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*Research Article*

# Synergistic Impact of *Lactobacillus plantarum* and *Bacillus coagulans* on Solid-State Fermentation of *Astragalus* and Effects of Fermentation Products on Disease Resistance of Crucian Carp (*Carassius auratus*)

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Dietary supplementation with fermentation products of *Astragalus* can enhance the immune function of aquaculture animals. We explored the synergistic effects of *Lactobacillus plantarum* and *Bacillus coagulans* on the solid-state fermentation of *Astragalus* to investigate whether feeding fermentation products to crucian carp can improve disease resistance. The optimal ratio of *Astragalus* + (*L. plantarum* + *B. coagulans*) (ALB), temperature, and number was 3 : 3, 37°C, and $1.0 \times 10^8$ CFU/g, respectively. After 48 h mixed fermentation, the number of probiotics increased to $2.2 \times 10^9$ CFU/g, pH decreased to 3.2, and high molecular weight proteins disintegrated into small molecular weight or soluble proteins. In addition, several holes appeared on the *Astragalus* surface. Compared with unfermented *Astragalus*, the content of *Astragalus* polysaccharide and calycosin increased by 3.82- and 1.31-fold, respectively, on fermentation and that of total *Astragalus* saponins decreased by 0.77-fold. Furthermore, on Cyprinid herpesvirus 2 (CyHV-2) and *Aeromonas veronii* (*A. veronii*) challenges, the cumulative survival rates of crucian carp fed common feed and ALB were 80.00% and 65.00%, respectively. Overall, feeding mixed fermentation products to crucian carp positively impacted their health and disease resistance. We believe that our results provide theoretical guidance for developing effective plant-based agents to prevent diseases in crucian carp.

## 1. Introduction

*Astragalus* is one of the largest and most diverse genera in the family Leguminosae. *Astragalus* spp. is widely used in traditional Chinese medicine. They are sweet in taste and slightly warm in nature, and they affect both the spleen and lung meridians [1]. Several studies have reported that *Astragalus* exerts various pharmacological effects, such as improving immune function [2], protecting the kidneys, and regulating antiviral responses [3]. The main active ingredients of *Astragalus* include, for example, *Astragalus* polysaccharide (APS) and total *Astragalus* saponins [4]. APS exerts antiviral [5], antistress, and antioxidant effects [6], all of which can evidently enhance immunity [7, 8].

The popularity of plant-derived antibacterial products has witnessed a rapid growth considering that they facilitate the healthy and sustainable development of aquaculture [9]. At present, *Lactobacillus* and *Bacillus* represent the most commonly used probiotics in aquaculture [10]. Dietary supplementation with *L. plantarum* has been observed to improve immunity, disease resistance, and survival in aquatic animals [11, 12]. Furthermore, feeding *B. coagulans*

to aquatic animals has been found to improve their growth, immune response, and antioxidant capability [10, 13]. Using the combination of probiotics and traditional Chinese medicines to achieve intestinal microecological balance has received much attention from the aquaculture industry [14–16]. Microorganisms with strong decomposition and transformation abilities ferment traditional Chinese medicines, resulting in the abundant production of secondary metabolites. The reaction conditions of this process are mild and characterized by high efficiency, low toxicity, and low residue [17–19]. To our knowledge, solid-state fermentation of *Astragalus* by *L. plantarum* and *B. coagulans* remains to be reported. In this study, we explored the synergistic effects of *L. plantarum* and *B. coagulans* on the solid-state fermentation of *Astragalus*, which served as the medicinal substrate. We investigated changes in APS, total *Astragalus* saponins, and calycosin during fermentation and also verified the effects of fermentation products on disease resistance in crucian carp (*Carassius auratus*). Our findings provide theoretical guidance for developing plant-based agents to prevent major diseases in crucian carp.

## 2. Materials and Methods

*2.1. Traditional Chinese Medicine, Bacteria, and Experimental Fish.* *Astragalus* (production batch no. 20210702) was purchased from Tongrentang Pharmaceutical Co., Ltd. (Beijing, China) and crushed through a 100-mesh sieve. *B. coagulans* (YFI-NJ2, CCTCC: M 2021313) and *L. plantarum* (YFI-7, CCTCC: M 2019656) were isolated from the intestines of healthy crucian carp (i.e., experimental fish) by the Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences [20]. Experimental fish (weight, $29 \pm 1$ g) were acquired from the Wuhan Academy of Agricultural Sciences. During the experiment, aquaculture water was maintained at $25^{\circ}C \pm 1^{\circ}C$, pH of $7.8 \pm 0.2$, and dissolved oxygen level of $6.5 \pm 0.5$ mg/L. Crucian carp were given standard commercial pellet feed (protein ≥28%, fat ≥3%, water ≤12%, and ash content ≤18%; particle size = 2 mm) twice a day (2% body weight each time).

