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Streptomyces kunmingensis XK9 and galactooligosaccharide synergistically enhance growth performance, nonspecific immunity and disease resistance in striped catfish (*Pangasianodon hypophthalmus*)

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Streptomyces kunmingensis XK9 and galactooligosaccharide synergistically enhance growth performance, nonspecific immunity and disease resistance in striped catfish (*Pangasianodon hypophthalmus*)

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Abbreviated title: The beneficial of synbiotic containing *Streptomyces kunmingensis* XK9 and galactooligosaccharide in *Pangasianodon hypophthalmus*

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Abstract

The present study evaluated the effectiveness of the synergy of *Streptomyces kunmingensis* XK9 and galactooligosaccharides (GOS) in improving growth performance, nonspecific immunity, and protection of striped catfish (*Pangasianodon hypophthalmus*). Seven feed regimens were tested in striped catfish fingerlings, including commercial pellets supplemented with preparations S_7, S_8, and S_9 containing XK9 at 10^7 CFU/mL, 10^8 CFU/mL, and 10^9 CFU/mL, respectively; G_5 containing 5% GOS; S_7G_5, S_8G_5, and S_9G_5 including 5% GOS and XK9 at different doses of 10^7 CFU/mL, 10^8 CFU/mL, and 10^9 CFU/mL, respectively. After 3 months of treatment with different dietary regimens, weight gain, specific growth rate, weight gain rate, and feed conversion ratio were greatly improved in fish-fed diets containing S_9, S_8G_5, and S_9G_5 ($p<0.05$). Nonspecific immune parameters, including total white blood cell, phagocytic activity, and phagocytic index, increased consistently in fish that were fed diets containing synbiotics S_8G_5 and S_9G_5 ($p<0.05$). Lysozyme and complement activity increased substantially in fish that were fed diets supplemented with S_9G_5 ($p<0.05$). The protective effect of XK9 and GOS on striped catfish was evaluated after 15 days of challenge with *Edwardsiella ictaluri*. The results

achieved show that the cumulative mortality rate sharply decreased in fish fed a diet containing the synbiotic S_9G_5, down 2.17 times compared to the control group; pathogen density was lowest in tanks of fish that were fed regimens containing S_8G_5, S_9G_5, and S_9 ($p<0.05$). The results suggest that synbiotics, including *S. kunmingensis* XK9 and GOS, have the potential to be applied in sustainable farming for the striped catfish industry.

Key words: *Streptomyces*, GOS, growth performance, immune enhancement, resistance to disease, striped catfish

Farming of the striped catfish *(Pangasianodon hypophthalmus)* is one of the world's most crucial inland aquaculture industries (Griffiths et al., 2010). Global striped catfish production has increased from 113.2 thousand tons in 2000 to 2.52 million tons in 2020 (accounting for 5.1% of farmed fish production) (FAO, 2022), of which catfish that provided from Vietnam accounted for over 75% of world production (Nguyen et al., 2017).

According to Au and Ngan (2023), striped catfish is one of the main aquaculture species in Vietnam, and it is exported to more than 140 countries and territories worldwide. However, in recent years, Vietnam's catfish industry has faced many challenges due to market fluctuations, fierce competition from other producing countries, trade and technical barriers, and diseases, among which infectious diseases cause severe damage to many catfish farming areas (Dzung et al., 2022). In order to minimize the impact of disease, it is necessary to address the fish health constraints based on scientifically proven and recommended methods. For years, antibiotics for treating and controlling infectious diseases in fish have been in wide use in aquaculture (Austin, 2017); however, the use of antibiotics should be under strict control and subject to regulatory measures because of drug resistance and residue-related issues (Harikrishnan et al., 2011). In response to reduced antibiotic use in fish, various alternative solutions such as vaccines, herbal medicines, and probiotics have been applied as practical approaches to prevent and treat infectious diseases of fish (Ayalew and Fufa, 2018).

Probiotics have been used in aquaculture to improve growth performance, reduce feed conversion ratio, treat environmental pollution, enhance immunity, and resist to pathogens (Subedi and Shrestha, 2020). Common microorganisms used as probiotics are *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bacillus* sp., *Bifidobacterium bifidum*, *Lactococcus lactis*, and *Saccharomyces cerevisiae* (Ouwehand et al., 2002). *Streptomyces* is known as a probiotic in aquaculture due to its ability to generate antibacterial compounds (Desriac et al., 2020), to produce siderophores that inhibit *Vibrio* sp. (You et al., 2005), and to secrete extracellular enzymes which decompose organic compounds, enhancing the growth of aquatic livestock (Augustine et al., 2015). It is also recorded to be resistant to viruses that cause white spot syndrome in shrimp (Jenifer et al., 2015).

Prebiotics are administered to livestock to supplement nutrients for the host and their intestinal microbiota (Collins et al., 2009). Many prebiotics such as xylooligosaccharides (XOS), galactooligosaccharides (GOS), fructooligosaccharides (FOS), mannanoligosaccharides (MOS), beta-glucan, and inulin have been shown to improve growth in many different fish species (Abdelmalek et al., 2015; Yilmaz et al., 2007; Grisdale- Helland et al., 2008). Prebiotics improve fish health (Eshaghzadeh et al., 2015; Hoseinifar et al., 2016),

induce beneficial transformation of the intestinal microbiota, including total bacteria and lactic acid bacteria (Guerreiro et al., 2017), and can also act as immunostimulants (Mo et al., 2015; Ghafarifarsani et al., 2021) and disease resistance components (Zhu et al., 2023).

