Effect of Bacillus amyloliquefaciens LSG2-8 on the intestinal barrier function of Rhynchocypris lagowskii

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Abstract

Probiotics have been widely used in aquaculture. This article aims to study the effect of *Bacillus amyloliquefaciens* LSG2-8 on the intestinal barrier function of *Rhynchocypris lagowskii. B. amyloliquefaciens* LSG2-8 were added to *R. lagowskii* basal diets (CK) as additives at four concentrations: 1.0×10^6 (D−6), 1.0×10^7 (D−7), 1.0×10^8 (D−8) and 1.0×10^9 (D−9) CFU/g by dry weight of basal diet. After a 56 days feeding experiment, the intestinal digestive enzymes and immune enzymes activity of *R. lagowski* on group D-6, D-7, D-8 and D-9 diet were significantly higher than the control ($p < 0.05$). In molecular experiments, we found that the levels of TGF-β mRNA, IL-10 mRNA, ZO-1 mRNA and Claudin-3 mRNA in group D-8 *R.. lagowskii* were significantly higher ($p < 0.05$) than the control and other groups. Furthermore, the levels of IL-1β and IL-8 mRNA of *R. lagowski* on group D-6, D-7, D-8 and D-9 diet were significantly lower than the control ($p < 0.05$). In addition, we found that *B. amyloliquefaciens* LSG2-8 can regulate the intestinal flora balance and improve the intestinal structure of *R. lagowskii*. In conclusion, *B. amyloliquefaciens* LSG2-8 can improve the intestinal barrier function of *R. lagowskii*,and can be used as a feed additive in aquaculture.

1. Background

The intestinal tract of animals plays a major role in the digestion and absorption of nutrients, and it is also an important barrier to maintain a relatively stable environment in the organism [1–4]. The intestinal barrier is mainly composed of biological barriers, chemical barriers, physical barriers and immune barriers, among which physical barriers and immune barriers are particularly important for animal intestines [5]. As many as 10^{14} kinds of microorganisms reside in the intestinal tract of animals. Different microbial communities form an interdependent and mutually restrictive relationship. A micro-ecological balance system is established in the intestinal tract of animals, which constitutes the intestinal biological barrier [6]. When the animal's diet composition, lifestyle, conditions and other external factors change, the animal's intestinal microecological balance will be destroyed, resulting in a decline in the colonization resistance of the intestine, and a large number of pathogens from the outside take the opportunity to colonize and invade, triggering disease[7]. A large number of studies have shown [8–12] that probiotics can effectively maintain the biological barrier function of fish intestines. Probiotics entering the fish body can adhere to the intestinal wall by means of the specific adhesion mechanism of the bacteria itself, and compete with pathogenic bacteria for nutrients and living space. It can also secrete organic acids such as acetic acid, lactic acid, and short-chain fatty acids, reduce the pH of the intestine, inhibit the growth and proliferation of pathogenic bacteria, regulate the intestinal flora, make the intestinal microecosystem in the best balance, and improve the intestinal biological barrier of fish. The chemical barrier is composed of mucins, mucopolysaccharides, glycolipids, bile, digestive enzymes and lysozymes that cover the mucous layer of intestinal epithelial cells. They have the functions of resisting the intrusion of harmful substances from the outside into the intestine, regulating the physiological functions of the intestine, and protecting the intestinal mucosa. The activity of digestive enzymes can reflect the ability of fish to digest and absorb feed nutrients, mainly including protease, amylase and lipase. They can decompose the macromolecular nutrients in the feed into small molecules that are beneficial to the

digestion and absorption of fish, so as to improve the utilization rate of feed and promote the growth and development of fish [13].

The intestinal physical barrier, also known as the mechanical barrier, which is closely related to the tight junctions of intestinal mucosal epithelial cells and is the most important in the function of the intestinal barrier.In order to maintain the integrity of the intestinal tract, epithelial cells need to continuously differentiate from stem cells into goblet cells, paneth cells or enteroendocrine cells. Paneth cells can secrete substances such as antibacterial peptides and lysozyme, regulate the intestinal flora and effectively resist the invasion of pathogens [14]. Goblet cells can play the role of lubricating and protecting the intestinal epithelium by secreting mucin, and can also serve as antigen-presenting cells to deliver luminal antigens to CD103 + dendritic cells, thereby promoting T cell development and enhancing animal cell immunity. Tight junctions are complexes composed of Claudin, Occludin, junction adherensive molecules (JAM), and tight junction proteins (ZOs). Among them, Occludin and occludin are the backbones of tight junctions. Tight junction proteins are the basis of tight junction support structures and are used to connect transmembrane proteins. They establish a sealing area between adjacent epithelial cells to prevent macromolecular substances such as toxins in the intestinal lumen from entering the blood, which has a certain adjustment effect on the permeability of water and nutrients at the same time [15, 16]. The connection between the intact intestinal mucosal cells and the intestinal mucosal cells together constitutes the physical barrier of the intestinal mucosa, thereby ensuring the absorption and utilization of nutrients by the body. If the integrity of the intestinal mucosal structure is destroyed, the entire intestinal function will be damaged, which will affect the absorption and utilization of nutrients by the animal body and decrease the growth rate [17, 18]. Numerous studies have reported [19–21] that adding probiotics to the diet of fish can significantly increase the number of intestinal villi, the thickness of the intestinal wall and the number of goblet cells, and enhance the physical barrier function of the intestine.

The immune barrier is the first line of defense against harmful substances from the outside world. It has a unique innate and acquired defense mechanism that can prevent pathogens from destroying animal bodies [22]. Intestinal-related lymphoid tissues, immune cells and cytokines play an important role in the intestinal immune barrier. In higher vertebrates, intestinal-associated lymphoid tissue is composed of scattered lymphatic tissue and organized lymphatic tissue, while in lower vertebrates such as fish, there is a lack of organized intestinal-associated lymphoid tissue and the digestive tract is scattered. The immune cells of fish mainly include lymphocytes, plasma cells, macrophages and granulocytes [23]. Immunoglobulin M synthesized by B lymphocytes is the main antibody in the serum of most fish and the main antibody to the antigen on the vertebrate B cell, which plays an important role in the body's immune response. Cytokines are small molecular peptides that are secreted by immune cells in the process of immune response and can regulate the immune function of the body [24–26]. Interleukin (IL) and transforming growth factor (TGF) are the main cytokines in fish. The interleukins in fish have both pro-inflammatory and anti-inflammatory effects. Among them, IL-8 is a pro-inflammatory chemokine, which is related to the promotion of neutrophil chemotaxis and degranulation [27, 28]. Interleukin-10 is a cytokine with anti-inflammatory effects. In monocytes or macrophages, IL-10 can reduce the production of inflammatory mediators and inhibit antigen presentation [29, 30].

Therefore, in this experiment, *B. amyloliquefaciens* LSG2-8 isolated from healthy *R. lagowskii* was added to the feed to explore the effects of *B. amyloliquefaciens* on the intestinal chemical barrier function, immune barrier function, biological barriers and physical barrier function of *R. lagowskii*.

2. Methods

2.1 Fish handing

The experiment was carried out in accordance with the research plan of the Institutional Animal Care and Use Committee of Jilin Agricultural University.

2.2 Diet preparation

The feed used fish meal as the animal protein source, dehulled soybean meal and wheat bran as the vegetable protein source, fish oil and corn oil as fat sources, flour and dextrin as energy sources, and was formulated into a basis with a crude protein content of 37% and a crude fat content of 6.56% feed (Table 1). The feed ingredients were crushed and passed through a 60-mesh sieve, and the quality was weighed according to the formula. Each feed preparation was mixed well and extruded through a 1.5 mm diameter laboratory presser machine. The pellets were air dried at room temperature, then evenly spray *B. amyloliquefaciens* LSG2-8 (Preserved in the laboratory of Jilin Agricultural University) with viable counts of 1.0×10^6, 1.0×10^7, 1.0×10^8 and 1.0×10^9 CFU/g respectively and stored in − 20°C freezer until use (BCD-258WDPM, Haier Group Co., Ltd.). The test diets were prepared every week to maintain the actual bacterial count. The bacterial count was determined twice in a week by plate counting method.

Table 1
Formulation and chemical composition of basal diet (g/Kg dry matter).

