Synergy of Astragalus polysaccharides and probiotics (Lactobacillus and Bacillus cereus) on immunity and intestinal microbiota in chicks

S. P. Li,*† X. J. Zhao,*1 and J. Y. Wang‡

*College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi, China, 712100; †College of Chinese Traditional Veterinary Medicine, Hebei Agriculture University, Dingzhou, Hebei, China, 073000; and ‡College of Science, Northwest A&F University, Yangling, Shaanxi, China, 712100

ABSTRACT Probiotics and Astragalus polysaccharides (APS) can modulate systemic immunity and intestinal microbiota in animals and human beings. It is still unknown if the combined application of probiotics and APS in feed has synergistic effects on immunity and intestinal microbiota. To address this issue, this study was designed to investigate the synergistic effects on immunity and intestinal microbiota in chicks. A total of 240 female Hy-Line chicks were assigned to 4 treatments. Four treatments were fed the same corn-soy meal control diet; however, treatments 2, 3, and 4 were supplemented (per kg of feed) with 220 mg of APS, 4 × 10¹⁰ cfu probiotics, and dual treatment, respectively. Parameters evaluated included serum Newcastle disease antibody titer, peripheral blood acid α-naphthyl acetate esterase-positive (ANAE+) T-lymphocyte percentage, immune organ relative weights, histological changes, and selected intestinal tract bacteria. Compared with the control, Newcastle disease antibody titer, ANAE+ T-lymphocyte percentage, immune organ relative weights, histological changes, as well as lactobacilli and Bacillus cereus numbers significantly increased (P ≤ 0.05); however, the Escherichia coli numbers in chicks of the treatments decreased (P ≤ 0.05). Interestingly, there were significant differences in ANAE+ T-lymphocyte percentage of dual treatments compared with APS treatment (P ≤ 0.05), and also in spleen index, bursa of Fabricius index, immune organ histological changes, and lactobacilli, Bacillus cereus, and E.coli numbers compared with the APS or probiotics treatments (P ≤ 0.05). The results showed that probiotics combined with APS administration in feed displayed synergistic modulation effects on immunity and intestinal microbiota, which is very important for the exploration of new prebiotics.

Key words: Astragalus polysaccharide, probiotic, immunity, intestinal microbiota, chick

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INTRODUCTION

Probiotics are defined as “living organisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition” (Lilly and Stillwell, 1965). Over the last 2 decades, probiotics (direct-fed microbial), including lactobacilli and Bacillus cereus cultures, have been used extensively. Probiotics have been proposed to exert their beneficial effects by maintaining a normal intestinal milieu, by stimulating the immune system, detoxifying colonic contents, lowering serum cholesterol concentrations, promoting lactose tolerance, and producing metabolites that are essential to maintain intestinal health (Lilly and Stillwell, 1965; Hooper et al., 2001; Salma et al., 2007; Willis et al., 2007; Walter et al., 2008).

Polysaccharides from natural sources are a class of macromolecules that can profoundly affect the immune system, and therefore have the potential as immunomodulators with wide clinical applications (Tzianabos, 2000). For example, the roots of Astragalus membranaceus (Huangqi) are among the most popular health-promoting herbs in China, and their use can be traced back more than 2,000 yr and were recorded in Shen Nong’s Materia Medica written in the Han Dynasty. Scientific investigation in the last 2 decades has revealed much insight into the pharmacological functions of different components of Huangqi, especially its polysaccharide fractions (Cui et al., 2003; Kim et al., 2008). It is well documented that polysaccharides derived from A. membranaceus, which have been used as immune enhancers (Chen et al., 2003; Qiu et al., 2007), also show antibacterial (Guo et al., 2004b), antiviral
Table 1. Ingredients and chemical composition of the basal pretreatment and treatment diets

<table>
<thead>
<tr>
<th>Item</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients (%)</td>
<td></td>
</tr>
<tr>
<td>Corn</td>
<td>66</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>23.8</td>
</tr>
<tr>
<td>Colesseed meal</td>
<td>3</td>
</tr>
<tr>
<td>Fish meal</td>
<td>1.1</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>2.3</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.6</td>
</tr>
<tr>
<td>CaHPO4·2H2O</td>
<td>1.85</td>
</tr>
<tr>
<td>Salt</td>
<td>0.3</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.03</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>0.02</td>
</tr>
<tr>
<td>Premix2</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
</tr>
</tbody>
</table>