The present study evaluates the synergistic effect of *Streptomyces kunmingensis* XK9 (*S. kunmingensis* XK9, or XK9) and GOS as the synbiotics in stimulating growth, enhancing immunity, protecting fish, and minimizing pathogenic bacteria in the pond environment, withan aim at application in the striped catfish farming industry.

Material and methods

Material

The microorganism strains used in this study were provided by Hanoi Open University, including *S. kunmingensis* XK9, gene bank code OR122317; *Edwardsiella ictaluri* E01, gene bank code ON459710.1, LD_{50} for fingerlings $10^{5,29}$ CFU/mL (data from Project ĐTĐL-31/21 funded by the Ministry of Science and Technology of Vietnam). *S. kunmingensis* XK9 was grown in tryptone soya broth (TSB) (Himedia) to reach a density of 10^{10} CFU/mL; *E. ictaluri* E01 was grown in brain heart infusion (BHI) (Himedia), and then adjusted to reach a density of 10^6 CFU/mL.

Striped catfish fingerlings, with an average weight of 11.5 g, were specifically chosen for this study. They were purchased from a local farm in Tan Chau district, An Giang province, Vietnam.

The fish pellets used for feed were produced by the Feed One company in Vietnam (F0128). Nutritional components are listed in Table 1.

Table 1. The nutritional components of the pellets for catfish

Contents	Dry weight (%)
Moisture	11.0
Crude protein	28.0
Crude lipid	5.0
Crude fiber	7.0
Calcium	1.0
Total phosphorus	1.0
Total lysine	1.3
Methionine and cysteine (total)	0.9

Source: https://feedone.com.vn/products/cam-cho-ca-tra-f-0128.

Methods

Experimental design

Before the trials, striped catfish were randomly distributed into 200 L glass tanks (previously disinfected with 30 mg/L chlorine) at a density of 30 fish per tank. The fish acclimated for 7 days under controlled conditions with dechlorinated water, continuous aeration, temperature (28 ± 0.5°C), pH (7.5 ± 0.25), and dissolved oxygen (≥ 6.0 mg/L); 30% of the water was changed daily. During the trial, fish were fed on demand, twice daily (10:00 a.m. and 3:00 p.m.) with the diets including pellets supplemented with *S. kunmingensis* XK9 and GOS (Anhui Elite Co., Ltd), details as follows: diet G_5 contained 5% GOS, S_7G_5 contained 10^7 CFU/g XK9 and 5% GOS, S_8G_5 contained 10^8 CFU/g XK9 and 5% GOS, S_9G_5 contained

10^9 CFU/g XK9 and 5% GOS, S_7 contained 10^7 CFU/g XK9, S_8 contained 10^8 CFU/g XK9, S_9 contained 10^9 CFU/g XK9. In the control tank, fish that were fed only commercial pellets, as mentioned above. The experiment was carried out for 3 months.

Growth performance and feed conversion ratio

Growth parameters include weight gain (WG), specific growth rate (SGR %/day), weight gain rate (WGR %), and feed conversion ratio (FCR) calculated according to the following equations:

$$WG\ (g) = \text{final weight}\ (g) - \text{initial weight}\ (g)$$

$$SGR\ (\%/\text{day}) = [\text{Ln final weight}\ (g/\text{fish}) - \text{Ln initial weight}\ (g)] \times 100/\text{days}$$

$$WGR\ (\%) = [\text{final weight}\ (g) - \text{initial weight}\ (g)] \times 100/\text{initial weight}\ (g)$$

$$FCR = \text{feed intake}\ (g)/\text{body weight gain}\ (g)$$

Blood samples collection

At the end of each month during the trial, three striped catfish were randomly selected from each tank and then transferred to 10 L glass tanks that were filled with 3 L of water containing 250 µg/mL tricaine methanesulfonate (MS-222) to anesthetize the fish (Matthew et al., 2022). Fish blood from the caudal vasculature was sampled into tubes coated with heparin (100 IU) for hematological assays. Blood samples were aliquoted in 2 vials; one was centrifuged at 4°C for 15 minutes to collect plasma for the lysozyme and complement activity assay, and the other was stored at 4°C to enumerate total white blood cell (WBC) and determine phagocytic activity (PA) and phagocytic index (PI).

Total white blood cell

The blood samples were collected and diluted in Natt-Herrick solution (1:1000). The WBC were counted using a Neubauer hemocytometer. The WBC was calculated as follows: WBC (cells/µL) =(n × d)/ v (n: number of WBC in four marginal squares; d: the dilution 1000; v: volume of counting area is 4 mm^2 × 0.1 mm = 0.4 mm^3 = 0.4 µL).

Phagocytic activity and phagocytic index

Phagocytic activity and phagocytic index were evaluated according to the procedure described by Paderes et al. (2013) with the following modifications: the anterior kidney of the experimental fish was collected and ground in RPMI solution (Sigma-Aldrich). The suspensions were centrifuged at 4°C for 5 minutes, 1,500 rpm. Using a micropipette, 50 µL of supernatant was placed onto glass slides and then incubated at 28°C for 60 minutes to let macrophages adhere to the slides. The slides were washed with Hank's solution (Sigma-Aldrich), and 50 µL of 10^7 CFU/mL *Saccharomyces cerevisiae* stained with Congo red was dropped into the area coated with macrophages. The incubation of the slides was continued at 28°C for 120 minutes for phagocytosis. The slides were washed with PBS (Merck), the reaction area was fixed with methanol, and stained with Giemsa dye. The phagocytosis process was observed under a light microscope (×100). The PA and PI were determined by enumerating 100 phagocytes per slide under the microscope. The following equations were calculated for PA and PI:

PA (%)= (Number of phagocytic cells with engulfed yeast/number of phagocytes) × 100
PI = (number of engulfed yeast cells/number of phagocytic cells with engulfed yeast) × 100

Lysozyme activity

The standardization of the lysozyme activity methodology was determined according to Ellis (1990), in which the lysozyme activity was evaluated by the lysis of *Micrococcus luteus* (*Micrococcus lysodeikticus,* Sigma-Aldrich). The reaction was performed on 96-well plates. The striped catfish plasma was heated at 56°C for 30 minutes to degrade the complement, which could lyse the reference bacteria. In the control wells, 10 µL of lysozyme solution at concentrations of 0, 2, 4, 8, and 16 µg/mL was added, followed by 200 µL of *Micrococcus luteus* suspension at a concentration of 0.6 mg/mL in phosphate buffer, pH 6.2. In the experimental wells, 10 µL of experimental fish plasma and 200 µL of *Micrococcus luteus* (concentration as above) were added. The reaction plates were incubated at 28°C for 120 minutes; the results were measured using a spectrophotometer, wavelength 495 nm. Lysozyme activity was calculated based on the lysozyme standard curve.

Complement activity

Complement activity was determined according to the procedure outlined by Sunyer and Tort (1995). The reaction was performed in the 96-well plates. 10 µL of sheep red blood cells (5×10^7 cells/mL) was pipetted in the wells. Fish plasma was diluted to 1:10, 1:100, 1:200, 1:500, 1:1000 and placed into wells containing sheep red blood cells. Positive (100% lysis) and negative controls (spontaneous lysis) were also processed in each well by replacing fish plasma with buffer or distilled water, respectively. The reaction plates were held on a shaker at 28°C for 120 minutes and then centrifuged at 4°C for 10 minutes to avoid unlysed cells. Supernatants were transferred to flat-bottom microtiter plates, and the result was determined by the enzyme-linked immunosorbent assay plate reader at the absorbance measured at 415 nm. Complement activity is expressed as alternative complement hemolysis (ACH50 value- unit mL), which is the volume of plasma required to lyse 50% of sheep erythrocytes under standard conditions.

Challenge experiment

After two weeks of acclimatization and feeding with diets supplemented with XK9 and GOS as described above, the striped catfish were challenged with an intraperitoneal injection of *E. ictaluri* (density 10^6 CFU/mL), injection volume 0.2 mL/fish. After that, the fish continued to be fed with the diets as before the challenge. The fish were not challenged and fed daily with commercial pellets without XK9 and GOS in the negative control groups. In contrast, the fish were challenged and fed daily with the pellet without XK9 and GOS in the challenged control group. All the fish were continuously cultivated for 15 days under the above conditions, and the clinical signs and mortality were observed and recorded twice daily. During the experiment, the water in the tanks was not changed. Freshly dead and moribund fish were collected for bacterial confirmation. Mortality was considered only when *E. ictaluri* was retrieved from experimentally challenged fish (Koch's postulates) (Eissa et al., 2023). The cumulative daily mortality was calculated as follows: daily mortality rate (%) = 100 x (the number of fish that died each day/ the initial number of the experimental fish).

Quantification of pathogens persisting in the challenge tanks

E. ictaluri density was enumerated according to the method of Tuttle et al. (2023), with modifications. 250 µL of water in fish-challenged tanks was collected at day 15 post-injection and was serially diluted (10-fold) up to 10^{-3}. 50 µL of serial dilutions were then overlaid on EIM (*E. ictaluri* medium), that is, the selective medium for *E. ictaluri* (Shotts and Waltman, 1990). After that, the plates were incubated at 28°C for 24 hours. On EIM agar plates, *E. ictaluri* colonies are small, translucent, and greenish; *E. ictaluri* colonies were picked and cryopreserved in a 50% glycerol stock at −80 °C; a representative colony was used to extract genomic DNA for later confirmation by 16s rRNA gene sequencing. The density of *E. ictaluri* in the water of the experimental tanks was calculated according to the formula: N (cell/µL) = (n/50) x d (N: the density of bacteria, n: the average of *E. ictaluri* colonies in a plate, d: the dilution).

Statistical analysis

All experiments were carried out in triplicate. The results were expressed as mean values with the corresponding standard error (SE). The data were controlled for normality by the Shapiro-Wilk normality test and homoscedasticity through the Levene test for homogeneity of variances. Datasets were assessed for statistical significance using one-way analysis of variance (ANOVA). A post hoc Tukey test was undertaken to identify statistical differences between dietary treatments (SPSS 22.0 Software). Differences were considered statistically significant when $p<0.05$.

Results

Growth performance

Data in Table 2 show that diets supplemented with synbiotics or with either *S. kunmingensis* XK9 or GOS 5% significantly improved the growth performance of catfish compared to the control group ($p<0.05$). The weight gain (WG), weight gain rate (WGR), and specific growth rate (SGR) were improved the most and were equivalent in fish-fed diets containing S_8G_5 and S_9G_5 ($p>0.05$). These results highlight the potential of these diets for further investigation. The growth parameters of catfish in the group that consumed a diet containing S_9 were not significantly different from the groups S_8G_5 and S_9G_5 ($p>0.05$). Conversely, the value of WG, WGR, and SGR of fish that were fed a diet containing synbiotic S_7G_5 was lower than the three groups (S_8G_5, S_9G_5, and S_9) mentioned above ($p<0.05$) and did not differ from the groups that fed diets supplemented with S_7, S_8, and G_5 ($p>0.05$).