Ingredients	Concentrations (g /Kg)
Fish meal	200.00
Soybean meal	330.00
Fish oil	20.00
Corn oil	20.00
Triticum aestivum	60.00
Flour	100.00
Dextrin	70.00
Corn protein flour	150.00
$CaHPO_4$	30.00
1% Compound premix	10.00
Lys	2.00
Met	5.00
50% Choline chloride	3.00

2.3 Experimental fish and breeding management

R. lagowskii was purchased from obtained from Niannianyouyu Aquaculture Co., Ltd (Meihekou City, Jilin Province, China.). The breeding experiment was carried out in the temperature-controlled single-cycle breeding system of the aquarium ecological room of Jilin Agricultural University. The fish were acclimated in glass aquaria for 15 d and fed a basal diet before the initiation of the experiment. Then, 450 fish having a mean body weight of 10 g were randomly distributed into 15 breeding barrel (30 fish per barrel) of 5 experimental groups. The CK group was the control group, and the D-6, D-7, D-8 and D-9 groups were the probiotic supplemented groups, which were 1.0×10^6, 1.0×10^7, 1.0×10^8 and 1.0×10^9 CFU/g respectively. The fish were fed twice a day with full food (8 am and 5 pm), and adopt artificial hand-spreading method. 50% of the water was changed every day in order to keep the water quality of the aquaculture water fresh. The feeding status of each barrel of fish was recorded every day and the activity status of the fish was observed. During the feeding experiment,keep the water temperature at 20℃ ± 1℃, ammonia nitrogen < 0.3 mg/L, nitrite < 0.05 mg/L, dissolved oxygen > 5 mg/L, and pH control at 7.5 ~ 8.5.

2.4 Sample collection

At the end of 60 days of the feeding trial, the fishes were fasted for 24 h and anesthetized using MS-222 (Sigma-Aldrich, India). Six fish from each group were sacrificed for enzymatic analysis. For immune-related and intestinal barrier genes expression, six fishes were randomly collected from the control group and the probiotic supplemented groups and sacrificed for their foregut, midgut and hindgut. The samples were immediately passed liquid nitrogenand, then stored in an ultra-low temperature refrigerator at -80 ℃ (BDF-86V936, Shandong Brocade Scientific Instrument Co., Ltd.). Then take 6 fish to carefully squeeze out the contents of the intestines (feces and undigested feed), divide them into foregut, midgut and hindgut, then wash the intestines with normal saline, and finally use general-purpose tissue fixative Fix the foregut, midgut and hindgut separately and store at 4 ℃ in the dark for future use.

2.5 Enzyme analysis

Appropriate amount of foregut, midgut and hindgut tissues were weighted and recorded their exact weights. The physiological saline was added according to the ratio of tissue sample to physiological saline 1 : 9. A 10% tissue homogenate was made in an ice-water bath, and centrifuged at 4000r/min for 10 min at 4 ℃, then the supernatant was carefully aspirated for determination. The kits purchased from Jiancheng Bioengineering Institute (Nanjing, China) were used for the measurements of digestive enzymes and immune enzymes activities, including:amylase, lipase, protease, lysozyme (LZM), alkaline phosphatase (AKP) and acid phosphatase (ACP).

2.6 Analysis of genes expression related to immune and intestinal barrier

Trizol (Shanghai Shenggong Biological Engineering Co., Ltd.) method was used to extract RNA from liver, foregut, midgut and hindgut. The concentration and purity of the isolated RNA were measured by UV spectroscopy at 260 nm and 280 nm in a Nanodrop (Thermo-scientific, USA). RNA integrity was further confirmed by Agarose Gel Electrophoresis and stored in an ultra-low temperature refrigerator at -80 ℃ (BDF-86V936, Shandong Brocade Scientific Instrument Co., Ltd.) for testing[31]. Fluorescence quantitative PCR adopted the Oen Step TB Green dye method, used Takara's One Step TB Green PrimeScript™ RT-PCR Kit II kit, and was carried out in the ABI (7500) RT-PCR system. The reaction system was 20μL, and the specific reaction systemwas shown in Table 2. The primer sequences of immune-related genes and gut barrier genes were shown in Table 3. All primers were designed by Premier 5.0 software and synthesized by Shanghai Shenggong Biotechnology Service Co., Ltd. All the target genes were analyzed with β-actin as the internal control. All reactions were done in triplicate. Then, the specificity and homogeneity of the PCR products were analyzed and confirmed by melt curve analysis post-amplification. The relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method [32].

Table 2
RT-PCR reaction.

Reagent	Volume(μL)
One Step TB Green RT-PCR Buffer 4(2 X)	10
PrimeScript 1 Step Enzyme Mix 2	0.8
PCR Forward Primer	0.8
PCR Reverse Primer	0.8
ROX Reference Dye (50 X)	0.4
Total RNA	2
RNase Free dH_2O	5.2
Total	20

Table 3
Primer sequence information of target gene.

Target gene	Primer sequence	Gen bank
β-actin-F	CGGTATCCATGAGACCACCT	AAB97964.1
β-actin-R	CTTCTGCATCCTGTCAGCAA	
TGF-β-F	TTGGGACTTGTGCTCTAT	EU099588
TGF-β-R	AGTTCTGCTGGGATGTTT	
IL-1β-F	CTGGAGCAATGCAATACAAA	AJ245635
IL-1β-R	AGGTAGAGGTTGCTGTTGGAA	
IL-8-F	ATGAGTCTTAGAGGTCTGGGTG	DQ453125
IL-8-R	ACAGTGAGGGCTAGGAGGG	
IL-10-F	AATCCCTTTGATTTTGCC	AB110780
IL-10-R	GTGCCTTATCCTACAGTATGTG	
ZO-1-F	CCTCAGACCACTCCAGACACTCTC	565459
ZO-1-R	TCGGCATCTTCCCACCATCTTAG	
Claudin-3-F	ATCACTCGGGACTTCTA	KF193859
Claudin-3-R	CAGCAAACCCAATGTAG	

2.7 Detection of the diversity of intestinal flora

In a sterile operating table, take a sample of *R. lagowskii*'s intestinal contents diluted with 0.5 mL of normal saline and place it in a 1.5 mL enzyme-free tube, quickly put it in liquid nitrogen and freeze it, and store it in a refrigerator at -80 ℃ for later use. The following operations were all done by Ovison Gene Technology Co., Ltd.: Select sequencing primers based on the sequence of the V3 ~ V4 region of the 16S rRNA gene. The purified amplified product was sequenced by Illumina PE250. By comparing the Silva ribosome database, the operational taxa (OUT) are classified based on the 97% similarity level, and the RDP classifier Bayesian algorithm is used for taxonomic analysis, at the Phylum and Genus classification levels Count the community composition of each sample, and use Mothur software and R language tools to analyze diversity, species composition and structure respectively. Use PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) to predict microbial gene function.

2.8 Observe the structure of intestinal tissue

The fixed intestinal tissue structure was made into paraffin sections according to the conventional method, and the sections were sealed with neutral gum after H.E staining. Observe and photograph the slices under a microscope, and record the fold height, lamina propria width and muscle layer thickness.

2.9 Statistical analysis

All parameters were performed in triplicate, and the results were presented as means ± SD (standard error). The results were subjected to one-way analysis of variance (ANOVA). Tukey's test was used to determine the significant differences between the variables at $p < 0.05$, and the data were analyzed by SPSS 20.0 software.

3. Results

3.1 Digestive enzymes analysis

In examining the effect of *B. amyloliquefaciens* LSG2-8 on digestive enzymes activity, we found that the activities of amylase, lipase and protease in the foregut, midgut and hindgut of group D-8 *R. lagowskii* significantly higher than the control group and other groups ($p < 0.05$; Table 4).

Table 4
The effect of *Bacillus amyloliquefaciens* LSG2-8 on the activity of amylase, lipase and protease of *Rhynchocypris lagowskii*.

Group	Amylase (U/mg),			Lipase (U/g)			Protease (U/mg)		
	foregut	midgut	hindgut	foregut	midgut	hindgut	foregut	midgut	hindgut
CK	0.83 ± 0.03^{b}	0.53 ± 0.02^{b}	0.44 ± 0.01^{b}	22.36 ± 0.36^{b}	11.63 ± 0.09^{b}	11.51 ± 0.10^{b}	61.41 ± 0.67^{c}	57.92 ± 0.27^{b}	55.71 ± 0.27^{c}
D-6	0.86 ± 0.03^{b}	0.55 ± 0.03^{b}	0.45 ± 0.01^{b}	23.01 ± 0.34^{b}	11.70 ± 0.10^{b}	11.70 ± 0.08^{b}	62.96 ± 0.20^{bc}	59.83 ± 0.55^{b}	56.08 ± 0.33^{c}
D-7	0.92 ± 0.02^{b}	0.60 ± 0.02^{ab}	0.54 ± 0.01^{a}	23.60 ± 0.10^{ab}	12.87 ± 0.40^{a}	12.65 ± 0.10^{a}	63.95 ± 0.33^{b}	62.68 ± 0.70^{a}	57.92 ± 0.41^{b}
D-8	1.20 ± 0.05^{a}	0.63 ± 0.03^{a}	0.56 ± 0.01^{a}	24.75 ± 0.23^{a}	13.44 ± 0.24^{a}	12.85 ± 0.06^{a}	66.03 ± 0.28^{a}	63.36 ± 0.32^{a}	60.00 ± 0.29^{a}
D-9	0.88 ± 0.09^{b}	0.54 ± 0.01^{b}	0.46 ± 0.02^{b}	22.90 ± 0.42^{b}	12.42 ± 0.24^{ab}	11.81 ± 0.04^{b}	61.88 ± 0.43^{c}	58.35 ± 0.63^{b}	56.25 ± 0.41^{c}

Note: Data were presented as the mean ± SD (n = 3). The means with different letters within the same column (line) were significant differences at the 0.05 probability level, and the means with the same letters within the same column were not significant differences.

