Growth performance, survival, blood chemistry, and immune gene expression of channel catfish (*Ictalurus punctatus*) fed probiotic-supplemented diets.

By

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Abstract

The channel catfish (*Ictalurus punctatus*) farming industry is the largest and one of the oldest aquaculture industries in the United States. Despite being an established industry, production issues stemming from disease outbreaks remain problematic for producers. Supplementing fish diets with probiotics to enhance the immune system and growth potential is one approach to mitigating disease. Although considerable laboratory data demonstrate efficacy, these results do not always translate to natural modes of disease transmission. Hence, the present work was conducted in the laboratory but incorporated flow-through water from large catfish pond production systems, allowing for natural exposure to pathogens. Two feeding trials were conducted in an 18-tank aquaria system housing two different sizes, 34.8 ± 12.5 g and 0.36 ± 0.03 g, of channel catfish. Channel catfish in the first trial were fed three experimental diets over six weeks. Commercial diets were top-coated with two selected spore-forming *Bacillus* spp. probiotics, *Bacillus velezensis* AP193 (1×10^6 CFU g^{-1}) and BiOWiSH (3.6×10^4 CFU g^{-1}), or a basal diet that contained no dietary additive. In the second eight-week trial, diets were top-coated with BiOWiSH at three concentrations (1.8, 3.6, and 7.3×10^4 CFU g^{-1}), along with one basal diet (no probiotic). At the completion of these studies, growth performance, survival, hematocrit, blood chemistry, and immune expression of interleukin 1β (*il1β*), tumor necrosis factor-alpha (*tnf-α*), interleukin-8 (*il8*), transforming-growth factor β1 (*tgf-β1*), and toll-like receptor 9 (*tlr9*) were evaluated using qPCR. Trial results revealed no differences ($p > 0.05$) among treatments concerning growth, survival, or hematological parameters. For immune gene expression, interesting trends were discerned, with substantial downregulation observed in *B. velezensis* AP193-fed fish for *il1β*, *tnf-α*, and *tlr9* expression within splenic tissue, compared to that of the basal and BiOWiSH diets ($p < 0.05$). However, the results were not statistically

significant for anterior kidney tissue in the first trial. In the second trial, varied levels of probiotic inclusion revealed no significant impact of BiOWiSH's products on the expression of *il1β*, *tnf-α*, *il8*, and *tgf-β1* in both spleen and kidney tissue at any rate of probiotic inclusion ($p > 0.05$). Based on these findings, more research on utilizing probiotics in flow-through systems with natural infection conditions is crucial to ensure consistency from a controlled laboratory scale to real-world practices.

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1. Introduction

Concentrated in the southern part of the US, the channel catfish (*Ictalurus punctatus*) industry is well-established in the domestic aquaculture sector. This industry has a long developmental history, socioeconomics, coupled with vital research and extension programs. Modernized practices, such as applying intensive aeration in production and improved breeding programs has propelled catfish farming to become one of the largest and oldest aquaculture sectors in the US (Hargreaves, 2002). Economically, among other farmed freshwater fish, the catfish industry including the hybrid catfish that make up over 50% generally contributed about $352 million U.S. dollars in sales annually in 2021 (United States Department of Agriculture: Stuttgart, 2021). This is a sizable contribution given that the majority of the industry's revenue comes from four major states: Mississippi, Arkansas, Alabama, and Texas. Despite their tolerance of poor water quality and resilience to several infectious agents, as well as well-established business models, expanding the production of catfish and other farmed aquatic animals for human consumption is fraught with challenges connected to biotic and abiotic factors, notably disease outbreaks. In fact, pathogenic infections such as bacterial, fungal, and parasitic diseases have caused very high mortality in catfish aquaculture, including motile *Aeromonas* septicemia (MAS), enteric septicemia of catfish (ESC), and columnaris disease (Bilodeau & Waldbieser, 2005; Mohammed & Peatman, 2018; J. Pridgeon & P. Klesius, 2011; Shoemaker, Olivares-Fuster, Arias, & Klesius, 2008; Wagner, Wise, Khoo, & Terhune, 2002). Despite antibiotics' considerable efficacy in preventing and managing both infectious and non-infectious diseases, concerns about antibiotic resistance, costs and residue accumulation may outweigh the advantages of antibiotics in the long run, making them less sustainable (H. Chen et al., 2018; H. Chen et al., 2015; Santos & Ramos, 2018; Watts, Schreier, Lanska, & Hale, 2017). Numerous strategies have been put