The diets containing the synbiotic XK9 and GOS or each of these ingredients also significantly impacted the feed conversion ratio (FCR) of striped catfish ($p<0.05$). The FCR of the fish that were fed diets containing S_8G_5 and S_9G_5 decreased sharply and equally ($p>0.05$) compared to the control group ($p<0.05$). The FCR in catfish fed the diets supplemented with S_8 and S_9 was slightly higher than the two groups, S_8G_5 and S_9G ($p<0.05$). The FCR in the fish that were fed diets to which synbiotic S_7G_5 was added was significantly higher than in the four groups mentioned above (S_8G_5, S_9G_5, S_8, and S_9) ($p<0.05$); however, this value was slightly lower than in catfish fed the diets supplemented with S_7 or G_5 ($p<0.05$). The FCR of catfish that

consumed the diet supplemented with only 5% GOS was the lowest compared to all fish groups that were fed diets containing XK9 and GOS ($p<0.05$) (Figure 1).

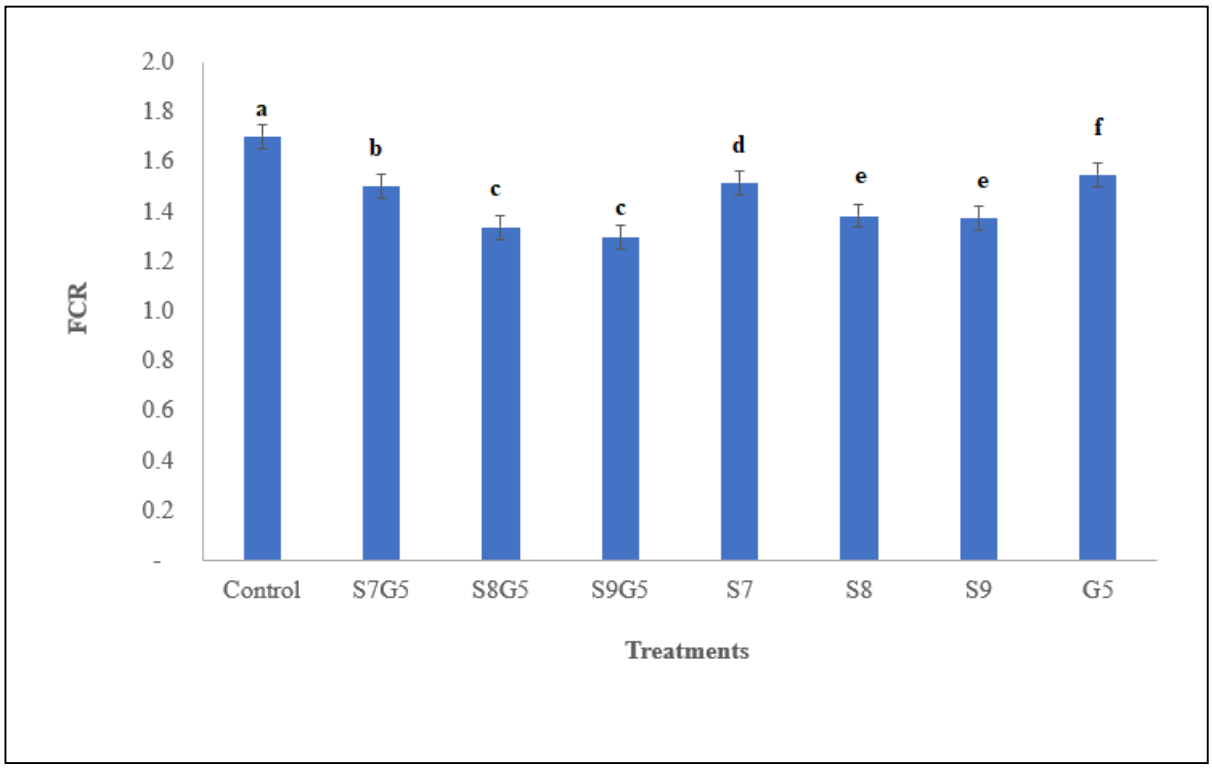

Figure 1. Effect of XK9 and GOS on feed conversion ratio

Data are presented as mean ± SE, n = 30.

a, b, c, d, e, f - values in columns with different letters differ significantly ($p<0.05$). Data was analyzed by one-way ANOVA and the Tukey test.

Abbreviations: FCR, feed conversion ratio; S_7G_5, the diet containing 10^7 CFU/g XK9 and 5% GOS; S_8G_5, the diet containing 10^8 CFU/g XK9 and 5% GOS; S_9G_5, the diet containing 10^9 CFU/g XK9 and 5% GOS; S_7, the diet containing 10^7 CFU/g XK9; S_8, the diet containing 10^8 CFU/g XK9; S_9, the diet containing 10^9 CFU/g XK9; G_5, the diet containing 5% GOS; control, the diet was only commercial pellets, without XK9 and GOS.