Calculated chemical analysis

| ME3 (MJ/kg) | 12.15 |
| CP3 (%)     | 18.25 |
| L-Lysine (%)| 0.32  |
| Methionine (%)| 0.87 |
| Calcium (%) | 0.81  |
| Total phosphorus (%)| 0.70 |

1The basal pretreatment and treatment diets were the same. Treatment diets were basal diet supplemented (per kg of feed) with 220 mg of Astragalus polysaccharides, 4 × 10^10 cfu probiotics, or dual treatment.
2Premix provided per kilogram of diet: vitamin A, 1,500 IU; vitamin D3, 200 IU; vitamin E, 10 IU; vitamin K, 0.5 mg; vitamin B1, 1.8 mg; vitamin B2, 3.6 mg; vitamin B6, 3.0 mg; vitamin B12, 0.009 mg; biotin, 0.15 mg; folic acid, 0.55 mg; pantothenic acid, 10.0 mg; niacin, 27 mg; choline (as choline chloride), 1,300 mg; iron (FeSO4·H2O), 80 mg; manganese (MnSO4·7H2O), 60 mg; zinc (ZnSO4·7H2O), 40 mg; copper (CuSO4·5H2O), 8 mg; iodine (KI), 0.35 mg; selenium (Na2SeO3), 0.15 mg.
3Analyzed composition.

Prebiotics are defined as nondigestible food ingredients that beneficially affect the host by selective stimulation of growth or activity of beneficial bacterial species in the colon, thus benefiting host health (Gibson and Robedorf, 1995). Carbohydrates, especially oligosaccharides and polysaccharides, have been used as prebiotics to influence the composition of the bacterial populations in the large intestine of several animal species (Baurhoo et al., 2007; Biggs et al., 2007; Canibe et al., 2007; Liu et al., 2007). Therefore, it has been hypothesized that some herb polysaccharides hold some promise as potential modifiers of the intestinal microbiota (Guo et al., 2004b) and thus interact with probiotics. This study was designed to test the interaction between probiotics and Astragalus polysaccharides (APS) on modulation of immunity and selected intestinal bacteria.

MATERIALS AND METHODS

Birds and Experimental Design

Two hundred forty 1-d-old female Hy-Line chicks were obtained from a local commercial hatchery. Subsequently, healthy birds were randomly allotted to 4 treatments based on average BW with 6 replicate pens per treatment and 10 chickens per pen. Four treatments were fed a same corn-soy meal control diet (Table 1), but treatments 2, 3, and 4 were supplemented (per kg of feed) with 220 mg of APS, 4 × 10^10 cfu probiotics, and dual treatment, respectively. All diets were formulated to meet the NRC (1994) nutrient requirements. The experiment was concluded when the birds were 6 wk of age. At d 7 of age, the chicks were vaccinated by ocular route with 0.2 mL of Newcastle disease (ND) live vaccine (China Animal Husbandry Industry Co. Ltd, Beijing, China).

APS Preparation

*Astragalus membranaceus* was purchased from local sources and produced in Xilingol (Inner Mongolia, China). *Astragalus* was dried overnight at 45°C and ground through a 1-mm sieve for APS, a water-soluble polysaccharide extraction. *Astragalus* polysaccharide was isolated from boiling water extract of *Astragalus*, followed by ethanol precipitation, dialysis, and protein depletion using the Sevag method (Staub, 1965). The product was a hazel-colored powder, and the yield of the polysaccharide fractions is 36.12% (wt/wt) following the phenol-sulfuric acid method reported previously (Ashwell, 1966). *Astragalus* polysaccharide was mixed with the bird diet separately according to the experimental design mentioned above. The supplement concentration was 220 mg/kg of the diet, which was considered the optimum concentration for enhanced health by a preliminary experiment.

Probiotics Preparation

The probiotic product (Xi’an CangHai Biologic Engineering Co. Ltd., Xi’an, China) comprised probiotic bacteria isolated from the cecum (*Lactobacillus*) of healthy adult chickens and soil (*B. cereus*). The product had a total bacterial count, expressed as colony-forming units of 1 × 10^12 cfu/kg of product.