3.2 Immune enzymes analysis

The results of the effect of *B. amyloliquefaciens* LSG2-8 on the immune enzymes activity of *R. lagowskii* were shown in Table 5. In examining the effect of *B. amyloliquefaciens* LSG2-8 on the immune enzymes activity, we found that the activities of lysozyme (LZM), alkaline phosphatase (AKP) and acid phosphatase (ACP) of group D-8 *R. lagowskii* significantly higher than the control group and other groups ($p < 0.05$).

Table 5
The effect of *Bacillus amyloliquefaciens* LSG2-8 on the activity of LZM, AKP and ACP of *Rhynchocypris lagowskii*.

Group	LZM (Ug/mg)			AKP (U/mg)			ACP (Ug/mg)		
	foregut	midgut	hindgut	foregut	midgut	hindgut	foregut	midgut	hindgut
CK	70.39 ± 0.09^{d}	70.85 ± 0.21^{c}	64.85 ± 0.12^{c}	13.43 ± 0.07^{c}	12.45 ± 0.10^{c}	12.30 ± 0.04^{c}	3.55 ± 0.05^{c}	2.89 ± 0.01^{c}	2.72 ± 0.06^{c}
D-6	75.30 ± 0.08^{c}	72.19 ± 0.12^{b}	67.33 ± 0.03^{b}	15.54 ± 0.05^{b}	13.47 ± 0.22^{b}	13.36 ± 0.05^{b}	4.05 ± 0.04^{b}	3.77 ± 0.11^{b}	3.50 ± 0.09^{b}
D-7	76.90 ± 0.51^{bc}	72.50 ± 0.06^{b}	70.02 ± 0.15^{a}	15.55 ± 0.55^{b}	13.53 ± 0.12^{b}	14.55 ± 0.16^{a}	4.08 ± 0.05^{b}	4.46 ± 0.04^{a}	3.69 ± 0.19^{b}
D-8	81.56 ± 0.51^{a}	74.45 ± 0.18^{a}	70.66 ± 0.26^{a}	18.72 ± 0.11^{a}	15.61 ± 0.13^{a}	14.81 ± 0.08^{a}	5.25 ± 0.06^{a}	4.49 ± 0.09^{a}	4.33 ± 0.12^{a}
D-9	77.73 ± 0.45^{b}	72.00 ± 0.27^{b}	66.98 ± 0.30^{b}	13.45 ± 0.06^{c}	13.46 ± 0.11^{b}	13.58 ± 0.07^{b}	3.81 ± 0.11^{bc}	3.75 ± 0.06^{b}	3.65 ± 0.03^{b}

Note: Data were presented as the mean ± SD (n = 3). The means with different letters within the same column (line) were significant differences at the 0.05 probability level, and the means with the same letters within the same column were not significant differences.

3.3 Analysis of the expression of genes related to immunity

The feed containing four levels of the *B. amyloliquefaciens* LSG2-8 was analyzed for immune-related expression. Genes such as TGF-β mRNA and IL-10 mRNA were up-regulated in response to *B. amyloliquefaciens* LSG2-8 supplementation (Fig. 1). TGF-β mRNA and IL-10 mRNA genes expressions in the group supplemented with 1×10^{8}CFU/ g were significantly increased compared to control and the other groups. In contrast, the expression of IL-8 mRNA and IL-1β genes were not significantly increased by the *B. amyloliquefaciens* LSG2-8 supplementation compared to control ($p < 0.05$) (Fig. 1).

3.4 Analysis of the expression of genes related to the intestinal barrier

The results of the effect of *B. amyloliquefaciens* LSG2-8 on the expression of intestinal barrier related genes of *R. lagowskii* were shown in Fig. 2. The results showed that the expression of intestinal barrier related genes such as ZO-1 mRNA and Claudin-3 mRNA in the intestinal tissue were significantly increased by the *B. amyloliquefaciens* LSG2-8 supplementation compared to control ($p < 0.05$).

3.5 Detection of the diversity of intestinal flora

The effect of *B. amyloliquefaciens* LSG2-8 on the intestinal microflora of *R. lagowskii* is shown in Fig. 3 and Fig. 4. At the phylum level, *Proteobacteria*, *Firmicutes*, *Bacteroides* and *Actinomycetes* are the dominant

populations. Among them, the abundance of *Proteobacteria* in the D-6, D-7, D-8 and D-9 groups was lower than that of the control group, the abundance of *Firmicutes* in the D-6, D-8 and D-9 groups higher than the control group, while the abundance of *Firmicutes* in the D-7 group is lower than that in the control group.The abundances of *Bacteroides* and *Actinomycetes* in the D-6, D-7, D-8 and D-9 groups were lower than those of the control group. Among them, the *Bacteroides* abundance in the D-8 group was the lowest, and the D-9 group The *Actinomycota* has the lowest abundance. At the genus level, the main intestinal flora are *Bacillus* (*Firmicutes*) and *Acinetobacter* (*Proteobacteria*).

The sparse curve of each sample is shown in Fig. 5. It can be seen from the figure that the dilution curve of each sample tends to be flat, indicating that the sequencing depth is reasonable and the sequencing volume is sufficient to cover most groups. From the Venn diagram, it can be seen that the proportion of OTU shared among the tested samples is relatively high (Fig. 6). PICRUSt predicts that the addition of *B. amyloliquefaciens* LSG2-8 to feed can reduce the relative abundance of microorganisms related to immune system diseases and metabolism-related diseases in the intestinal tract of *R. lagowskii*, and can increase the relative abundance of microorganisms related to digestive system functions (Fig. 7).

3.6 Observe the structure of intestinal tissue

The influence of *B. amyloliquefaciens* LSG2-8 on the intestinal structure of *R. lagowskii* is shown in Table 6 and Fig. 8. It can be seen from the figure that the tissue structure of the foregut, midgut and hindgut of the *R. lagowskii* is clear and complete, the intestinal villi are developed, the mucosa is clear, and there is no shedding. It can be seen from the table that, compared with the control group, the probiotic supplement group can significantly increase the fold height, lamina propria width and muscle thickness of the *R. lagowskii's* intestinal tract ($p < 0.05$).

Table 6
Effects of *Bacillus amyloliquefaciens* LSG2-8 on the intestinal tissue of *Rhynchocypris lagowskii.*

Group	Fold height(μm)			Lamina propria width (μm)			Muscle layer thickness(μm)		
	foregut	midgut	hindgut	foregut	midgut	hindgut	foregut	midgut	hindgut
CK	1014.53 ± 1.77^{d}	716.27 ± 2.33^{b}	743.45 ± 1.87^{c}	17.27 ± 0.83^{b}	10.34 ± 0.45^{b}	12.55 ± 0.36^{b}	26.13 ± 1.27^{c}	27.43 ± 0.53^{c}	16.44 ± 0.56^{c}
D-6	1037.28 ± 1.59^{c}	648.83 ± 2.76^{d}	680.13 ± 3.02^{d}	21.75 ± 0.48^{a}	11.60 ± 0.15^{b}	11.71 ± 0.15^{bc}	35.91 ± 1.04^{b}	27.33 ± 0.56^{c}	23.38 ± 0.36^{c}
D-7	1033.90 ± 1.75^{c}	667.87 ± 0.87^{c}	852.04 ± 3.90^{a}	17.81 ± 0.99^{b}	14.82 ± 0.77^{a}	12.39 ± 0.23^{b}	27.36 ± 0.53^{c}	29.69 ± 0.55^{b}	23.68 ± 0.46^{c}
D-8	1226.03 ± 3.19^{a}	729.67 ± 3.88^{a}	845.93 ± 1.06^{a}	19.49 ± 0.35^{ab}	13.99 ± 0.42^{a}	19.15 ± 0.21^{a}	41.21 ± 0.47^{a}	34.57 ± 0.27^{a}	28.49 ± 0.76^{c}
D-9	1083.24 ± 2.19^{b}	665.85 ± 2.28^{c}	828.02 ± 0.62^{b}	17.52 ± 0.16^{b}	10.85 ± 0.41^{b}	10.71 ± 0.10^{c}	25.67 ± 1.43^{c}	28.12 ± 0.35^{bc}	21.39 ± 0.53^{c}

Note: Data were presented as the mean ± SD (n = 3). The means with different letters within the same column (line) were significant differences at the 0.05 probability level, and the means with the same letters within the same column were not significant differences.

