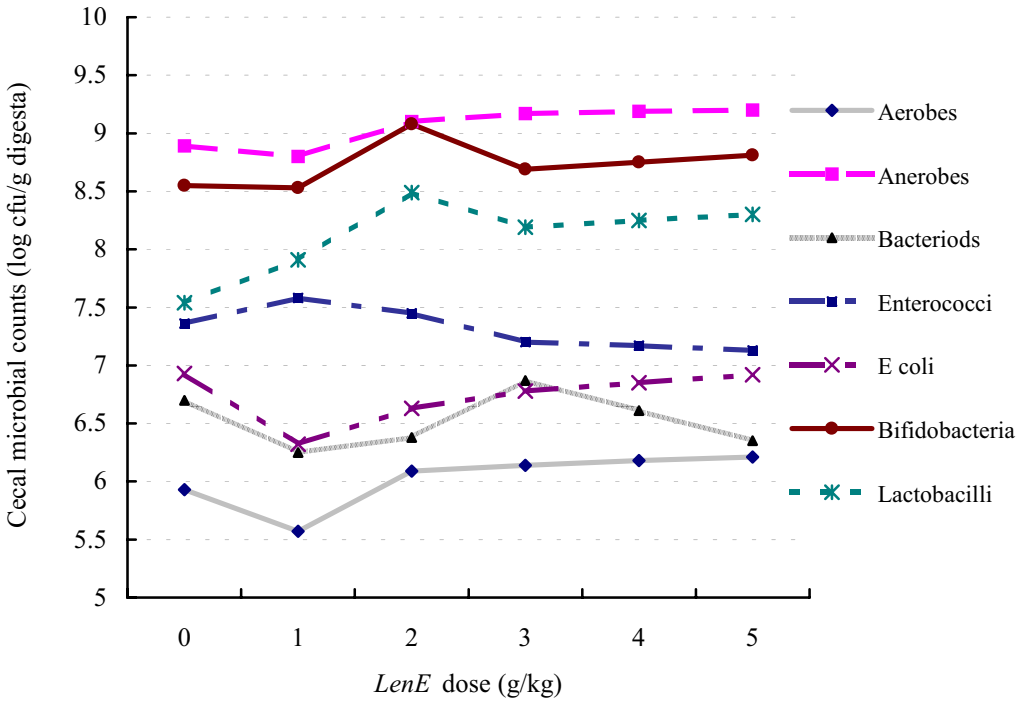



**Figure 5.** Cecal microbial counts in chickens one-week after feeding different levels of *Lentinus edodes* polysaccharide extract (*LenE*).

### *Immune Responses*

The immune status of the host is known to play an important role in its ability to resistant 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).