*2.2. Strain Activation and Morphological Observation.* Lyophilized *L. plantarum* and *B. coagulans*, stored in glycerin at −80°C, were quickly thawed and inoculated in sterilized de Man–Rogosa–Sharpe (MRS; HopeBio, Qingdao, China) and brain heart infusion (BHI, Difco, Detroit, MI, USA) broths. *L. plantarum* and *B. coagulans* were cultured at 37°C with agitation (180 rpm) for 24 h. Subsequently, 5% of this suspension was inoculated into sterilized MRS and BHI broths. On a thermostatic culture for 24 h, the concentration was adjusted to $1.0 \times 10^8$ CFU/mL. A part of this bacterial suspension was dried and fixed on a glass slide for Gram staining (Jiancheng, Nanjing, China) [17], while the other part was fixed in 2.5% glutaraldehyde, rinsed with phosphate-buffered saline, dehydrated through an ethanol gradient, freeze-dried, sprayed with gold [21], and observed under a scanning electron microscope (SEM) (Hitachi, Tokyo, Japan).

*2.3. Experimental Grouping and Fermentation Conditions.* This study included the following experimental groups: *Astragalus*, *L. plantarum*, *B. coagulans*, *Astragalus* + *L. plantarum*, *Astragalus* + *B. coagulans*, *Astragalus* + (*B. coagulans* + *L. plantarum*) in a ratio of 4 : 2, *Astragalus* + (*B. coagulans* + *L. plantarum*) in a ratio of 3 : 3, and *Astragalus* + (*B. coagulans* + *L. plantarum*) in a ratio of 2 : 4. After 48 h fermentation in an incubator maintained at 37°C, the number of probiotics in the solid-state fermentation medium of each group was detected to identify the optimal ratio of *Astragalus* and probiotics.

*2.4. Morphological Observation of Astragalus.* After fermentation for 48 h, 200 μg of fermentation product was fixed in 2.5% glutaraldehyde at 4°C for >6 h. Subsequently, the sample was rinsed with phosphate-buffered saline, dehydrated through an ethanol and tert-butanol gradient, freeze-dried, sprayed with gold, and photographed under a SEM.

*2.5. pH Changes in Astragalus Fermentation.* Our preliminary results demonstrated the effects of fermentation on *Astragalus*, *L. plantarum*, and *B. coagulans*. *L. plantarum* + *Astragalus*, *B. coagulans* + *Astragalus*, *Astragalus* + (*B. coagulans* + *L. plantarum*) in a ratio of 4 : 2, and *Astragalus* + (*B. coagulans* + *L. plantarum*) in a ratio of 3 : 3 were used to continue fermentation. Bacterial suspensions were transferred into separate 15 mL centrifuge tubes, sealed, and fermented at 37°C for 7 days. Fermentation product (200 μg) was extracted from each group at 0 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, and 168 h, and then mixed with 0.2 mL of water. Subsequently, pH was measured using an acidometer (Mettler–Toledo, China).

*2.6. SDS-PAGE.* Unfermented *Astragalus*, *L. plantarum* + *Astragalus*, *B. coagulans* + *Astragalus*, and *Astragalus* + (*B. coagulans* + *L. plantarum*) in a ratio of 3 : 3 were used to continue fermentation for 1 week. Fermentation product (0.2 g) was frozen overnight in liquid nitrogen and ground, proteins were then extracted and protein concentration was measured using a BCA protein assay kit (Biyuntian, Shanghai, China) every day. And 50 μg of protein was boiled for 10 minutes, run for 1.5 h at 80–100 V, adsorbed with Coomassie brilliant blue for 1 h and decolorized, and then, gels were analyzed by a gel imaging system (Bio-Rad, Hercules, CA, USA). A predyed protein marker (10–180 kDa) (Boshide, Wuhan, China) was used in this experiment.

*2.7. Determination of Active Ingredients in Astragalus Fermentation.* Unfermented *Astragalus*, *L. plantarum* + *Astragalus*, *B. coagulans* + *Astragalus*, and *Astragalus* + (*B. coagulans* + *L. plantarum*) in a ratio of 3 : 3 were used to continue fermentation for 48 h. Fermentation products were then dried in an oven at 60°C, and the content of APS, total *Astragalus* saponins, and calycosin was determined [22–24].