Table 2. Effect of XK9 and GOS on striped catfish growth performance

Dietary groups	FW (g)	WG (g)	SGR (%/day)	WGR (%)
S_7G5	$40.13^{a} \pm 0.29$	$28.60^{a} \pm 0.26$	$1.40^{a,c} \pm 0.00$	$248.67^{a} \pm 2.51$
S_8G_5	$44.67^{b} \pm 0.53$	$33.07^{b} \pm 0.53$	$1.50^{b} \pm 0.00$	$287.97^{b} \pm 4.63$
S_9G_5	$44.60^{b} \pm 0.40$	$33.10^{b} \pm 0.40$	$1.50^{b} \pm 0.00$	$287.57^{b} \pm 3.43$
S_7	$39.03^{a} \pm 0.33$	$27.53^{a} \pm 0.33$	$1.33^{a,c} \pm 0.33$	$239.4^{a} \pm 2.90$
S_8	$42.67^{c} \pm 0.33$	$31.17^{c} \pm 0.33$	$1.47^{b,c} \pm 0.03$	$271.00^{c} \pm 0.00$
S_9	$43.23^{b,c} \pm 0.17$	$31.73^{b,c} \pm 0.17$	$1.50^{b} \pm 0.00$	$276.07^{b,c} \pm 1.33$
G_5	$39.23^{a} \pm 0.13$	$27.73^{a} \pm 0.13$	$1.40^{a,c} \pm 0.00$	$241.00s^{a} \pm 1.10$
Control	$33.70^{d} \pm 0.10$	$22.20^{d} \pm 0.10$	$1.20^{d} \pm 0.00$	$193.33^{d} \pm 0.92$

Data are presented as mean ± SE, n = 30.

a, b, c, d - values in columns with different letters differ significantly ($p<0.05$). Data was analyzed by one-way ANOVA and the Tukey test.

Abbreviations: FW, final weight; WG, weight gain; WGR, weight gain rate; SGR, specific growth rate; S_7G_5, the diet containing 10^7 CFU/g XK9 and 5% GOS; S_8G_5, the diet containing 10^8 CFU/g XK9 and 5% GOS; S_9G_5, the diet containing 10^9 CFU/g XK9 and 5% GOS; S_7, the diet containing 10^7 CFU/g XK9; S_8, the diet containing 10^8 CFU/g XK9; S_9, the diet containing 10^9 CFU/g XK9; G_5, the diet containing 5% GOS; control, the diet was only commercial pellets, without XK9 and GOS.

Table 3. Effect of XK9 and GOS on nonspecific cellular immunity of striped catfish

Dietary groups	Total white blood cell (10^5 cell/μL)			Phagocytic activity (%)			Phagocytic index		
	M1	M2	M3	M1	M2	M3	M1	M2	M3
S_7G5	$2.14^{a} \pm 0.03$	$2.16^{a} \pm 0.03$	$2.19^{a} \pm 0.03$	$60.68^{a} \pm 0.41$	$60.42^{a} \pm 0.42$	$60.21^{a} \pm 0.21$	$2.56^{a} \pm 0.12$	$2.52^{a} \pm 0.05$	$2.59^{a} \pm 0.04$
S_8G_5	$2.31^{b} \pm 0.02$	$2.28^{b} \pm 0.02$	$2.32^{b} \pm 0.01$	$84.72^{b} \pm 0.21$	$84.64^{b} \pm 0.24$	$84.60^{b} \pm 0.17$	$3.26^{b} \pm 0.08$	$3.43^{b} \pm 0.05$	$3.38^{b} \pm 0.02$
S_9G_5	$2.40^{b} \pm 0.04$	$2.35^{b} \pm 0.03$	$2.34^{b} \pm 0.04$	$85.79^{b} \pm 0.37$	$85.82^{b} \pm 0.44$	$86.65^{b} \pm 0.77$	$3.38^{b} \pm 0.06$	$3.50^{b} \pm 0.07$	$3.52^{b} \pm 0.08$
S_7	$2.07^{a} \pm 0.01$	$2.05^{c} \pm 0.01$	$2.09^{a} \pm 0.02$	$59.60^{a,c} \pm 0.15$	$60.36^{a} \pm 0.46$	$61.86^{a} \pm 0.32$	$2.19^{c} \pm 0.05$	$2.25^{c} \pm 0.02$	$2.12^{c} \pm 0.03$

S_8	2.15^{a} ± 0.01	2.16^{a} ± 0.02	2.14^{a} ± 0.01	76.14^{d} ± 0.54	75.02^{c} ± 1.14	75.96^{c} ± 1.01	2.12^{c} ± 0.05	2.18^{c} ± 0.06	2.15^{c} ± 0.05
S_9	2.15^{a} ± 0.02	$2.14^{a,c}$ ± 0.03	2.13^{a} ± 0.01	79.59^{e} ± 0.88	79.06^{d} ± 0.42	78.63^{c} ± 0.24	$2.35^{a,c}$ ± 0.05	$2.37^{a,c}$ ± 0.05	2.44^{a} ± 0.05
G_5	1.62^{c} ± 0.00	1.60^{d} ± 0.01	1.60^{c} ± 0.04	58.09^{c} ± 0.21	57.53^{e} ± 0.30	58.03^{a} ± 0.53	1.60^{d} ± 0.12	1.61^{d} ± 0.03	1.56^{d} ± 0.05
Control	1.12^{d} ± 0.02	1.12^{e} ± 0.01	1.11^{c} ± 0.00	50.30^{f} ± 0.30	50.51^{f} ± 0.51	50.26^{d} ± 0.58	1.34^{e} ± 0.01	1.32^{e} ± 0.01	1.35^{d} ± 0.02

Data are presented as mean ± SE, n = 3.

a, b, c, d, e, f - values in columns with different letters differ significantly ($p<0.05$). Data was analyzed by one-way ANOVA and the Tukey test.

Abbreviations: M, month after dietary treatment; S_7G_5, the diet containing 10^7 CFU/g XK9 and 5% GOS; S_8G_5, the diet containing 10^8 CFU/g XK9 and 5% GOS; S_9G_5, the diet containing 10^9 CFU/g XK9 and 5% GOS; S_7, the diet containing 10^7 CFU/g XK9; S_8, the diet containing 10^8 CFU/g XK9; S_9, the diet containing 10^9 CFU/g XK9; G_5, the diet containing 5% GOS; control, the diet was only commercial pellets, without XK9 and GOS.