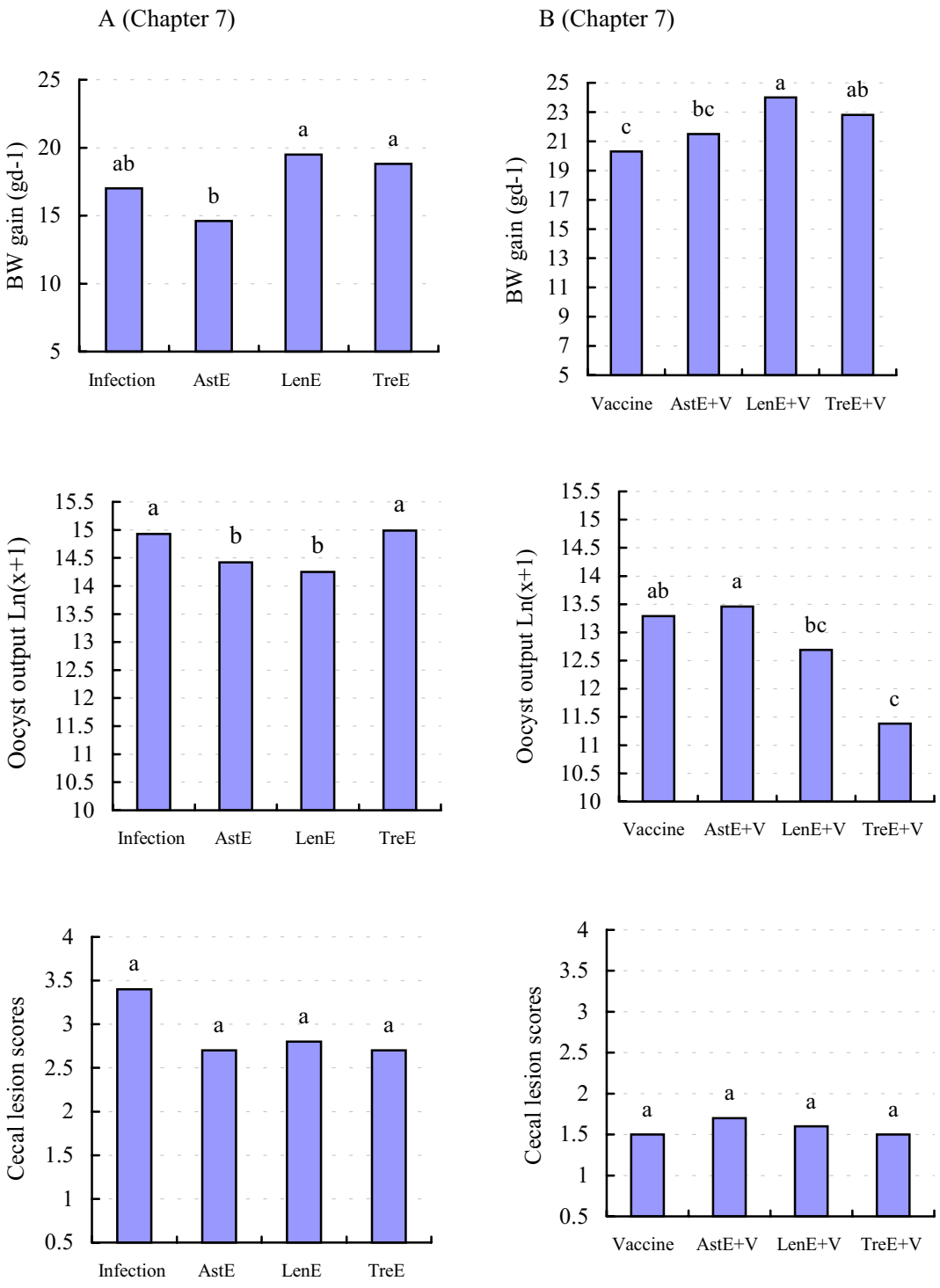



**Figure 6.** Growth and immune response of *E. tenella* infected chickens fed with the polysaccharide extracts (A) (*LenE = Lentinus edodes* extract*; TreE = Tremella fuciformis* extract*; AstE= Astragalus membranaceus* extract) or the extract plus vaccine treatments (B) (*LenE*+V = *Lentinus edodes* 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).

