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Bacillus velezensis AP193 exerts probiotic effects in channel catfish (*Ictalurus punctatus*) and reduces aquaculture pond eutrophication

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ABSTRACT

Ictalurus punctatus (channel catfish) is an economically important farmed fish particularly in the southeastern United States. Aquaculture sustainability is threatened by disease pressure and the eutrophic conditions resulted from intensive fish farming. Previous research identified *Bacillus velezensis* strains that reduced mortality due to bacterial pathogens when used as a feed amendment. This study was conducted to determine the effects of *B. velezensis*-amended feed on catfish growth performance, pond water quality, and on the microbiomes in fish intestines and pond water. *B. velezensis* AP193 was evaluated in a ten-week pond trial, with four replicate ponds per probiotic treatment or control group. Feed amended with *B. velezensis* AP193 induced a 40.4% or 32.6% increase in growth relative to control feed in fingerling catfish that originated from aquaria or raceways, respectively. No significant differences were observed in the catfish intestinal microbiota or the pond microbiota due to probiotic-amended feed. The water quality was improved in ponds in which fish were fed with the probiotic-amended feed, as significant reductions were found in total phosphorus (19%), total nitrogen (43%) and nitrate (75%). These data suggest that *B. velezensis* AP193 can promote catfish growth and improve pond water quality when used as a feed amendment.

1. Introduction

Due to its rapid growth rate, low cost, and proficient reproduction capabilities, the channel catfish (*Ictalurus punctatus*) is an economically important aquaculture species, particularly in the southeastern United States (Agriculture, 2003). For maximized productivity of the aquaculture system, fish feeding efficiency is extremely important. Traditionally, forage fisheries have been exploited for the production of fish meal, but the rapid depletion of wild fisheries (Naylor et al., 2009) has led to the use of soybean meal as an alternative (Da et al., 2013). However, feed conversion ratios (FCRs) are much lower in fish with the use of plant protein resulting in up to a 15% deterioration of fish growth performance compared to a fish meal-based diet (Sales, 2009). Phosphorus found in plant protein sources are unusable by fish, and phytate, a common storage component of phosphorus, can serve as an anti-nutrient in chelating iron resulting in anemia (Zhu et al., 2014). Unused phytate and other feed-derived nutrients will ultimately be released as fish waste and contribute to the eutrophication of the aquaculture pond ecosystem (Cho and Bureau, 2001).

Phytase is a phosphohydrolase that catalyzes the hydrolysis of phytate, allowing for phosphorous availability for absorption (Kumar et al., 2012). This enzyme is found in many microorganisms, which are being exploited for supplementation in feed. To supplement high feed demands, production facilities have been created to ferment phytase from microorganisms, many of which are already regarded as probiotics (Askelson et al., 2014). For this reason, providing the fish with probiotics can potentially reduce eutrophication, induce weight gain, and be a viable option to promote sustainable aquaculture management practices. Eutrophication due to feed-derived phytate and other nutrients can result in blooms of algae and cyanobacteria (Anderson et al., 2002; Boyd, 2015; Kumar et al., 2012) Due to the ability of cyanobacterial taxa to synthesize and release toxins into the water column,

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they can be devastating to fish production (Sevrinreyssac and Pletikosic, 1990). In addition to hepatotoxins and neurotoxins, some cyanobacteria and other bacterial taxa produce the metabolites, 2-methylisoborneol (MIB) and geosmin, that result in unwanted off-flavors in catfish (Vanderploeg et al., 1992).

Another factor responsible for significant economic losses in aquaculture is due to disease (Stentiford et al., 2017). One traditional treatment for disease is the use of antibiotics, and there are currently only three antibiotics approved by the FDA for their use in food catfish (Benbrook, 2002). However, with growing concern over the use of antibiotics due to the development of pathogen multi-drug resistance (Patil et al., 2016), it is important to seek alternative means of treatment. Probiotics can reduce fish mortality due to pathogens by direct antagonism via synthesis of secondary metabolites, by competitive exclusion, and/or by activation of the innate immune system (Balcazar et al., 2006; Macfarlane and Cummings, 1999; Wang et al., 2008). *Bacillus* spp. have good potential as probiotics for aquaculture application due to their ability to form endospores, allowing for a long shelf life and survival from exposure to gastric acid (Casula and Cutting, 2002; Hong et al., 2005). Furthermore, strains within the *B. subtilis* group, which includes *B. velezensis* (previously described as *B. amyloliquefaciens* subsp. plantarum (Dunlap et al., 2016)), have not been associated with disease.

Previous research evaluated a collection of 160 *Bacillus* spp. strains for their antimicrobial activity against bacterial and fungal fish pathogens and evaluating the impact on mortality after challenge (Ran et al., 2012). The 21 *Bacillus* spp. strains that showed production of secondary metabolites that inhibited the growth of fish pathogens were then tested for their survival and persistence in the catfish intestine and protection against infection by *E. ictaluri* (Ran et al., 2012). In Nile tilapia (*Oreochromis niloticus*), a specific strain *B. velezensis* AP193 showed protection against infection by *Aeromonas hydrophila* (Addo et al., 2017b) or *Streptococcus iniae* (Addo et al., 2017a). Out of the 21 strains tested, the five strains that indicated the greatest enhancement of growth and best in vitro antagonistic activity against *E. ictaluri*, *S. iniae*, and *A. hydrophila* were selected for further testing. This study evaluated the efficacy of these probiotic strains when used as a feed additive in a 10-week aquaria trial testing their growth promoting capabilities and protective effects against *E. ictaluri*. Further, this study also presents the effects that feeding *B. velezensis* AP193 has in 1) promoting the growth of channel catfish, 2) determining its impact on the microbiome in fish intestines and ponds and 3) assessing its effects on pond water quality.

2. Materials & methods

2.1. Animal welfare statement

All Channel catfish challenges were conducted under the approval of the Animal Care and Use Committee (IACUC) of Auburn University in accordance with U.S. welfare guidelines for the humane care and use of laboratory animals.

2.2. Bacterial growth conditions

B. velezensis strains used in this study were from a collection of soil and catfish intestine-isolated bacteria (Ran et al., 2012). As described previously, each *B. velezensis* strain was grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA) at 28 °C. *E. ictaluri* S97-773 was grown in TSB or on TSA at 26 °C (Ran et al., 2012).

