Evaluation of dietary immunostimulants and probiotics on growth performance and health indices of channel catfish, tilapia, and shrimp

by

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Abstract

Channel catfish, Nile tilapia, and whiteleg shrimp are three major aquaculture species worldwide. Growth performance and disease mortality are common bottlenecks within these culture systems. Feed supplementation with microorganisms or their byproducts is one approach to mitigate these challenges. Two studies investigated the effects of a commercial *Saccharomyces cerevisiae*-derived DVAQUA (postbiotic) at 0, 1, or 4 % inclusion levels in channel catfish and Nile tilapia diets over a 10-week feeding period. Immune-related gene expression, lysozyme activity, blood chemistry, and growth metrics were measured at the end of the feeding period. Tilapia were subsequently challenged by immersion of *Streptococcus iniae* (ARS-98-60) and catfish by immersion of *Edwardsiella ictaluri* (S97-773) or virulent *Aeromonas hydrophila* (ML09-119) to assess the potential of the postbiotic against common aquaculture pathogens. Nile tilapia resistance to *S. iniae* infection appeared to increase proportionally to the inclusion of DVAQUA, with up to 19 % relative percent survival for the 4 % inclusion (P=0.054). There was no significant mortality reduction for channel catfish with both pathogens (P>0.05). The impact of DVAQUA on channel catfish growth performances and blood chemistry was also measured over a 6-month feeding period. No differences were observed across lysozyme activity, blood chemistry, and growth metrics. In a third trial, Whiteleg shrimp were fed a *Bacillus velezensis* AP193 wild-type strain (1×10^7 CFU g^{-1}), an AP193 high-antimicrobial-producing strain (1×10^7 CFU g^{-1}), or BiOWiSH Feedbuilder Syn3®, a commercial *B. subtilis* probiotic (2×10^5 CFU g^{-1}) amended diet for two months. Shrimp were then challenged by immersion with *Vibrio parahaemolyticus* (A3). No significant differences were observed in growth performance, immune-related gene expression, or blood chemistry at the end of the feeding period. There was no apparent mortality reduction at the end of the pathogen challenge (P=0.988) among the different treatment groups. Further research

is needed to evaluate different concentrations of probiotics and their performance in high-production aquaculture systems.

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List of Abbreviations

ABW	Average body weight
AHPND	Acute hepatopancreatic necrosis disease
ALB	Albumin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMR	Antimicrobial resistance
AMY	Amylase
ANOVA	Analysis of variance
BHIA	Brain heart infusion agar
BHIB	Brain heart infusion broth
BUN	Urea nitrogen
BW	BiOWiSH ® FeedBuilder Syn3
Ca^{+2}	Calcium
cDNA	Complementary deoxyribose nucleic acid
CFU	Colony forming unit
CRE	Creatinine
DFO	Deferoxamine mesylate
DNA	Deoxyribose nucleic acid
DO	Dissolved oxygen
ESC	Enteric septicemia of catfish
FCR	Feed conversion ratio
GLOB	Globulin

GLU	Glucose
H2	*B. velezensis* AP193 mutant strain
K^+	Potassium
MAS	Motile *Aeromona*s septicemia
MOS	Mannan oligosaccharides
Na^+	Sodium
OD	Optical density
PAMP	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PER	Protein efficiency ratio
PHOS	Phosphorus
PO	Phenoloxidase
PRRs	Pattern recognition receptors
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RPM	Revolutions per minute
SGR	Specific growth rate
TAN	Total ammonia nitrogen
TBIL	Total bilirubin
TP	Total protein
TSA	Tryptic soy agar
TSB	Tryptic soy broth
WG	Weight gain

WT	*B. velezensis* AP193 wild-type strain
YYS	Yeast or yeast subcomponents
βG	β-glucan

Chapter 1:

Assessment of Yeast-based Immunostimulants in Cultured Catfish and Tilapia

1.1. Abstract

Channel catfish and Nile tilapia are two aquaculture species of great importance. Intensive production is often hindered by poor growth performance and disease mortality. This study aimed to evaluate the potential of a fermented yeast product, DVAQUA, on channel catfish and Nile tilapia growth performances and disease resistance. In a first trial, channel catfish were fed 0, 1, 2, or 4 g kg^{-1} of DVAQUA over a 6-month feeding period in a flow-through system. No significant differences were observed across lysozyme activity, blood chemistry, and growth metrics at the end of the feeding trial (P>0.05). In a second trial, channel catfish and Nile tilapia were fed 0, 1, or 4 g kg^{-1} of DVAQUA inclusion levels diets over a 10-week feeding period in a recirculated system. To assess the potential of the postbiotic against common aquaculture pathogens, juvenile catfish were subsequently challenged by immersion of *Edwardsiella ictaluri* (S97-773) or virulent *Aeromonas hydrophila* (ML09-119) and tilapia by immersion of *Streptococcus iniae* (ARS-98-60). Immune-related gene expression, lysozyme activity, blood chemistry, and growth metrics were measured at the end of the feeding period, but no differences were observed across the different metrics. There was no significant mortality reduction for channel catfish with both pathogens (P>0.05). Nile tilapia resistance to *S. iniae* infection appeared to increase proportionally to the inclusion of DVAQUA, with up to 19 % relative percent survival for the 4 g kg^{-1} inclusion (P=0.054). More research is needed to evaluate the effect of higher concentrations of DVAQUA and its impact on other aquaculture pathogens. These results suggest that this postbiotic may be beneficial in protecting Nile tilapia against *S. iniae* infection.

1.2. Introduction

Catfish and tilapia are two aquaculture species of considerable commercial importance, representing 9 and 11% of the global pisciculture production in 2021 (FAO, 2023). Expansion of the sector has been limited by infectious diseases and their impact on production yields (Rodger, 2016). One of the primary etiologic agents of piscine streptococcosis in marine and freshwater aquaculture worldwide is *Streptococcus iniae* (Agnew and Barnes, 2007; Heckman et al., 2020). The annual worldwide monetary loss was underestimated initially at $100M (Shoemaker, Klesius, and Evans 2001). Chinese producers, who represent the majority (26%) of global tilapia production ($13B), have reported losses of 30–80% due to streptococcsis (Ye et al., 2011; Chen et al., 2012). Motile *Aeromonas* septicemia (MAS) and enteric septicemia of catfish (ESC), respectively, caused by *Aeromonas hydrophila* and *Edwardsiella ictaluri*, are common pathogenic infections that hinder catfish aquaculture at both food-size and fingerlings stages (Wise et al., 2015; Mohammed and Peatman 2018; Griffin et al., 2020). According to the 2020 Alabama Catfish Disease Survey, MAS and ESC were responsible for 2.7 and 0.4 million pounds in losses, respectively. Furthermore, all three pathogens can infect tilapia and catfish (Chen et al., 2012; Soto et al., 2012; Lee and Wendy, 2017).

Oral administration of antibiotics in fish feeds is the most common treatment against bacterial infection. Ready access to antibiotics and limited availability of veterinary support result in uninformed and often unrecorded disease control, which poses a considerable threat to developing a sustainable aquaculture industry. This has led to increased antimicrobial resistance (AMR), which decreases the treatment efficacy for common bacterial diseases. Additionally, antibiotic residues in food fish and their products can lead to import refusals and negative impacts on international trade (Watts et al., 2017; Chen et al., 2020). Furthermore, studies have confirmed that the contamination of water bodies and sediments with residues of antibiotics causes stress on the aquatic environment (Hossain et al., 2017; Li et al., 2012).

Fish vaccination, as part of an informed veterinary health program, can lead to the success of antimicrobial reduction, such as the one achieved during the growth of Norway's aquaculture industry (Barnes et al., 2022). As an inexpensive and eco-friendly alternative to conventional methods, various medicinal plant compounds have been used for disease control since they enhance nonspecific immunity (Jeyavani et al., 2022). Other antibiotic alternatives are being developed, such as bacteriophage therapy or quorum quenching (Pereira et al., 2022; Shaheer et al., 2021). Probiotics and postbiotics have gained popularity as a scalable, economical, and practical treatment method for fish health (Bondad-Reantaso et al., 2023). Many benefits can be derived from their use, such as inhibition of harmful bacteria, synthesis of essential nutrients, enhancement of the host immune system, or reduction of eutrophic conditions (Santos et al., 2019; Thurlow et al., 2019; Knipe et al., 2021). Probiotics can leverage the interdependence between gut microbial composition and metabolite profiles to regulate host metabolisms like growth and immunity (Simón et al., 2021; Xia et al., 2020).

The yeast *Saccharomyces cerevisiae* contains various immunostimulating compounds such as β-glucans, nucleic acids, oligosaccharides, or chitin. β-glucans are complex polysaccharides that can be components of the cell wall of yeast and fungi and are therefore recognized by pattern recognition receptors (PRRs) as pathogen-associated molecular patterns (PAMPs). Thus, β-glucans have immunomodulatory properties in many organisms, inducing nonspecific protection against infections and stress, and they can be used as part of both prophylactic and therapeutic strategies (Pogue et al. 2021). Mannan oligosaccharides (MOS) of *S. cerevisiae* are undigestible for fish but can stimulate the growth of bacterial species that inhabit the digestive tract (Del Valle, Bonadero, and Fernández-Gimenez 2023). DVAQUA is an *S. cerevisiae* fermentation postbiotic composed of multiple compounds obtained by proprietary fermentation technology.

To assess the effects of this novel yeast product on channel catfish, a 6-month feeding trial was performed using flow-through pond water where growth performance and blood chemistry were measured. The potential of DVAQUA as an immunostimulant was evaluated after a 2-month feeding period followed by infection challenge with virulent *A. hydrophila* and *E. ictaluri* for channel catfish and with *S. iniae* for Nile tilapia. Growth performance, survival, blood chemistry, lysozyme activity, and immune gene expression were measured and compared against a yeast-free diet.

1.3. Materials and methods

1.3.1. Experiment A: Catfish long-term growth trial

Channel catfish fingerlings of 1.7 g average body weight were stocked in 60L aquariums (100 fish per aquarium) supplied with pond water in a flow-through system. Fish were fed four different diets containing 0, 1, 2, or 4g kg^{-1} of DVAQUA, and each diet was distributed to five aquaria as experimental replicates. The diet was 43.1 % protein, 7.3 % lipid, 4.6 % fiber, and 8.5 % ash. Animals were fed to apparent satiation, and distributed feed was recorded. Water temperature and dissolved oxygen were measured twice daily (YSI-55 digital oxygen/temperature meter, YSI corporation, Yellow Springs, Ohio, USA). Total ammonia nitrogen (TAN) concentration was measured weekly using colorimetric analysis (YSI 9300 photometer, YSI corporation, Yellow Springs, OH, USA). To maintain an appropriate density in the aquaria during the growth period, animals from the same treatment were pooled together at the 9^{th}, $16^{th,}$ and 20^{th}

week, and aquaria were evenly restocked with fewer individuals to a density of 13, 9 and 12 kg m^3 respectively. Catfish were bulk-weighed every month to measure growth performance metrics:

Growth rate (%) = 100 × (final weight − initial weight)/initial weight

Specific growth rate = 100 x (ln final weight – ln initial weight) / days of feeding

Thermal growth coefficient = (final weight /3 − initial weight /3)/Σ (Temp × days) × 100

Survival rate (%) = 100 × Total number of animals harvested / Total number of animals stocked

Feed conversion ratio (FCR) = Feed given (as is)/weight gain (wet weight)

Protein efficiency ratio = weight gained / weight of protein consumed

1.3.2. Experiment B: Channel catfish juvenile short-term growth trial and disease challenge

Channel catfish fingerlings (n=125) of 11.2±0.08g average body weight were stocked in 120L tanks in a recirculated system. Fish were fed on three different diets containing 0, 1, or 4 g kg^{-1} of DVAQUA, and each diet was distributed to three tank replicates using the same diet listed above. Animals were fed to apparent satiation for 50 days, and distributed feed was recorded. Total ammonia nitrogen (TAN) was measured every week, while dissolved oxygen (DO) and temperature were measured daily in the morning and afternoon. Fish were bulk-weighed at the end of the feeding period to measure growth performance metrics, as described above.