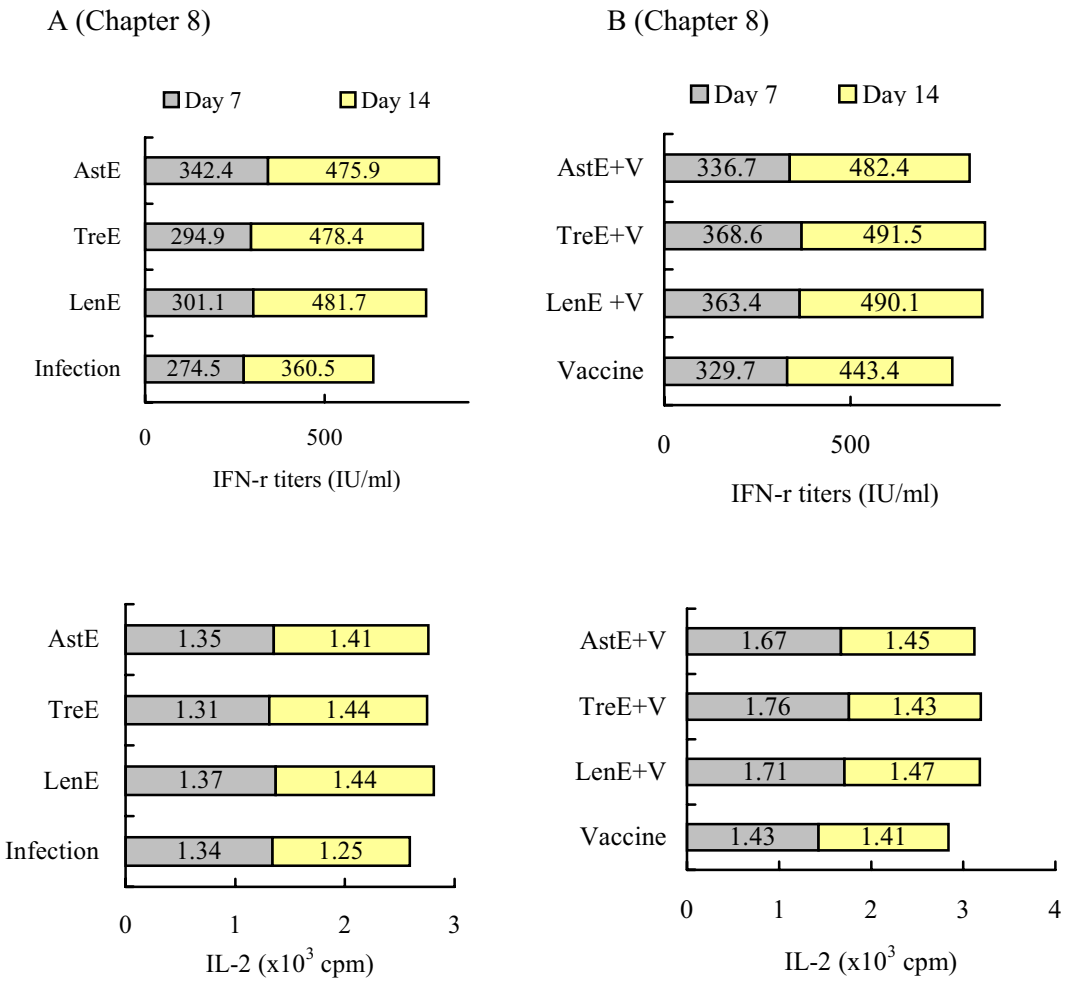



**Figure 7.** Cytokines IFN-γ and IL-2 secretion of *E. tenella* infected chickens fed with the polysaccharide extracts (*LenE = Lentinus edodes* extract*; TreE = Tremella fuciformis* extract*; AstE= Astragalus membranaceus* extract) (A) or the extract plus vaccine treatments (B) (*LenE*+V = *Lentinus edodes* extract plus vaccine; *TreE*+V = *Tremella fuciformis* extract plus vaccine; *AstE*+V = *Astragalus membranaceus* extract plus vaccine). [abc]: Means within different treatments with no common superscript differ significantly ($P<0.05$).