2.3. Spore-amended feed preparation

B. velezensis spores were prepared with some modification by the method described by Kenny and Couch (Kenney and Couch, 1981). *B. velezensis* strains were grown in TSB overnight at 28 °C. The cell suspension was then spread onto spore preparation agar (peptone 3.3 g/L, beef extract powder 1.0 g/L, NaCl 5.0 g/L, K2HPO4 2.0 g/L, KCl 1.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.25 g/L, $MnSO_4$ 0.01 g/L, lactose 5 g/L, agar 15 g/L), and incubated at 28 °C for 5 to 7 days. To collect the spores, 5 mL of sterile water were added to the plate, then scraped using a sterile loop, and poured into a sterile tube. The spore suspension was then incubated at 80 °C for 15 min to kill any vegetative cells. The concentration of the spore suspension was determined by serial dilution and plating onto TSA followed by incubation at 30 °C overnight. The final concentration of spores was altered to $\sim 1 \times 10^9$ colony forming units (CFUs)/mL. Then the spore suspension was added to fish feed at 8% (*v/w*) for a final concentration of 4×10^7 CFU/g of feed, which was consistent with previous published literature and has been regarded as the appropriate dose to elicit probiotic effects in previously performed channel catfish trials (Ran et al., 2012). Commercial feed was spray-coated at 8% of the dry weight with the spore suspension described above, as well as coated with 5% menhaden fish oil at 3% of the dry weight of feed. Prepared feed stocks were stored at 4 °C until used.

2.4. Aquaria study conditions

Channel catfish fingerlings were obtained from Auburn University North Fisheries Unit. Each fish was roughly 4 months old and weighed approximately 20 g and had no immediate history of infection and no previous history of *E. ictaluri* infections. The fingerlings were batched weighed and were randomly assigned to their designated feed treatment with five replicate tanks per treatment and 30 fish per replicate tank. Experimental grow-out tanks included the same 60 L aquaria used during the previously conducted *Bacillus* trials (Ran et al., 2012), each containing 45 L of de-chlorinated Auburn, Alabama, city water supplied through a flow through system at a rate of 0.6 L/min. The water temperature was kept at 25–28 °C for the duration of the trial. Fish were acclimated in experimental tanks while being fed standard catfish fingerling feed for three days prior to initial feeding with experimental feed. During the feeding trial, fish were fed once a day at approximately 3% of the total biomass. After acclimation, fish from each unit were counted and batch-weighed biweekly during the feeding trial. The amount of feed applied to each tank was adjusted every two weeks by the updated weight information. The feeding lasted for 10 weeks, which was deemed necessary based on previously performed challenges (unpublished data). At the end of the trial, fish were batch weighted. The body weight gain and FCR were calculated as:

$$\text{Weight gain} = (\text{Wf}–\text{Wi})/\text{Wi}$$

$$\text{FCR} = \text{F}/(\text{Wf}–\text{Wi})$$

where Wf and Wi were final and initial mean weight (g) per fish in a tank. F is the cumulative amount of feed (g) given to one fish in a tank.

The fish were also evaluated for their levels of protection against *E. ictaluri* infection. After the last batch weighing, fish were further fed with the respective diet for 2 days. Then aliquots of the fish were challenged by *E. ictaluri* 18 h post the last feeding. Each treatment group consisted of five replicates of 60 L aquaria containing 16 fish, except for the control group that only contained 4 replicate aquaria. Fish were challenged by immersion for 1 h in 10 L of water containing 5.2×10^6 CFU/mL of *E. ictaluri* S97–773. *B. velezensis* spore-amended feeding was not interrupted and was continued for one-day post-challenge due to the cessation of feeding by the fish. Fish mortality was recorded daily for seven days and the final mean mortality for each treatment was used to determine the effects of feeding with each *B. velezensis* strain. Representative dead fish were dissected and the presence of *E. ictaluri* were confirmed by microbiological examination of kidney and liver swabs on TSA. The identity of the recovered *E. ictaluri* was confirmed by 16S rRNA gene sequencing. All remaining fish were euthanized after day 7 of the challenge through an overdose of MS-222 (> 250 mg/L) and incinerated. The strain that indicated the best

protective effects against ESC, along with the highest enhancement in growth performance, was further tested for its efficacy in ponds.

2.5. Pond study conditions

An average of 861 fingerling catfish with a total average weight of 26.28 kg were released into each of the eight 0.04 ha ponds. Each randomized pond represented one replicate for each of the two treatments used in the study. Therefore, four replicate ponds were stocked for feeding the probiotic and four replicate ponds were stocked for feeding a control diet. Each pond contained two separate groups of fish that were grown to fingerlings in either pond raceway systems or in aquaria. To distinguish the two batches of fish from one another the adipose fins were clipped from the fish that were raised in aquaria prior to release into each replicate pond. For each pond, an average of 461 fish were released from the raceway origin and an average of 400 fish were released from the aquaria origin. The aquaria derived fish were roughly 3.5 months old and weighed roughly 15 g and the raceway derived fish were roughly six months old weighed roughly 40 g. Once fish were stocked in each replicate pond, fish were fed once daily at approximately 2% of the total fish biomass for ten weeks. Based on the average biomass that was calculated for each group of four ponds, a volumetric 1% feed amount per pond was determined. The fish were acclimated to the ponds for two weeks prior to the trial and fed approximately 0.5% of average biomass of control feed a day. Each pond was fed 0.5% of average biomass at a time and if all the feed was eaten quickly then another 0.5% was given (up to 2%) until feeding behavior was observed to subside. To determine fish growth performance, 100 fish were randomly pulled from the overall population at 10 weeks and weighed. All fish were starved for 48 h prior to harvesting. The remainder of the fish were euthanized with an overdose of MS-222 (> 250 mg/L) and incinerated.

2.6. Fish intestine and pond sampling and DNA isolation

For the aquarium study, intestine samples were obtained just before the start of probiotic feeding or after ten weeks of probiotic-amended feeding. Intestinal tissue samples were obtained aseptically by dissecting the anterior intestine starting at the end of the pyloric stomach region (posterior of the pyloric sphincter) and continuing to the end of the posterior region of the intestine through the rectum just anterior to the anal vent. Three fish were sacrificed through an overdose of MS-22 (> 250 mg/L) and the intestine samples were combined to give one sample. The intestine samples were then homogenized in sterile water and frozen at −80 °C until the DNA extraction was performed. For the initial time point, which consisted only of control samples, there were 23 replicates of fish intestinal samples, each containing pooled samples from three fish, used for DNA isolation. For the ten-week time point, replicate pooled intestine samples were taken from each treatment group ($n = 5$) in each aquarium.