At the end of the 50-day growth period (c.f. 1.3.2), fish from each treatment were pooled and transferred to a system supplied with dechlorinated and UV-sterilized city water. Each 50-L tank was stocked with 25 fish from the respective diet, and each diet was either exposed to *E. ictaluri*, virulent *A. hydrophila,* or pathogen-free culture media (mock-challenged). Each modality of infection was replicated three times, while the mock treatments were unique (Figure 1A). Diets were maintained during the entire challenge. Mortality was recorded, necropsies were performed on 20 % of the total daily mortality, and reisolated colonies were confirmed by PCR for each pathogen.

Edwardsiella ictaluri (S97-773) was revived from a frozen glycerol stock onto brain heart infusion agar (BHIA; Becton Dickinson, Sparks, MD, USA) according to the manufacturer's recommendation to ensure purity. After 48 h of growth, three colonies were inoculated into 20 mL of brain heart infusion broth (BHIB; Becton Dickinson, Sparks, MD, USA) and grown at 28 °C and 175 rpm in a shaker. After 18 h of incubation, 100 µL of culture were transferred to 250 mL of BHIB and incubated at 28°C and 175 rpm for 18 h. The optical density (OD) of the inoculum was measured at 600nm and adjusted to 1.095 (8.55×10^8 CFU mL^{-1}) through dilution with sterile BHIB. Immersion of the pathogen was performed statically in 10 L of water with 4 mL of bacterial inoculum for 30 min, after which the water flow was resumed to 0.3 L min^{-1}.

Virulent *Aeromonas hydrophila* (ML09-110) bacterial culture for the immersion challenge was grown according to Peatman et al. (2018). Briefly, bacteria were revived from frozen glycerol stock onto tryptic soy agar for *Aeromonas hydrophila* (TSA; Becton Dickinson, Sparks, MD, USA) according to the manufacturer's recommendation to ensure purity. After 24 hours of growth, a single colony was inoculated into 20 mL of tryptic soy broth (TSB; Becton Dickinson, Sparks, MD, USA) and grown at 28 °C and 150 rpm in a shaker for 24 h. The culture was then expanded into 1L TSB under the same conditions described above for 18h. All TSB media were supplemented with iron chelator (xenosiderophore) deferoxamine mesylate (DFO; Sigma, St. Louis, MO, USA) at a final concentration of 0.4 mM. The final OD was read at

600nm and adjusted to 2.037 (1.68×10^9 CFU mL^{-1}). Immersion of pathogen was performed after clipping fish adipose fin under static conditions within 10 L of water. A total of 100 mL of bacterial inoculum was added to the tank for 60 min, after which the water flow was resumed to 0.3 L min^{-1}.

1.3.3. Experiment C: Nile tilapia juvenile short-term growth trial and disease challenge

Nile tilapia fingerlings of 5.7g average body weight were stocked into 21, 60 L aquaria at a density of 20 fish per aquaria in a recirculating system. Fish were fed three diets containing 0, 1, or 4g kg^{-1} of DVAQUA, and each diet was distributed to seven aquaria. Animals were fed to apparent satiation for 69 days, and distributed feed was recorded. As mentioned above, fish were bulk weighed at the end of the feeding period to measure growth performance. At the end of the 69-day growth trial, fish from each treatment were pooled and transferred to a system supplied with dechlorinated and UV-sterilized city water. Each tank was stocked with 13 fish from the respective diet, and each diet was either exposed to *Streptococcus iniae* or pathogen-free media (mock). Each modality of infection was replicated three times, while the mock treatments were unique (Firgure 1B). Diets were maintained during the entire challenge. Mortality was recorded, necropsies were performed on 20 % of the daily mortality, and reisolated colonies were confirmed via PCR for the specific pathogen.

Streptococcus iniae (ARS-98-60) was revived from frozen glycerol stock onto sheep blood agar to ensure purity. After 24 hours of growth, one colony was inoculated into 5ml of TSB and grown at 28 °C and 175 rpm in a shaker for 24 hours. The OD of the inoculum was measured at 540nm and adjusted to 1.572 through dilution with sterile TSB. Two ten-fold (4 mL into 36 mL TSB) serial dilutions were performed to obtain a bacterial inoculum at 1.04×10^7

CFU mL^{-1}. Fish were challenged by intraperitoneal injection of 0.1 mL of bacterial inoculum or sterile TSB.

1.3.4. Gene expression analysis by real-time PCR

At the end of catfish and tilapia growth trials (1.3.2 and 1.3.3), fish were sampled for anterior kidney and spleen. The organs were kept in DNA/RNA Shield ™ (Zymo Research, Irvine, CA, USA) and stored overnight at 4 °C prior to storage at -20 °C. Organs were thawed on ice prior to RNA extraction with Quick-RNA Miniprep Kit (Zymo, USA). RNA concentrations were diluted to 50 ng uL^{-1} and transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). Final cDNA was diluted to 1.25 ng uL^{-1} and stored at 25 °C. Each 10 uL qPCR reaction was carried with the following components: 5 uL of SYBR™ Green PCR Master Mix (Thermo Fisher Scientific), 0.4 uL of forward primer (10mM), 0.4 uL of reverse primer (10mM), 2.2 uL of nuclease-free water, and 2 uL of sample cDNA (1.25 ng uL^{-1}).

Experiments B and C utilized four genes of interest and two genes of reference. The efficiencies of the primers were determined at 100 ± 10 % for each gene (Table 1). Each PCR was performed with duplicate samples, and the cycling conditions were 2 min at 50 °C, 2 min at 95 °C followed by 15 s at 95 °C, 15 s at 58 °C and 30 s at 72 °C for 40 cycles. A melt curve analysis was performed after amplification to verify the accuracy of each amplicon and non-template controls (nuclease-free water in lieu of cDNA). The relative abundance of interested genes was calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

1.3.5. *Sera lysozyme activity and blood biochemistry*

Blood samples were collected at the end of catfish and tilapia growth trials (*Experiment A: Catfish long-term growth trial*; *Experiment B: Channel catfish juvenile short-term growth trial* and *Experiment C: Nile tilapia juvenile short-term growth trial*). Fish were anesthetized with 100 mg L^{-1} tricaine methanesulfonate (MS-222; Syndel, Ferndale, WA, USA) buffered in rearing water, and blood samples collected with sterile syringes from the caudal vein around the caudal peduncle into 1.7 mL microcentrifuge tubes. Samples were left to clot overnight at 4°C before centrifuging 5 min at 10,000 x *g* for sera collection into a sterile 1.7 mL microcentrifuge tube. Sera was stored at -20 °C until processing. In a 96-well microtiter plate, 250 µL of 0.25 mg mL^{-1} suspension of *Micrococcus lysodeikticus* (Sigma Chemical, St Louis, MO, USA) in 0.05 mol L^{-1} sodium phosphate buffer (pH 6.0) was added to 10 µL of the serum. The OD_{590} reading was recorded at 0 and 25 min of incubation at 35 °C. A unit of lysozyme activity was defined as the reduction in absorbance after incubation (Lange et al., 2001).

Sera samples were also analyzed using a VetScan® VS2 analyzer (Abaxis Inc., Union City, CA). A total of 100 µL of serum was used to determine the following parameters using a Comprehensive Diagnostic Rotor: Albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase (AMY), total bilirubin (TBIL), urea nitrogen (BUN), calcium (Ca^{+2}), phosphorus (PHOS), creatinine (CRE), glucose (GLU), sodium (Na^{+}), potassium (K^{+}), total protein (TP), and globulin (GLOB).

1.3.6. *Statistical analysis*

All data were analyzed using IBM SPSS Statistics for Windows, version 27 (IBM Corp., Armonk, N.Y., USA) or GraphPad 10 (GraphPad Software, Boston, MA, USA). 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. If data did not meet the assumptions of ANOVA, through the use of a Shapiro-Wilk test (normality) or a Brown-Forsythe test (equivalent variances), a nonparametric Kruskal-Wallis test was performed. A significance level (alpha) of a = 0.05 was used for all statistical analyses.

1.4. Results

1.4.1. Growth performance

In experiment A, after 180 days of treatment, fish fed 4 g kg^{-1} of DVAQUA displayed reduced growth performances and feed utilization compared to the control diet, ABW was decreased by 8 % (P=0.001), FCR increased by 11% (P=0.015) and PER increased by 13 % (P=0.010; Table 2. Growth performance of channel catfish (180 days) fed different levels of yeast product, stocked at 1.70 ± 0.02g (Mean ± SEM) initial body weight [Experiment A].Table 2). None of the growth metrics were significantly different in Experiment B (P > 0.05; Table 3). The survival rate was significantly higher (P=0.026) in channel catfish fed 1g kg^{-1} of DVAQUA compared to those provided 4 g kg^{-1}; however, the maximum deviation across treatment was 4 %. There was no difference in growth metrics between tilapia fed a control or yeast-amended diet, regardless of the inclusion level (experiment C) (Table 4). The survival rate was 4 % higher when tilapia were fed 1g kg^{-1} compared to those fed a control diet, and this result was statistically significant (P=0.041).

1.4.2. Infection challenge

In experiment B, catfish mortality following *E. ictaluri* exposure proportionally increased with the DVAQUA inclusion level; however, this was insignificant (P=0.623). The different inclusion rates of the additive in the channel catfish diet did not result in significantly different survival rates against *A. hydrophila* (Figure 1A). In experiment C, Nile tilapia survival increased with increasing DVAQUA inclusion rate in the fish diet; however, this result was not statistically significant (P=0.054) (Figure 1B).

1.4.3. Lysozyme activity and blood biochemistry

Channel catfish showed increased serum lysozyme activity by 4 % and 2 % at 1 and 4 g kg^{-1} of DVAQUA inclusion rate, respectively. However, these results were not significantly different from the control group (P=0.170) (Figure 2). Nile tilapia serum lysozyme activity decreases as the inclusion of DVAQUA increases in the diet. Lysozyme activity decreased by 5 % at 1g kg^{-1} (P=0.676) and by 15 % at 4g kg^{-1} (P=0.028; Figure 3). In Experiment A, channel catfish fed 2 g kg^{-1} had a significantly higher serum glucose level compared to the other treatment groups (P=0.041). Groups provided 1 and 4 g kg^{-1} did not present significantly higher glucose levels than the control diet (Table 5). In experiment B, channel catfish displayed a significantly higher (P=0.021) total serum protein level with higher postbiotic concentration compared to the postbiotic-free diet (Table 6). In experiment C, Nile tilapia fed with the highest postbiotic concentration showed a significantly lower potassium level compared to the group that did not receive postbiotics (P=0.026). Total bilirubin was significantly higher when tilapia received postbiotics; however, there was no difference between the two postbiotic concentrations (P=0.006) (Table 7).

1.4.4. Immune gene expression

In channel catfish, an increase in postbiotics in the feed resulted in an increasing upregulation of *il8* in the kideny tissue (P=0.012) and *igd* in the spleen tissue (P=0.009) with the highest increase occuring at 4g kg^{-1}. *Igd* tend to be downregultated in kidney tissue as the concentration of postbiotic increases however this was not statistically significant (*P*= 0.931). Gene expression of *igm* in kidney tissue was downregulated at 4g kg^{-1} compared to 2g kg^{-1} however, the DVAQUA-treated groups were not different from the control group (*P*=0.037). The same trend was observed with *il1β* in the spleen tissue (*P*=0.035) (Figure 6).