*2.8. Challenge Test.* Two hundred crucian carp were randomly divided into five groups (40 fish/group). After feeding for four weeks, the five groups were divided into three subgroups: in the first subgroup, the fin of each experimental fish was intraperitoneally injected with 0.2 mL *Aeromonas veronii* ($1.0 \times 10^7$ CFU/mL), in the second subgroup, each experimental fish was intraperitoneally injected with 0.2 mL of viral suspension of Cyprinid herpesvirus 2 ($1.0 \times 10^6$ copies/mL), and in the third subgroup, experimental fish were intraperitoneally injected with phosphate-buffered saline (i.e., the control). The number of dead crucian carp was recorded every 24 h for 14 consecutive days. Diseased fish were collected, and then, pathogen detection and identification were performed.

*2.9. Statistical Analysis.* Values represent mean ± standard deviation. Data were analyzed with GraphPad Prism 8.0 (Version X, La Jolla, CA, USA) by one-way analysis of variance, followed by the Bonferroni test. $P < 0.05$ was considered statistically significant. All tests included at least three biological replicates.

## 3. Results

*3.1. Morphological Observation.* *L. plantarum* showed growth on MRS agar, while *B. coagulans* showed growth on BHI agar. On Gram staining, *L. plantarum* as well as *B. coagulans* cells appeared purple (Gram-positive) (Figures 1(a) and 1(b)). Optical microscopy revealed single or pairs of bacterial cells for both. The SEM showed that *L. plantarum* and *B. coagulans* cells were rod-shaped and nonflagellated (Figures 1(c) and 1(d)).

*3.2. Number of Probiotics after Fermentation.* *Astragalus* was inoculated with *L. plantarum* and *B. coagulans* at $1.0 \times 10^8$ CFU/g. *L. plantarum* and *B. coagulans* proliferation levels were different in different experimental groups. The number of *L. plantarum* decreased to $8.0 \times 10^7$ CFU/g in the group A + L, and that of *B. coagulans* increased to $1.3 \times 10^8$ CFU/g in the group A + B. Interestingly, the number of *B. coagulans* reached $1.0 \times 10^8$ CFU/g on BHI agar in 48 h, but in the group A + B, the number of *B. coagulans* reached to $1.3 \times 10^8$ CFU/g in 24 h. Among the six groups, and group A + LB (3 : 3) showed the highest number of probiotics ($2.2 \times 10^9$ CFU/g), followed by group A + LB (2 : 4) ($2.5 \times 10^8$ CFU/g), group A + B ($1.3 \times 10^8$ CFU/g), group A + L ($7.9 \times 10^7$ CFU/g), group A + LB (4 : 2) ($4.8 \times 10^7$ CFU/g), and group A (0 CFU/g) (Figure 2).

*3.3. pH Changes in Astragalus Fermentation.* The pH of *Astragalus* was 6.2–6.4 when inoculation with *L. plantarum* or *B. coagulans*. After 24 h fermentation, the pH of all groups decreased to ≤4.0, after 24 h, there was little change in pH. After 48 h of fermentation, compared with other experimental groups, the pH of group A + LB (3 : 3) was the lowest at 3.3 (Figure 3).

*3.4. Changes in Proteins at Different Fermentation times.* Proteins from different suspensions were extracted and subjected to SDS-PAGE. Protein bands of fermented suspensions were darker than those of unfermented *Astragalus* probably because *L. plantarum* and *B. coagulans* proteins were simultaneously extracted (Figure 4(a)). After 24 h fermentation, bands at 35–40 kDa showed obvious differences, in comparison with unfermented *Astragalus* and histones were completely degraded in the *L. plantarum* and mixed fermentation groups. In the case of the *B. coagulans* group, the band color became lighter (Figure 4(b)). Numerous proteins were enriched at around 25 kDa. After 48 h of fermentation, protein bands of all sizes appeared pale (Figure 4(c)).

*3.5. Morphological Observation of Astragalus.* Fermentation for different durations had different effects on the morphology of *Astragalus*. After 24 h of fermentation, a large number of bacterial cells were found to be attached to the surface of *Astragalus*. Furthermore, tiny holes were evident on the surface of *Astragalus* (Figure 5(a)). After 48 h fermentation, these holes became clearly visible, and bacteria cells were attached to them (Figure 5(b)). As evident from higher magnification, holes on the *Astragalus* surface showed different shapes and sizes, with many penetrating the surface and several showing extremely small diameters (Figure 5(c)).

