Project Title: Probiotics in Yellow Perch and Tilapia Culture [Termination Report]
Key Word(s): Aquaculture Drugs
Total Funds Committed: $240,000
Initial Project Schedule: September 1, 2012 to August 31, 2014
Current Project Year: September 1, 2015 to August 31, 2016
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Reason for Termination: Project objectives completed and funds have been terminated.

Project Objectives:

1. Characterize the microbial community of early ontogeny of yellow perch and tilapia during growout phase in control (laboratory) setting and compare to practical industry conditions (minimum of 2 farms for each species).
2. Isolate bacteria that possess the characteristics resulting in inhibition of pathogenic *Vibrio* and *Aeromonas* species.
3. Compare commercial probiotics to those isolates identified in Objective 2.
4. Establish culture of axenic fish model to evaluate probiotics and inoculants which possess disease inhibition.

Project Summary

Yellow perch larvae were cultured in high density (30-40 per L; 7.9-10.6 per gal) using live zooplankton for 17 days. The average rate of survival through the entire experimental period was 32.0±7.6% and the swim bladder inflation rate was 35.8±20.6%. The average juvenile weight was 24.5±5.0 mg (0.86±0.18 oz) and average growth rate 29.4±1.6% day-1. Fish were then subjected to treatments with isolated probiotic strains of bacteria and potential pathogenic bacteria isolates. Isolates from adult yellow perch were used to further characterize by heat shock challenge and determine their inhibitory potential against common fish pathogens, *Vibrio anguillarium* and *Aeromonas salmonicida*. Of the eight isolates tested all but three isolates showed inhibition of *Vibrio*, while there appears to be only a weak inhibition to *Aeromonas*.

University of Minnesota investigators validated 16S rRNA surveying in yellow perch. Significant technical effort was put towards finding a DNA extraction procedure and PCR conditions suitable for analysis of intestinal contents from farmed yellow perch and tilapia, and compared them to several other fish species from fish farms and wild. To address isolated probiotic from Ohio State University (OSU) yellow perch and commercial probiotics a feeding experiment was performed with yellow perch juveniles that included dietary treatments with yellow perch isolates and controls, and unchallenged fish. *Flavobacterium columnare* challenge was performed by adding final bacterial density of 108/ml. The final survival rates for treatments did not differ significantly (93- 100%). There were no mortalities during columnaris exposure and no disease symptoms were observed in the following 17 days. An affordable axenic apparatus was constructed for use in the culture of fish larvae and juveniles by modification of existing flaws in equipment described in the literature. Check valves were added to all incoming and outgoing water lines to limit backflow, reducing the risk of contamination through exposure to the external environment. All chambers have been placed on a 6-position magnetic stir plate to keep water mixed within the

chambers. This prevented “hypoxic” zones, maintained live zooplankton (rotifers) in suspension, as well as allow for more efficient removal of metabolites and detritus from the system. The system was tested with hybrid cichlid (*Cichlasoma synspilum*, female x *Amphilophus citrinellum* male) and performance (survival and growth) was comparable to fish reared in open system.

Technical Summary and Analysis

The results of the yellow perch larvae/juveniles rearing are indicative of the highest performance. The proposed studies include comprehensive characterization of the microbiota of the yellow perch digestive tract and surrounding water in production facilities of the North Central Region (NCR). These results will be used to identify cultures of probiotic bacteria that are inhibitory to yellow perch pathogens. It is expected that probiotic strains that can protect yellow perch juveniles from infection by at least two common pathogens, *Aeromona*s and *Vibrio* species without negative effects on the host fish, will be identified. Therefore, the probiotics identified in this study can potentially contribute to sustainable development of the aquaculture industry and securing an organic produce status for fish.