In Nile tilapia, *il8* (*P*=0.540), *il1β* (*P*=0.334) and *il6* (*P*=0.449) were downregulated in the kidney tissue with increasing concentration of additive in the feed. In the spleen tissue, 1g kg^{-1} tend to have a higher upregulation effect than 4g kg^{-1} for both *il8* and *tnfα* genes however, this was not significant (*P*>0.05) (Figure 7).

1.5.Discussion

1.5.1. Growth performance

The chemical composition of whole yeast cells depends on yeast strains, culture medium, growth conditions, and subsequent post-fermentation processing (Øverland and Skrede, 2017). Concerning the protein content, yeast presents a composition similar to soy protein and, depending on the processing after fermentation, is between 30 to 60 % protein (Bertolo et al., 2019). Yeast can be applied as a functional and technological ingredient in the food industry.

In our study, the growth performances of channel catfish and Nile tilapia were not impacted by *S. cerevisiae* fermentate at 1, 2, or 4 g kg^{-1} inclusion rate. (Welker et al., 2007) found similar results in channel catfish fed yeast or yeast subcomponent supplementation between 0.1 and 2 %. This was also reported by (Ozório et al., 2012) in tilapia fed up to 10 % *S. cerevisiae* inclusion rate; however, FCR started to significantly improve above 15 % compared to the control diet. Ozório et al. (2010) reported that yeast could replace 50 % (190 g kg^{-1}) of fishmeal protein in a pacu diet, at which level FCR did not differ compared to a control diet. Still, the growth rate and feed intake were significantly higher with the yeast additive. Similar results were observed with *S. cerevisiae* in a tilapia diet with doses as low as 2 g kg^{-1} (Abdel-Tawwab et al., 2020), suggesting that *S. cerevisiae* can be an effective natural protein source and improve the palatability of tilapia feed.

Contrary to our results, (Asadi Rad et al., 2012) found that FCR in Nile tilapia fingerlings was significantly improved at >0.5 g kg^{-1} of dry yeast supplementation. The same observation was made by Andriamialinirina et al. (2020), where 1 % yeast hydrolysate had significantly elevated weight gain (WG), specific growth rate (SGR), protein efficiency ratio (PER) and lower feed conversion ratio (FCR) compared to the control group. This suggests that the subsequent post-fermentation processing may significantly impact the final product's efficacy. Furthermore, the non-digestible components of yeast-based postbiotic can be used as a substrate for growth by both beneficial and harmful bacteria of the fish gut microbiome, unless the postbiotic favor exclusively the former type of bacteria, a positive impact on the host may not be evident.

1.5.2. Infection challenge

During the past few years, the traditional use of antibiotics in aquaculture has been criticized due to the potential development of antibiotic-resistant bacteria, antibiotic residues in fish flesh, and the destruction of microbial populations in the culture environment. As an alternative to antibiotics, postbiotics have attracted extensive scientific and commercial attention in aquaculture (Jamal et al., 2020). Yeasts are a good source of β-glucan which is well-known for its role in immunostimulation in fishes and other vertebrates. Hence, yeasts are commercially applied in aquaculture as a postbiotic to induce immune responses in host fishes by binding pathogen-associated molecular patterns (PAMP) to pattern recognition receptors (PRR). Such PAMP-PRR interactions and the downstream signaling are responsible for the enhancement of the activities of the immune system in fishes. In this mechanism, professional antigen-presenting cells such as macrophages are activated by β-glucan to engulf pathogens and to process and present antigens more efficiently by phagocytosis and autophagy, which, in turn, activate other immune cells. Similarly, interleukin and cytokines produced by activated macrophages have been reported to activate T cells, B cells, NK cells, and other macrophages for better defense against pathogens (Rodrigues et al., 2020). The absence of yeast or yeast subcomponent stimulated immunity can result from a loss of immune cell stimulation during the recovery period or from 'glucan overload'. Elevated levels of yeast intake, either through high dietary concentrations or extended feeding (>4 weeks), can cause an overload of glucan receptors on phagocytic cells reducing their ability to phagocytose bacteria in fish (Couso et al., 2003). Therefore, reducing the yeast or yeast subcomponents (YYS) concentration or feeding duration may be needed to see YYS-stimulation of immunity. Decreased immunity through elevated dietary intake of YYS has been reported in African catfish (Yoshida et al., 1995). Results from our study suggest that *S. cerevisiae* supplementation may be more effective at reducing mortality caused by gram-positive bacteria. However, yeast subcomponents from different commercial feed additives consisting mainly of β-glucan or oligosaccharide did not improve the disease resistance of Nile tilapia against *S. iniae, E. tarda* (Shelby et al., 2009), or *A. hydrophila* infection (Berto et al. 2016). In channel catfish fed 0.1 to 2% of yeast-supplemented feed, survival from *E. ictaluri* infection was not significantly different from the control group when animals received 4 weeks of postbiotics treatment (Welker et al., 2007). This was also the case with a 2-week pre-challenge feeding duration; however, a shorter 1-week feeding duration had significantly higher survival to ESC infection in relation to the control diet (Welker et al., 2012).

This suggests that a shorter treatment period could potentially improve the effectiveness of DVAQUA.

The vAh challenge model creates a direct port of entry into the fish organism through the wound. This allows the pathogen to bypass the gut membrane, which is the target of the postbiotic and where the immune response may potentially be enhanced. Therefore, another model with an oral exposure to the pathogen would allow for a better response from the fish immune system.

1.5.3. Lysozyme activity and blood biochemistry

Similar to our findings, yeast whole cells or subcomponents fed at 0.1 to 2 % inclusion rate in channel catfish diet had no significant impact on the lysozyme activity (Welker et al., 2007, 2012). This is opposed to (Abu-Elala et al., 2018), where a 0.2 % inclusion level of yeast β-glucans and MOS fed to Nile tilapia significantly improved lysozyme activity. Yeast hydrolysate supplementation also considerably increased serum lysozyme activity at 1 % and 3 % inclusion rates, suggesting an influence on the nonspecific immune response in Nile tilapia (Andriamialinirina et al., 2020). A similar study by Bu et al. (2019) tested 10, 20, and 30 g kg^{-1} inclusion rates with Ussuri catfish but only reported a significant increase in lysozyme activity at 10 g kg^{-1} compared to the control group. In this present experiment, only serum lysozyme was analyzed to characterize the systemic reaction of the animal to the immunostimulant. However, the gut is the target organ for yeast-based feed additives. Therefore, analysis of intestinal lysozyme content could present important differences in future research.

Contrary to our result, alanine aminotransferase (ALT), total protein (TP), and albumin (AL) were found to increase with increasing yeast extract levels (0.5 and 1.0 %) in a tilapia diet (Hassaan, Soltan, and Ghonemy 2014). This is possibly due to the lower inclusion levels used in our study. However, Andriamialinirina et al. (2020) found no significant difference in alanine aminotransferase (ALT), total protein (TP), or globulin (GLOB) with tilapia fed 1 and 3 % which can potentially be explained by post-fermentation hydrolysis of the yeast. Studies that assessed identical yeast product and inclusion rates found a significant change in the blood chemistry of rainbow trout (Tahmasebi-Kohyani et al., 2012) but not of beluga sturgeon (Yousefi et al., 2012).

In African catfish fed diets containing different levels of autolyzed brewer's yeast, ALP was not significantly different across treatments, but ALT was significantly lower in fish that received 3 and 6 g kg^{-1} in comparison to the 10 g kg^{-1} and control groups (Adeoye et al., 2020). Determining plasma or serum enzyme activity levels of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) may indicate hepatic function and status in fish. Elevation in the serum ALT enzyme activity can indicate liver damage or inflammation to environmental contaminants, disease, stress, and nutrients (Wang, 2007; Kumar, et al., 2011).

1.5.4. Immune gene expression

The gut is the primary target for immunosaccharides, especially in the case of immune modulation. The interaction between yeast immunosaccharides and pattern recognition receptors (PRRs), such as β-glucan (βG) receptors expressed in the macrophage cell wall, initiates a cascade of reactions involving cytokines, interleukins and tumor necrosis factors (Selim and Reda, 2015). Furthermore, mannoligosaccharides (MOS) are not digested by intestinal enzymes and are a substrate for lactic acid-producing bacteria, which can out-compete pathogenic gram-negative bacteria (Abu-Elala and Ragaa, 2015). Bu et al. (2019) found that Ussuri catfish fed 10 g kg^{-1} of yeast additive displayed significant down-regulation of *il1β* and *il8* but upregulated *igm*. Groups fed 20 and 30 g kg^{-1} were not significantly different from the control. A similar result was found by Feng et al. (2022) with juvenile largemouth bass where yeast culture significantly up-regulate the expression of anti-inflammatory cytokines (*tgfβ1*) and down-regulate pro-inflammatory cytokines genes (*il8*, *il1β*, *tnfα*) in the intestine. However, opposite results were reported by Andriamialinirina et al. (2020), where the expression of *il1β*, *il10*, and *tnfα* was significantly up-regulated in tilapia fed 1 or 3. % yeast hydrolysates compared to the control group. Furthermore, the low yeast concentration presented a significantly higher gene expression than the high yeast concentration. The same upregulation was found at 0.1 or 0.2 % of βG and MOS mix (Immunowall®) in a Nile tilapia diet with pro-inflammatory cytokines and immune-related gene (*tnfα* and *il1β*) versus the control group (Abu-Elala et al., 2018). Based on this literature, the gene expression differs according to the fish species, yeast form, and inclusion rate in the diet.

1.5.5. Conclusion

Several pieces of literature approved the ameliorating effects of dietary yeast products on aquatic animal health. Still, these effects are varied according to the fish species, age, and the chemical composition and solubility of the yeast products. In our experimental conditions, channel catfish growth performances did not improve after a short or long-term feeding period. Yeast-based feed additives that enhance the fish's resistance to pathogens through stimulation of the animal immune response may require shorter and intermittent application to avoid the overstimulation of the fish immune system. The immunostimulant performances may be more accurately evaluated through a challenge model with oral infection of the animal.

1.6. References

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Tables

Table 1. Primers used for real-time qPCR analysis of immune genes of interest. CCF=channel catfish, NT=Nile tilapia.

Gene	Species	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Size (bp)	Reference
il1β	CCF	GTGTAAGCAGCAATCCAGTCA	CAAGCACAGAACAGTCAGGTAT	180	Wang et al. 2021
il8	CCF	TGGGGAGAAGGAAAAGCAGAG	TGAACAGGAGGCACTTGGAGG	111	Jiang et al. 2017
igM	CCF	CTGTGCAAAGCGCCCCGAAATC	CTCCCGCTCGCATCCTTCCATAT	142	Xu et al. 2019
igD	CCF	AAAGTGGCTCCCTGAATCTGT	ACTGGTGCTCTTCTCAACTGG	187	Xu et al. 2016
il1β	NT	TGCACTGTCACTGACAGCCAA	ATGTTCAGGTGCACTATGCGG	113	Choi et al. 2007
il6	NT	ACAGAGGAGGCGGAGATG	GCAGTGCTTCGGGATAGAG	165	Zhou et al. 2020
il8	NT	GCACTGCCGCTGCATTAAG	GCAGTGGGAGTTGGGAAGAA	135	Ming et al. 2013
tnfα	NT	GAGGTCGGCGTGCCAAGA	TGGTTTCCGTCCACAGCGT	119	Chen et al. 2016
ef1α	CCF	GTTGAAATGGTTCCTGGCAA	TCAACACTCTTGATGACACCAAC	118	Zhu et al. 2017
actb	CCF	CCGTGACCTGACTGAATACC	GCCCATCTCCTGCTCAAAG	139	Zhao et al. 2013
actb	NT	TGGTGGGTATGGGTCAGAAAG	CTGTTGGCTTTGGGGTTCA	217	Yang et al. 2013
ef1α	NT	GCACGCTCTGCTGGCCTTT	GCGCTCAATCTTCCATCCC	250	Yang et al. 2013

Table 2. Growth performance of channel catfish (180 days) fed different levels of yeast product, stocked at 1.70 ± 0.02g (Mean ± SEM) initial body weight [Experiment A].