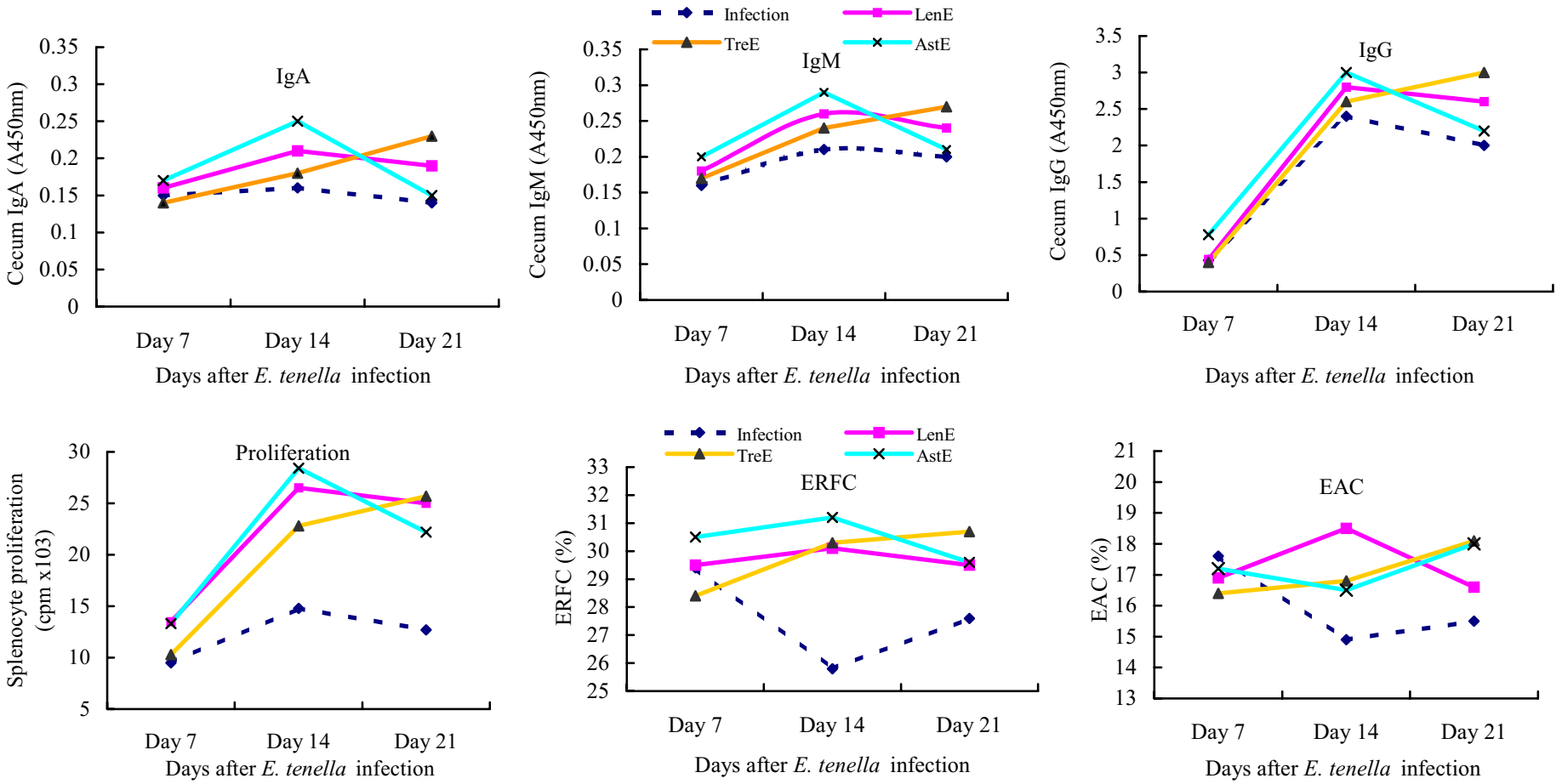



**Figure 8.** Immuno-modulation of the polysaccharide extracts (*LenE = Lentinus edodes* extract*; TreE = Tremella fuciformis* extract*; AstE= Astragalus membranaceus* extract) in chickens infected with *E. tenella*. Cecum samples were diluted for specific IgA (1: 2), IgM (1: 1) and IgG (1: 8). A 450 = absorbance (A) at 450 nm; ERFC = erythrocyte rosette forming cells; EAC = erythrocyte-antibody-complemental 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 cocciodial 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 coccidian-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)

| | China | World | China contribution (%) |
|---|---|---|---|
| Poultry production in 2002 | | | |
| Number of chickens ($x10^9$) | 3.92 | 15.42 | 25 |
| Total meat production (mt) | 67.32 | 242.63 | 28 |
| Poultry meat production (mt) | 13.52 | 72.24 | 19 |
| Poultry meat/total meat (%) | 20 | 30 | |
| 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)

*Chickens 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 7days) 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

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

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 promoters in animals. Researchers should identify these components and study their efficacy.