For the pond study, intestine samples were obtained just before the start of amended feeding and again after ten weeks of probiotic-amended feeding. Fish were sacrificed, then the intestine from the end of the stomach to the anus was aseptically removed. The intestine samples were then homogenized in sterile water, and DNA was immediately extracted. For the initial time point, consisting of fish prior to the initiation of feeding, samples were taken from two fish in each of the eight ponds ($n = 16$). For the ten week-post feeding initiation time point, there were eight independent replicates for both the control and AP193 groups and each sample was used for DNA isolation.

Pond water samples were collected in 50 ml sterile tubes at times zero, four and ten weeks post-feeding initiation. The samples were then filtered through a 0.2 μm filter, and DNA was extracted from the filter. For the initial time point, which consisted only of control samples there were eight replicates. For the four and ten-week post-feeding time points there were four replicates for both the control and AP193 groups. All samples were used for DNA isolation.

For both aquarium and pond studies, fish intestine DNA was isolated using a stool extraction kit (E.Z.N.A.® Stool DNA Kit, Omega Bio-Tek, Inc., Norcross, GA) according to manufacturer instructions. Pond water DNA was isolated using a water extraction kit (PowerWater® DNA Isolation Kit, MO BIO Laboratories Inc., Carlsbad, CA) according to manufacturer instructions.

2.7. Pond water quality analyses

Pond water samples were collected every two weeks from time zero to week ten, for a total of six-time points and stored at −80 °C for later use. However, due to the loss of product from prolonged storage, a total of 24 samples were used during the final analyses. Samples were placed within 50 mL conical tubes and were immediately stored at −80 °C until further analysis. Water samples were analyzed using standard protocols as follows: total ammonia nitrogen (TAN) by the salicylate method (Bower and Holmhansen, 1980; Le and Boyd, 2012); nitrite-nitrogen by the diazotization method (Boyd and Tucker, 1992); nitrate-nitrogen was measured by the Szechrome NAS reagent method (Van Rijn, 1993); and total nitrogen (TN) and total phosphorus (TP) were analyzed by ultraviolet spectrophotometric screening method with Aquamate Model AQA 2000E (Thermo Fisher Scientific, Suwanee, GA, USA) and ascorbic acid methods, respectively, following digestion in potassium persulfate solution (Gross et al., 1999).

2.8. 16S rRNA gene sequencing and analyses

16S rRNA gene sequencing was conducted using an Illumina MiSeq next-generation sequencer (San Diego, CA). The V4 variable region (515 bp–806 bp) was PCR amplified with a unique barcode identifier included in the forward primer. The amplicons were then pooled to an equimolar concentration and the Illumina TruSeq DNA library preparation protocol was followed according to manufacturer instructions. The sequences were analyzed using the software program Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010). For the aquarium study, sequences were trimmed using CLC genomics workbench (CLC bio, Boston, MA), ambiguous base calls were removed, and all sequences < 200 bp were omitted. Sequences were then analyzed for taxa relative abundance using QIIME. Libraries were separated according to barcode, and only sequences with a minimum quality score of 10 were used. QIIME was used to identify operational taxonomic units (OTUs) by the "uclust" method with a divergence of 3% (Edgar, 2010). OTUs were aligned by to the Greengenes Core reference alignment by PyNAST (Caporaso et al., 2010; DeSantis et al., 2006). QIIME was then used to summarize OTUs according to treatment and generate a table of phylum relative abundance data. The relative abundance data from QIIME was used to construct graphs with GraphPad Prism (GraphPad Software, La Jolla, CA). For the pond study, sequencing was performed by Molecular Research LP (Shallowater, TX). Sequences were trimmed, ambiguous base calls were removed, and sequences < 150 bp was omitted. OTUs were generated by clustering at 3% divergence, and taxonomically classified by alignment to the Greengenes Core, RDPII, and NCBI (Agarwala et al., 2016; Benson et al., 2017; Cole et al., 2003; DeSantis et al., 2006). Additional ordination analysis of beta diversity, specifically Principal Coordinates Analysis (PCoA) using the Bray-Curtis distance, was conducted via the Phyoseq package in R (McMurdie and Holmes, 2013) and visualized using the R packages ggplot2 (v2.2.1.9000), scales (v0.4.1) and grid (Ginestet, 2011). 16S rRNA gene sequence reads were submitted to the short-read archive database at the National Center for Biotechnical Information (NCBI) within the BioProject accession # PRJNA418129, with catfish intestinal microbiome samples having accession numbers SAMN08105202 to SAMN08105264, and the pond water microbiome samples having accession numbers SAMN08016039 to SAMN08016062.

2.9. Cultivation of B. velezensis AP193 from pond water

To assess the presence of strain AP193 within pond water samples, a 15 mL sample was taken from each pond from surface water selected randomly, on a bimonthly basis. Two separate 100 μL samples were removed from each pond sample, with one sample heat-treated at 80 °C for ten minutes before serial dilution and plating onto Tryptic Soy Agar (TSA), while the other replicate sample was serially diluted and plated onto TSA without heat inactivation. The inoculated plates were incubated for 24 h at 30 °C. After 24 h the CFUs/ml of pond water were determined and any colonies with a colony morphology indicative of *B. velezensis* AP193 were selected for molecular confirmation. Each pure culture was used for DNA isolation using a E.Z.N.A.® Bacterial DNA Isolation kit (Omega Bio-Tek, Inc., Norcross, GA). A *B. velezensis* AP193-specific primer set was designed that targeted a genetic locus not found within any of the other *B. velezensis* strains with an available genome sequence ($n = 32$), with the C20_157F primer (5′- ATCGCATTTGGAT GTGGATT) and the C20_157R primer (5′- CGTTTCTGAATGGCGCT TAT). The PCR thermal cycling conditions consisted of 5 min at 94 °C, followed by 25 cycles of a touchdown PCR with 30 s at 94 °C, 30 s at 68 °C to 60 °C (5 cycles at 2 °C decreasing increments) and 1 min at 72 °C. The PCR results were resolved by agarose gel electrophoresis and any PCR amplicon was purified using an E.Z.N.A Cycle Pure kit (Omega Bio-Tek) and Sanger sequenced using the C20_157F primer at the Auburn Sequencing and Genomics laboratory to confirm the identity of the recovered bacterial isolate as *B. velezensis* AP193.

2.10. Statistical analyses

To analyze the differences between probiotic fed and control fed treatments the aquaria and pond growth performance and mortality data were subjected to one-way analysis of variance (ANOVA) and two-way ANOVA, respectively, followed by Tukey for multiple comparison procedure by mean. A two-way ANOVA was conducted to determine the significance of origin and treatment effects and their interaction during the pond trials. The ANOVA and Tukey comparisons were carried out using Statistical Analysis System version 9.3 (SAS Institute, Inc., Cary, NC, USA) and R version 3.4 (R foundation for Statistical Computing, Vienna, Austria). Water quality data were analyzed for means and standard deviation by ANOVA on ranks followed by Tukey for multiple comparison procedure by means of SigmaPlot version 11.0 statistical software (Aspire Software International, Ashburn, VA, USA).