4. Discussion

The intestine of an animal is an organ that communicates with the outside world. The traditional concept believed that it only has the function of digestion and absorption, and did not participate in pathological changes outside the organ. With the deepening of research, everyone realized that the intestinal tract also has a barrier function to prevent harmful substances such as toxins, anti-nutritional factors and bacteria in the intestinal cavity from invading the body. The intestine is not only an important nutrient digestion and absorption organ in the body, but also an important immune and endocrine organ in the body. As the site most frequently exposed to microorganisms in the external environment, the intestine is not only the main site for pathogenic microorganisms and toxins to invade the body, but also the first important barrier to prevent intestinal pathogenic microorganisms from infecting the body [33–36]. The complete intestinal barrier includes four parts:physical barrier, chemical barrier, biological barrier and immune barrier. Each has different molecular regulation mechanisms and biological functions, and is organically combined through their respective signal pathways to jointly defend against foreign antigens from invading the body [37–39].

Digestive enzymes are the main element that constitutes a chemical barrier. The growth of the body is positively related to the digestion and absorption of nutrients. The intestine is the main place where the body absorbs nutrients. Various digestive enzymes in the intestine play an important role in the digestion of nutrients. Therefore, increasing the activity of digestive enzymes is an effective way to improve the digestion

of nutrients [40, 41]. Sogarrd proposed for the first time that probiotics can improve animal digestive enzyme activity [42]. At present, a large number of studies have shown that probiotics can significantly increase the activities of protease, amylase and lipase in aquatic animals, and increase the utilization rate of feed [43–45]. Wang et al. [46] added *Bacillus* and photosynthetic bacteria to *Cyprinus carpio* fine feed and found that the intestinal protease, lipase and amylase activities in the experimental group were significantly increased ($p < 0.05$). Wu et al. [47] fed *Bacillus subtilis* Ch9 to grass carp, the intestinal protease and amylase activity of the experimental group was significantly higher than the control group after two weeks ($p < 0.05$). Suzer et al. [48]found that the protease, lipase and amylase activities of *Sparus aurata* after ingesting probiotic eutrophic biological diet were higher than the control group. Consistent with the results of previous studies, this study found that adding *B. amyloliquefaciens* LSG2-8 to the diet can significantly increase the activity of amylase, lipase and protease in the intestinal tract of *R. lagowskii.* At present, there are different mechanisms for probiotics to improve the activity of digestive enzymes in the intestines of animals. The generally accepted concept is that probiotics produce extracellular enzymes while exerting their effects in the intestinal tract, which may be one of the reasons for the results of this experiment. In addition to the enzymes contained in the animal body itself, probiotics can produce digestive enzymes such as proteases, lipases and amylases during the metabolism process, thereby increasing the host's intestinal digestive enzyme activity, assisting the body in digesting organic substances, and increasing the conversion rate of food. In addition, when the probiotics enter the digestive tract of aquatic animals with the bait, they can sprout and grow into metabolically active cells in the digestive tract, adjust the structure of the intestinal microbial community, improve the microecological environment, and optimize the physiological activities of the intestinal tract, thereby indirectly promote the body's synthesis and secretion of digestive enzymes.

Non-specific immunoenzyme activity and cytokines are the main indicators to measure the body's immune barrier function. Important lysosomal enzymes such as alkaline phosphatase, acid phosphatase and lysozyme play an important role in the non-specific immune response. Alkaline phosphatase is a regulatory enzyme that participates in many important functions in all organisms. It can destroy the surface molecular composition of harmful substances, accelerate the degradation of harmful substances, and prevent the reproduction of germs [49]. Acid phosphatase is an enzyme that catalyzes the hydrolysis of phosphate monoesters under acidic conditions. It is mainly involved in the metabolism of phosphate esters and also has the functions of regulating metabolism, energy conversion and signal transduction. As a marker enzyme of lysosomes, studies have found that acid phosphatase is closely related to the normal performance of lysosomal physiological functions [50]. Lysozyme plays an important role in the forefront defense mechanism of fish against infectious pathogens. When pathogens invade, they can stimulate the increase in the concentration of lysozyme in the fish. Lysozyme causes the rupture of pathogenic cells by enzymatically hydrolyzing mucopolysaccharides in the cell wall of pathogens, especially for Gram-positive bacteria or certain specific bacterial cells [51]. In this experiment, we found that adding *B. amyloliquefaciens* LSG2-8 to the diet at a concentration of 1.0×10^8 CFU/g can significantly increase the activity of LZM, AKP and ACP in the intestinal tract of *R. lagowskii* ($p < 0.05$). Consistent with the results of this study, Yang et al. [52] reported that *Bacillus velezensis* JW can significantly increase the activity of AKP and ACP in *Carassius auratus* ($p < 0.05$). Li et al. [53] found that *Lactobacillus plantarum* can significantly increase the activities of AKP and ACP in sea cucumbers ($p < 0.05$), but has no significant effect on lysozyme ($p > 0.05$). This

difference may be related to environmental factors such as species differences, feed formulations, and the types of probiotics used. At the same time, a large number of studies have confirmed that adding a certain amount of probiotics to the material can improve the immunity of farmed fish [54, 55]. Probiotics can improve fish immunity may be due to the fact that the added probiotics reduce the ammonia nitrogen content in the water body and create a relatively good living environment for fish. Furthermore, probiotics can also produce antibacterial active substances, which can resist the colonization and invasion of pathogenic bacteria and improve the immunity of fish.

Cytokine, as an indispensable mediator that secreted from immune cells with regulating the immune response, repairing damaged tissues and defensing against infection. Interleukin (IL) and transforming growth factor (TGF) are the main cytokines in fish. The interleukins in fish have both pro-inflammatory and anti-inflammatory effects. IL-1β and IL-8 are widely accepted as two important pro-inflammatory factors that commonly used as indicator genes in response to bacterial and viral invasion [56, 57]. IL-10 and TGF-β are pleiotropic anti-inflammatory factors that are effective at relieving inflammation by inhibiting release of inflammatory cytokines [58]. Many experiments have proved that probiotics can regulate and induce the immune factors of the intestinal mucosal cells of animals. Christensen et al. [59] found 6 strains of *Lactobacillus*, namely *Lactobacillus Reuteri* DSM12246, *Lactobacillus plantarum* Lb1, *Lactobacillus fermentum* Lb20, *Lactobacillus casei* CHCC3137), *Lactobacillus plantarum* 299v and *Lactobacillus johnsonii* La1 can induce the increase of the expression of IL-12 and tumor necrosis factor TNF in the intestinal dendritic cells of mice to varying degrees. In fish probiotics research reports, different probiotics will also regulate the differential expression of some immune factors in the intestine. Previous studies have shown that dietary administration with 1.0×10^9CFU/g *L. brevis* JCM 1170 and *L. acidophilus* JCM 1132 significantly increased ($p < 0.05$) TGF-β gene expression of hybrid tilapia [60]. Reyes-Becerril et al. also through probiotics in vivo experiments that when 1.0×10^6 CFU/g active *Debaryomyces hansenii* was added to feed golden-head sea bream for 4 weeks, found that the expression of immune genes in the intestine of sea bream (including Hep, IgM, TCR-β, CSF-1R, TNF-α and IL-1β) were down-regulated [61]. In the present study, supplementation of *B. amyloliquefaciens* LSG2-8 1.0×10^8 CFU/g diets significantly up-regulated the expression of IL-10 mRNA and TGF-β mRNA, and down-regulated the expression of IL-8 mRNA and IL-1-β mRNA in the foregut, midgut and hindgut of *R. lagowskii*. However, Kim et al. found that two active probiotics, *Carnobacterium maltaromaticum* B26 and *Carnobacterium divergens* B33 were isolated from healthy rainbow trout intestines. They all mixed with the intestinal cells of rainbow trout for 6 h and 12 h, except for the complement C3 gene, other intestinal cytokines (such as IL-1β, IL-8, TNF-α and TGF-β) were not found to be up-regulated [62]. The minor differences may be related to the species variation, feed formula, the probiotics species used and other environmental factors.

The intestinal physical barrier plays an important role in resisting the invasion of pathogenic bacteria, maintaining the balance of intestinal flora, and protecting the health of the body. The integrity of the physical barrier includes the integrity of the tight junctions between the intestinal mucosal cells and the integrity of the intestinal mucosal epithelial cells. Zo-1 is an important tight junction protein, which is involved in the maintenance of polarity and material transport in epithelial cells [63, 64]. Claudin-3 is a transmembrane protein, which is essential for tight junctions between intestinal mucosal cells [65, 66].