## REFERENCES

Andreason, M. 2000. Consequences of the ceased used of growth promoters presented at DVHS 2000, 4th and 5th May 2000, Denmark.

Banfield, M. J., R. P. Kwakkel, M. Groeneveld, R. A. Ten Doeschate, and J. M. Forbes. 1999. Effects of whole wheat substitution in broiler diets and viscosity on a coccidial infection in broilers. Br. Poult. Sci. 40 (Suppl): S58-9.

Breed, D.G. J., J., Dorresten, T. P. M., Shetters, L.V. D., Waart, E., Rijke and A. N., Vermeulen. 1997. Peripheral blood lymphocytes from Eimeria tenella infected chickens produce gamma-interferon after stimulation *in vitro*. Parasite Immunology Oxford, 19(3): 127-135.

Breves G., L., Szentkuti, and B., Schroeder. 2001. Effects of oligosaccharides on functional parameters of the intestinal tract of growing pigs. DTW Deutsche Tieraerztliche Wochenschrift, 108(6): 246-248.

Brown, R. H. 1996. The living gut must be considered when trying to eliminate pathogens. Feedstuffs, 68:14.

China International Fair on Traditional Chinese Medicine and Natural herbal medicine, 2003. http://tcm.expo.net.cn

Ensminger, M. E., J. E., Oldfield and W. W., Heinemann. 1990. Feeds and Nutrition, The Ensminger publishing company, Clovis, California, USA, 1544pp.

FAOSTAT. 2003. FAO Statistical Databases. http://apps.fao.org/.

Gao, Q. P., M. K., Killie, H.C., Chen, R. Z., Jiang, R., Seljelid, Q. P., Gao, H. C., Chen, and R Z., Jiang. 1997. Characterization and cytokine-stimulating activities of acidic heteroglycans from Tremella fuciformis. Planta Medica, 63(5): 457-460.

Gardiner, K. R., P. J. Erwin, N. H. Anderson, J. G. Barr, M. I. Halliday, and B. J. Rowlands. 1993. Colonic bacteria and bacterial translocation in experimental colitis. Br. J. Surg. 80:512-516.

Gibson, G. R., and M. B., Roberfroid, 1995. Dietary modulation of the human colonic microflora: introducing the concept of prebiotics. J. Nutr. 125:1401.

Girard, F., G., Fort, P., Yvoré, and P., Quéré. 1997. Kinetics of specific IgA, IgM and IgG production in the duodenal and caecal mucosa of chickens infected with E. Acervulina and E. tenella. Inter. J. Parasit. 27(7): 803-809.

Grizard, D., and C., Barthomeuf. 1999. Non-digestible oligosaccharides used as prebiotic agents: Mode of production and beneficial effects on animal and human health. Reprod. Nutri. Develop. 39(5-6):563-588.

Hu, T. J., B. T., Fanf, and Q. Z., Huang. 1995. Effect of 8301 polysaccharide on spleen IL-2 production in rats. Chinese J of Vet. Science and Technology (in Chinese), 25(1): 28.

Hu, T. J., J. L., Liang, and F. S., Cheng. 1998. The use of 8301 polysaccharide as an adjuvant in coccidial infected chickens. J. of Grass and Livestock (in Chinese), 6: 5-7.

Huang, N. L. 1982. Cultivation of Tremella fuciformis in Fujian, China. Mushroom Newsl. Trop. (in Chinese), 2(3): 2-5.

Huang, Q. S., G. B., Lu, Y. C., Li, and J. H., Guo. 1982. Study on the polysaccharides of "Huang Qi" (Astragalus mongholicus Bung). Acta Pharma. Sinica (in Chinese), 17(3): 200-206.

Hughes, R. J., M., Choct, A., Kocher, and R. J., Van Barneveld. 2000. Effect of food enzymes on AME and composition of digesta from broiler chickens fed on diets containing non-starch polysaccharides isolated from lupin kernel. Bri. Poult. Sci. 41(3): 318-323.