evaluated to limit antibiotic usage, some of which use probiotics and herbal extracts or innovative methods such as vaccination or interference of quorum sensing via probiotics (Citarasu, 2009; N. V. Hai, 2015; Harikrishnan, Balasundaram, & Heo, 2011; G. Kumar et al., 2019; J. W. Pridgeon & P. H. Klesius, 2011; Reuter, Steinbach, & Helms, 2016; Ringø et al., 2020).

Among the possible antibiotic alternatives, feed additives, particularly probiotics, are economical, relatively simple to administer, and scalable depending on the size of the production operation. Probiotics have shown promise in preventing and managing pathogenic agents, contributing to better water quality, promoting animal health, and accelerating growth (Edwards, 2015; Li & Boyd, 2016; Van Hai, 2015). Probiotic amendments have demonstrated usefulness in various farming systems for many species ranging from teleost fish to crustaceans in improving innate immunity, competing for limiting factors, and decreasing the population of pathogenic bacteria to a tolerable density that limits illness risk, particularly by generating a healthy gastrointestinal microbiota that promotes fish growth (V. Kumar, Sinha, Makkar, De Boeck, & Becker, 2012; Luo, Bai, & Chen, 2014; Ringø & Song, 2016; Shelby, Lim, Yildirim-Aksoy, & Klesius, 2007). Various bacterial candidates have been identified and isolated for aquaculture application, which *Bacillus* spp. is most dominant within the commercially-available products, especially for dietary inclusion along with water-amended products using nitrifying bacteria (Gatesoupe, 1999). *Bacillus* spp. have been shown to reside in the intestinal tracts of several aquatic species, demonstrating its capacity to occupy the animal gut, and *Bacillus* spp. isolates derived from soybean or other plant rhizospheres may be well suited as amendments for soy-based fish feed (Ran etal., 2012; Kuebutornye, Abarike, & Lu, 2019). Although there are numerous accounts of significant proof of efficacy in the laboratory, these findings are not always transferable to production-scale settings or relevant to natural routes of disease transmission.

Interestingly, studies have shown that the efficiency of probiotics is significantly impacted by environmental conditions, which can either impair or negate the benefits of a microorganism-enriched diet (Ibrahim, Ouwehand, & Salminen, 2004; Srisapoome & Areechon, 2017; Thurlow et al., 2019).

In order to assess the effects of probiotics on channel catfish growth performance, survival, blood chemistry, and immune gene expression, two feeding trials using catfish production pond water were conducted. The flow-through water from the effluent of large catfish pond production system was utilized to more closely approximate traditional pond-rearing conditions.

2. Materials and Methods

2.1 Diet Preparation

The basal diet (BD) was formulated to 32% protein and 6.5% lipid (Table 1). The BD was made at Aquatic Animal Nutrition Laboratory at the School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University (Auburn, AL, USA), utilizing standard fish feed procedures. The pre-ground dry ingredients and oil were weighed and then incorporated for 15 min in a food mixer (Hobart Corporation, Troy, OH, USA). The mixture was then mixed with hot water to get a pellet-ready consistency. Diets were pressure-pelleted with a 3-mm die on a meat grinder.