Principal Accomplishments

Objective 1. — Yellow Perch larvae used in 2014 experiments were bred from several 5- 6 year old females from the OSU aquaculture facility and males either from the same source or from Millcreek Perch Farm (Marysville, Ohio). The batch produced for intensive rearing in the OSU aquaculture greenhouse facility originated from egg ribbons that were released and fertilized within the broodstock tank on April 23 and 25, 2014. For Phase I, 50-L (13.2 gal) conical tanks were initially stocked with 1628+ 340 (n=9) larvae/tank. This phase began with the first feeding of larvae at 3 days-post-hatching (dph) and continued throughout the first 10 days of exogenous feeding. The system was equipped with a constant inflow of evaporated sea salt and *Nannochloropsis* algae paste. After 10 days of feeding, 300 larvae were randomly sampled from each tank and moved to the indoor laboratory facility.

Phase II lasted for 7 days fish were reared in nine 60-L cylindrical tanks with constant inflow of water. Temperature remained at 17.2±0.2 ˚C (63 ±32 ˚F) throughout this phase. The rotifers *Brachio*nus, a continuous culture maintained at aquaculture lab, and *Artemia* nauplii were hatched from cysts prior to enrichment. During the second phase, fish were initially provided with *Artemi*a, then transitioned to Otohime A® diet. The average rate of survival was 32.0±7.6%. Swim bladder inflation rate was 35.8±20.6% at the end of the second phase. The average juvenile weight was 24.5±5.0 mg (0.86±0.18 oz). The results suggest that the growth of yellow perch larvae/juveniles is greater in the ethyl ester fatty acids (EE)-enriched groups than the triglyceride fatty acids (TAG)-enriched groups, especially during the first 10 days of exogenous feeding.

Findings of DNA extraction and PCR analysis of intestinal content from yellow perch are presented in Technical Update section (Figure 1). The image displays 16S amplicon sequencing from homogenized intestinal tissue from three fish, and a corresponding water tank sample. With the exception of one distal intestinal sample, intestinal samples were predominantly phyla Fusobacteria and Proteobacteria, respectively. While the predominant genus in these samples was *Cetobacterium* spp., the Proteobacteria were substantially more diverse, including genera such as *Plesiomonas, Ralstonia*, and *Aeromonas* among many others. Actinobacteria were mostly

classified as *Mycobacterium* spp. The water tank sample predominantly contained sequences classified as Mycobacterium, Azospira, Pedobacter, and Cetobacterium.

Objective 2. — Bacterial community profiling of wild and farmed fish in the upper Midwest. The analysis was performed using hierarchical clustering which takes into account species presence and abundance, then performs unsupervised clustering based on these parameters (Figure 2). Clear trends emerge which showed association of perch intestinal microbiota with farmed walleye but not with other farmed fishes.

Potential probiotic bacteria were isolated from the intestinal tract of yellow perch collected in OSU aquaculture laboratory. Isolates were challenged by heat shock to further determine their inhibitory potential against common fish pathogens, *Vibrio anguillarium* and *Aeromonas salmonicid*a. To test their direct inhibitory abilities to the two pathogens, investigators first streak plated on agar with our isolates, heat shocked and cross streaked with the pathogenic species. Of the eight isolates tested all but three isolates showed inhibition of *V. anguillarium* but only weak inhibition to *A. salmonicida*. Once it was determined that the isolates have probiotic potential in-vitro to the selected pathogens, the 16S rRNA gene of the isolates was sequenced. Results indicated that five of the six isolates are strains of *Lactococcus lactis* and one isolate was classified to Pseudomonas.