Jensen, B. B. 1993. The possibility of manipulating the microbial acting in the digestive tract of monogastric animals. 44th Annual Meeting of the European Association for Animal Production Session IV. pp. 16-19.

Korakli, M., M. G., Gaenzle, and R. F., Vogel. 2002. Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye, and exopolysaccharides produced by Lactobacillus sanfranciscensis. J. Appl. Microbiol. 92(5):958-965.

Li, C. M. 1998. Chinese Herb Medicine Feed Additives (in Chinese), pp. 11-235, Beijing: Chinese Agricultural University press.

Li, G. X., E. P., Lillehoj, and H. S., Lillehoj. 2002. Interleukin-2 production in SC and TK chickens infected with Eimeria tenella. Avi. Dis. 46 (1): 2-9.

Liang, J. L., T. J., Hu, and B. T., Fan. 1995. Experiment of 8301 polysaccharide inducing IFN production in rats. J. of Traditional Chinese Vet. Medicine (in Chinese), 4: 12-13.

Lillehoj, H. S. 1998. Role of T lymphocytes and cytokines in coccidiosis. Inter. J. Parasitol. 28: 1071-1081.

Lillehoj, H. S., S. Y., Kang, L., Keller, and M., Sevoian. 1989. Eimeria tenella and E. Acervulina:

lymphokines secreted by an avian T cell lymphoma or by sporozoite-stimulated lymphocytes protect chickens against avian coccidiosis. Exp. Parasitol. 69: 54-64.

Liu, M., J. Z., Li, F. Z., Kong, J. Y., Lin, and Y., Gao. 1998. Induction of immunomodulating cytokines by a new polysaccharide-peptide complex from culture mycelia of Lentinus edodes. Immunopharmacology, 40(3): 187-198.

Long, P. L. 1984. Coccidiosis Control: Past, Present and Future. British Poultry Science, 25: 3-18.

Ma, X. Q., Q., Shi, J. A., Duan, T. T., Dong, and K. W., Tsim. 2002. Chemical analysis of Astragalus Radix (Huangqi) in China: a comparison with its adulterants and seasonal variations. J Agric Food Chem. 50(17): 4861-6.

Macfarlane, G. T., and J. H., Cummings. 1991. The colonic flora, fermentation and large bowel digestive function. In: The Large Intestine: physiology, Pathophysiology and Disease (Phillips, S. F., Pemberton J. H. and Shorter R.G., eds.), pp. 51-92. Raven Press, New York, NY.

Martin, A., H. S., Lillehoj, B., Kaspers, and D., Bacon. 1994. Mitogen-induced lymphocyte proliferation and interferon production induced by coccdia infection. Avi. Dis. 38: 262-268.

Martin, A., H. S., Lillehoj, B., Kaspers, and L. D., Bacon. 1993. Antigen-specific T cell proliferation following coccdia infection. Poult. Sci. 72: 2084-2094.

Miles, R. O., D. M., Janky, and R. H., Harms. 1984. Virginiamycin and broiler performance. Poult. Sci. 63:1218-1221.

Min, S. D. 1996. Medicinal value of fungi. Acta Edulis Fungi. 3(4): 55-64.

Miyamoto, T., W., Min, and H. S., Lillehoj. 2002. Kinetics of interleukin-2 production in chickens infected with Eimeria tenella. Comparative Immunology Microbiology and Infectious Diseases, 25(3): 149-158.

Newman, K. E. 1995. Mannan-oligosaccharides: natural polymers with siginificant impact on the gastrointestinal microflora and immune system. In: Biotechnology in the feed industry. Proc. Alltech's Tenth Annual Symposium. T. P. Lyons and K. A. Jaques(Eds):pp. 167-174. Nottingham University Press, Nottingham.

Ottenhoff, T. H., and T., Mutis. 1995. Role of cytoxic cells in the protective immunity against and immunopathology of intracellular infections. European Journal of Clinic Investigation, 25: 371-377.