2.2 Probiotics

Spores of the two probiotic strains were sprayed onto the BD as a topcoat. For *Bacillus velezensis* AP193, 0.025 g kg^{-1} of a lyophilized spore stock determined to be 4×10^{10} colony forming units (CFU) per g was suspended in 10 mL of distilled, deionized water and sprayed onto feed for a final concentration of 1×10^6 CFU g^{-1} (Table 2, B-AP). For BiOWiSH, the *Bacillus subtilis* FeedBuilder Syn3 spore stock was 7.2×10^7 CFU g^{-1} (BiOWiSH Technologies Inc., Cincinnati, OH, USA), which was suspended in distilled, deionized water according to

TABLE 1. Formulation and proximate composition of basal diet used in the feeding trials (% as is).

Ingredients[1]	Basal diet	Amino acids[1]	Basal diet
Poultry meal[a]	6.00	Alanine	1.60
Soybean meal[b]	55.50	Arginine	2.34
Menhaden fish oil[c]	3.59	Aspartic Acid	3.53
Corn Starch[d]	3.46	Cysteine	0.49
Corn[e]	28.00	Glutamic Acid	5.77
Mineral premix[f]	0.50	Glycine	1.64
Vitamin premix[g]	0.80	Histidine	0.86
Choline chloride[h]	0.20	Hydroxylysine	0.08
Rovimix Stay-C[i]	0.10	Hydroxyproline	0.25
CaP-dibasic[j]	1.85	Isoleucine	1.62
Proximate composition[1] (g/100g as is)		Lanthionine	0.04
Crude protein	33.7	Leucine	2.63
Moisture	6.57	Lysine	2.08
Crude Fat	4.85	Methionine	0.52
Crude Fiber	4.24	Ornithine	0.04
Ash	6.63	Phenylalanine	1.68
		Proline	1.76
		Serine	1.13
		Taurine	0.17
		Threonine	1.17
		Tryptophan	0.42
		Tyrosine	1.16
		Valine	1.76

[a]Tyson Foods, Inc., Springdale, AR, USA.

[b]De-hulled Solvent Extracted Soybean Meal, Bunge Limited, Decatur, AL, USA.

[c]Omega Protein Inc., Houston, TX, USA.

[d]MP Biomedicals Inc., Solon, OH, USA.

[e]Faithway Feed Co., Gunterville, AL, USA.

[f]Trace mineral premix (g/100g premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.250; Ferrous sulfate, 4.000; Magnesium sulfate anhydrous, 13.862; Manganese sulfate monohydrate, 0.650; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate heptahydrate, 13.193; Alpha-cellulose, 67.964.

[g]Vitamin premix (g/kg premix): Thiamin HCl, 0.438; Riboflavin, 0.632; Pyridoxine HCl, 0.908; Ca-Pantothenate, 1.724; Nicotinic acid, 4.583; Biotin, 0.211; folic acid, 0.549; Cyanocobalamin, 0.001; Inositol, 21.053; Vitamin A acetate, 0.677; Vitamin D3, 0.116; Menadione, 0.889; dL-alpha-tocoperol acetate, 12.632; Alpha-cellulose, 955.589.

[h]VWR Amresco, Suwanee, GA, USA.

[i]Stay-C® (L-ascorbyl-2-polyphosphate 35% Active C), Roche Vitamins Inc., Parsippany, NJ, USA.

[j]VWR Amresco, Suwanee, GA, USA

[l]Analysis conducted by University of Missouri Agricultural Experimental Station Chemical Laboratories (Columbia, MO, USA) (Results are expressed on g/100g of feed as is, unless otherwise indicated).

manufacturer's specifications, and sprayed onto feed for a final concentration in the first experiment of 3.6×10^4 CFU g^{-1} (Table 2, B-BW). In the second experiment, the final concentrations of the *B. subtilis* FeedBuilder Syn3 on feed were 0 (basal diet), 1.8, 3.6, and 7.2×10^4 CFU g^{-1} (Table 2; B-BW-L, B-BW, B-BW-H).