*3.6. Content of Active Ingredients on Astragalus Fermentation.* After 48 h of mixed fermentation, APS content was 3.82-fold higher than that in group A. In contrast, the content of total *Astragalus* saponins in groups AL and AB was 0.36- and 0.23-fold lower than in group A, and in group ALB, it was 0.77-fold lower than in the group A. The content of calycosin was relatively lower in group AL and higher in groups AB and ALB, being 1.42- and 1.31-fold more than that in group A, respectively (Figure 6).

*3.7. CyHV-2 and A. veronii Challenge.* After feeding co-fermentation products for 28 days, crucian carp were challenged with *A. veronii* and CyHV-2. We found that their cumulative survival rate in group ALB was the highest, reaching 65.00% after an *A. veronii* challenge. After the CyHV-2 challenge, the group ALB showed the least number of deaths, with the cumulative survival rate being 80.00%. In comparison with fish in the control group, fish in the experimental groups showed a higher cumulative survival rate. On the CyHV-2 and *A. veronii* challenges, the cumulative survival rates of crucian carp fed common feed + A, common feed + AL, common feed + AB, and common

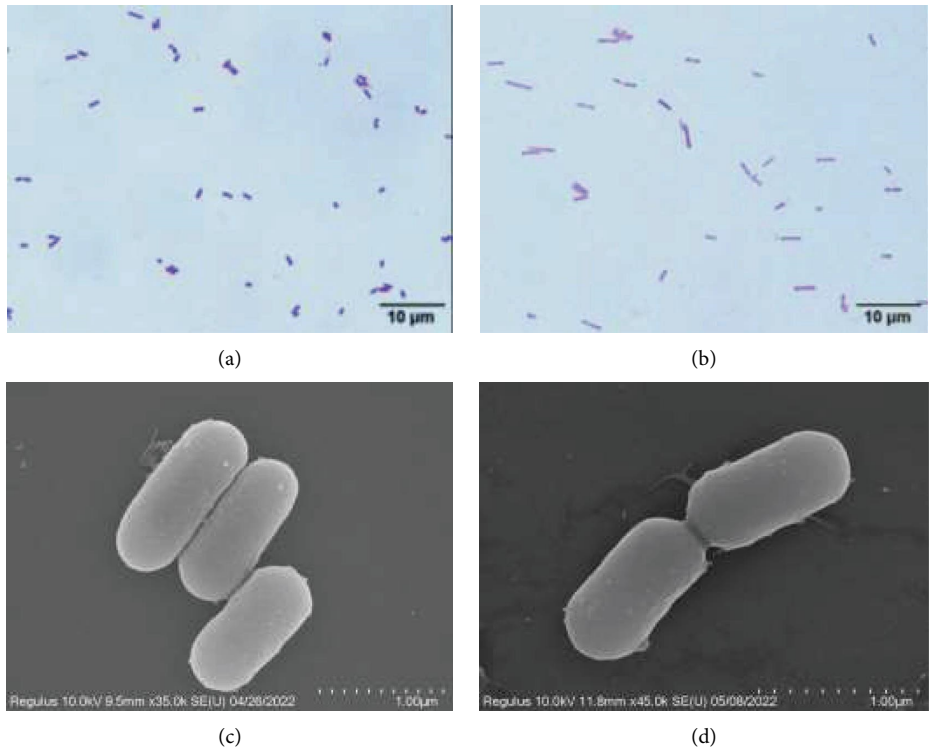


FIGURE 1: Morphological observation of *L. plantarum* and *B. coagulans*. (a) Gram staining of *L. plantarum* (scale: 10 $\mu$m). (b) Gram staining of *B. coagulans* (scale: 10 $\mu$m). (c) SEM of *L. plantarum* (scale: 1 $\mu$m). (d) SEM of *B. coagulans* (scale: 1 $\mu$m).