3. Results

3.1. Catfish growth and disease susceptibility in aquaria

Four *B. velezensis* strains (AB01, AP79, AP143 and AP193) that had been previously observed to have the best efficacy in inhibiting *E. ictaluri* infections and in persisting within the catfish intestine were selected for a ten-week aquaria study to assess their effects on catfish growth performance. Of the four strains, only the diet amended with *B. velezensis* AP143 indicated little to no effects on fish weight gain or FCR compared to the control (Table 1). Fish fed with a diet amended with the other three *Bacillus* strains did show an improved weight gain and FCR compared to the control (Table 1). The weight gain in fish fed a *B. velezensis* AP193-amended feed showed the most weight gain at 6.78 g per fish and had an equivalent FCR to AP79 which was 1.04 (Fig. 1A). The one-way ANOVA and Tukey Multiple Comparison Test showed that the differences between treatment groups were not significant ($P > .05$).

Table 1
Channel catfish growth and FCR in aquaria ($N = 5$).

Treatment	Weight gain per fish (g) in aquaria (mean ± SE)	FCR in aquaria
Control	6.25 ± 0.17	1.09 ± 0.01
AB01	6.64 ± 0.36*	1.07 ± 0.04
AP79	6.58 ± 0.18*	1.04 ± 0.01
AP143	6.32 ± 0.15*	1.09 ± 0.02
AP193	6.78 ± 0.15*	1.04 ± 0.03

* $P > .05$ compared to control.

Following ten weeks of control or probiotic-amended feeding, fish were challenged by immersion with *E. ictaluri*. Fish that were fed with feed amended with *B. velezensis* AB01 or AP143 showed a 6% or 11% increase in percent mortality, respectively, when compared with the control ($P > .05$). On the other hand, fish that were fed with feed amended with *B. velezensis* AP79 were observed to have a 12% decrease in percent mortality when compared to the control ($P > .05$). Among all of the treatment groups, the fish fed with feed amended with *B. velezensis* AP193 had the lowest mean mortality of 47.8%, which was 23% lower than the control group mortality of 62.1% ($P = .07$) (Table 2). While these results did not reveal a statistically significant change in fish growth performance or disease control in aquaria, *B. velezensis* AP193 had been previously observed to provide significant decreases in fish mortality due to infection with *E. ictaluri* (Ran et al., 2012) or with *A. hydrophila* (Addo et al., 2017b), and was therefore selected for evaluation in a replicated pond study.

3.2. Analysis of catfish intestinal microbiota from fish grown in aquaria

Successful PCR amplification of the V4 region of the 16S rRNA gene was achieved for all samples of AP193-, AP79-, and AP143- amended feed, while there were two successful PCR amplifications for the control, and four successful PCR amplifications for AB01-amended feed. The analysis of the intestinal microbiota from catfish raised in replicate aquaria indicated the predominance of the bacterial phyla *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, and *Proteobacteria* (Fig. 2). Similar to previously published results based on 16S rRNA gene sequences, a high relative abundance of *Fusobacteria* taxa was observed within the catfish intestinal microbiota (Larsen et al., 2014). While *Fusobacteria* taxa were dominant within fish in the control group at time zero, a significant 36% decrease in their relative abundance was observed by the ten-week time point ($P < .05$). In contrast, in all of the treatment groups fed with probiotic-amended feed, the relative abundance of the *Fusobacteria* taxa did not decrease to the same extent as in control fish. For all treatment groups, the relative abundance of *Proteobacteria* taxa was observed to increase over time, and this was especially evident for control fish and fish fed with strain AP143 (relative abundance increased 22%; $P < .05$). All significant changes in the relative abundance of bacterial taxa at the genus level are listed in Supplemental Table 1.

3.3. Catfish growth in ponds

Fish fed with the feed amended with *B. velezensis* AP193 spores were observed to have a significant increase in their weight gain compared to the control fish in the pond study (Fig. 1B; $P = .04$). The aquaria-origin fish in the AP193-fed ponds had an average weight gain of 40.08 g compared to the average weight gain for fish in control ponds of 28.55 g, which was a 40.4% increase relative to control fish. The raceway-origin fish had an overall better growth relative to aquaria-origin counterparts, with the fish in control ponds exhibiting an average weight gain of 44.78 g and the fish in ponds fed with AP193 observed to have an average weight gain of 59.37 g, which was a 32.6% increase relative to control fish. There was a significant difference in weight gain between the two fish populations ($P = .01$). Fish losses due to bird predation precluded reporting FCRs from the pond study. The differences in average weight in fish in the control ponds and the AP193-fed ponds at week zero and at week ten were determined and revealed that there was no difference in average weight initially, but that there was an increase in the average weight of the AP193-fed fish relative to