Table 4. Effect of XK9 and GOS on lysozyme and complement activity of striped catfish

Dietary groups	Lysozyme activity (μg/mL)			Complement activity		
	M1	M2	M3	M1	M2	M3 (U/mL)
S_7G5	174.85^{a} ± 2.71	177.21^{a} ± 3.65	$172.6^{a,e}$ ± 1.37	22.36^{a} ± 0.44	22.42^{a} ± 0.33	22.55^{a} ± 0.28
S_8G_5	224.13^{b} ± 4.88	221.30^{b} ± 2,43	229.45^{b} ± 2.19	25.90^{b} ± 0.13	26.24^{b} ± 0.34	26.56^{b} ± 0.35
S_9G_5	252.28^{c} ± 6.58	251.96^{c} ± 5.40	246.77^{c} ± 2.41	27.75^{c} ± 0.71	27.85^{c} ± 0.34	27.40^{b} ± 0.36
S_7	149.37^{d} ± 0.84	150.90^{d} ± 0.91	140.02^{d} ± 3.54	19.97^{d} ±0.15	19.90^{d} ± 0.15	20.62^{c} ± 0.38
S_8	$161.30^{a,d}$ ± 0.23	162.13^{d} ± 0.71	161.50^{e} ± 0.83	$23.03^{a,b}$ ± 0.11	22.84^{a} ± 0.16	$23.19^{a,d}$ ± 0.62
S_9	183.12^{a} ± 1.83	184.38^{a} ± 2.23	182.00^{a} ± 3.94	24.58^{b} ± 0.20	24.80^{e} ± 0.33	24.76^{d} ± 0.36
G_5	137.53^{d} ± 1.42	136.10^{e} ± 1.87	135.18^{f} ± 1.75	16.04^{d} ± 0.24	15.17^{f} ± 0.13	15.47^{e} ± 0.31
Control	121.25^{e} ± 0.78	118.64^{f} ± 1.76	122.08^{g} ± 0.99	12.82^{e} ± 0.26	12.68^{g} ± 0.36	12.76^{f} ± 0.28

Data are presented as mean ± SE, n = 3.

a, b, c, d, e, f, g - values in columns with different letters differ significantly ($p<0.05$). Data was analyzed by one-way ANOVA and the Tukey test.

Abbreviations: M, month after dietary treatment; S_7G_5, the diet containing 10^7 CFU/g XK9 and 5% GOS; S_8G_5, the diet containing 10^8 CFU/g XK9 and 5% GOS; S_9G_5, the diet containing 10^9 CFU/g XK9 and 5% GOS; S_7, the diet containing 10^7 CFU/g XK9; S_8, the diet containing 10^8 CFU/g XK9; S_9, the diet containing 10^9 CFU/g XK9; G_5, the diet containing 5% GOS; control, the diet was only commercial pellets, without XK9 and GOS.

Table 5. Effect of XK9 and GOS on *E. ictaluri* density in the water of experimental tanks

Dietary groups	S_7G5	S_8G_5	S_9G_5	S_7	S_8	S_9	G_5	Challenged control
E. ictaluri (CFU/ mL)	$568.00^{a} \pm 7.21$	$148.00^{b} \pm 2.31$	$103.00^{b} \pm 2.91$	$666.67^{a} \pm 35.28$	$266.00^{c} \pm 4.16$	$173.33^{b,c} \pm 7.69$	$2960.00^{d} \pm 41.6$	$5160.00^{e} \pm 34.64$

Data are presented as mean ± SE, n = 30.

a, b, c, d, e - values in columns with different letters differ significantly ($p<0.05$). Data was analyzed by one-way ANOVA and the Tukey test.

Abbreviations: S_7G_5, the diet containing 10^7 CFU/g XK9 and 5% GOS; S_8G_5, the diet containing 10^8 CFU/g XK9 and 5% GOS; S_9G_5, the diet containing 10^9 CFU/g XK9 and 5% GOS; S_7, the diet containing 10^7 CFU/g XK9; S_8, the diet containing 10^8 CFU/g XK9; S_9, the diet containing 10^9 CFU/g XK9; G_5, the diet containing 5% GOS; challenged control, fish were challenged and fed daily the diet was only commercial pellets, without XK9 or GOS.

Enhancement of nonspecific immunity

Synergism of XK9 and GOS or one of these components improved non-specific immune parameters in striped catfish, including WBC, PA, PI, lysozyme activity and complement activity compared to the control group ($p < 0.05$). During the three-month experiment, the values of WBC, PA, and PI were found to be highest in the groups fed diets supplemented with synbiotics S_8G_5 and S_9G_5 ($p<0.05$) and lowest in group G_5 ($p<0.05$) (Table 3). Meanwhile, lysozyme activity and complement activity were highest in the group fed the diet supplemented with synbiotic S_9G_5 ($p<0.05$) (Table 4). The data in Table 3 indicate that the density of WBC in fish that were fed diets containing synbiotic S_7G_5 or containing only XK9 at different concentrations (S_7, S_8, S_9) was equivalent ($p>0.05$) (Table 3). The PA value in group S_7G_5 was equivalent to group S_7 throughout the experimental period ($p>0.05$) and was substantially lower than groups S_8 and S_9 ($p<0.05$). The PI value of group S_7G_5 was equivalent to group S_9 ($p>0.05$) and significantly higher than groups S_7 and S_8 ($p<0.05$). For nonspecific humoral immune parameters such as lysozyme activity (La) and complement activity (Ca), the data in Table 4 show that the La values of groups S_7G_5 and S_9 are equivalent ($p>0.05$) and significantly higher than groups S_7 and S_8 ($p<0.05$). Meanwhile, the Ca values of groups S_7G_5 and S8 were similar ($p>0.05$) and consistently lower than those of group S_9 during the 3 months of experimental follow-up ($p<0.05$).