Probiotics can regulate the permeability of the intestinal mucosal barrier by regulating the expression of tight junction proteins. Previous studies have shown that colitis mice eat compound probiotics, the expression levels of ZO-1, Claudin, Occludin and other genes in the intestine were all up-regulated. The results showed that the composite probiotics can protect the integrity of the intestinal mucosal barrier by reducing the permeability of the intestinal mucosa [67]. The probiotic *Lactobacillus brevis* SBC8803 and *Lactobacillus rhamnosus* GG and their supernatants could increase the expression of tight junction proteins in the intestinal tract of mice with alcoholic liver disease and humans [68–70]. Similarly, in this experiment, we found that *B. amyloliquefaciens* LSG2-8 could significantly up-regulate the expression of ZO-1 mRNA and Claudin-3 mRNA genes in the intestinal tract of *R. lagowskii*,thereby reducing the permeability of the intestinal tract and improving the integrity of the intestinal mucosal barrier, which was consistent with the results of Jiang et al [71]. The mechanism of probiotics regulating the physical barrier of the intestine may be as follows: probiotics can prevent pathogenic bacteria from being fixed in the intestine, promote mucus secretion in the intestinal mucosa and increase the thickness of the mucosal layer, thereby improving the barrier function of the intestinal physical membrane.

The digestive ability of fish is closely related to its intestinal tissue structure. A complete intestinal tissue structure is the prerequisite for improving the digestive ability of fish, and the study of intestinal morphological characteristics is also the main way to understand whether the physiological condition of fish is normal [72]. The intestine of fish is not as complicated as the intestine of mammals, and has obvious differentiation. It is mainly composed of four parts: mucosal layer, submucosal layer, muscle layer and serosal layer. The increase in the height of the folds on the mucosal layer can expand the absorption surface area of nutrients, which is more conducive to the digestion and absorption of nutrients, and the mucosal layer is the main adsorption site for the flora in the intestine [73]. Therefore, the structural changes of the mucosal layer can be observed to understand the influence of *B. amyloliquefaciens* LSG2-8 on the intestinal structure of *R. lagowskii*. The main role of the intestinal muscularis is to promote intestinal peristalsis, which is composed of smooth muscle, so increasing the thickness of the intestinal muscularis can indirectly promote the digestion and absorption of nutrients by fish. The results of this experiment showed that the height of the intestinal fold, the width of the lamina propria and the thickness of the muscle layer of *R. lagowskii* with *B. amyloliquefaciens* LSG2-8 were higher than those of the control group. This shows that the addition of *B. amyloliquefaciens* LSG2-8 can promote the increase of intestinal fold height, lamina propria width and muscle layer thickness of specific cultured subjects, and can improve the structure of intestinal tissue.

Intestinal microbes play an important role in the regulation of animal nutrition metabolism, immunity and diseases. From the sparse curve of the sample, it can be seen that the sequencing results of this experiment basically cover all the microorganisms in the intestine of *R. lagowskii*, and from the Venn diagram, it can be seen that the proportion of OTU shared between each sample is relatively high, which shows that under the same environmental conditions, the similarity of the microbial flora in the intestinal tract of *R. lagowskii* fed with different levels of *B. amyloliquefaciens* LSG2-8 was higher. We also found that feeding different levels of *B. amyloliquefaciens* LSG2-8 *R. lagowskii's* gut microbes are mainly composed of *Proteobacteria*, *Firmicutes*, *Bacteroides* and *Actinomycetes*. This result is consistent with some research findings. The level composition of the intestinal microbiota of carp and grass carp is similar [74, 75]. Among them, *Firmicutes*

and *Bacteroides* play an important role in the process of carbohydrate metabolism and nutrient absorption [76]; *Actinomycetes* are mostly effective antibiotics, and their most important role is to produce and extract antibiotics. In addition, it can also recover biological materials with complex classification [77].The normal distribution of beneficial and harmful bacteria in the intestines of fish is in a dynamic balance. When the body is attacked by pathogens, the dynamic balance of intestinal flora will be broken, resulting in a sharp increase in the number of harmful bacteria in the intestine. The number of beneficial bacteria is decreasing, which leads to a decline in the body's immune function and disease outbreaks [78, 79]. Studies have found that the potential pathogens that cause bacterial diseases in aquaculture animals are widely distributed in the intestines of fish [80, 81]. Similarly, in this experiment, we found that there are *Aeromonas* bacteria in the intestines of *R. lagowskii* and *Flavobacterium*, *Aeromonas* and *Flavobacterium* are common conditional pathogens. In the prediction of PICRUs, we found that the addition of *B. amyloliquefaciens* LSG2-8 to feed can reduce the relative abundance of microorganisms related to immune system diseases and metabolism-related diseases in the intestinal tract of *R. lagowskii's* stalk. It shows that *B. amyloliquefaciens* LSG2-8 can maintain the balance of the intestinal flora of *R. lagowskii* by promoting the survival and reproduction of beneficial bacteria and inhibiting the growth of harmful bacteria. And we speculate that *B. amyloliquefaciens* LSG2-8 may inhibit the growth of pathogenic bacteria by secreting antibacterial substances and competing with pathogens for living space and nutrients. However, its mechanism of action on intestinal flora still needs further study.

5. Conclusion

In this experiment, we conclude that *B. amyloliquefaciens* LSG2-8 could promote the chemical barrier function, physical barrier function and immune barrier function of intestine of *R. lagowskii.* The specific performance was that *B. amyloliquefaciens* LSG2-8 increased the digestive and immune enzyme activities in the intestinal tract of *R. lagowskii*, up-regulated the expression of immune-related genes and intestinal barrier genes, regulated the balance of intestinal flora and improved intestinal structure. Hence, *B. amyloliquefaciens* LSG2-8 could be used in aquaculture, and it seems we should probably focus on the development of probiotics isolated from the cultured fish species rather than using other sources probiotics in aquaculture.

6. Abbreviations

Junction adherensive molecules (JAM), Interleukin (IL), Transforming growth factor (TGF), Lysozyme (LZM), Alkaline phosphatase (AKP), Acid phosphatase (ACP), Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), *Bacillus amyloliquefacien*s (*B. amyloliquefaciens*), *Rhynchocypris lagowskii* (*R. lagowskii*)

Declarations

Ethics approval and Consent to participate

The experiment was carried out in accordance with the research plan of the Institutional Animal Care and Use Committee of Jilin Agricultural University.

Consent to publication

All authors agree to participate in the publication of this article.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing Interest

The authors have no relevant financial or non-financial interests to disclose.

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Authors' Contributions

All authors contributed to the study conception and design. DMZ put forward the research direction. YKC designed the entire experiment in detail. ZXG collects data and understands the research status at home and abroad. Material preparation was performed by YRZ. GQW guided the whole experiment process. Data collection was performed by QY. QJW analyzed the data. EM polished the article language. The first draft of the manuscript was written by MNY and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

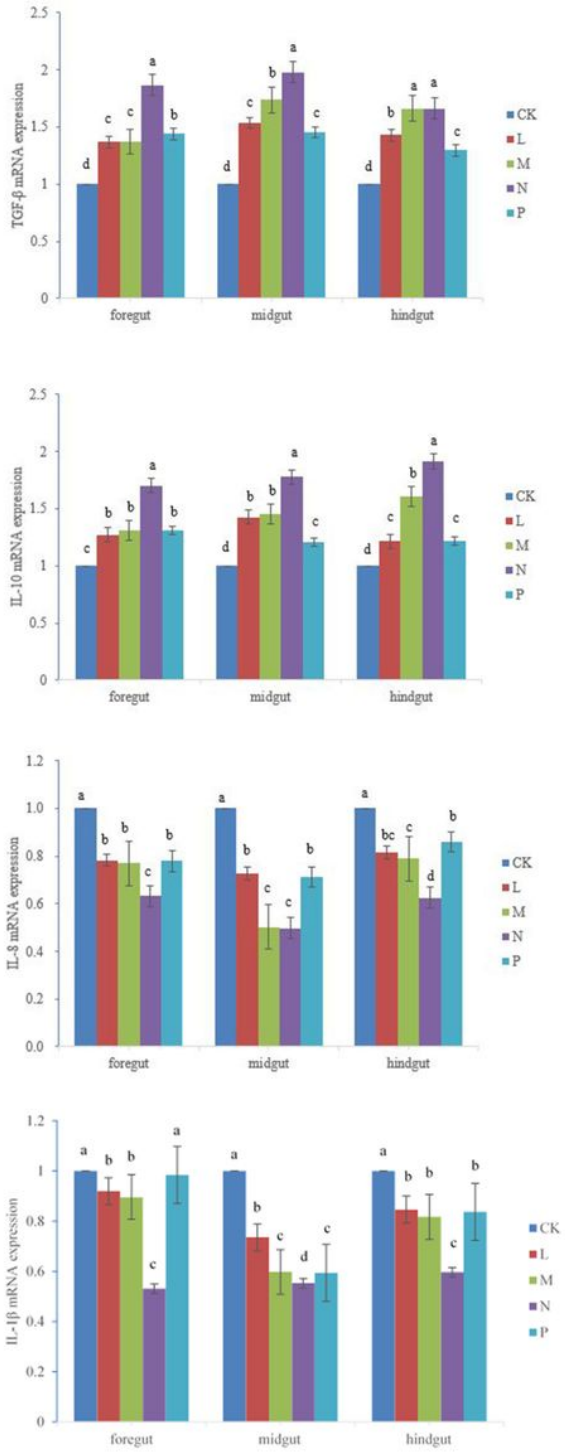

Figure 1

The effect of *Bacillus amyloliquefaciens*LSG2-8 on the expression of TGF-β mRNA, IL-10 mRNA, IL-8 mRNA and IL-1β mRNA of *Rhynchocypris lagowskii*