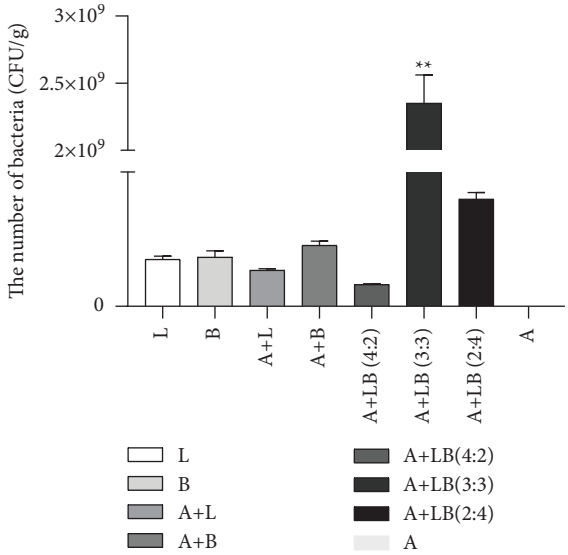


FIGURE 2: Number of viable bacteria (CFU/g) across different experimental groups. L: *L. plantarum*, B: *B. coagulans*, A + L: *Astragalus* + *L. plantarum*, A + B: *Astragalus* + *B. coagulans*, A + LB (4 : 2): *Astragalus* + (*B. coagulans* + *L. plantarum*) in a ratio of 4 : 2, A + LB (3 : 3): *Astragalus* + (*B. coagulans* + *L. plantarum*) in a ratio of 3 : 3, *A* + LB (2 : 4): *Astragalus* + (*B. coagulans* + *L. plantarum*) in a ratio of 2 : 4, and A: unfermented *Astragalus*. $^{**}P < 0.01$.

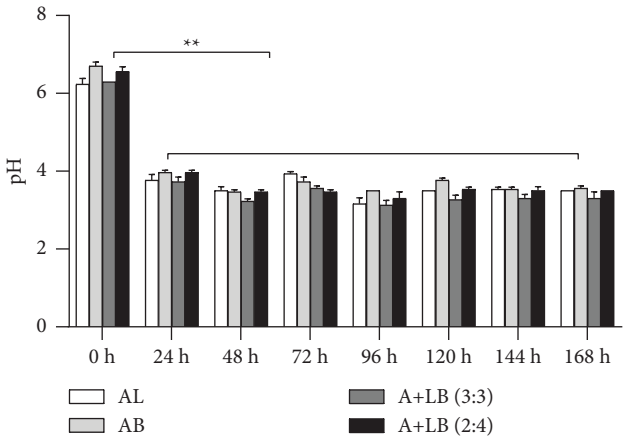


FIGURE 3: pH at different fermentation times. AL: *Astragalus* + *L. plantarum*, AB: *Astragalus* + *B. coagulans*, A + LB (3 : 3): *Astragalus* + (*B. coagulans* + *L. plantarum*) in a ratio of 3 : 3, and A + LB (2 : 4): *Astragalus* + (*B. coagulans* + *L. plantarum*) in a ratio of 2 : 4. $^{**}P < 0.01$.

feed + ALB were 55.00% and 40.00%, 65.00% and 55.00%, 60.00% and 50.00%, and 80.00% and 65.00%, respectively (Figure 7).

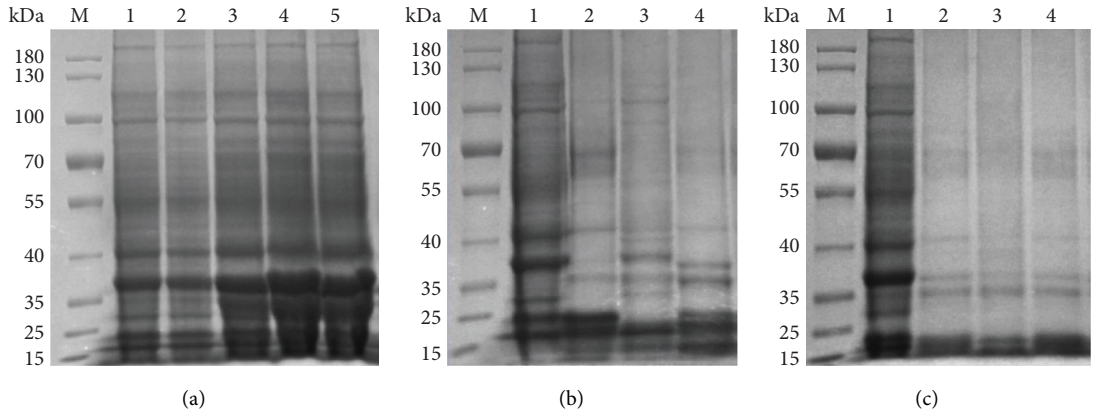


FIGURE 4: Changes in proteins before and after fermentation. (a) Before fermentation, bands 1 and 2 represent group A and bands 3, 4, and 5 represent groups AL, AB, and ALB, respectively. After fermentation for (b) 24 h and (c) 48 h, bands 1, 2, 3, and 4 represent groups A, AL, AB, and ALB, respectively.