Sampling	Metric	0 g kg^{-1}	1 g kg^{-1}	2 g kg^{-1}	4 g kg^{-1}	*P*-value
Day 0-35	ABW (g)	7.1 ± 0.3	7.6 ± 0.3	6.9 ± 0.1	7.2 ± 0.5	0.479
	FCR	0.89 ± 0.06	0.85 ± 0.02	0.84 ± 0.02	0.89 ± 0.02	0.664
	PER	0.31 ± 0.02	0.3 ± 0.01	0.3 ± 0.01	0.31 ± 0.01	0.638
	GR (%)	316 ± 16	325 ± 5	332 ± 14	327 ± 14	0.855
	SGR	4.06 ± 0.13	4.37 ± 0.08	4.02 ± 0.07	4.3 ± 0.16	0.139
	TGC	1.96 ± 0.31	1.98 ± 0.13	2.25 ± 0.18	2.14 ± 0.27	0.792
	Survival	66 ± 10	59 ± 3	74 ± 6	66 ± 8	0.553
Day 35-62	ABW (g)	16.1 ± 1.4	17.5 ± 0.5	14.2 ± 0.6	16 ± 0.9	0.155
	FCR	1.28 ± 0.09	1.2 ± 0.05	1.38 ± 0.02	1.33 ± 0.09	0.341
	PER	0.45 ± 0.03	0.42 ± 0.02	0.49 ± 0.01	0.47 ± 0.03	0.315
	GR (%)	256 ± 24	258 ± 12	221 ± 6	246 ± 23	0.478
	SGR	2.36 ± 0.22	2.45 ± 0.14	2.12 ± 0.11	2.33 ± 0.26	0.671
	TGC	1.26 ± 0.07	1.27 ± 0.05	1.13 ± 0.06	1.12 ± 0.14	0.484
	Survival	99 ± 3	96 ± 2	96 ± 1	94 ± 10	0.934
Day 62-99	ABW (g)	25.2 ± 1.4	28 ± 0.5	25.1 ± 0.7	25.6 ± 0.3	0.078
	FCR	1.13 ± 0.07	1.13 ± 0.04	1.19 ± 0.06	1.21 ± 0.05	0.601
	PER	0.4 ± 0.02	0.39 ± 0.02	0.42 ± 0.02	0.42 ± 0.02	0.655
	GR (%)	169 ± 2	162 ± 1	172 ± 4	167 ± 3	0.055
	SGR	1.54 ± 0.03	1.43 ± 0.02	1.61 ± 0.07	1.5 ± 0.05	0.068
	TGC	1.94 ± 0.06	1.82 ± 0.04	1.98 ± 0.09	1.9 ± 0.06	0.343
	Survival	100 ± 0	99 ± 1	99 ± 0	100 ± 0	0.221
Day 99-113	ABW (g)	26.3 ± 1.2[a]	30.1 ± 0.8[b]	26.6 ± 0.7[a]	27.7 ± 0.4[ab]	**0.012**
	FCR	2.8 ± 0.63	2.2 ± 0.28	4.54 ± 0.81	2.75 ± 0.54	0.112
	PER	0.98 ± 0.22	0.77 ± 0.1	1.59 ± 0.28	0.96 ± 0.19	0.112
	GR (%)	109 ± 2	109 ± 1	105 ± 1	108 ± 2	0.759
	SGR	0.25 ± 0.06	0.25 ± 0.03	0.17 ± 0.03	0.24 ± 0.04	0.774
	TGC	0.93 ± 0.21	0.96 ± 0.13	0.28 ± 0.22	0.9 ± 0.17	0.066
	Survival	100 ± 0[a]	100 ± 0[a]	97 ± 1[b]	100 ± 0[a]	**0.003**
Day 113-143	ABW (g)	37.8 ± 0.9[a]	38.2 ± 0.7[a]	36.3 ± 0.5[ab]	34.9 ± 0.3[b]	**0.007**
	FCR	1.24 ± 0.19	1.11 ± 0.04	1.2 ± 0.04	1.5 ± 0.08	0.096
	PER	0.44 ± 0.07	0.39 ± 0.01	0.42 ± 0.01	0.53 ± 0.03	0.108
	GR (%)	133 ± 4	133 ± 2	130 ± 1	124 ± 2	0.133
	SGR	0.86 ± 0.08	0.85 ± 0.05	0.78 ± 0.03	0.65 ± 0.04	**0.047**
	TGC	1.17 ± 0.13	1.15 ± 0.08	1.08 ± 0.02	0.92 ± 0.06	0.205
	Survival	98 ± 1	98 ± 1	99 ± 1	100 ± 0	0.455

Day 143-180	ABW (g)	82.9 ± 1.5[a]	84.1 ± 1.3[a]	76.7 ± 1.6[b]	76.5 ± 0.7[b]	**0.001**
	FCR	0.92 ± 0.02[a]	0.96 ± 0.02[ab]	0.98 ± 0.01[ab]	1.02 ± 0.02[b]	**0.015**
	PER	0.32 ± 0.01[a]	0.34 ± 0.01[ab]	0.34 ± 0[ab]	0.36 ± 0.01[b]	**0.010**
	GR (%)	218 ± 2	217 ± 5	209 ± 3	216 ± 3	0.272
	SGR	2.31 ± 0.04	2.32 ± 0.07	2.2 ± 0.04	2.31 ± 0.05	0.342
	TGC	2.69 ± 0.05	2.67 ± 0.08	2.48 ± 0.07	2.56 ± 0.05	0.103
	Survival	99 ± 1	98 ± 1	98 ± 1	97 ± 1	0.698

[1]*Different letters indicate a significant treatment difference (P < 0.05).*

Table 3.Growth performance of channel catfish cultured in a recirculating system for two months and fed different levels of yeast product, stocked at 11.2±0.08g initial body weight. Values are represented as mean ± SEM of 5 replicate tanks [Experiment B].

Metric	0 g kg^{-1}	1 g kg^{-1}	4 g kg^{-1}	*P*-value
ABW (g)	39.8 ± 1.2	39.1 ± 0.5	41.5 ± 1.3	0.339
FCR	0.94 ± 0.04	0.95 ± 0.01	0.88 ± 0.02	0.309
PER	0.37 ± 0.01	0.38 ± 0	0.36 ± 0.01	0.309
GR (%)	252 ± 9	250 ± 8	272 ± 7	0.165
SGR	2.52 ± 0.05	2.51 ± 0.04	2.63 ± 0.04	0.170
TGC	0.052 ± 0.001	0.052 ± 0.001	0.055 ± 0.001	0.368
Survival (%)	98 ± 0 ^{ab}	96 ± 1 ^{a}	99 ± 0 ^{b}	0.026

Table 4. Growth performance of Nile tilapia cultured in a flow-through system for two months fed different levels of yeast product, stocked at 5.7 ± 0.1 g initial body weight. Values are represented as mean ± SEM of 5 replicate tanks [Experiment C].

Metric	0 g kg^{-1}	1 g kg^{-1}	4 g kg^{-1}	*P*-value
Initial ABW	5.7 ± 0.1	5.7 ± 0.1	5.7 ± 0.1	0.976
Final ABW	130 ± 3	129 ± 2	121 ± 3	0.064
Feed (g)	2331 ± 65	2288 ± 27	2221 ± 27	0.226
FCR	0.93 ± 0.02	0.89 ± 0.02	0.93 ± 0.02	0.324
PER	0.39 ± 0.01	0.37 ± 0.01	0.39 ± 0.01	0.304
GR (%)	2194 ± 53	2153 ± 46	2029 ± 69	0.131
SGR	9.21 ± 0.07	9.16 ± 0.06	8.99 ± 0.1	0.126
TGC	0.172 ± 0.002	0.170 ± 0.001	0.165 ± 0.002	0.060
Survival (%)	96 ± 2[a]	100 ± 0[b]	99 ± 1 [b]	0.041

[1]*Different letters indicate a significant treatment difference ($P < 0.05$).*

Table 5.Serum blood chemistry and enzyme activity from channel catfish cultured in a flow-through system for six months fed different levels of yeast product. Values are represented as mean ± SEM of 4 replicate tanks [Experiment A].

Metric	0 $g.kg^{-1}$	1 $g.kg^{-1}$	2 $g.kg^{-1}$	4 $g.kg^{-1}$	*P*-value
ALB ($g\ dL^{-1}$)	1.9 ± 0.06	1.96 ± 0.07	1.92 ± 0.12	2 ± 0.08	0.845
ALP ($U\ L^{-1}$)	65 ± 2	71 ± 3	68 ± 3	59 ± 13	0.678
ALT ($U\ L^{-1}$)	13.8 ± 0.6	12.8 ± 0.2	12.8 ± 0.4	14 ± 0.3	0.085
AMY ($U\ L^{-1}$)	53 ± 3	55 ± 4	53 ± 2	52 ± 3	0.929
Ca^{+2} ($mg\ dL^{-1}$)	14 ± 0.5	14 ± 0.2	13.6 ± 0.6	13.3 ± 0.5	0.667
PHOS ($mg\ dL^{-1}$)	15.8 ± 0.4	15.4 ± 0.5	14.9 ± 0.2	14.9 ± 0.3	0.319
GLU ($mg\ dL^{-1}$)	91.2 ± 3.6 ^{a}	92.8 ± 4.9 ^{a}	106.8 ± 4 ^{b}	100.4 ± 2.7 ^{ab}	0.041
Na^{+} ($mmoL\ L^{-1}$)	141 ± 1	142 ± 1	141 ± 1	142 ± 1	0.736
TP ($g\ dL^{-1}$)	3.58 ± 0.11	3.62 ± 0.06	3.58 ± 0.14	3.72 ± 0.1	0.761

Table 6. Serum blood chemistry and enzyme activity from channel catfish cultured in a recirculated system for two months fed different levels of yeast product. Values are represented as mean ± SEM of 5 replicate tanks [Experiment B].

Metric	0 g kg^{-1}	1 g kg^{-1}	4 g kg^{-1}	*P*-value
ALP (U L^{-1})	82 ± 3	82 ± 7	86 ± 8	0.878
ALT (U L^{-1})	9.5 ± 1.5	9 ± 0.6	9.7 ± 1.9	0.783
AMY (U L^{-1})	72 ± 2	62 ± 4	69 ± 5	0.721
GLU (mg dL^{-1})	107 ± 8	86 ± 11	101 ± 12	0.465
Na^+ (mmoL L^{-1})	144 ± 1	148 ± 2	148 ± 2	0.128
K^+ (mmoL L^{-1})	2.2 ± 0.4	2.4 ± 0.2	2.7 ± 0.5	0.693
TP (g dL^{-1})	3.8 ± 0 [a]	3.9 ± 0.1 [ab]	4 ± 0 [b]	**0.021**

[1]*Different letters indicate a significant treatment difference (P < 0.05).*

Table 7. Serum blood chemistry and enzyme activity from Nile tilapia cultured in a recirculated system for two months fed different levels of yeast product. Values are represented as mean ± SEM of 5 replicate tanks [Experiment C].