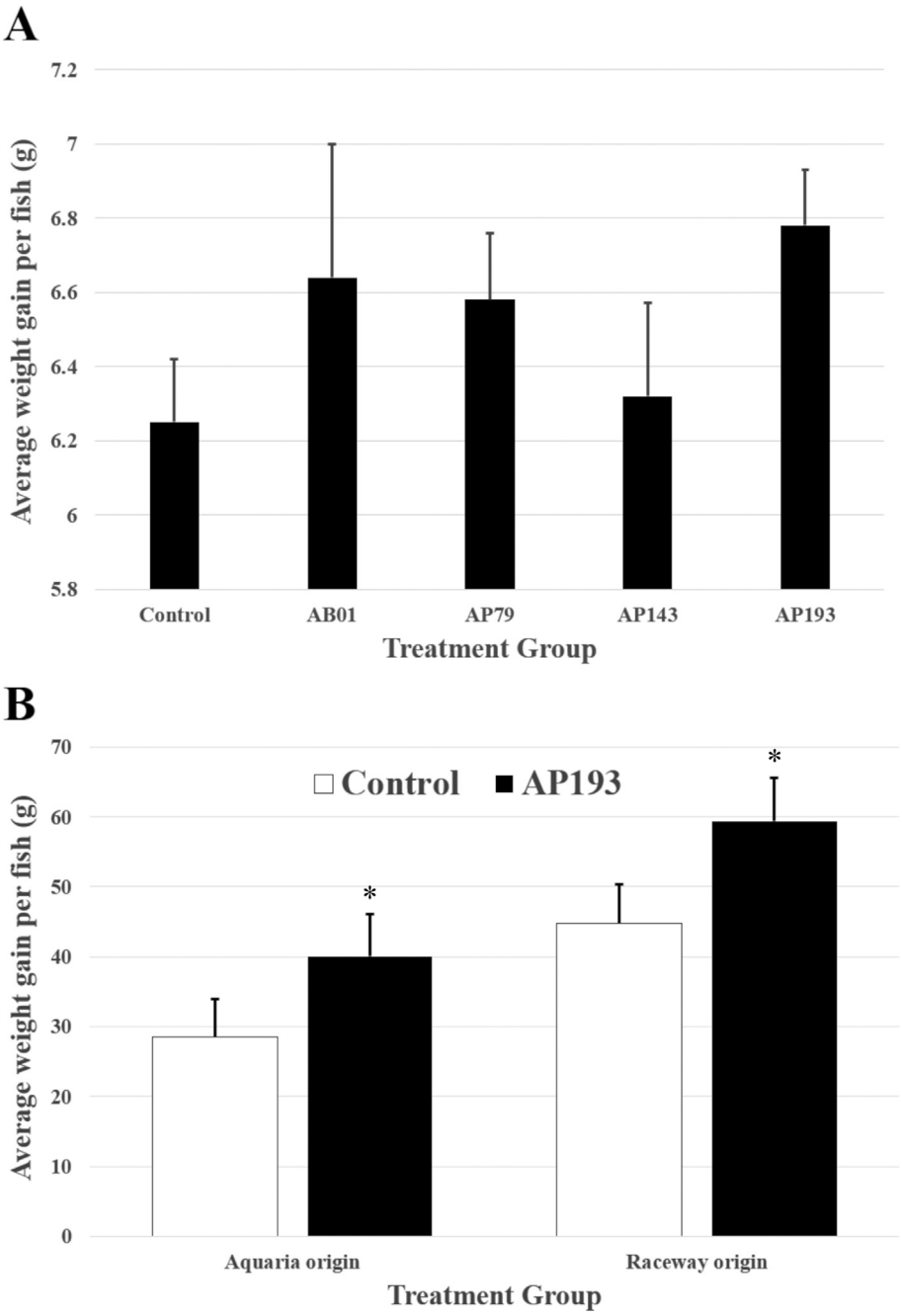

Fig. 1. Growth performance of channel catfish after 10 weeks of probiotic feeding in (Panel A) aquaria or (Panel B) ponds. For the pond study fish were obtained from either aquaria or from a raceway, and fins were clipped to distinguish between the two origins. All data is presented as the average weight gain per fish ± SE of 5 replicate tanks or 4 replicate ponds for Panel A and Panel B, respectively. Asterisk labeled means ($P < .05$ compared to control). Labeled means of fish populations from two origin populations during the pond trial ($P < .05$ compared to control).

Table 2
Mean percent mortality of channel catfish fingerlings challenged with *E. ictaluri* S97-773 after being fed *B. velezensis* probiotic strains for 10 weeks (N = 5).

Treatment	Percent mortality (Mean ± SE)
Control	62.1 ± 8.07
AB01	65.6 ± 3.63
AP79	54.6 ± 5.29
AP143	69.8 ± 1.61
AP193	47.8 ± 3.00*

* $P = .07$ compared to control.

control fish by week ten ($P = .04$) (Fig. 1B).

3.4. Analysis of catfish intestinal microbiota from fish grown in ponds

The analysis of the intestinal microbiota from catfish raised in ponds indicated the predominance of the bacterial phyla *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, and *Proteobacteria* (Fig. 3A). A significant 14.9% decrease in *Bacteroidetes* taxa relative abundance was observed for fish with the *B. velezensis* AP193-amended diet relative to the initial time point ($P < .05$). This decrease in *Bacteroidetes* taxa included reductions in the relative abundance of the genera *Paludibacter* (decreased by 2.8%), *Parabacteroides* (decreased by 4.4%), *Barnesiella* (decreased by 5.3%) and *Dysgonomonas* (decreased by 2.7%). Additionally,

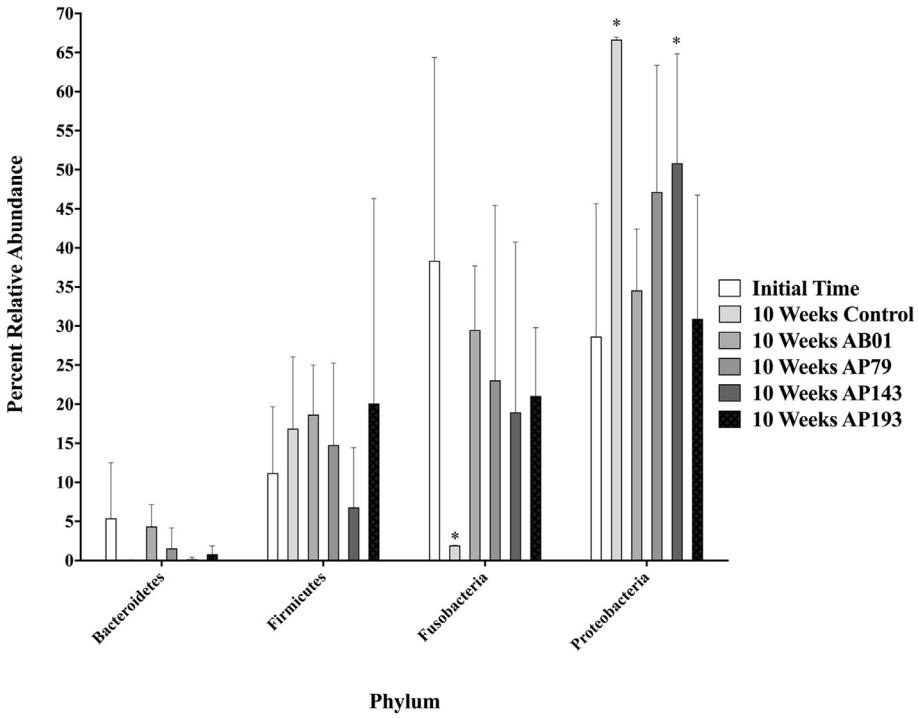

Fig. 2. Phylum relative abundance based on 16S rRNA gene sequences amplified from channel catfish intestinal microbiota for the initial time and after 10 weeks in the aquarium study. All error bars represent the standard error between the pooled replicate intestine samples for each treatment. Asterisk labeled means ($P < .05$ compared to 10 weeks control time point).