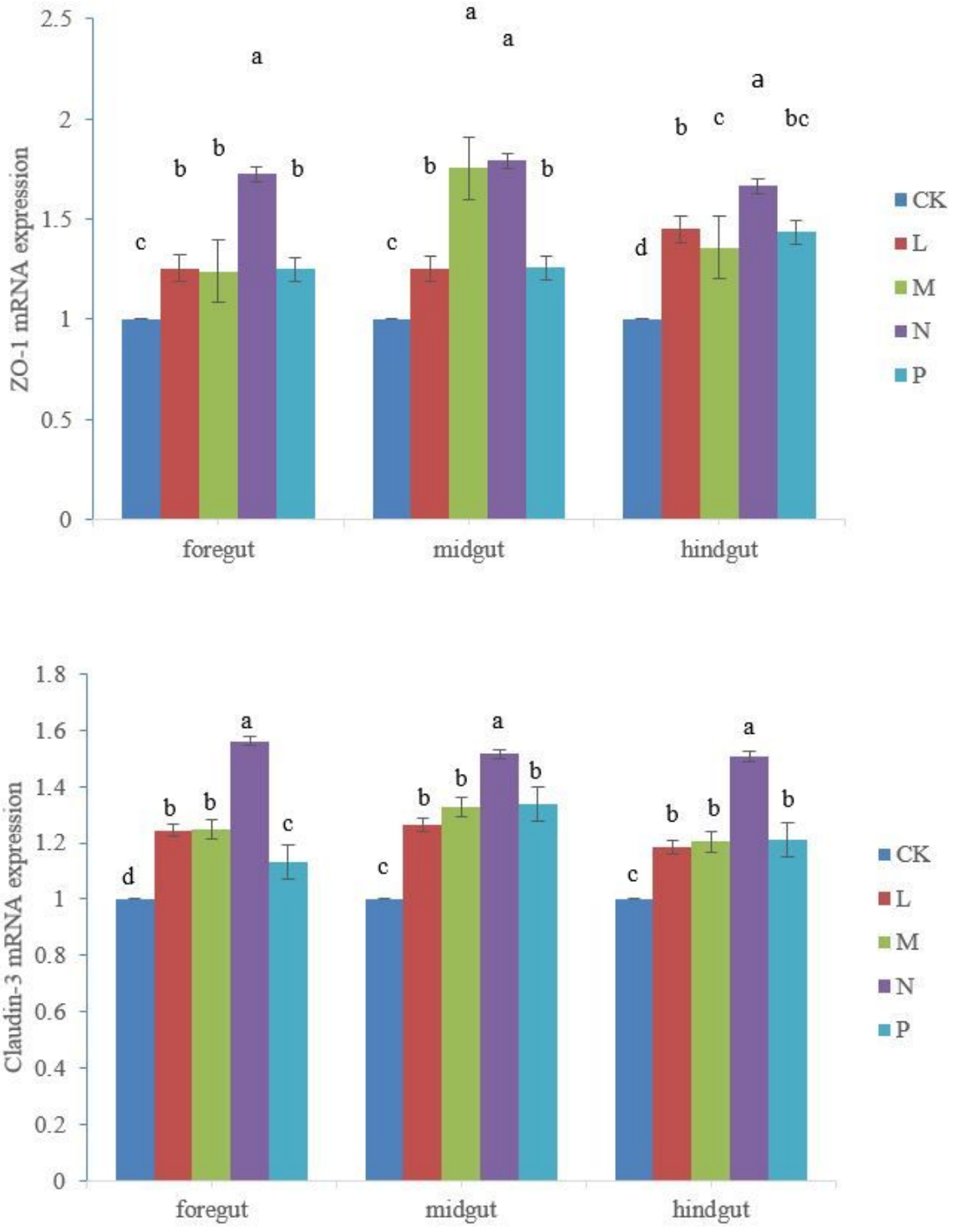

Figure 2

The effect of *Bacillus amyloliquefaciens*LSG2-8 on the expression of ZO-1 mRNA and Claudin-3 mRNA of *Rhynchocypris lagowskii*

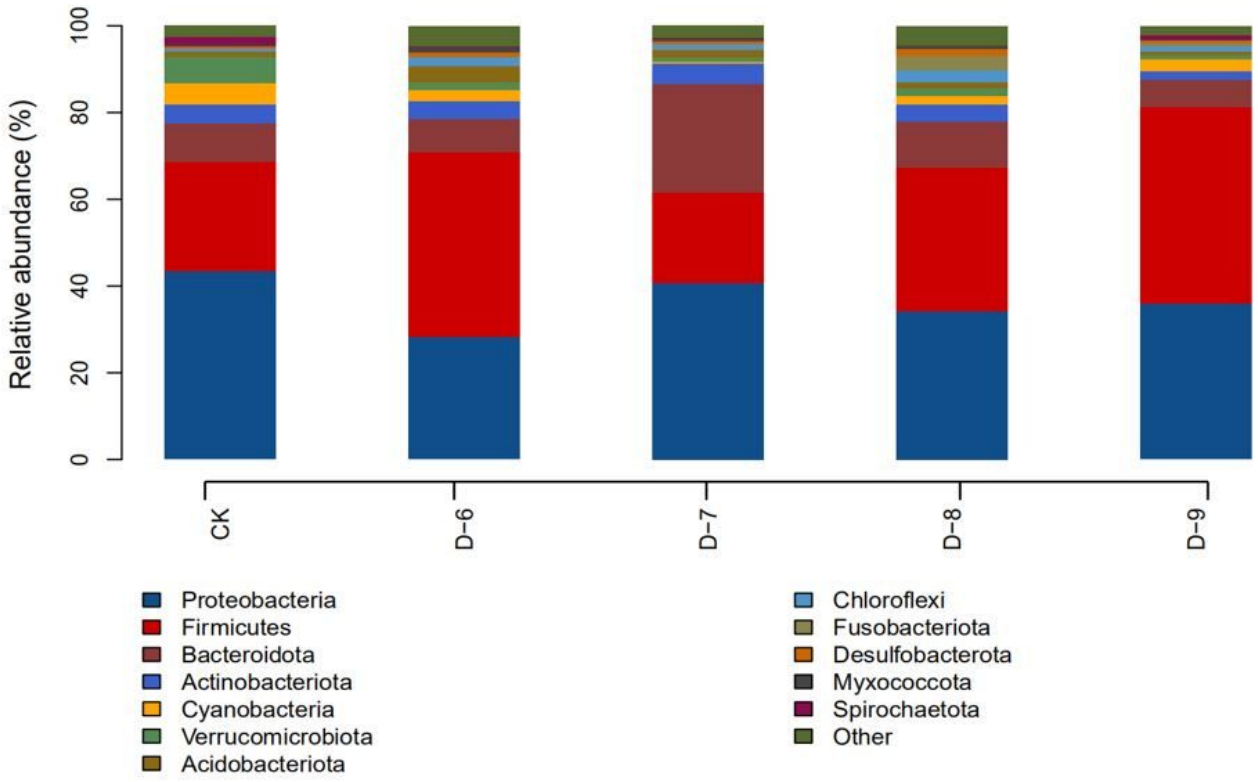

Figure 3

The effect of *Bacillus amyloliquefaciens* LSG2-8 on the phylum level in the intestine of *Rhynchocypris lagowskii*