Metric	0 g kg^{-1}	1 g kg^{-1}	4 g kg^{-1}	*P*-value
ALP (U L^{-1})	22 ± 4	24 ± 4	26 ± 3	0.694
ALT (U L^{-1})	9 ± 0.7	8.8 ± 0.5	7 ± 0.5	0.089
AMY (U L^{-1})	12 ± 2	14 ± 2	12 ± 2	0.898
GLU (mg dL^{-1})	83 ± 8	82 ± 8	80 ± 4	0.841
Na^+ (mmoL L^{-1})	152 ± 2	155 ± 2	154 ± 3	0.785
K^+ (mmoL L^{-1})	6.2 ± 0.8 ^{a}	4.5 ± 0.6 ^{ab}	3.7 ± 0.4 ^{b}	**0.026**
TP (g dL^{-1})	3.9 ± 0.1	3.9 ± 0.2	3.9 ± 0.2	0.855
ALB (g dL^{-1})	2.3 ± 0.1	2.2 ± 0.2	2.3 ± 0.1	0.658
TBIL (mg dL^{-1})	0.3 ± 0.03 ^{a}	0.45 ± 0.03 ^{b}	0.42 ± 0.02 ^{b}	**0.006**
GLOB (g dL^{-1})	1.6 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	0.941

[1]*Different letters indicate a significant treatment difference (P < 0.05).*

Figures

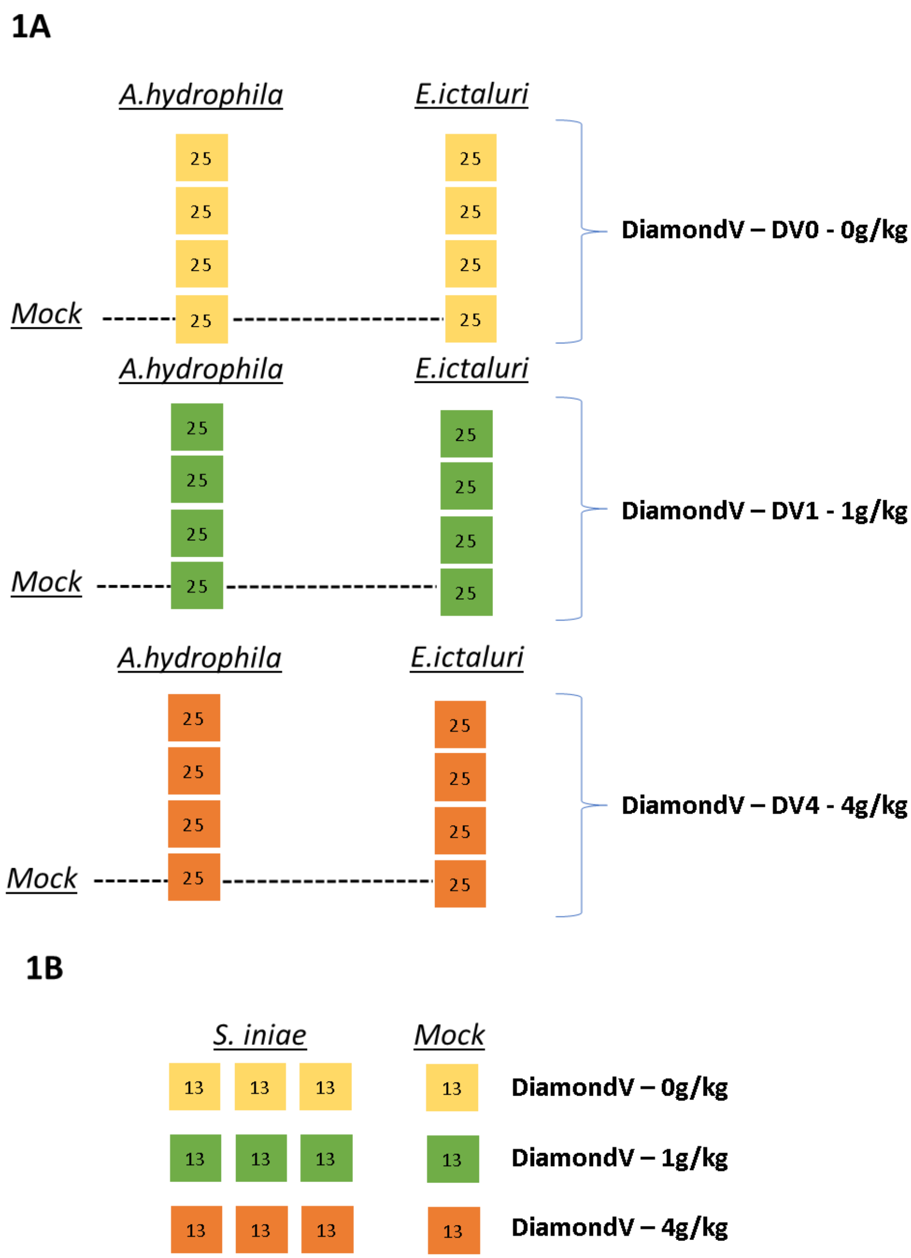

Figure 1. Disease challenge layout for channel catfish (A) and Nile tilapia (B).

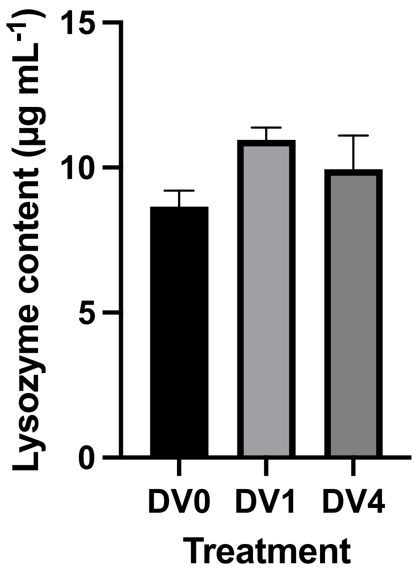

Figure 2. Serum lysozyme activity (µg mL^{-1}) from channel catfish fed different levels of commercial yeast product (*P* = 0.170). Values are represented as mean ± SEM of 5 replicate tanks [Experiment B].

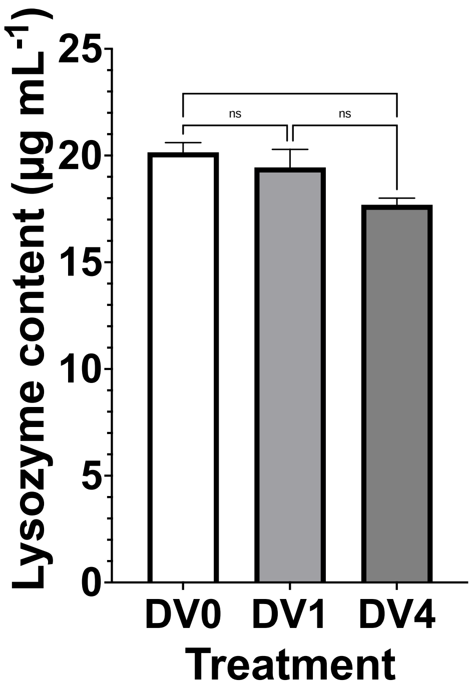

Figure 3. Serum lysozyme activity (µg mL^{-1}) from Nile tilapia fed different levels of a dietary yeast product ($P = 0.031$). Values are represented as mean ± SEM of 5 replicate tanks [Experiment C].

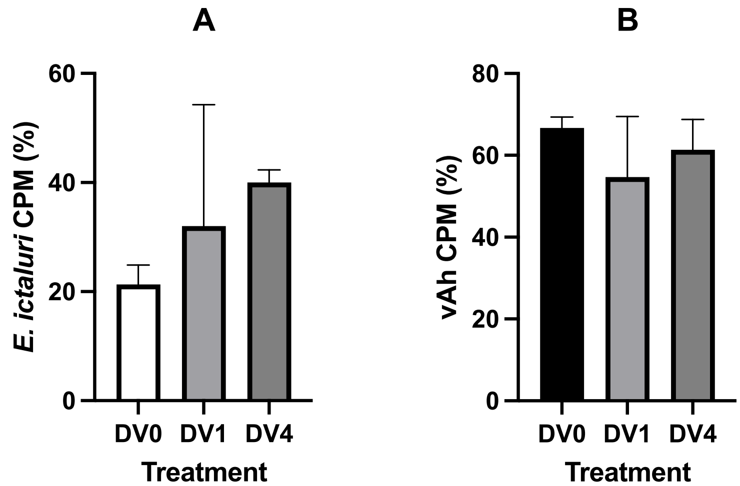

Figure 4. Cumulative percent mortality from *E. ictaluri* (A) (*P* = 0.623) or vAh (B) (*P* = 0.697) infections in channel catfish fed different levels of a commercial yeast product. Each infection group had three tanks (n=3), and each mock group had one tank (n=1). No mortalities were reported in the mock-challenged catfish. Values are represented as mean ± SEM [Experiment B].

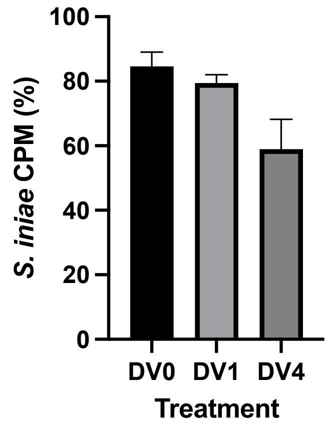

Figure 5. Cumulative percent mortality from *S. iniae* infection in Nile tilapia fed different levels of dietary yeast product. The infection group had three tanks (n=3), and the mock group had one tank (n=1) (*P* = 0.054). Values are represented as mean ± SEM [Experiment C].

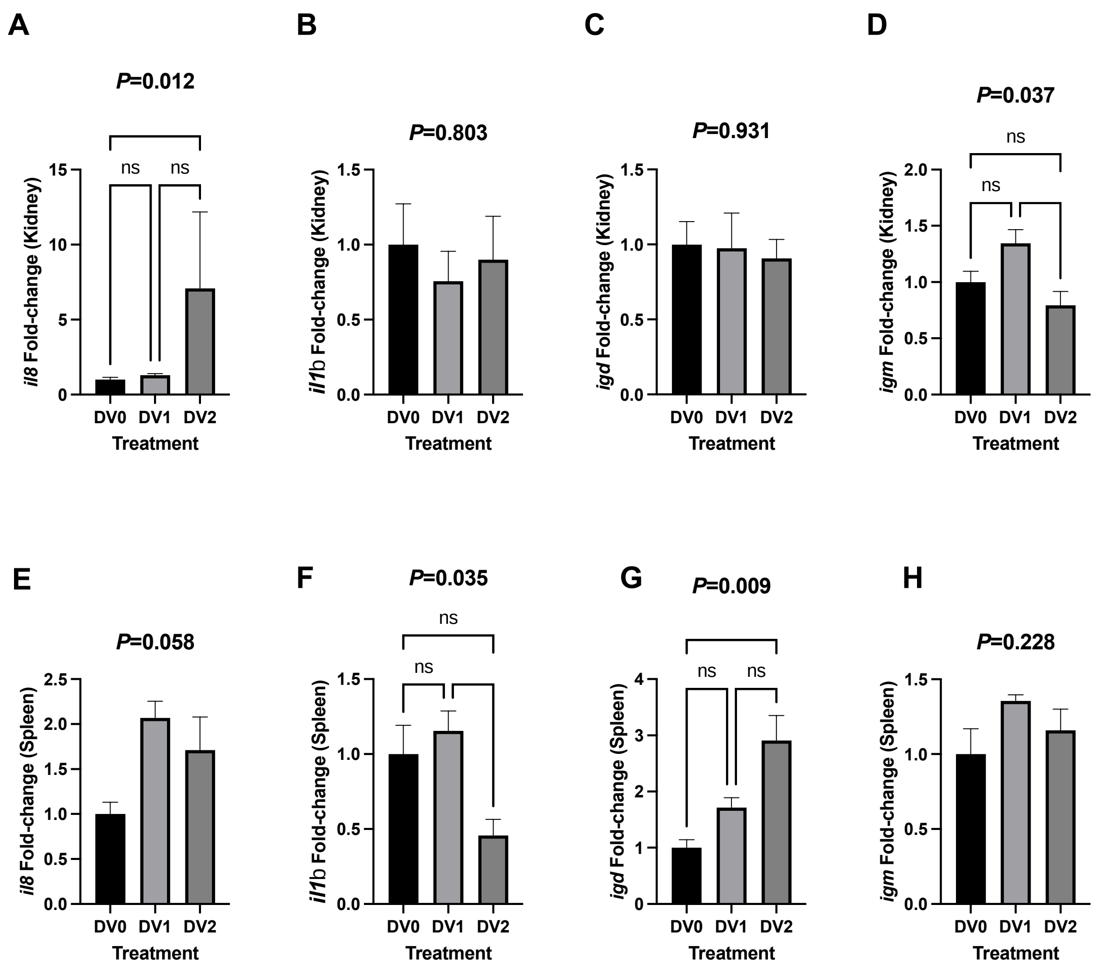

Figure 6. Kidney (K; A-D) and spleen (S; E-H) gene expression of channel catfish different levels of a dietary yeast product. Values are represented as mean ± SEM [Experiment C].