Pang, F., Y. X., Feng, and D. Y., Zhang. 1995. Review on current research of Astragalus mongholicus Bga. World Phytomedicines, 10(3): 110-115.

Peterson, R. A., G. H., Carpenter, W. T., Jones, and J. A., kla, 1991. Effect of tetracycline hydrochloride and oxytetrocycline hydrochloride given via drinking water on early mortality of broiler chicks from twenty-eight-week-old dams. Poult. Sci. 70(4):1040-1042.

Qi, Z. S. 1987. Review on chemical study of Astragalus membranacea Radix. Chinese Traditional and Herbal Drugs (in Chinese), 18(5): 41-29.

Rana N., and Tikaram, S. M. 2001a. In-vivo assessment of drug resistance in field isolates of *Eimeria tenella*. Indian Journal of Animal Science, 71(7): 627-630.

Rana N., and Tikaram, S. M. 2001b. Performance index based evaluation of anticoccidials against field strains of *Eimeria tenella* in broiler chickens. Indian Journal of Animal Science, 71(7): 623-626.

Rolfe, R. D. 1991. Population dynamics of the intestinal tract. In: Colonization control of human enteropathogens in poultry (Blankenship, L.C., ed.) Academic Press; Inc. San Diego, CA, pp. 59-75.

Rosen, G. D. 1995. Antibacterals in poultry and pig nutrition. In: Boitechnology in animal feeds and animal feeding. R. J. Wallance and A. Chesson (Eds): 143-172. VCH, Weinhiem.

Smith, H. W. 1965. The development of the flora of the alimentary tract in young animals. J. Pathol. Bacteriol. 90: 495-513.

Suo, X. and G. Q., Li. 1998. Coccidia and Coccidiosis of Domestic Fowl (in Chinese). Chinese Agriculture University Press. Beijing.

Wallach, M., N. C., Smith, C. M. D., Miller, J., Eckeri, and M. E., Rose. 1994. Eimeria Maxima: Elisa and western blot analyses of protective sera. Parasit. Immuniol. 16: 377-383.

Williams, B. A., S. Tamminga, and M. W. A. Verstegen. 2000. Fermentation kinetics to assess microbial activity of gastro-intestinal microflora. Pages 97-100 in An EAAP Satellite Symposium on Gas Production: Fermentation Kinetics for Feed Evaluation and to Assess Microbial Activity. British Society of Animal Science. Aug. 18-19. Wageningen, Netherlands.

Williams, R. B. 1999. A compartmentalised model for the estimation of the cost of coccidiosis to the world's chicken production industry. Inter. J. Parasitol. 29(8): 1209-1229.

Xia, E. N. and Q. H., Cheng. 1988. Isolation, analysis and bioactivities of Tremella fuciformis fruit body polysaccharides. Acta Mycologica Sinica, 7(3): 166-174.

Yang, J., M. C., Wu, and S. H., Zhang. 1999. Study on isolation, purification and characteristics of polysaccharide Le-III from the fruit body of Lentinus edodes. J. of Huazhong Agricultural University (in Chinese with English summary), 18(2):188-190.

Yang, J., M. C., Wu, and S. H., Zhang. 2001. Isolation, purification and characterization of polysaccharides from the fruit body of Lentinus edodes (Berk.) J. of Plant resources and Environment (in Chinese with English summary), 10 (2): 4-6.

Yun, C. H., H. S., Lillehoj, J. T., Zhu, and W. G., Min. 2000. Kinetic differences in intestinal and systemic interferon-γ and antigen-specific antibodies in chickens experimentally infected with

Eimeria maxima. Avi. Dis. 44 (2): 305-512.

Zhao, R. H., H. Y., Zhang, and T., Tiao. 1995. The resources and stock of the common use Chinese herb medicine in China. J. of Chinese Medicine (in Chinese), 20(12): 712.

Zheng, Z., D., Liu, C., Song, C., Cheng, and Z., Hu. 1998. Studies on chemical constituents and immunological function activity of hairy root of Astragalus membranaceus Radix. Chin J Biotechnol. (in Chinese with English summary), 14(2): 93-7.