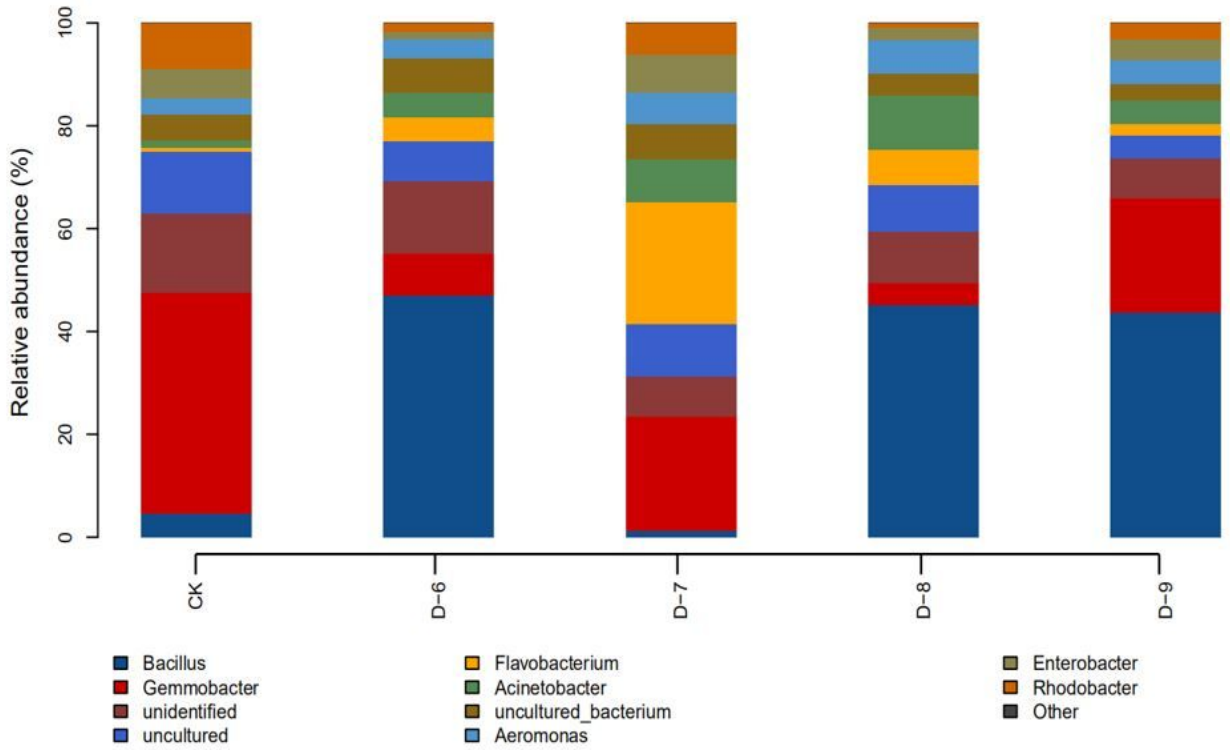

Figure 4

The Effect of *Bacillus amyloliquefaciens* LSG2-8 on the genus level in the intestine of *Rhynchocypris lagowskii*

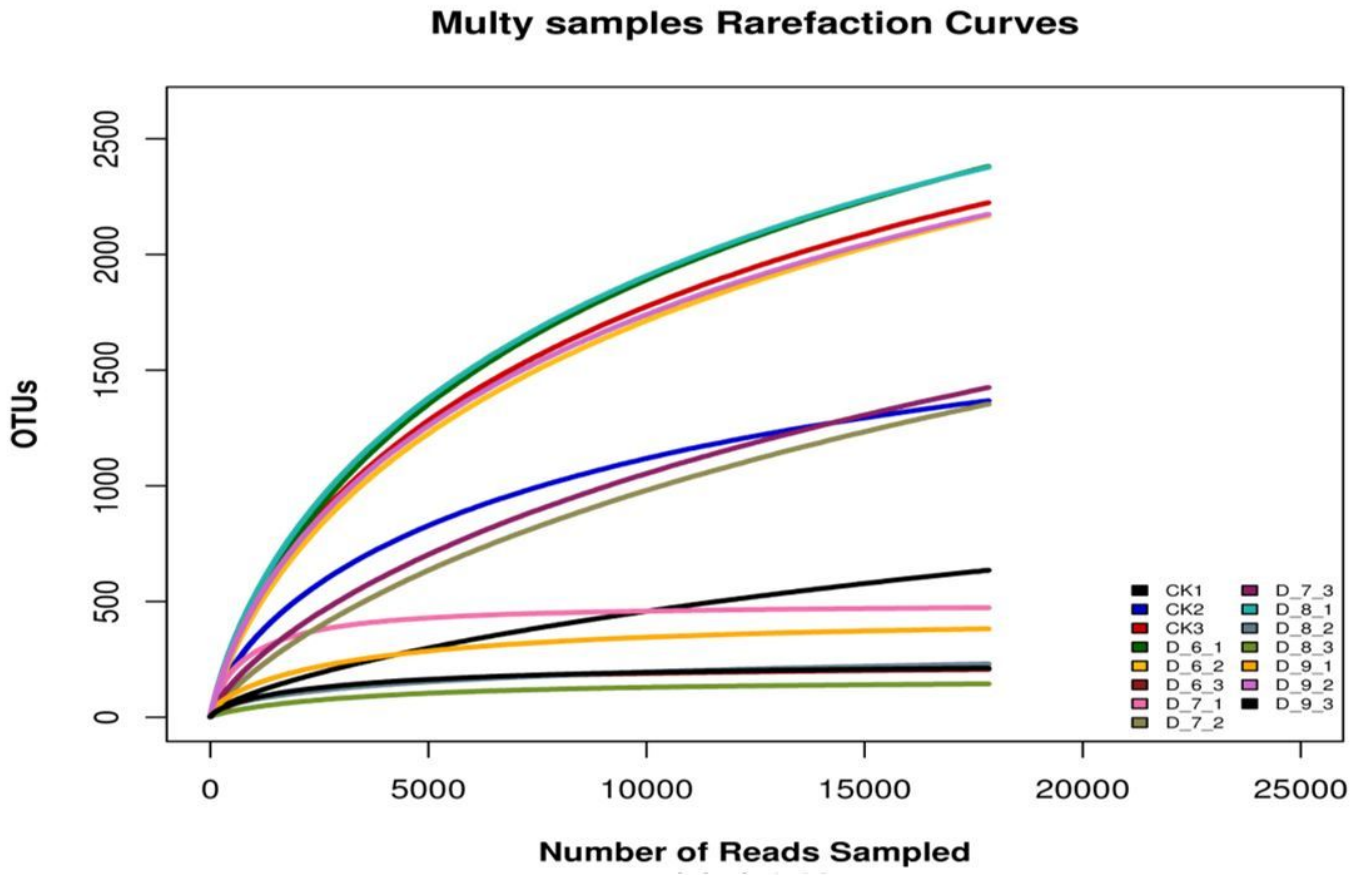

Figure 5

The spare cures of different sample

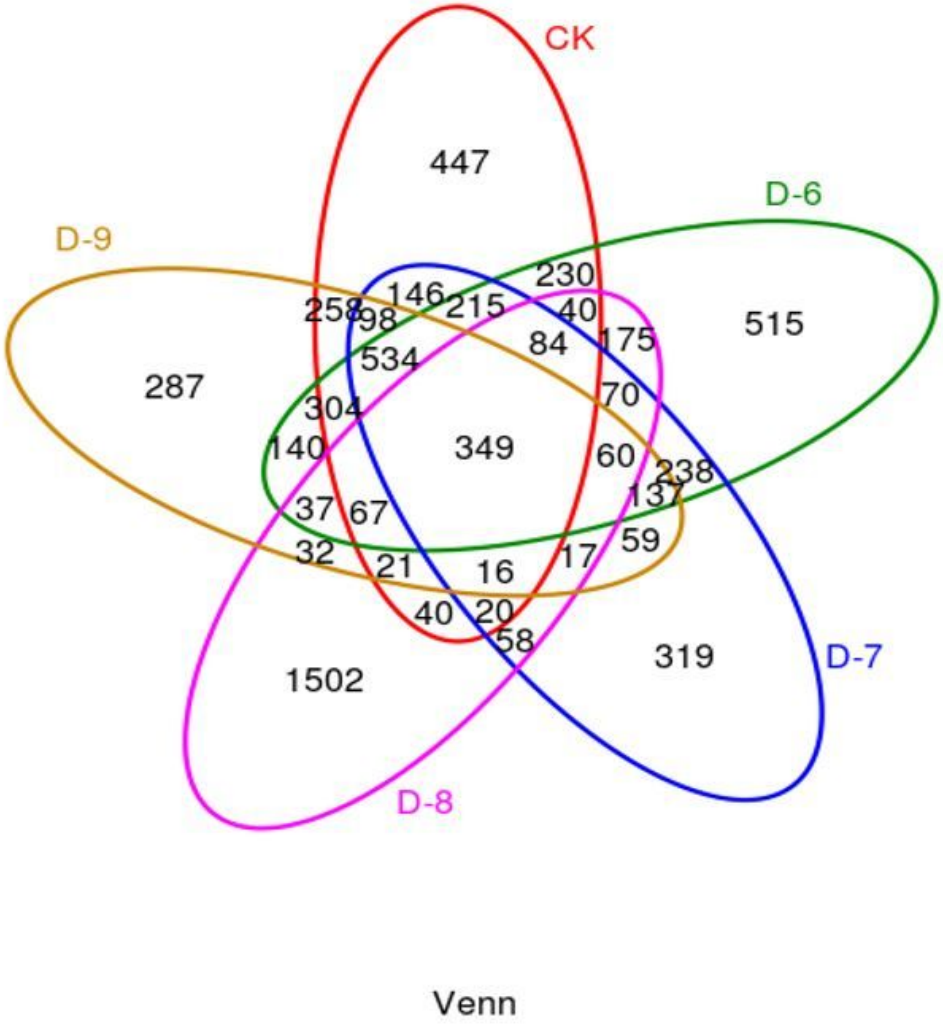

Figure 6

The Venn diagram of microbial flora in the intestine of *Rhynchocypris lagowskii*