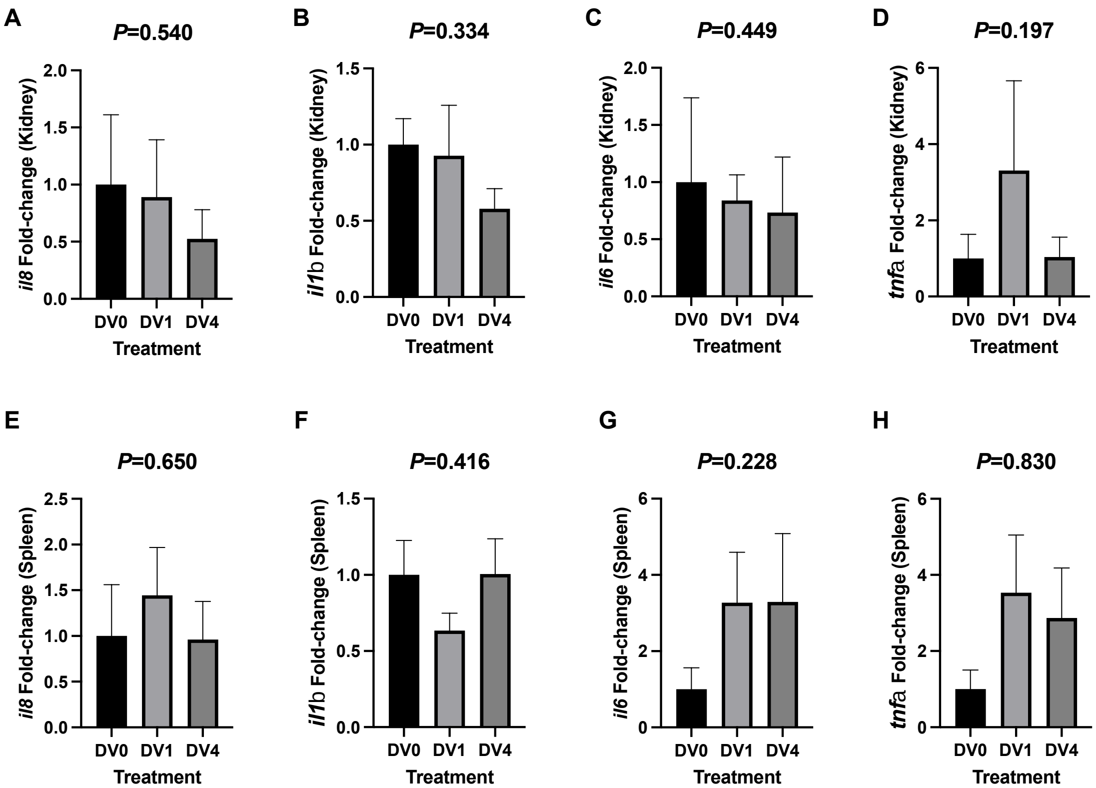

Figure 7. Kidney (K; A-D) and spleen (S; E-H) gene expression of Nile tilapia fed different levels of a dietary yeast product. Values are represented as mean ± SEM [Experiment C].

Chapter 2:

Application of *B. velenzensis* as a probiotic in Pacific whiteleg shrimp

2.1. Abstract

Whiteleg shrimp (*Penaeus vannamei*) is an aquaculture species of considerable commercial importance. Growth performance and disease susceptibility are the main constraints of the industry. Acute hepatopancreatic necrosis disease (AHPND) outbreaks caused by *Vibrio parahaemolyticus* can yield up to 100 % mortality rate. White leg shrimp post-larvae of 1.70 ± 0.02 g were cultured in pre-established biofloc and fed a *Bacillus velezensis* AP193 wild-type strain (1×10^7 CFU g^{-1}), an AP193 high-antimicrobial-producing strain (1×10^7 CFU g^{-1}), or BiOWiSH Feedbuilder Syn3®, a commercial *B. subtilis* probiotic (2×10^5 CFU g^{-1}) amended diet for two months. No significant differences were observed in growth performance, immune-related gene expression, or blood chemistry at the end of the feeding period. Shrimp were subsequently challenged by immersion with *Vibrio parahaemolyticus* (A3) in the biofloc. There was no apparent mortality reduction at the end of the pathogen challenge (P=0.997) among the different treatment groups. More research is needed to evaluate different concentrations of probiotics and their performance in biofloc or clear-rearing system conditions.

2.2. Introduction

Whiteleg shrimp (*Penaeus vannamei*) is an aquaculture species of considerable commercial importance as it represented 53% of the global crustacean production, with 6 million tons ($36.5 billion) produced in 2021 (FAO, 2023). Expansion of the sector has been limited by the water quality and disease susceptibility in intensive production settings (Pérez-Sánchez et al., 2018).

Vibrio spp. are ubiquitous in many aquatic environments, including aquaculture systems, and can be isolated from many healthy crustaceans (de Souza Valente and Wan, 2021). Pathogenic *Vibrio* spp. may cause high production losses in Penaeus farms. Vibriosis diseases such as acute hepatopancreatic necrosis disease (AHPND) were responsible for more than US$ 44 billion in losses from 2010 to 2016, combined from China, Malaysia, Mexico, Thailand, and Vietnam, including losses at the farm gate and those resulting from a drop in feed sales and exports (Tang and Bondad-Reantaso, 2019). AHPND is known to be caused by strains of *Vibrio parahaemolyticus* that contain a unique virulence plasmid (Tran et al., 2013). Outbreaks can cause up to 100% mortality in adults and postlarvae (Schryver et al., 2014; Soto-Rodriguez et al., 2015).

Bacterial infections are most commonly treated by oral administration of antibiotics in shrimp feeds. Disease control unsupervised by veterinary support results in uninformed and often unrecorded antibiotics use, posing a considerable threat to developing a sustainable aquaculture industry. This has led to increased antimicrobial resistance (AMR), which decreases the treatment efficacy for common bacterial diseases. Additionally, antibiotic residues in aquaculture productions can lead to import refusals and negative impacts on international trade (Watts et al., 2017; Chen et al., 2020). Finally, contamination of water bodies and sediments with residues of antibiotics causes stress on the aquatic environment (Li et al., 2012; Hossain et al. 2017).

Growing global concerns about AMR have prompted the search for sustainable approaches for disease prevention and control in aquaculture. Although shrimp do not possess elements of adaptive immunity, priming of the innate immune response can exhibit memory-like protein, which opens the potential of a vaccine-like solution for shrimp (Chang et al., 2018; Amatul-Samahah et al., 2020). Phytotherapy, as an inexpensive and eco-friendly alternative to conventional methods, uses a variety of medicinal plant compounds that enhance nonspecific immunity with proven efficiency in disease control (Jeyavani et al., 2022). Other alternatives are being developed to attenuate the virulence of aquaculture pathogens, such as bacteriophage therapy and quorum quenching (Zhao et al., 2015; Quiroz-Guzmán et al., 2018; Shaheer et al., 2021; Pereira et al. 2022) . The concept of probiotics is one of the promising areas for research and development that has gained popularity for its scalable, economical, and practical treatment method in shrimp (Kumar et al.. 2016; Pérez-Sánchez et al., 2018).

Based on their mechanisms of action, probiotics can create a hostile environment for pathogens, synthesize essential nutrients, modulate the host's immune response to infection, and reduce the eutrophication of aquaculture systems (Santos et al., 2019; Thurlow et al., 2019; Knipe et al., 2021). The intestinal microbiota provides a nutritional benefit as well as protection against pathogens and contributes to the differentiation of immune responses, which opens the possibility of its manipulation through the use of probiotics (Xie et al., 2019; Ringø et al. 2022).

Bacillus velezensis is a plant growth-promoting bacterium that can inhibit plant pathogens. However, based on its properties, it is emerging as a probiotic in animal feed. The characteristics and potential functions of different strains of *B. velezensis* have been widely evaluated (Khalid et al., 2021). Spores of *B. velezensis* do not cause toxicity or damage to the host and can successfully propagate as part of the gut microbiota (Zhang et al. 2019). Previous studies have demonstrated the antimicrobial property and protection of the probiotic *B. velezensis* AP193 against multiple aquatic pathogens such as *E. ictaluri* (Ran et al., 2012) in channel catfish or *A. hydrophila* (Addo et al., 2017a) and *S. iniae* (Addo et al., 2017b) in Nile tilapia. Furthermore, *Bacillus* spp. commonly produces phytase, which catalyzes the sequential hydrolysis of phytate, the main phosphorus storage form in plant-based foods. It is an anti-nutrient with low digestibility and high chelating capacity, limiting mineral bioavailability in aquaculture feed (Priyodip et al., 2017). The potential of AP193 as an immunostimulant was evaluated after a one-month feeding period followed by an infection challenge with *V. parahaemolyticus*. Growth performance, survival, blood chemistry, lysozyme activity, and immune gene expression were measured and compared against a probiotic-free diet.

2.3. Materials and methods

2.3.1. Shrimp growth trial

Whiteleg shrimp (n=40 per tank) of 0.75 g average body weight were stocked in individual 150-L tanks with pre-establish biofloc and supplemental aeration. The shrimp were fed a standard feed diet (35 % protein, 7 % lipid, 4 % fiber) coated with four different treatments, *B. velezensis* AP193 wild type strain (WT), AP193 mutant with higher difficidin production (H2), BiOWiSH ® FeedBuilder Syn3 (BW) or water (CTL). Each treatment was attributed to six replicated tanks for one month. The daily feed was calculated according to the formula of (Garzade Yta et al., 2004):

$$Feed(daily) = (Number\ of\ shrimp \times Expected\ weekly\ growth\ rate \times Expected\ FCR)/7$$

The feeding rates were calculated, assuming a feed conversion ratio (FCR) was 1.4 and 1 g of weight gain per week. Shrimp in all treatments received equal amounts of feed throughout the experiment. The feed was manually offered four times (~ 0830, 1100, 1400, and 1700 h) per day.

Molasses was added directly into the shrimp culture once a day, 2 h after the first daily feeding, with an estimated C:N ratio of 15. The C:N ratio was calculated based on the following assumptions: feed protein is 35 %; 16 % of protein is nitrogen; approximately 40 % of ingested N is excreted by the shrimp; and 40 % of molasses is carbon, so the addition of molasses is approximately 14.3 g per 10 g feed.