Samples

On 21 and 42 d of age, 6 birds from each treatment (1 bird from each replicated pen) were randomly selected and transferred to a germ-free room and held without feed with water available ad libitum for 8 h. Blood samples were collected from the chicks by cardiac puncture and directly aliquoted into 2-mL sterile vials, using liquid sodium heparin (1,000 US pharmacopeia units/mL, ZaoZhuang HuaBao Pharmacy Co. Ltd., Shanghai, China) as an anticoagulant [to obtain the whole blood for α-naphthyl acetate esterase-positive (ANAE+) T-lymphocyte percentage test], or were allowed to clot for 4 h. After centrifugation (3,000 × g × 10 min), the serum was aliquoted into 1-mL vials and stored at −20°C for ND antibody titers determined by means of a microhemagglutination inhibition test (Fu and Liu, 1997). Then chicks were killed by cervical dislocation in a germ-free isolation chamber sterilized by
UV radiation. The cecum and ileum were removed from each bird, and the fresh excreta were gently squeezed and carefully collected in sterilized 5-mL sterile vials for enumeration of microbial populations. In addition, spleen, bursa of Fabricius, and thymus were weighed quickly for immune organ relative weights and then collected in the 10% (vol/vol) formalin for routine paraffin section.

**Enumeration of Peripheral Blood T Lymphocytes**

Peripheral blood T-lymphocyte counts were determined by the histochemical demonstration of ANAE on blood films according to the method of Lin (1999). The lymphocytes with reddish-brown reaction products were regarded as being ANAE⁺.

**Microbial Populations Enumeration**

Fresh ileac and cecal samples (0.5 g) were diluted with 9.5 mL of sterilized distilled water and vortexed until a pH of 6.0 was obtained. One gram of wet sample was diluted with 10 mL of distilled water, of which 1 mL was transferred into 9 mL of sterilized distilled water. Samples were serially diluted from 10⁻¹ to 10⁻⁷. One-tenth milliliter of each diluted sample was coated 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 log₁₀ per gram of fresh sample. The bacterial groups and species determined included lactobacilli (LBS agar), *Escherichia coli* (MacConkey agar), and bifidobacteria (bifidobacterium agar composed of tomato juice, 400 mL; dissoluble amyllum, 0.5 g; peptone, 15 g; yeast extract, 2 g; glucose, 20 g; sodium chloride, 5 g; Tween-80, 1 mL; 5% cysteine, 0.5 mL; liver extract, 80 mL; agar powder, 20 g; and distilled water, 520 mL; pH = 7.0) incubated at 37°C for 72 h.

**Statistical Analysis**

All results were presented as means. Experimental data were analyzed using the SPSS for Windows statistical package program, version 8.0.0 (SPSS Inc., Chicago, IL). Comparisons of the means were performed using Duncan's multiple range test. Significance was defined as a P-value of ≤0.05%.

**RESULTS**

**Serum ND Antibody Titer**

The treatments significantly increased (P ≤ 0.05) ND antibody titer during the experiment (Figure 1). Although not significant, the dual treatments were numerically greater (7.3 ± 0.8 and 6.3 ± 0.8, respectively) on d 21 and 42, respectively, than those of the APS treatments (6.8 ± 0.8 and 7.0 ± 0.6, respectively) or probiotics treatments (5.7 ± 0.5 and 5.5 ± 0.5, respectively).

**Peripheral Blood ANAE⁺ T-Lymphocyte Percentages**

The ANAE⁺ T-lymphocyte percentages of the treatments were all significantly greater (P ≤ 0.05) than that of the control except APS treatment on 21 d (Figure 2). In Figure 2, we also found that ANAE⁺ T-lymphocyte percentage of dual treatment (34.1 ± 1.7 and 37.4 ± 1.5) at d 21 and 42, respectively, was numerically greater than the probiotic treatment and significantly greater than the APS treatment (P ≤ 0.05).

**Immune Organ Relative Weights and Histological Change**

As illustrated in Table 2, all immune organ relative weights of dual treatment were significantly greater (P ≤ 0.05) than that of the control, even than that of APS or probiotics treatment except for thymus index. In addition, immune organ relative weights of APS and probiotics treatments were significantly increased (P ≤ 0.05) when compared with the control group during the whole experiment except spleen index on 42 d and bursa of Fabricius index on 21 d.