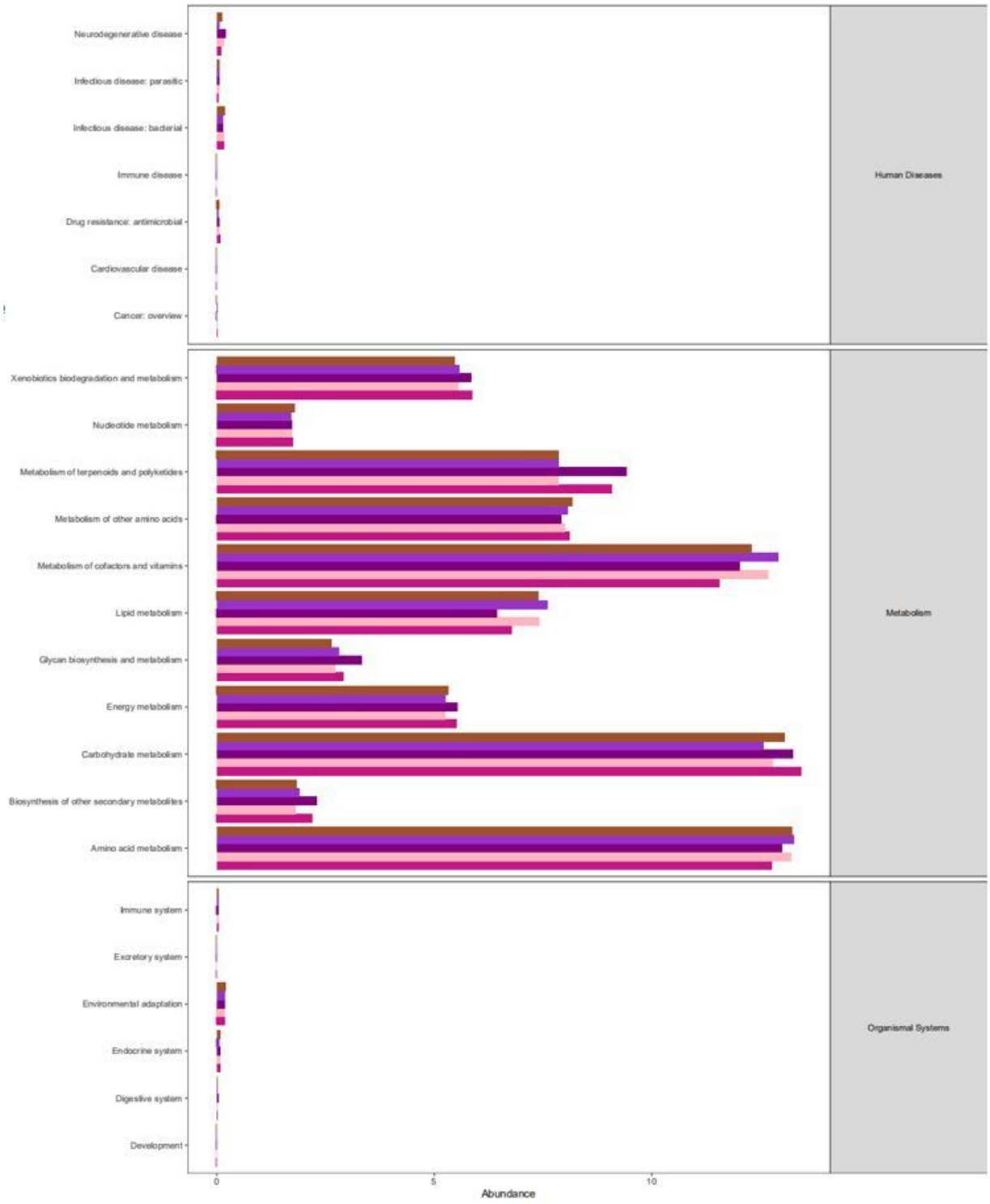

Figure 7

The KEGG second-level distribution map predicted by PICRUSt of the intestinal microorganisms of *Rhynchocypris lagowskii*

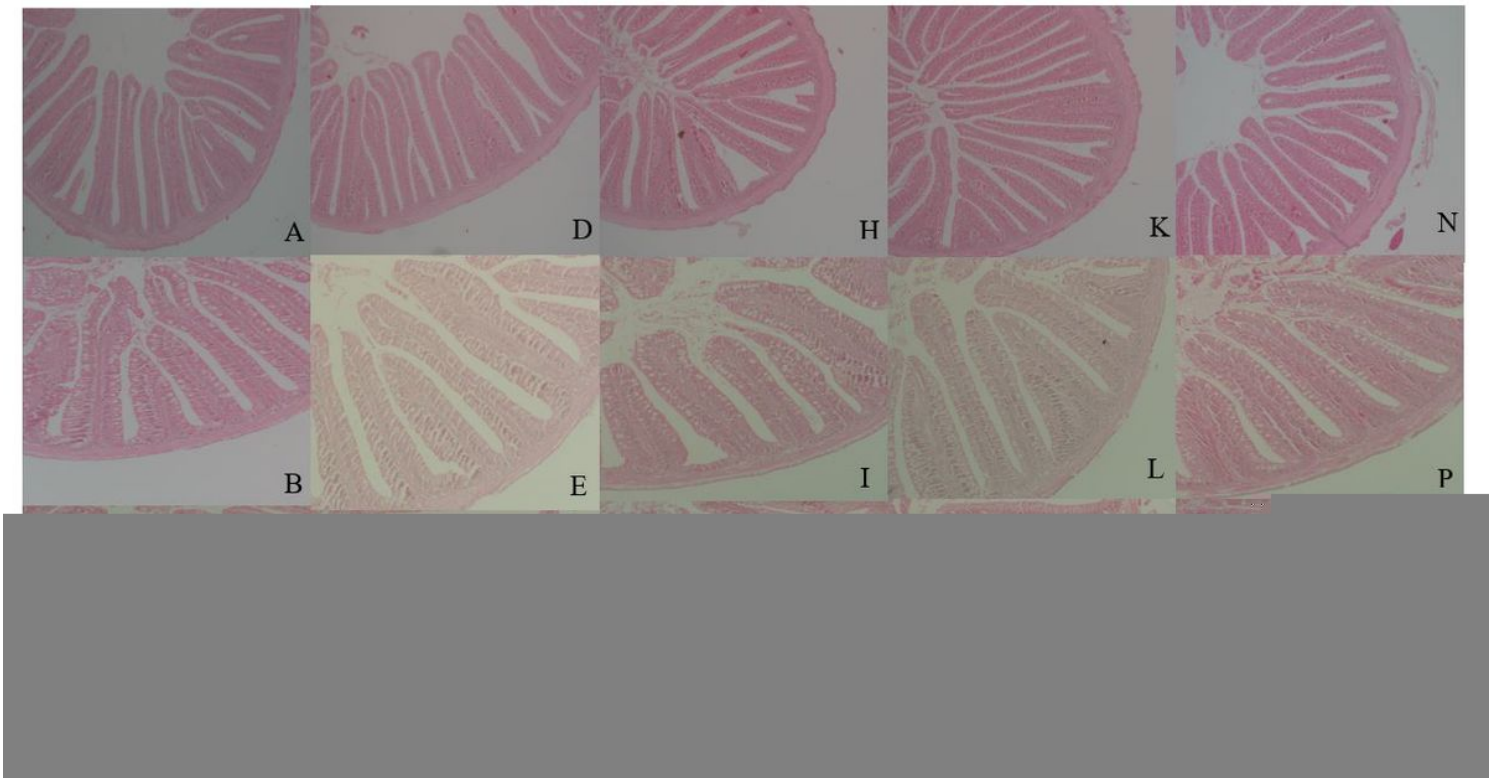

Figure 8

The Effect of *Bacillus amyloliquefaciens* LSG2-8 on the intestinal morphology of *Rhynchocypris lagowskii* (100×)

Note: A, B, and C are the foregut, midgut, and hindgut of *Rhynchocypris lagowskii* in control, D, E, F are the foregut, midgut, and hindgut of *Rhynchocypris lagowskii* in group D-6, H, I, J are the foregut, midgut, and hindgut of *Rhynchocypris lagowskii* in group D-7, K, L, M are the foregut, midgut, and hindgut of *Rhynchocypris lagowskii* in group D-8, N, P, Q are the foregut, midgut, and hindgut of *Rhynchocypris lagowskii* in group D-9.

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