For both WT and H2 treatments, *B. velezensis* AP193 spore suspension (1.25 x 10^8 CFU mL^{-1}) was spray coated onto feed pellet at 8 % (v/w) for a final concentration of 1×10^7 CFU $g^{-1}$of feed, which was consistent with previous published literature and has been regarded as the appropriate dose to elicit probiotic effects (Thurlow et al. 2019; Addo et al. 2017b). For BW treatment, 2 g of spore powder (1 x 10^9 CFU g^{-1}) was resuspended in 800 mL of distilled, deionized water, and spore suspension (2.5 x 10^6 CFU mL^{-1}) was spray coated onto a feed pellet at 8 % (v/w) for a final concentration of 2.0×10^5 CFU g^{-1} of feed according to manufacturer recommendation. The control or basal diet without probiotic amendment was coated with distilled water. The diets were then air-dried for at least 4 h, stored at 4 °C, and used within five days of mixing.

During the experimental period, dissolved oxygen (DO) and temperature were measured twice daily using a YSI 650 multi-parameter instrument (YSI, Yellow Springs, OH, USA) for each individual tank. Salinity was measured weekly using the same instrument. pH was tested weekly using a waterproof pH Testr 30 (Oakton instrument, Vernon Hills, IL, USA). Water samples were collected and filtered through a 45μm membrane filter to monitor total ammonia nitrogen (TAN) and nitrite-nitrogen weekly.

After one month of feeding, all the animals were counted and bulk-weighed on a digital scale (Ohaus Scout Pro 4000g; Ohaus Corporation, Parsippany, NJ, USA) to measure growth performance indices:

Growth rate (%) = 100 × (final weight − initial weight)/initial weight

Specific growth rate = 100 x (ln final weight – ln initial weight) / days of feeding

Thermal growth coefficient = (FBW1/3 − IBW1/3)/Σ (Temp × days) × 100

Survival rate (%) = 100 × Total number of animals harvested / Total number of animals stocked

2.3.2. Shrimp disease trial

At the end of the feeding period, shrimp from the same feeding treatment were pooled together and redistributed with 15 L of corresponding biofloc into 40 L aquaria at a density of 14 shrimp per aquaria. *Vibrio parahaemolyticus* (strain A3; kindly provided by Dr. Craig Shoemaker) was previously revived from cryostock onto chrome agar (CHROMagar™ Vibrio) for 24 h. One colony was then cultured in 100 mL of tryptic soy broth (TSB; Becton Dickinson, Sparks, MD, USA) supplemented with 2 % salt for 6 h at 150 rpm and 30 °C. A 10-mL inoculum (8.6×10^{8} CFU mL^{-1}) was added to the tank to obtain 5.7×10^{5} CFU mL^{-1} during immersion for 30 min. The mock-challenged tank received 10 mL of sterile TSB. At the end of the immersion period, 15 L of biofloc was added to maintain a total volume of 30 L during the experimental period (7 days). Respective diets and feeding regimes (2.1. growth trial) were maintained during the entire challenge. Temperature and dissolved oxygen were measured twice a day. After one week, shrimp were harvested to record their survival.

2.3.3. Gene expression analysis by real-time PCR

At the end of growth trials (2.1 Shrimp growth trial), shrimp gut was sampled from three animals per tank. The tissues were kept in DNA/RNA Shield ™ (Zymo Research Inc., USA) and stored overnight at 4 °C prior to storage at -20 °C. Organs were thawed on ice prior to RNA extraction with Quick-RNA Miniprep Kit (Zymo Research Inc., USA). RNA concentrations were diluted to 10 ng uL^{-1} and transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). Final cDNA was diluted to 1.25 ng uL^{-1} and stored at 25 °C. Each 10 uL qPCR reaction was carried with the following components: 5 uL of SYBR™ Green PCR Master Mix (Thermo Fisher Scientific), 0.5 uL of forward primer (10mM), 0.5 uL of reverse primer (10 mM), and 4 uL of template cDNA (1.25 ng uL^{-1}). The relative expression of four immune-related genes, *tnfα* (tumor necrosis factor-alpha), *tgfβ1* (transforming growth factor beta 1), *proPO* (prophenoloxidase), superoxide dismutase (*sod*), and two reference genes, ribosomal protein L21 (*l21*) and beta-actin (*actb*) were determined in the gut tissue. The efficiencies of the primers were determined at 100 ± 10 % for each gene. Each PCR was performed with duplicate samples, and the cycling conditions were 2 min at 50 °C, 2 min at 95 °C followed by 15 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C for 40 cycles. A melt curve analysis was performed after amplification to verify the accuracy of each amplicon and non-template controls (nuclease-free water in lieu of cDNA). The relative abundance of interested genes was calculated by using the $2{-}\Delta\Delta Ct$ method (Livak and Schmittgen 2001). Experiments B and C utilized four genes of interest and two genes of reference.

2.3.4. Blood biochemistry

At the end of growth trial (2.3.1 Shrimp growth trial), shrimp hemolymph was sampled from three animals per tank. Hemolymph was withdrawn from the pericardial cavity of the shrimp using a 25-gauge needle and 1-cc syringe inserted beneath the carapace at the cephalothorax-abdominal junction (Roy et al., 2009). The 1 mL syringe was preloaded with 0.3 mL of an anti-coagulant and used to collect approximately 0.3 mL of hemolymph. To maintain semi-quantitative data, the weight of anticoagulant and hemolymph were determined gravimetrically. The anticoagulant solution (Liu et al., 2004) contains 30 mM sodium citrate tribasic dihydrate (Sigma S4641); 0.34 M sodium chloride (NaCl); 10 mM EDTA – ethylenediaminetetraacetic acid (Sigma, E9884); in deionized (DI) water. Concentrations of the hemolymph constituents were measured using Abaxis, VetScan® VS2 analyzer (Union City, CA). Samples from the same tank were pooled together, and a total of 100 μL of hemolymph was used to determine the following parameters using a Comprehensive Diagnostic Rotor: albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase (AMY), total bilirubin (TBIL), urea nitrogen (BUN), calcium (Ca^{+2}), phosphorus (PHOS), Creatinine (CRE), glucose (GLU), sodium (Na^{+}), potassium (K^{+}), total protein (TP), and globulin (GLOB).

2.3.5. Statistical analysis

All data were analyzed using IBM SPSS Statistics for Windows, version 27 (IBM Corp., Armonk, N.Y., USA) or GraphPad 10 (GraphPad Software, Boston, MA, USA). 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. If data did not meet the assumptions of ANOVA, through the use of a Shapiro-Wilk test (normality) or a Brown-Forsythe test (equivalent variances), a nonparametric Kruskal-Wallis test was performed. A two-way ANOVA, with the main effects of Diet and Challenge, was used to analyze endpoint mortality to discern the influence of dietary treatment and challenge among groups. For all statistical analyses, α-priori was set to 0.05.

2.4. Results

2.4.1. Growth performance

The average body weight of the animals was 3 (WT), 7 (H2), and 22% (BW) higher at the end of the feeding period in comparison to the control group. However, this was not significant (*P*=0.356) (Table 9). Survival rates were below 60 % and not significantly different across the treatment groups (*P*=0.971). No significant differences (*P*>0.05) were observed in water quality parameters across the different treatments during the experiment (Table 10).

2.4.2. Blood biochemistry

Alanine aminotransferase phosphorus, glucose, and potassium levels were higher in the groups that received probiotics than in the control group; however, this was insignificant (*P*>0.05). Amylase was lower when shrimp were fed with probiotics, but this was not significantly different from the control group (*P*=0.355).

2.4.3. Gene expression

The gene expression data did not show significant changes in any of the immune genes evaluated (*P*>0.05). Still, both BW and H2 treatments displayed upregulations for all the genes compared to the control group. WT treatment showed downregulation of all the genes except tgfb1 compared to the control group (Figure 9).

2.4.4. Infection challenge

Mortality rates were not statistically different across dietary treatments when shrimp were exposed to *V. parahaemolyticus* (*P*=0.997). Shrimp exposed to sterile inoculum had a minimum average mortality rate of 33 %, and there was a difference in mortality found between the mock-challenge and challenged shrimp (*P*=0.020) from the two-way ANOVA (Figure 8). The interaction of the main effects (Diet x Challenge) was not significant (*P*=0.984).

2.5. Discussion

2.5.1. Growth performance

A previous study by Madani et al. (2018) found improved weight gain, total length, specific growth rate, FCR, and survival in *L. vannamei* raised in a flow-through system and fed diets supplemented with 10^4-10^8 CFU g^{-1} of *B. licheniformis* and *B. subtilis* mix. Similar results were observed with *B. coagulans* ATCC 7050 at 10^6 – 10^8 CFU g^{-1} with a 50 % water exchange every two days (Amoah et al., 2019). Improved growth performances were also found with *B. subtilis* UTM 126 at 1 x 10^5 CFU g^{-1} and 50 % daily water exchange (Balcázar et al., 2007). In a system with a biweekly exchange rate, *B. subtilis* strains L10 and G1 mixed at 1 x 10^8 CFU g^{-1} improved the growth and survival rates but not at 1 x 10^5 CFU g^{-1} (Zokaeifar et al., 2012). The same probiotic used in our study (*B. velezensis* strain AP193) demonstrated significantly improved weight gain in channel catfish (Thurlow et al., 2019).

Although several studies have demonstrated the beneficial effects of probiotics on the growth performance of shrimp (Wang, 2007; Liu et al., 2009; Shen et al., 2010), the exact mechanism of action is not well understood. The first explanation could be related to the action of competitive exclusion, by which probiotics may create a hostile environment for pathogen colonization. Another possible explanation for the improvement of the shrimp growth factors by *B. subtilis* may be due to the induction of digestive enzymes, such as protease and amylase. Furthermore, the strain of probiotic, concentration in the feed, and the water exchange rate of the system can potentially influence the growth performance during the treatment.

The low survival (<60 %) and absence of differences in growth performances across the treatments cannot have been caused by the water quality, as those parameters were within the acceptable range (Table 10). This outcome can potentially be attributed to the intrinsic qualities of our biofloc (density, chemistry, microbiome, etc.), which were not monitored in our experiment and could have eclipsed any potential effect of the probiotic treatments.

2.5.2. Blood chemistry

L. vannamei fed diets supplemented with 1×10^4 and 1×10^8 CFU g^{-1} of *B. subtilis* and *B. lichenformis* did not present higher albumin concentrations but showed higher total protein and glucose serum concentrations (Madani et al., 2018). Similar results were observed with *B. coagulans* at 1×10^8 CFU/g (Amoah et al., 2019).

Contrary to our results, in Zokaeifar et al. (2012) both groups fed 1×10^5 and 1×10^8 CFU g^{-1} of *B. subtilis* strains mix showed significantly increased amylase levels compared to the control diet, this can be explained by the different probiotic strains and the clear water rearing system used. Alanine aminotransferase (ALT) was significantly lower in shrimp fed with *Bacillus subtilis* and *Bacillus lichenformis* compared to the control group (Naderi et al., 2017). Several other studies revealed that the beneficial functions of immunostimulants lowered ALT activity in the liver of fish or hepatopancreas of shrimp (Pan et al., 2003; Anand et al., 2017; Ayiku et al., 2020). According to (Mohankumar and Ramasamy 2006), its level increases with hepatopancreatic damage or during stress like transportation and DO depletion. Biofloc, apart from being a source of nutrient-rich autotrophic and heterotrophic microbial components, is also a source of detritus and fecal components. The latter is rich in ash contents which at higher levels can interfere with the physiological condition of the shrimp.