Protection of catfish from disease and quantification of pathogens persisting in the challenge tanks

After 15 days of the experiment, mortality was not observed in the negative control group (data not shown in Figure 2); in contrast, mortality cumulated at its peak on the eighth day (86.67%) in the challenge control group. In the groups of fish fed the diets supplemented with synbiotics, the cumulative mortality decreased substantially compared to the challenged control group, in which the lowest mortality rate was observed in group S_9G_5, a decrease of 2.17 times compared to the challenged control group. The mortality rate of the S_8G_5 and S_7G_5 groups decreased by 1.86 times and 1.37 times, respectively. *Streptomyces* has proven effective in protecting fish from *E. ictaluri* infection; compared to the challenged control group, the cumulative mortality rate in groups S_7, S_8, and S_9 decreased by 1.18 times, 1.30 times, and 1.37 times, respectively. Meanwhile, GOS added separately to the diet did not clearly show the ability to protect fish infected with *E. ictaluri*; the cumulative mortality rate of group G_5 was reduced by 1.08 times compared to the challenged control (Figure 2).

At the end of the challenge test, *E. ictaluri* was not detected in the water of the negative control tank. In contrast, the density of *E. ictaluri* was highest in the challenged control tanks (Table 5). Pathogen density was found to be lowest in the water of tanks where the fish were fed diets containing S_8G_5, S_9G_5, and S_9 ($p<0.05$). XK9 exhibited a significant role in minimizing the persistence of pathogens in challenged tanks; pathogen density in tanks S_8G_5, S_9G_5, and S_9 did not show a statistical difference ($p>0.05$); similarly, pathogen density in tanks S_7G_5 and S_7 did not differ significantly ($p>0.05$).

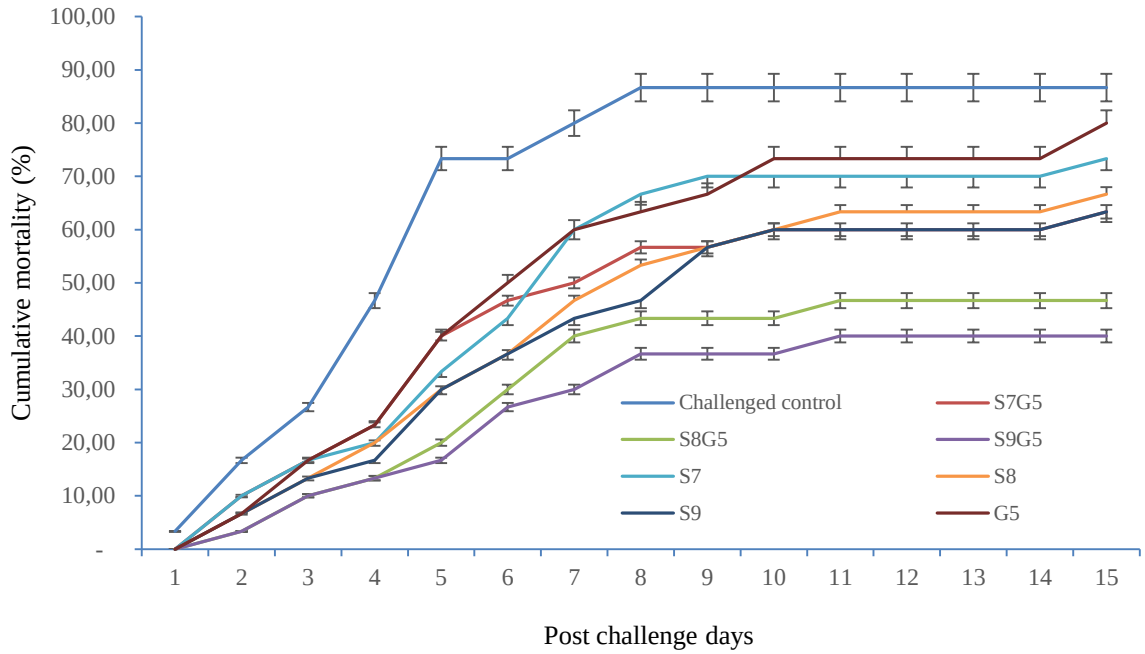

Figure 2. Effect of XK9 and GOS on the cumulative mortality of challenged fish

Data are presented as mean ± SE, n = 30.

Abbreviations: S_7G_5, the diet containing 10^7 CFU/g XK9 and 5% GOS; S_8G_5, the diet containing 10^8 CFU/g XK9 and 5% GOS; S_9G_5, the diet containing 10^9 CFU/g XK9 and 5% GOS; S_7, the diet containing 10^7 CFU/g XK9; S_8, the diet containing 10^8 CFU/g XK9; S_9, the diet containing 10^9 CFU/g XK9; G_5, the diet containing 5% GOS; challenged control, fish were challenged and fed daily the diet was only commercial pellets, without XK9 or GOS.