Paludibacter and *Parabacteroides* spp. also showed significant decreases (1.4% and 1.0%, respectively) at ten weeks for fish with the AP193-amended diet as compared to the ten-week control group. All other significant changes in the relative abundance of bacterial taxa are listed in Supplemental Table 2. There was a 20.3% increase in the relative abundance of *Proteobacteria* taxa in these probiotic-fed fish ($P < .05$) at ten weeks as compared to the initial time. However, while many *Proteobacteria* taxa increased in their relative abundance over time with probiotic feeding, *Pseudomonas* spp. were observed to decrease in their relative abundance over time by 2.2% in fish fed with a control diet and by 6.6% in fish fed with an AP193-amended diet (Supplemental Table 2). Furthermore, there was a significant decrease of *Pseudomonas* spp. relative abundance of 4.4% when comparing the ten-week control diet with the ten-week AP193-amended diet. In both control and probiotic-fed fish, a significant decrease in the relative abundance of *Firmicutes* taxa was observed from time zero to week ten, with the control group having decreased by 16.8% and the AP193-amended diet group decreasing by 16.6%.

Additionally, the PCoA plot indicated clustering of the intestinal microbiota based on time, as can be seen when comparing initial time versus the ten-week time point for the control and AP193-amended diets (Fig. 3B). However, no separation by treatment group was observed, as can be seen by the close clustering of the ten-week control samples together with samples from the AP193-amended diet.

3.5. Analysis of pond water microbiota

The analysis of pond water microbiota indicated the predominance of the bacterial phyla *Fusobacteria*, *Chloroflexi*, *Firmicutes*, *Verrucomicrobia*, *Cyanobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* (Fig. 4A). No significant change in the relative abundance of bacterial taxa affiliated with the above phyla was observed across treatment groups or over time. Additionally, the PCoA plot indicated clustering of the different treatments, but with no significant difference based on either time or treatment (Fig. 4B).

3.6. Detection of strain AP193 from pond water

While no AP193-like colonies were detected in pond water samples at earlier time points, at the eighth-week time point the probiotic fed ponds and the control ponds had means of 25 CFU/mL and 10 CFU/mL for colonies with an AP193-like colony morphology, respectively. Of the four colonies tested from each pond, 100% of the colonies isolated from the probiotic fed ponds were positively identified as strain AP193, whereas only 50% of the control pond isolated colonies were positive with the AP193-specific primer set (unpublished data). There was no observable difference in AP193 colony detection after week 8.

3.7. Pond water quality

There were differences between the control and probiotic fed ponds for total phosphorus, total nitrogen, and nitrate-nitrogen (Table 3). Total phosphorus was lower in concentration in treated ponds than control ponds, with means of 0.110 mg/L and 0.136 mg/L, respectively ($P = .014$). Total nitrogen was also lower in treated ponds, 0.195 mg/L, than in control ponds, 0.344 mg/L ($P = .025$). Nitrate-nitrogen also followed this trend with the greatest difference with 0.013 mg/L in treated ponds and 0.051 mg/L in control ponds. There were no differences found between the treatments for total ammonia nitrogen and nitrite-nitrogen ($P > .05$).

4. Discussion

The four best-performing *Bacillus* spp. strains (AB01, AP79, AP143 and AP193) from previous studies were selected for further study for their potential as probiotics for use in catfish aquaculture. All four of these strains were found to be affiliated with *B. velezensis* based on phylogenetic analyses (Hossain et al., 2015), without any predicted virulence factors (unpublished data). It was previously observed that strain AP193 expresses the antibiotic, difficidin, and that the production of this polyketide is critical for AP193-mediated biocontrol activity in plants (Hossain et al., 2015). The previous study observed that strain AP193 mutants deficient in difficidin synthesis (Δ*sfp* or Δ*dfnD*) were also completely lacking in the ability to inhibit the in vitro growth of *E.*