2.5.3. Gene expression

The prophenoloxidase-activating system is activated through recognition molecules in the hemolymph of invertebrates. The prophenoloxidase activating system is a non-self-recognition system in invertebrates that can recognize and respond to intruders via lipopolysaccharides or peptidoglycan from bacteria and β-glucans from fungi (Söderhäll and Cerenius, 1998). Up-regulation of *proPO* resulted in increased PO activity in shrimp fed *L. plantarum*-supplemented diet, which enhanced the resistance against *V. alginolyticus* (Chiu et al., 2007). Both $1x10^5$ and $1x10^8$ CFU g^{-1} concentrations of *B. subtilis* resulted in significant upregulation of *proPO* compared to the control diet (Zokaeifar et al., 2012).

2.5.4. Infection challenge

No reduction of mortality post-infection was observed across the different probiotic diets in our experiment. However, in Amoah et al. (2019), *B. coagulans* ATCC 7050 supplementation as low as $1x10^6$ CFU g^{-1} significantly reduced mortality against *V. parahaemolyticus* infection. This is potentially due to a different challenge model (i.e. injection) and the rearing of animals in clear water. compared to the control group. Similar result was found with *B. subtilis* UTM 126 (Balcázar et al., 2007) and *B. subtilis* strains L10 and G1 (Zokaeifar et al. 2012). Furthermore, *B. velezensis* strains have shown increasing resistance against *V. harveyi* in hybrid grouper (Li et al., 2019) and *A. salmonicida* in Atlantic salmon (Wang et al., 2019).

In a previous study by Gustilatov et al. (2022), biofloc inoculum added at 1:10 of the total volume 24 h before the challenge test produced a significantly higher survival rate than the control tank. It was also observed that a $1x10^5$ CFU mL^{-1} concentration of *V. parahaemolyticus* did not result in significantly higher mortality than the mock group. Similar observations were made between biofloc and clear water challenges (Shinn et al., 2018).

Therefore, it appears that the calibration of the biofloc and pathogen concentrations is necessary to observe the potential impact of a prophylactic. Our challenge model by immersion of *V. parahaemolyticus* could have allowed the pathogen to bypass the gut membrane, which is where AP193 spores can germinate and establish. This can explain the homogeneity in shrimp mortality across the treatments. A challenge model with oral exposure to the pathogen would allow for a better evaluation of the probiotic efficacy *in vivo.*

2.6. Conclusion

Although *B. velezensis* AP193 has shown efficacy in mortality reduction against multiple fish pathogens, it did not improve the survival of shrimp against *V. parahaemolyticus* in our challenge conditions. Adding the probiotic to the diet did not significantly enhance growth performances and the shrimp immune response. The biofloc quality may have a nonnegligible impact during feeding and infection trials. Future research should include a qualitative evaluation of the biofloc, such as the density and bacterial populations.

2.7. References

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Tables

Table 8. Primers used for real-time qPCR analysis.

Name	**Forward primer (5’ to 3’)**	**Reverse primer (5’ to 3’)**	**Reference**
TNF-α	AAAGAGGAACGTGGTCATGG	CACTCCTTTCCCCACTGTGT	Rahimnejad et al. 2018
TGF-β1	AACCATGCCCTTGTGCAAAC	CTTTGGGGGAACCTCGGTC	Rahimnejad et al. 2018
proPO	TACATGCACCAGCAAATTATCG	AGTTTGGGGAAGTAGCCGTC	Zheng et al. 2017
SOD	GCAATGAATGCCCTTCTACC	CAGAGCCTTTCACTCCAACG	Xie et al. 2018
β-actin	GAGCAACACGGAGTTCGTTGT	CATCACCAACTGGGACGACATGGA	Rahimnejad et al. 2018
L21	GTTGACTTGAAGGGCAATG	CTTCTTGGCTTCGATTCTG	Stephens et al. 2012

Table 9. Growth performance of whiteleg shrimp cultured in biofloc system with one month of probiotics diet, stocked at 40 shrimp per tank with an initial weight at 0.75±0.02g (Mean ± SEM) initial body weight.

	CTL	BW	WT	H2	*P*-value
ABW Day 0 (g)	0.72 ± 0.02	0.76 ± 0.01	0.75 ± 0.02	0.76 ± 0.02	0.393
ABW Day 33 (g)	2.33 ± 0.11	2.85 ± 0.18	2.4 ± 0.13	2.5 ± 0.36	0.356
Feed (g)	351.6 ± 0.5	350.6 ± 0.7	350.5 ± 0.5	350.3 ± 0.6	0.440
Weight gain (%)	68.6 ± 1.7	73 ± 1.5	68.5 ± 2	64.9 ± 6.6	0.493
TGC	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.517
Survival (%)	45 ± 6	52 ± 9	48 ± 14	46 ± 10	0.971
SGR	2.33 ± 0.11	2.64 ± 0.11	2.33 ± 0.12	2.24 ± 0.32	0.496

Table 10. Water quality of whiteleg shrimp cultured in biofloc system with one month of probiotics diet, stocked at 40 shrimp per tank with an initial weight at 1.70 ± 0.02 g (Mean ± SEM) initial body weight.

	CTL	BW	WT	H2	*P*-value
TAN (mg L^{-1})	0.3 ± 0.06	0.23 ± 0.03	0.36 ± 0.07	0.21 ± 0.03	0.110
Nitrite (mg L^{-1})	0.44 ± 0.08	0.49 ± 0.09	0.49 ± 0.09	0.44 ± 0.07	0.943
Alkalinity	49.6 ± 10	56.9 ± 9.1	41.7 ± 9.6	48.6 ± 9.5	0.732
pH	7.91 ± 0.08	7.99 ± 0.08	7.9 ± 0.08	7.85 ± 0.07	0.631
Salinity (ppt)	11.65 ± 0.09	11.65 ± 0.09	11.75 ± 0.11	11.69 ± 0.09	0.868
DO (mg.L^{-1})	6.78 ± 0.1	6.71 ± 0.11	6.92 ± 0.08	6.81 ± 0.08	0.482
Temperature (°C)	25.8 ± 0.1	25.7 ± 0.1	25.7 ± 0.1	25.7 ± 0.1	0.875

Table 11.Serum blood chemistry and enzyme activity from whiteleg shrimp cultured in a recirculating system for two months and fed different levels of probiotics. Values are represented as mean ± SEM of 5 replicate tanks [Experiment B].

	CTL	BW	WT	H2	*P*-value
ALT (U L^{-1})	18.4 ± 2	18.6 ± 4	20.5 ± 3.6	20.7 ± 1.7	0.935
AMY (U L^{-1})	80 ± 34	71 ± 32	24 ± 5	41 ± 7	0.355
PHOS (mg dL^{-1})	2.5 ± 0.6	2.8 ± 0.6	3 ± 0.4	2.8 ± 0.6	0.890
GLU (mg dL^{-1})	36 ± 5	47 ± 6	38 ± 4	45 ± 4	0.332
K^+ (mmoL L^{-1})	9.3 ± 0.5	9.8 ± 1.5	10.6 ± 1.7	11.1 ± 0.7	0.678

Figures

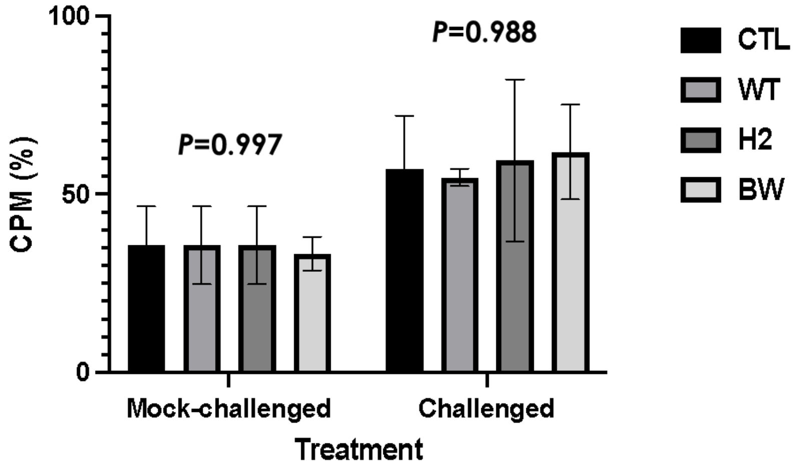

Figure 8. Cumulative percent mortality of whiteleg shrimp after 7 days of infection challenge with *V. parahaemolyticus* (P=. Values are represented as mean ± SEM [Experiment C].

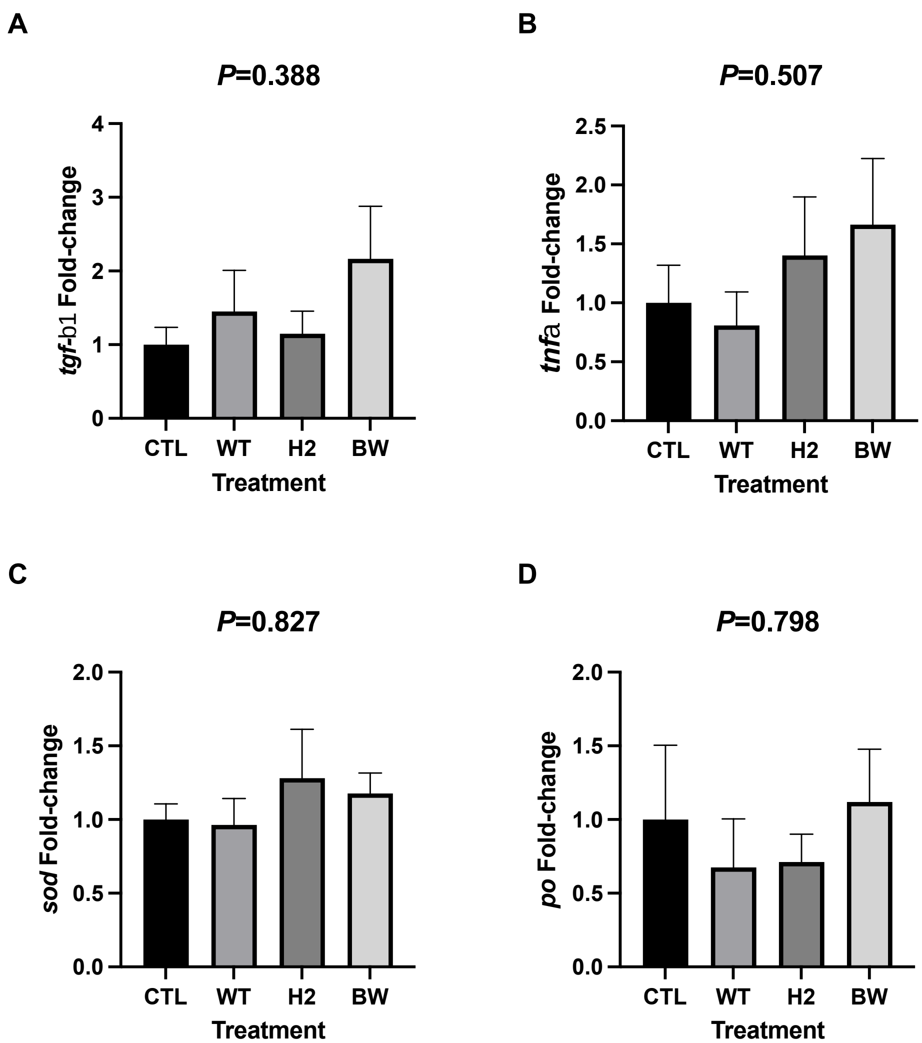

Figure 9. Gene expression of whiteleg shrimp intestine tissue fed different probiotics. Values are represented as mean ± SEM [Experiment C].