Histological changes were observed in immune organs collected from the chicks (Figure 3). There was an increase in area expansion in the thymus and thymus corpuscles at d 42. In the bursa of Fabricius, lymph follicle area expansion in the plica and lymphocyte numbers increased in dual treatment. Additionally, compared with the control, there were clear changes in white
pulp area and splenic lymphonoduli in the treatments. Splenic corpuscle numbers were increased in the dual treatment.

**Bacterial Enumeration**

In this study, conventional microbiological techniques using selective agar media were used to analyze the composition of ileum and cecum digesta samples. Lactobacilli, bifidobacteria, and *E. coli* counts of intestine are presented in Table 3. Compared with the control, lactobacilli and bifidobacteria numbers of the treatments (except the APS vs. control for lactobacilli on d 42) significantly increased (*P* ≤ 0.05), but *E. coli* numbers significantly decreased in ileum and cecum (*P* ≤ 0.05). The bacteria numbers of the dual treatment were significantly different when compared with the probiotics and APS treatment (*P* ≤ 0.05), with the exception being lactobacilli at d 21 and *E. coli* on d 21 and 42 in the ileum when the dual treatments was compared with the probiotic treatment.

**DISCUSSION**

**Immunostimulation**

In the present study, ND antibody titers and ANAE⁺ T-lymphocyte percentage were significantly improved in the treatments compared with the controls. The ND antibody titer is used as an indicator of specific humoral immunity. The ANAE⁺ T-lymphocyte percentage is, in fact, the T-lymphocyte percentage, and thus is used as an indicator of cellular immunity (Matutes and Catovsky, 1982). Therefore, the results indicate improvement of humoral and cellular immunity in chicks while feeding with probiotics, APS, and dual diet. The beneficial effects of probiotics treatment on immunity are in agreement with a large number of other researchers using probiotics in broilers (Haghighi et al., 2006; Xu and Li, 2007). However, little work has been done on APS. Chen et al. (2003) reported that an APS diet had no effect on ND microhemagglutination inhibition titers in broilers. This is not consistent with the present study and may be a result of the concentrations of APS used and differences in the rearing environments.

Interestingly, the dual treatment showed a greater numerical effect on humoral and cellular immunity than the probiotics and APS treatments. However, only a few immunologic parameters were studied, and the differences were not significant (*P* ≥ 0.05).

There were significant increases in thymus, bursa of Fabricius, and spleen relative weights in the dual-fed treatment compared with the probiotics and APS treatments with the exception of the thymus. These data suggest that synergism between the probiotics and APS enhanced the development of immune organs. This could also be seen by histological changes. In the

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**Table 2. Effects of different supplement on immune organ relative weights of chicks**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spleen relative weights</th>
<th>Thymus relative weights</th>
<th>Bursa of Fabricius relative weights</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21 d</td>
<td>42 d</td>
<td>21 d</td>
</tr>
<tr>
<td>Control</td>
<td>1.36</td>
<td>1.17</td>
<td>3.49</td>
</tr>
<tr>
<td>APS</td>
<td>1.64</td>
<td>1.25</td>
<td>4.74</td>
</tr>
<tr>
<td>Probiotics</td>
<td>1.66</td>
<td>1.39</td>
<td>4.75</td>
</tr>
<tr>
<td>Probiotics + APS</td>
<td>1.94</td>
<td>1.83</td>
<td>5.19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Contrasts</th>
<th>Probability level of the contrasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS vs. control</td>
<td>0.041* 0.264 0.015* 0.023* 0.134 0.034*</td>
</tr>
<tr>
<td>Probiotics vs. control</td>
<td>0.035* 0.187 0.013* 0.018* 0.095 0.038*</td>
</tr>
<tr>
<td>Probiotics + APS vs. control</td>
<td>0.014* 0.029* 0.008* 0.022* 0.041* 0.009*</td>
</tr>
<tr>
<td>Probiotics + APS vs. APS</td>
<td>0.038* 0.039* 0.195 0.694 0.045* 0.042*</td>
</tr>
<tr>
<td>Probiotics + APS vs. probiotics</td>
<td>0.040* 0.048* 0.182 0.784 0.047* 0.046*</td>
</tr>
</tbody>
</table>

1APS = *Astragalus* polysaccharide.

2The results are given as means (n = 6, 1 bird per replicate) for all treatments.