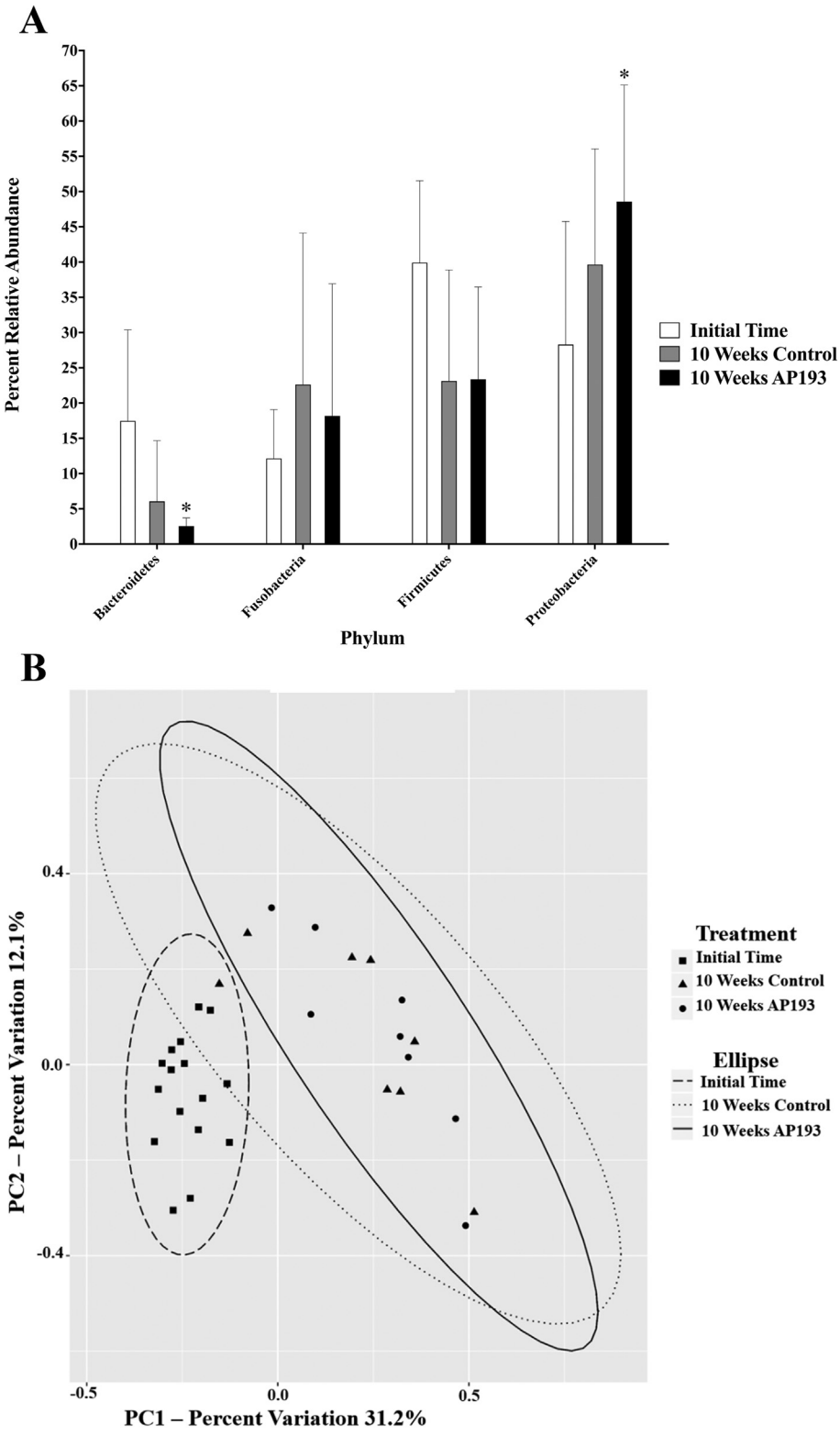

Fig. 3. (Panel A) Phylum relative abundance based on 16S rRNA gene sequences amplified from channel catfish intestinal microbiota for the initial time and after 10 weeks of feeding in the pond study; (Panel B) PCoA plot with 95% confidence interval ellipses, based on Bray-Curtis distance. All error bars represent the standard error between the pooled replicate intestine samples for each treatment. Asterisk labeled means ($P < .05$ when compared to initial time point).

ictaluri, further supporting the hypothesis that difficidin production is important for *E. ictaluri* disease control while leaving open the possibility that other mechanisms (e.g., competitive exclusion, stimulation of fish immune competence) were also involved.

In this study, a significant increase was observed in the growth performance of catfish fed with AP193-amended feed in pond trials, with 40.4% and 32.6% increases in average weight gain for the two populations of fish (aquaria or raceway sourced, respectively) used in the pond study ($P = .04$). Furthermore, multiple comparisons between the two populations of fish indicated that the raceway-reared fish had a

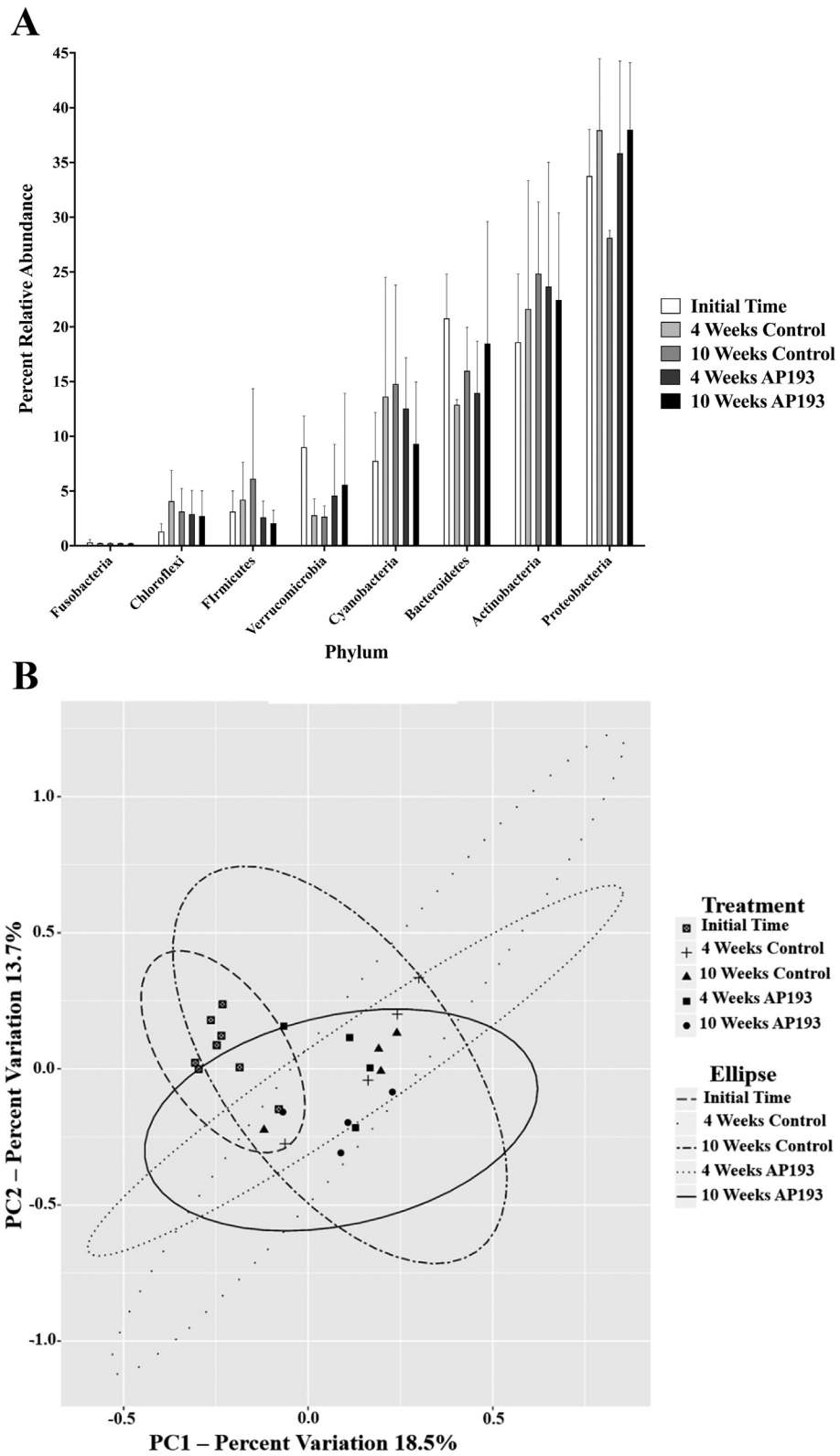

Fig. 4. (Panel A) Phylum relative abundance based on 16S rRNA gene sequences amplified from pond water microbiota for the initial time, 4, and 10 weeks after the start of feeding; (Panel B) PCoA plot with 95% confidence interval ellipses, based on Bray-Curtis distance. All error bars represent the standard error between the replicate water samples of each treatment.

significantly higher weight gain compared to the aquaria reared fish ($P = .01$); however, the differences in weight gain may be due to the differences in starting weights of the two different fish populations. Future research will explore the mechanism(s) by which *B. velezensis* AP193 and other probiotic strains may improve fish growth, including potentially by enhancing feed conversion efficiency and/or by decreasing the levels of the anti-nutrient phytate within the plant-based diet. In support of this latter hypothesis, *B. velezensis* AP193 was

Table 3
Mean concentrations (mg/L) of water quality parameters in control ponds and ponds with catfish fed with control or AP193-amended feed. Labeled means in a column without a common letter differ ($P < .05$) ($N = 24$).