2.3 Water Quality

Dissolved oxygen was maintained near saturation using air stones in each culture tank, and the sump tank using a standard airline connected to a regenerative blower. During the trial, dissolved oxygen (DO), temperature, and salinity were monitored twice daily using a YSI 55 multi-parameter instrument (YSI, Yellow Springs, OH, USA). Total ammonia N (TAN) and nitrite-N were measured twice per week using YSI 9300 photometer (YSI, Yellow Springs, OH, USA). The pH of the water was measured twice weekly during the experimental period using the EcoSense pH10A (YSI, Yellow Springs, OH, USA).

2.4 Experiment A: Probiotic Assessment

The first 6-week experiment took place in a biosecure wet lab at E. W. Shell Fisheries Center of Auburn University, Auburn, Alabama, from March to May 2021. Ten juvenile channel catfish (34.8 ± 12.5 g) were randomly stocked into twelve aquaria (75 L) in a flow- through system utilizing natural water sourced from channel catfish production ponds. Catfish were hand-fed twice daily at ~4% body weight, and the ration was adjusted every 2 weeks. The probiotic spores were top coated on fish feed with a final concentration of *B. velezensis* AP193 at 1×10^6 CFU g^{-1} (B-AP), and for BiOWiSH, the final concentration was 7.2×10^4 CFU g^{-1} (B-BW). The control or basal diet without probiotic amendment was coated with distilled water. The diets were then air-dried for at least 12 h, stored at 4 °C, and used within 3 days of mixing. Each experimental diet

TABLE 2. Experimental diets abbreviations of probiotic types, inclusion levels, and concentrations fed to channel catfish.

Diet abbreviations	Probiotic	Dietary inclusion level (g kg^{-1})	Product stock concentration (CFU g^{-1})	Product concentration on feed (CFU g^{-1})
Experiment A				
BD				
B-AP	*B. velezensis*	0.025	4.0×10^{10}	1.0×10^{6}
B-BW	*B. subtilis*	0.5	3.6×10^{7}	3.6×10^{4}
Experiment B				
BD				
B-BW-L	*B. subtilis*	0.25	1.8×10^{7}	1.8×10^{4}
B-BW	*B. subtilis*	0.5	3.6×10^{7}	3.6×10^{4}
B-BW-H	*B. subtilis*	1.0	7.2×10^{7}	7.2×10^{4}

was administered to 6 replicate tanks for the study duration.

The study tanks received water from channel catfish production ponds with a mean water flow rate of 1 L min^{-1}. During the trial, the water quality was within range for normal growth (6.78 ± 0.13 mg L^{-1} dissolved oxygen, 0.36 ± 0.11 mg L^{-1} total ammonium nitrogen (TAN), 0.03 ± 0.02 mg L^{-1} nitrite, 0.14 ± 0.03 g L^{-1} salinity, and pH 8.36 ± 0.71), except for temperature (20.01 ± 0.33 °C) (Boyd, Romaire, & Johnston, 1979).

At the end of the feeding trial, fish were bulk weighed, and three fish were randomly collected from each tank, anesthetized with buffered tricaine methanesulfonate (MS-222), and bled from the caudal vein with a 1 mL syringe, and then fish were euthanized, and the spleen and anterior kidney tissues were collected.

Blood samples were collected in a 1.5 mL microcentrifuge tube without anticoagulant for biochemistry analysis. For hematocrit analysis, blood was collected in heparinized soda-lime glass micro-hematocrit capillary tubes (DWK Life Sciences LLC, Milville, NJ, USA) that were wax-sealed (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). Spleen and kidney tissues were collected and preserved in DNA/RNA Shield (Zymo Research, Irvine, CA, USA) within 1.5 mL microcentrifuge tubes for gene expression analysis. All growth metrics were calculated as follows:

$$Final\ weight\ (FW, g)\ = \frac{Total\ biomass\ (g)}{Number\ of\ fish\ at\ termination\ (g)}$$

$$Percent\ weight\ gain\ (\ WG; \%) = \frac{Final\ weight\ (g) - Initial\ weight\ (g)}{Initial\ weight\ (g)}\ x\ 100$$

$$Survival\ rate\ (SR; \%) = \frac{1 - Total\ recorded\ mortalities}{Number\ of\ fish\ at\ the\ start\ of\ the\ study}\ x\ 100$$

$$Thermal - unit\ growth\ coefficient\ (TGC)$$

$$= \frac{Final\ weight^{1/3} - Initial\ weight^{1/3}}{Temperature\ (°C)\ x\ Days}\ x\ 1000$$