3The results are calculated as immune organ weight (mg) per BW (kg).

*P* ≤ 0.05.

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**Figure 2. Effects of different treatments on peripheral blood acid α-naphthyl acetate esterase (ANAE)-positive T-lymphocyte percentages.** Bars represent means for the 6 replicates (pens) per treatment ± SD. Within the same day, bars with different letters (a, b, c) differ significantly (*P* ≤ 0.05). APS = *Astragalus* polysaccharide.
present study, histological changes of bursa of Fabricius and spleen were very pronounced, such as lymph follicle area increases in bursa of Fabricius and white pulp area expansion. Because lymph follicle of the bursa of Fabricius and white pulp of spleen are both major sites of lymphocytes, the changes suggest that the dual treatment elicited lymphocytes formation in bursa of Fabricius and spleen.

Although there were no significant difference between dual treatment and probiotics or APS treatment on immunity, histological changes of immune organs suggested a synergism between APS and probiotics enriched with lactobacilli and bifidobacteria.

**Predominant Intestinal Microbiota**

Intestinal microbiota plays an important role in the health status of host animals and is the first barrier against pathogens from food. In general, intestinal bacteria may be divided into species that exert either beneficial (such as lactobacilli and bifidobacteria) or harmful (such as *E. coli*) effects on host health (Macfariane

![Figure 3](image-url). Histological changes in immune organs (hematoxylin and eosin-stained section, A, B viewed at 400× magnification, and C, D, E, F viewed at 100× magnification). A. Control, arrow indicates thymus corpuscle; B. dual treatment (probiotics combined with *Astragalus* polysaccharide), arrow indicates thymus corpuscle, visible expansion; C. control, arrows indicate lymph follicle in plica of bursa of Fabricius; D. dual treatment, arrows indicate lymph follicle, visible area expansion in lymph follicle, and amount increases in lymphocyte; E. control, arrow indicates white pulp; F. dual treatment, arrows indicate splenic corpuscle, visible amount increases in splenic corpuscle.
and Cummings, 1991). Therefore, a common approach to maintain host health is to increase the number of desirable bacteria to inhibit colonization of invading pathogens (Rolfe, 1991). In this study, a snapshot profile of ileum and cecum microbiota composition was generated.

The probiotic product, APS, and dual treatment resulted in a beneficial modulation of the microbiota, as evidenced by the significant ($P \leq 0.05$) increases in the concentrations of beneficial bacteria numbers (lactobacilli and bifidobacteria) and decreases in the concentrations of harmful bacteria numbers ($E. coli$). Although some studies lack positive effects (Priyankarage et al., 2003), the probiotic products were always beneficial to promote the balance of intestinal microbiota (Chichlowski et al., 2006; Shen et al., 2006; Matsuzaki et al., 2007). It is difficult to directly assess different studies using probiotics, because the efficacy of a probiotic application depends on many factors (Ewing and Cole, 1994; Patterson and Burkholder, 2003) such as species composition and viability, administration level, application method, frequency of application, bird age, environmental stress factors, and so on. However, data are still scarce on APS. To the best of our knowledge, similar research was only carried out by Guo et al. (2004b), who administrated APS and other polysaccharides in chickens infected with avian Mycoplasma gallisepticum, and found that these polysaccharides significantly increased numbers of bifidobacteria and lactobacilli and decreased $E. coli$ numbers. Our results are similar to their reports. Therefore, APS and other polysaccharides hold promise as potential modifiers of intestinal microbiota in chicks.

In addition, a surprising finding was that, in general, the beneficial changes in the microbiota of the dual treatment were significantly different when compared with the APS or probiotics treatment ($P \leq 0.05$). This supports a linkage between APS and probiotics, and suggests that the enhanced effect on intestinal microbiota of APS may result from its role as a prebiotic.

The mechanism by which APS improved the role of probiotics is uncertain. Overall, the results in this work showed that probiotics combined with APS in feed displayed synergistic modulation effects on immunity and intestinal microbiota. It would seem that APS is a potential alternative to the use of probiotics possibly acting as prebiotics. However, further study is needed to elucidate the mechanism (i.e., how APS affects the balance of intestinal microbiota and improves the effects of probiotics).

**ACKNOWLEDGMENTS**

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**REFERENCES**