Water quality parameter	*P* value	Control (Mean ± SE)	AP193 treatment (Mean ± SE)
Total phosphorus (mg/L)	0.014	0.136 ± 0.010a	0.110 ± 0.013b
Total nitrogen (mg/L)	0.025	0.344 ± 0.051a	0.195 ± 0.025b
Total ammonia nitrogen (mg/L)	0.829	0.142 ± 0.014a	0.137 ± 0.012a
Nitrite-nitrogen (mg/L)	0.945	0.004 ± 0.001a	0.004 ± 0.001a
Nitrate-nitrogen (mg/L)	0.044	0.051 ± 0.019a	0.013 ± 0.005b

observed to express phytase activity that was greater than or comparable to that of the other *B. velezensis* strains (unpublished data). The hydrolysis of the phosphate groups associated with phytate, mediated by a probiotic-expressed phytase, could result in more iron availability to support fish growth as well as less phosphate excreted from fish.

Significant reductions in total phosphorus, total nitrogen, and nitrate-nitrogen levels were observed in ponds containing channel catfish fed with AP193 that indicate beneficial, pond-wide effects on water quality. Nitrogen is required for plant growth, but excessive concentrations of nitrate-nitrogen and ammonium in ponds can contribute to dense phytoplankton blooms containing cyanobacterial blooms leading to toxic eutrophication and fish "off-flavor" (Boyd, 1982). While a previous study did not show any efficacy in the application of a bacterial amendment directly to pond water (Li and Boyd, 2016), in this study the probiotic applied via feed did result in improved water quality. Soy-based fish feed contains high levels of phytate, which is inositol-hexaphosphate (Cao et al., 2007; Storebakken et al., 1998). Previous research has determined that a hypervirulent strain of *A. hydrophila* causing epidemic outbreaks of motile *Aeromonas* septicemia has the ability to use *myo*-inositol as a sole carbon source, which suggests that the presence of high levels of inositol in the diet could contribute to *A. hydrophila* pathogenesis (Hossain et al., 2013). In addition, genomic analysis of *B. velezensis* AP193 indicates that this strain contains a phytase gene (Hossain et al., 2015), and has been observed to express phytase activity. Thus, *B. velezensis* AP193 has the capacity to degrade the phytate present within feed, potentially resulting in phosphorus uptake by the host and/or intestinal microbiota. Reduced phosphorus excretion into the pond water lessens pond water eutrophication, and presumably also reduces levels of bioavailable inositol that may contribute to *A. hydrophila* pathogenesis. This suggests that *B. velezensis* AP193 could reduce the severity of *A. hydrophila* outbreaks by means of competitive exclusion. Subsequent studies will investigate the benefit of feeding fish with feed amended with *B. velezensis* AP193 in reducing mortality associated with virulent *A. hydrophila*.

The addition of *B. velezensis* AP193 as a probiotic resulted in no significant changes in the intestinal microbiota, as compared to a 36% decrease in *Fusobacteria* over time for control samples, as well as an increase of *Proteobacteria* by 38% and 22% over the ten-week study for control and AP143 treatments, respectively. This indicated that despite the addition of high levels (4×10^7 CFU/g feed) of AP193 amended to channel catfish feed, the fish intestinal microbiota remained stable throughout the ten-week study, in contrast to that observed with the control diet or even with the AP143 treatment. The absence of a decrease in the relative abundance of *Fusobacteria* taxa or an increase in *Proteobacteria* taxa in response to AP193 dietary amendment suggests that the probiotic does not interfere with bacteria already present within the catfish intestine, and that AP193 may have a stabilizing effect on the structure of the intestinal microbial assemblage. *Fusobacteria* are gram-negative anaerobic, rod-shaped bacteria that produce butyrate and are known to be commensal microbiota in the channel catfish intestine (40). Since butyric acid has been observed to inhibit fresh water fish pathogens (Nuez-Ortin et al., 2012), maintaining the level of *Fusobacteria* taxa within the channel catfish intestine may be beneficial. A high relative abundance of *Fusobacteria* taxa within the fish intestine was observed in this study and in an earlier report (Larsen et al., 2014); however, this study did not show as high of a relative abundance of *Fusobacteria* as was observed previously. Interestingly, while *Fusobacteria* relative abundance dropped precipitously within the control fish intestinal microbiota over the course of ten weeks, probiotic-fed fish had levels of *Fusobacteria* taxa that were only moderately decreased at the ten-week time point. The high relative abundance of *Fusobacteria* was not observed in pond water, indicating that *Fusobacteria* taxa are natural inhabitants of the catfish gastrointestinal tract.

It is interesting to note the decrease in *Firmicutes* spp. over time in the pond study, particularly for the AP193 treatment, especially considering that *Firmicutes* spp. were added to the diet of the fish. Perhaps AP193 outcompeted its closely related bacteria for resources and/or attachment within the intestine. However, due to the low phylogenetic resolution afforded by 16S rRNA gene sequences, we cannot conclude based on these sequence data that AP193 was specifically detected from this ribotype analysis. The use of higher resolution phylogenetic analyses and/or strain-specific primer sets in future studies could track the relative abundance of this or other strains within the intestinal microbiota.

No significant differences were observed in the pond water microbiota based on a culture-independent based analysis of 16S rRNA gene amplicon relative abundance. Due to lower resolution of this genetic marker, we could not determine any changes in the relative abundance of *B. velezensis* or strain AP193. We therefore used a culture-dependent approach to determine the levels of *B. velezensis* AP193 in pond water at different time points. The low levels of AP193 (25 CFU/ml) detected in water from ponds in which fish were fed with an AP193-amended diet indicates that some level of the probiotic is present within the pond ecosystem. The observation that strain AP193 was isolated from a control pond, albeit at low levels (5 CFU/ml), could be due to cross-contamination of ponds or due to PCR primer cross-reactivity with other *B. velezensis* strains. These results collectively indicate that feeding channel catfish feed amended with *B. velezensis* AP193 did not significantly alter the pond water microbial assemblages and that low levels of the probiotic are present in pond water after prolonged feeding.

In conclusion, the addition of AP193 to channel catfish feed resulted in an observed stabilization of the intestinal microbiota composition over time. Additionally, the probiotic AP193 resulted in improved pond water quality. These results suggest that AP193 is a viable candidate as a channel catfish probiotic to promote fish growth and reduce aquaculture pond eutrophication, warranting further study in larger scale production ponds over longer time periods.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2018.11.051.

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