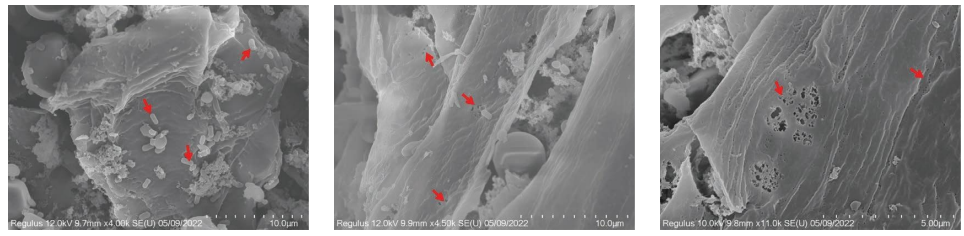


FIGURE 5: Morphological observation of *Astragalus* fermented for different durations. (a) After fermentation for 24 h probiotics (red arrows) were attached to the surface of *Astragalus*. (b) Holes (red arrows) became evident on *Astragalus* surface after fermentation for 48 h. (c) Higher magnification clearly showed these holes (red arrows) after fermentation for 48 h.

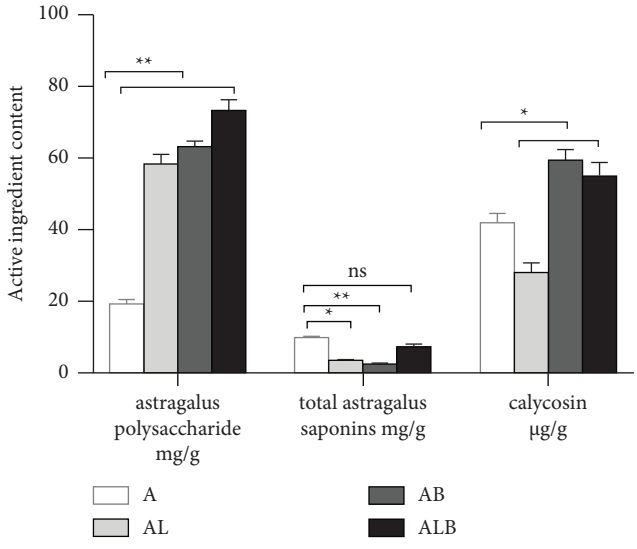


FIGURE 6: Changes in contents of active ingredients on *Astragalus* fermentation. A: unfermented *Astragalus*, AL: *Astragalus* + *L. plantarum*, AB: *Astragalus* + *B. coagulans*, and ALB: *Astragalus* + (*B. coagulans* + *L. plantarum*) in a ratio of 3 : 3. $^{*}P < 0.05$; $^{**}P < 0.01$.

## 4. Discussion

*Astragalus* can be used as a nutrient substrate to cultivate lactic acid bacteria. It has been illustrated that *Astragalus* extract promotes the growth of *Bifidobacterium* and *Bifidobacterium bifidum*, increasing their numbers to $4.59 \times 10^9$ CFU/mL and $2.67 \times 10^9$ CFU/mL, respectively [25]. Contrary to the results of previous studies, we herein found that the number of *L. plantarum* cells decreased to $8.0 \times 10^7$ CFU/g in group AL. This could be attributed to the absence of some nutrients or the high abundance of *L. plantarum*. Besides, the content of some *Astragalus* components may be too high, which may inhibit the growth of lactic acid bacteria. The number of *B. coagulans* (also known as *Weizmannia coagulans*) [26] cells increased to $1.3 \times 10^8$ CFU/g. Trace elements and polysaccharides produced during the fermentation process of *Astragalus* [27, 28] could have promoted *B. coagulans* growth. Upon mixed fermentation, the number of probiotics increased to $2.2 \times 10^9$ CFU/g. These preliminary results indicate that *Astragalus* interacts with *L. plantarum* and *B. coagulans*, further studies are warranted to elucidate the mechanism underlying this interaction.