# 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 coccdial 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 voerbespaarder (‘groeibevorderaar’). Het gebruik van deze middelen veroorzaakt echter een imbalans in de maagdarmflora en er kunnen resistente 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 verstrekkende 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) gedissecteerd 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: 3.5: 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 verschilden 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 microbieële populatie, daarbij gebruikmakend van microflora afkomstig uit de blinde darmen van kuikens 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 microbieële 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 *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 *TreS* de laagste indexwaarde en *AstS* de hoogste. Voor de extracten gold een variatie tussen de 23 en 80%. Hierbinnen vertoonde *LenE* de laagste indexwaarde en *AstE* de hoogste. De 'similarity index' van de DGGE bandpatronen was duidelijk lager bij *LenE* dan bij *LenS*, vergeleken met het inoculum en de blanco's, en hoger bij *AstE* dan bij *AstS*. Er was geen verschil in index tussen *TreS* en *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 *in vitro* verschillen ook aantoonbaar zouden zijn in een *in vivo* experiment werd in een groeiproef 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 *LenE*-gesupplementeerde kuikens lieten een betere groei en vc zien dan *TreE*- en *AstE*-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 *Aviaire Mycoplasma Gallisepticum* (AMG) en verdeeld over 9 proefgroepen: *LenE* supplementatie in doseringen van 1, 2, 3, 5 en 10 g/kg, *TreE* en *AstE* 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 èn 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* geinfecteerde 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保正动物的健康生长，特别是对仔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、益生素和其它饲料添加剂已成为该领域的研究热点。

在初试中，我门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+胰脏+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 添加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 对肝脏和肠道各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份（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。

根据中文文献，来自食用菌 - 香菇 *Tremella fuciformis* (*TreS*) 和银耳 *Lentinus edodes* (*LenS*)，以及植物药 - 黄芪 *Astragalus membranacea Radix* (*AstS*) 的免疫活性多糖似乎是很有潜力的抗生素促生长剂替代品。食用菌和植物药类多糖 （作为益生素）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的作用。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环境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况。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*）。用酚-硫酸法测定多糖提取物的总糖含量。用 HPAEC 测定多糖的单糖组份。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（$NH_3$）的产量。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 $NH_3$ 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品相比，*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)；安普霉素组（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数量。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 $9x10^4$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记分高于疫苗组，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（十二直肠、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+嗉囊、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 肉鸡分为 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，多糖提取物组的特异性 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统和免疫系统的作用机制。另外，阐述了中药多糖在家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 化性质如蛋白含量、总糖含量和单糖组份。
2. 离体发酵试验结果表明，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

Fucun Guo was born on 14th of June, 1964 in Lanzhou, Gansu province, P. R. China. She obtained her BSc degree in Veterinary Medicine in July 1986 at Gansu Agricultural University. After graduation, she was employed by the Institute of Traditional Chinese Veterinary Medicine, Chinese Academy of Agricultural Sciences. She worked as a researcher in the fields of Traditional Herbal Medicine and Animal Nutrition and Health. In 1997, she got a fellowship from Netherlands Fellowship Program (NFP) and enrolled as an Msc student of Wageningen University. She obtained her Master's degree in Animal Nutrition in Jan.1999. After graduation, she continued her work in Institute of Traditional Chinese Veterinary Medicine, Chinese Academy of Agricultural Sciences. From 2000, she started her PhD study in Animal Nutrition Group of Wageningen University. During this period, she also worked as a senior researcher at Key Laboratory of Veterinary Pharmaceutics of China Agricultural Ministry in Institute of Animal Science and Veterinary Pharmaceutics, Chinese Academy of Agricultural Sciences.

Cover design: Wankun Li
Printing: Grafisch Service Centrum Van Gils B.V., Wageningen, The Netherlands

This study was financially supported partly by Alltech Inc, USA, International Foundation for Science (IFS) and Orffa Netherlands Feed B.V.. Thanks for their financial support, which gave me the opportunity to carry out the experiments.