Discussion

In the present study, *S. kunmingensis* XK9 and GOS were added to catfish diets to improve growth performance, increase feed efficiency, enhance immunity and disease resistance, and reduce the persistence of those pathogens in water. Either of these two ingredients added to catfish diets undoubtedly improved the above parameters; however, the synergism of XK9 and GOS improved significantly all the observation parameters. These results are in parallel with Mohammad et al. (2022) who found that *Cyprinus carpio* fed a diet containing *Streptomyces chartreusis* as a probiotic demonstrated remarkably improved growth response and feed utilization. Another study reported that *Xiphophorus helleri* fed a diet supplemented with *Streptomyces* sp. showed improved performance and feed efficiency (Dharmaraj and Dhevendaran, 2010). Furthermore, *Streptomyces antibioticus* contributed significantly higher growth in freshwater catfish *Heteropneustes fossilis* (Das et al., 2021). The present findings show a more favorable picture of the effectiveness of prebiotics in synbiotics on the weight gain of catfish compared to the study of Wei et al. (2022), who reported that the inclusion of XOS and GOS did not significantly improve the weight gain of *Oreochromis niloticus* but significantly decreased the feed conversion ratio of the fish. The results of this study are similar to previous reports that *Penaeus monodon*-fed diets supplemented with *Streptomyces fradiae* and *Bacillus megateriumas* showed improved growth performance and fed efficiency (AftabUddin et al., 2018). Another report by Kaya et al. (2022) demonstrated that

the synergistic effect of *Bacillus clausii* and GOS led to significantly higher growth, improved FCR, and survival rates of *Neocaridina davidi*. The results of this study obtained from catfish fed the diets S_8, S_9, S_8G_5, and S_9G_5 also demonstrated the superior benefit of XK9 on the FCR of catfish compared to previous research reported by Boi et al. (2021), which showed that the FCR of farmed catfish in Soc Trang province, Vietnam, fluctuated from 1.45 to 1.65.

The results indicate that the inclusion of synbiotics of *S. kunmingensis* XK9 and GOS improved various nonspecific immune parameters such as total white blood cells (WBC), phagocytic activity (PA), phagocytic index (PI), lysozyme activity, and complement activity of catfish. The present findings are consistent with previous research conducted by Gunapathy et al. (2019), who found that *Labeo rohita* that were fed diets supplemented with probiotics and symbiotics had enhanced immune parameters, including WBC, PA, complement activity, and lysozyme activity. The report of Mohammad et al. (2022) showed that *Streptomyces chartreusis* significantly increased the lysozyme activity of *Cyprinus carpio*. Galactooligosaccharide have also been superior to other prebiotics in improving nonspecific immune parameters, including lysozyme and alternative complement activity in *Cyprinus carpio* (Seyed et al., 2016a). Also, taking a research-oriented approach to the effects of GOS on immunity enhancement in fish, Seyed et al. (2016b) reported on its ability to elicit positive effects of lysozyme activity and complement activity in *Rutilus frisii kutum* fry.

Regarding the synbiotics XK9 and GOS, besides being evaluated for improving growth performance and enhancing nonspecific immunity in catfish, they should also be studied to protect catfish from pathogens and control pathogens in the cultivated environment. In Vietnam, bacillary necrosis disease caused by *E. ictaluri* is the main cause of severe damage to catfish aquaculture (Phu et al., 2016), so in this study, *E. ictaluri* was selected as a pathogen to challenge catfish. The present findings suggest that XK9 plays a crucial role in protecting against disease. The effectiveness of controlling the mortality of challenged fish and pathogen concentrations in the water was greatly influenced by the dose of XK9. In the present trial, the lowest cumulative mortality was observed in fish fed the S_9G_5 diet, and the persistence of pathogen concentrations was lowest, with no significant differences in the tanks of fish that were fed the diets S_9, S_8G_5, and S_9G_5. The present results are confirmed by previous research conducted by Wenbin et al. (2021), who reported that the *Streptomyces virginiae* strain W18 reduced the mortality of *Carassius auratus* challenged with *Aeromonas veronii*. Similarly, research by Augustine et al. (2015) indicated that the *Streptomyces rubrolavendulae* strain M56 reduced the mortality rate of *Penaeus monodon* post-larvae infected with *Vibrio* sp. Furthermore, Xu et al. (2022) described that GOS significantly reduced mortality in hybrid sturgeon challenged with *Aeromonas hydrophila*.

Culture plating methods are considered highly standardized techniques for water safety monitoring (Tiwari et al., 2021). This technique is widely used to identify viable microbes. Monitoring the survival of infectious pathogens and determining if pathogens persist within commercial ponds is paramount because they are potentially the cause of disease. Referring to the study of Tuttle et al. (2023), *E. ictaluri* persisted in experimental tanks and was enumerated on selective EIM agar. The study results showed that at 15 days post-challenge, *E. ictaluri* was detected in the challenged control tanks with a concentration of 5.16×10^4 CFU/mL, similar to the data reported by Tuttle et al. (2023). The sharp decrease in *E. ictaluri* concentrations in

challenged tanks fed diets S_9, S_8G_5, and S_9G_5 showed that *S. kunningensis* XK9 effectively controlled the persistence of pathogens in the environment where infected fish lived.

The present findings show the positive synergistic impact of *S. kunningensis* XK9 and GOS in improving various parameters of the striped catfish, such as growth performance, feed conversion ratio, nonspecific immunity, disease resistance, and reduced pathogen persistence in the cultivating environment. The results suggest that supplementation with synbiotics, including 10^8 CFU/mL - 10^9 CFU/mL XK9 and 5% GOS, is recommended to be added to fish feed as a beneficial solution to increase the efficiency of industrial catfish.

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Institutional Review Board Statement

The experimental protocol of this study was approved by the Animal Ethics Committee of Hanoi Open University (protocol code 101/TB-HĐTVĐĐĐV, and date of approval is March 18, 2021).

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