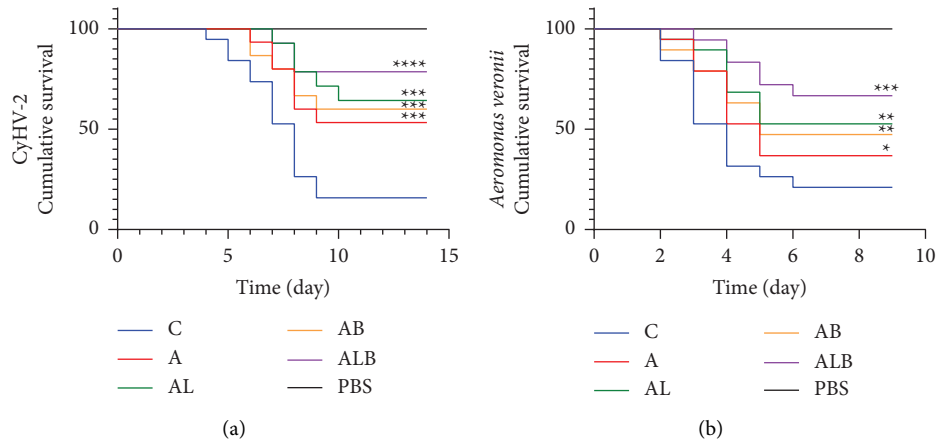


Figure 7: Cumulative survival rates of crucian carp on CyHV-2 and *A. veronii* challenge. C: common feed, A: common feed + *Astragalus*, AL: common feed + *Astragalus* + *L. plantarum*, AB: common feed + *Astragalus* + *B. coagulans*, and ALB: common feed + *Astragalus* + *L. plantarum* + *B. coagulans*.

Probiotics, in an appropriate ratio, can exert synergistic effects on fermentation substrates [29]. A single strain may offer an incomplete enzyme system, imposing certain limitations on the transformation of fermentation substrates. Thus, mixed fermentation systems comprising diverse strains are preferred [30]. In this study, *L. plantarum* and *B. coagulans* showed a good symbiotic relationship. *B. coagulans* is a facultative anaerobe, whereas *L. plantarum* is an anaerobe. *B. coagulans* consumes oxygen and provides a more suitable environment for *L. plantarum* proliferation during fermentation. An inappropriate ratio is bound to reduce the acid production rate and hinder the progress of fermentation [31, 32]. Our results demonstrated that probiotics actively proliferate when mixed in an appropriate ratio; furthermore, *L. plantarum* and *B. coagulans* exert synergistic effects.

A lower pH is favorable to fermentation and inhibits the growth of miscellaneous bacteria during the process. *Astragalus* fermentation with lactic acid bacteria produces a sour taste, which is more conducive to animal feeds [33]. Generally, lactic acid bacteria cannot utilize soluble carbohydrates in fermentation substrates, but they can utilize lactose to produce lactic acid, resulting in lower pH levels. A study observed that on fermenting *Astragalus* with *L. plantarum* and *Enterococcus faecium*, the decrease in pH to below 5.0 was significant from days 6 to 30 [33]. Another study reported that co-fermentation with *Bacillus subtilis* and *L. plantarum* lowered pH levels to around 5.0 [34]. Herein, the pH of *Astragalus* decreased to <4.0 after 24 h of fermentation, and it decreased even further within 48 h of fermentation. The rapid decrease in pH could be due to the fermentation mixture containing a high number of *L. plantarum* and *B. coagulans*, which are lactic acid bacteria. In addition, during the process of fermentation, the production of high-volume of organic acids could further reduce pH.

Amino acids are essential for normal growth and development as well as for the immune system [35]. Protein components of *Astragalus* have significant antioxidant activities. Relative to amino acids, peptides obtained by hydrolysis of proteins extracted from plants are more easily absorbed and utilized [36]. Hydrolyzed proteins or small molecular weight peptides have various physiological functions, such as antioxidation, lowering blood pressure, and enhancing immunity [37]. Soluble protein production can be enhanced by, for example, optimizing bacterial growth conditions or using a combination of different bacteria [38]. In this study, after 48 h of fermentation, the experimental group showed more prominent protein bands, while a few protein bands disappeared. The results at 72 h, 96 h, 120 h, 144 h, 168 h, and 48 h were the same. It is plausible that high molecular weight proteins were gradually degraded into small molecular weight or soluble proteins, which are more easily absorbed and utilized by animals. Our SDS-PAGE findings indicated that changes in proteins were closely related to the type and proportion of bacterial cells and the duration of fermentation.

Polysaccharides can be fermented and utilized by gut microbes as prebiotics, thereby regulating the composition of the gut microbiota and promoting human health. Fermentation of *Astragalus* with *L. plantarum* M-9 has been found to significantly increase polysaccharide levels [39]. Compared to unfermented *Astragalus*, *Astragalus* + *L. plantarum* fermentation increased APS content by 2.3-fold [33]. Furthermore, the APS content in group ALB was 3.82-fold higher than that in group A. Compared with unfermented *Astragalus* the content of APS in fermented *Astragalus* was significantly increased. Cellulase is secreted during the growth of *L. plantarum* and *B. coagulans*. A part of cellulose is degraded into soluble polysaccharides, increasing in the dissolution rate of APS. It could also be that probiotics produced some extracellular polysaccharides, resulting in an increase in the overall polysaccharide content.