2.5 Experiment B: Growth and Flow-Through with Juvenile Channel Catfish

The second 8-week growth trial was conducted from August to October 2021, using fingerling channel catfish (0.36 ± 0.03 g) randomly stocked into 18, 105 L fiberglass tanks in a flow-through system with the stocking density at 40 fish tank^{-1} using a natural water source from channel catfish production ponds. Fish were hand-fed twice daily at ~4% body weight, and the ration was adjusted every 2 weeks. There were four experimental diets, basal diet, and three inclusion levels of BiOWiSH FeedBuilder Syn3, with a final dosage on feed of 1.8×10^4, 3.6×10^4, and 7.2×10^4 CFU g^{-1} top-coated on feed. The inclusions represented 50, 100, and 200% of the recommended dose (B-BW-L, B-BW, and B-BW-H, respectively). The diets were left air-dried for at least 12 h, stored at 4 °C, and used within 3 days. The experimental diet was administered to 4 replicate tanks for 0.25 g kg^{-1} and 0.5 g kg^{-1} inclusion levels, while 0 g kg^{-1} and 1 g kg^{-1} had five replicate tanks for the study duration.

The study tanks received water from channel catfish production ponds, with the mean water flow rate at 1 L min^{-1}. Similar to the first trial, the water quality was within range for the normal growth for channel catfish (6.48 ± 0.04 mg L^{-1} dissolved oxygen, 0.14 ± 0.03 mg L^{-1} total ammonia nitrogen, 0.03 ± 0.01 mg L^{-1} nitrite, 0.19 ± 0.09 g L^{-1} salinity, 8.06 ± 0.09 pH, and temperature (27.51 ± 0.19 ◦C) (Boyd et al., 1979).

At the end of the feeding trial, fish were bulk-weighed, and three fish were collected, as previously described, for blood, spleen, and kidney samples. All growth parameters were calculated as similar to the first trial, with the addition of:

$$Feed\ conversion\ ratio\ (FCR) = \frac{Feed\ fed\ for\ the\ entire\ study\ (g)}{Biomass\ gained\ during\ study\ (g)}\ x\ 100$$

2.6 Hematocrit Analysis

Wax-sealed capillary tubes were spun down using a hematocrit IEC Clinical Centrifuge (International Equipment Co., Needham Heights, MA, USA) in 5 min using the instrument setting. The hematocrit percentage results were then read using a micro-capillary reader (International Equipment Co., Needham Heights, MA, USA).

2.7 Serum Biochemistry Analysis

Blood samples were allowed to clot at 4 ◦C overnight, followed by centrifugation at 15,000× *g* for 5 min to collect serum. Three serum samples from each tank were then pooled into one 100 µL composite sample. The serum biochemical parameters (alkaline phosphatase, alanine transaminase, gamma-glutamyl transferase, bile acids, total biliru- bin, albumin, blood urea nitrogen, and cholesterol) were determined by using Abaxis VetScan Mammalian Liver Profile on the Abaxis VetScan VS2 analyzer (Zoetis, Union City, CA, USA).

2.8 qPCR Gene Expression Analysis

RNA of spleen and kidney samples were extracted and purified using Quick-RNA Miniprep Kit (ZYMO Research, Irvine, CA, USA). Sample concentration was measured us- ing a NanoDrop Onec microvolume spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Extracted RNA samples were then diluted and standardized to 50 ng µL−1. All samples were then converted into cDNA using the High-Capacity cDNA Reverse Tran- scription Kit (Applied Biosystems, Waltham, MA, USA), according to the manufacturer's instructions. A total of 20 µL was used in the reaction, which included 2 µL of 10× R.T. buffer, 0.8 µL of 25× dNTP Mix, 2 µL of 10× R.T random primers, 1 µL of multiscribe reverse transcriptase, and 4.2 µL of