Saponins possess various pharmacological activities, such as immune regulation and multiorgan protection, and exert hypoglycemic, antiviral, and antitumor effects [40]. Gilthead sea bream (*Sparus aurata*) in the grow-out phase

showed high tolerance for saponins, indicating their ability to tolerate dietary plant feedstuffs containing such antinutrients [41]. Saponins reportedly increase ATP and ADP content in the brain tissue and increase the survival rate of nerve cells [42]. In this study, we observed that the content of total *Astragalus* saponins decreased after fermentation, which could be caused by bacterial decomposition. Probiotics in the digestive tract selectively decompose saponins into aglycones and sugars. Aglycone is produced by glycosides after probiotic hydrolysis, and saponins decreased significantly in polarity. Aglycone is easier to absorb by the intestine; it then enters the blood to play a pharmacodynamic role [43].

Calycosin is one of the main active components of *Astragalus*. It exerts obvious antioxidant, antiviral, immunosuppressive, and other pharmacological effects [44]. Calycosin glucoside is metabolized and hydrolyzed into aglycone calycosin in the intestine by intestinal microflora. Calycosin evidently promotes vascular endothelial regeneration, protects endothelial cells, and reduces apoptosis [45, 46]. Calycosin-7-O-$\beta$-D-glucopyranoside inhibits angiotensin II-induced endothelial cell apoptosis and reduces the expression of apoptosis genes, thereby alleviating endothelial cell damage [47]. Herein, calycosin content was found to decrease in group AL and increase in groups AB and ALB. It appears that *B. coagulans* produces higher levels of calycosin during fermentation. In the mixed fermentation system, the growth of *B. coagulans* could be inhibited and that of *L. plantarum* could be promoted, which might explain why the content of calycosin in group ALB was relatively lower than that in group AB.

The active ingredients of Chinese herbal medicine mainly include polysaccharides, flavonoids, and saponins. They are closely related to the immune functions of aquatic animals and can reportedly improve barrier defense. A study investigated the anti-CyHV-2 effect of different Chinese herbal medicines and found that adding 1% *Astragalus* to the basal feed improved the antiviral ability to as high as 70% [48]. Another study found that *Astragalus* root extract markedly improved the survival rate of common carp infected with *Aeromonas hydrophila* [49]. In this study, consistent with the findings of earlier studies, the cumulative survival rates of crucian carp in the mixed fermentation group on CyHV-2 and *A. veronii* challenges were considerably higher than those in the control and single fermentation groups. This may be because the abundance of *L. plantarum* was detected to increase after fermentation. *L. plantarum* releases the bactericidal peptide lactobacilli, which effectively inhibits the growth of pathogenic bacteria such as *Escherichia coli* and *Salmonella* [50]. Another reason could be the drastic increase (>3 times) in APS content, which consequently promoted the growth of intestinal probiotics, improved stabilization of bacterial flora in the intestine, and maintained the antioxidant and immune systems of crucian carp [51]. Collectively, these effects could have increased the resistance of crucian carp to CyHV-2.

## 5. Conclusions

Mixed fermentation of *Astragalus* with *L. plantarum* and *B. coagulans* in a ratio of 3 : 3 at $1.0 \times 10^8$ CFU/g for 48 h increased the number of probiotics to $2.2 \times 10^9$ CFU/g, degraded high molecular weight proteins into small molecular weight or soluble proteins, and increased the content of APS and calycosin. Our data suggest that feeding crucian carp with a combination of commercial pellets and fermentation products mixed in an appropriate ratio can improve disease resistance.

## Data Availability

The data that support the results in this study are available from the corresponding author upon a reasonable request.

## Ethical Approval

All experimental procedures were conducted according to guidelines of the appropriate Animal Experimental Ethical Inspection of Laboratory Animal Centre, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences (ID: YFI2022-zhouyong-06).

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Authors' Contributions

Lisha Shi conceptualized the study, developed the methodology, investigated the study, and wrote the original draft. Mingyang Xue wrote the original draft. Chen Xu performed formal analysis. Nan Jiang performed formal analysis. Yuding Fan reviewed and edited the article. Jianwu Chen investigated the study. Wei Liu investigated the study. Yeying Wu investigated the study. Lingbing Zeng reviewed and edited the article. Yong Zhou conceptualized the study and developed the methodology.

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