nuclease-free water. The cDNA was synthesized using a MiniAmp Plus thermal cycler (Applied Biosystems, Carlsbad, CA, USA). The thermal program was set at 25°C for 10 min, 37 °C for 120 min, and 85 °C for 5min. The RNA with a 25 ng μL^{-1} concentration was diluted to reach the concentration of 2.5 ng μL^{-1}. Experiment A utilized four genes: *il1β* (interleukin 1 beta), *tnf-α* (tumor necrosis factor alpha), *tlr9* (toll-like receptor 9), and *tgf-β1* (transforming growth factor beta 1) with a housekeeping gene (*18s* rRNA), while Experiment B used four genes *il1β*, *tnf-α*, *il8*, and *tgf-β1* with two housekeeping genes: *ef1α* (elongation factor 1 alpha) and *actb* (beta-actin) (Table 3). The efficiencies of the primers were determined by performing five serial dilutions, with a dilution ratio of 1:10, to achieve 90% to 110% efficiency for each gene. Totals of 5 µL of Powerup SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA, USA), 0.5 µL of each forward and reverse primer (stock concentration of 100 µM), 2 µL of nuclease-free water, and 2 µL of cDNA sample were used in each 10 µL reaction. Each sample was analyzed in duplicate, along with a negative control (nuclease-free water in place of a cDNA template). QuantStudio 5 Real-time PCR (Applied Biosystems, Carlsbad, CA, USA) was used for all runs, with cycle settings of 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 30 s. All relative quantifications were calculated according to the comparative Ct method ($2^{-\Delta\Delta Ct}$) (Schmittgen & Livak, 2008).

2.9 Statistical Analysis

All data were analyzed using R Version 4.2.1 (R Foundation for Statistical Computing, Vienna, Austria). The natural log transformation was performed on two parameters for blood biochemistry (ALP and ALT), and all relative gene expressions to meet the normality requirement (Bruce, Ma, Sudheesh, & Cain, 2021; Manera & Britti, 2006) . Outliers were detected and removed by using Dixon's test. Residuals were tested for normality using the Shapiro-Wilk

test and equivalent variances using Bartlett's test. Analysis of Variance (ANOVA) was used to compare treatment results, and significant outcomes were tested post hoc using Tukey's Honest Significant Difference for multiple comparisons. An *a priori* alpha value of a = 0.05 was used for all statistical analyses. The pooled standard error (PSE) was calculated as follows:

$$Pooled\ standard\ error\ (PSE) = \frac{Root\ mean\ squared\ error}{\sqrt{Average\ number\ of\ replicate}}$$

3. Results

3.1. Growth Performance

After six weeks, the two diets amended with *B. velezensis* B-AP and B-BW *B. subtilis* probiotics of Experiment A revealed no statistically significant changes ($p > 0.05$) in the final weight, percent weight gain, survival rate, or thermal-unit growth coefficient among treatments (Table 4). Experiment B yielded similar results after eight weeks, with no discernible difference for varying levels of inclusion in the final weight ($p = 0.122$), percent weight gain ($p = 0.090$), survival rate ($p = 0.715$), feed conversion ratio ($p = 0.228$), and thermal-unit growth coefficient ($p = 0.123$) (Table 5).

3.2. Hematological and Blood Serum Parameters

Similar patterns were identified for hematocrit and serum biochemistry parameters in Experiment A, despite various tendencies for bile acids, in which the basal diet presented the lowest level and comparatively lower hematocrit quantity for the *B. velezensis*-supplemented diet (B-AP). However, there was no significant difference discovered ($p = 0.462$**;** Table 6). Experiment B revealed a similar tendency for bile acids, which decreased with the B-BW treatment. In contrast, hematocrit fluctuated at different levels of probiotics inclusion, but there was no statistically significant difference ($p = 0.570$) (Table 7).