Objective 3.— Preparation of isolates for in vivo experiment included isolate V9 and commercial probiotic 2B. Volume of each culture was adjusted to yield 10^9 cfu/ml and cultures were freeze dried prior to processing into the fish feed. The test consisted of two sets of 12 aquaria that were open (challenge) or semi-recirculating (control). Feeding experiment was performed with yellow perch juveniles (0.08 g). The following dietary treatments were included in the study: commercial diet (control), diet with yellow perch isolated probiotic, diet with commercial probiotic, and yeast and krill based diet. One day before the bacterial challenge fish were divided into 30 fish per tank (designated for the challenge) and the remaining fish were distributed into a parallel system (no challenge). *Flavobacterium columnare* isolation was performed using infected fish (approx. 50% of the external body area infected). Samples were transferred onto a plate with beef extract/agar medium. The colonies were identified (yellowish with not- defined ragged edges). One plate was used to confirm bacterial strain by DNA sequencing method. The bacterial culture from the vial from which the plate had been streaked was used to inoculate additional cultures. In order to carry out columnaris challenge, bacterial culture was added to each tank to 8 provide final bacterial density of 10^8/ml. The desired colonies were found in the challenged group but not in the uninfected group. The density of bacterial colonies in challenged tanks was estimated as $8.7*^{105}$ CFU/ml. The results at the completion of the feeding experiment indicated the largest weight was observed in fish that were fed the control diet (0.57±0.02 followed by probiotic supplemented groups, 0.51±0.13, 0.42±0.03, and 0.23±0.02 g in experimental diet. The final survival rates for treatments after the challenge were 100, 98, 96, and 93%, respectively. There were no mortalities during the 24-h columnaris incubation period. No disease symptoms were observed due to introduction of columnaris bacteria. To date, construction and refinement of the axenic fish model needs to be completed for this objective.

Objective 4. — Investigators have constructed an affordable axenic apparatus (Figure 3) for use in the culture of fish larvae and juveniles by modification of existing flaws in equipment described

in the literature. Check valves were added to all incoming and outgoing water lines to limit backflow, reducing the risk of contamination through exposure to the external environment. All chambers have been placed on a 6-position magnetic stir plate to keep water mixed within the chambers. This prevented "hypoxic" zones, maintained live zooplankton (rotifers) in suspension, as well as allow for more efficient removal of metabolites and detritus from the system. The system was tested with zebrafish (*Danio rerio*) larvae (size comparable to yellow perch larvae) and hybrid cichlid (*Cichlasoma synspilum*, female x *Amphilophus citrinellum* male). Zebrafish embryos were injected into the system and hatched in the chambers. However, survival was compromised and experiments were terminated at this point. Hybrid cichlid, the size of newly hatched fish comparable to Nile tilapia acclimated well in chambers and performance (survival and growth) was comparable to fish reared in open system. Survival of fish in axenic system was 81.2+10% and fish increased weight from 2.3 mg (prior to first feeding) to 14.5+2.8 mg within 7 days at 28^{o}C (82.4 ^{o}F).

Impacts

Yellow perch are often stocked at high densities under environmentally stressed conditions that often result in increased number of diseases. Fish culture operations in the North Central Region (NCR) have all experienced disease outbreaks on occasion, resulting in significant monetary loss. Good husbandry practices can significantly reduce but not eliminate such outbreaks. Given that most aquaculture in the NCR occurs in ponds, administering chemotherapeutic drugs is not economically feasible because the large amount of water in individual ponds precludes treating the water and individual fish from many NCR species often cease or reduce feeding once infected by a pathogen. The industry has long recognized that feeding a nutrient complete diet is a good husbandry practice and that inclusion of probiotics that increase resistance to common pathogens would enhance the effectiveness of such a diet. A cost- effective reduction in fish losses will increase the economic viability of all culture operations within NCR.

The proposed studies included comprehensive characterization of the microbiota of the yellow perch digestive tract and surrounding water in production facilities of the North Central Region (NCR). These results will be used to identify cultures of probiotic bacteria that are inhibitory to yellow perch pathogens. It is expected that probiotic strains that can protect yellow perch juveniles from infection by at least two common pathogens, *Aeromonas* and *Vibrio* species without negative effects on the host fish, will be identified. Potential probiotic bacteria were isolated from the intestinal tract of yellow perch collected in OSU aquaculture laboratory. The probiotics identified in this study can potentially contribute to sustainable development of the aquaculture industry and securing an organic produce status for fish.

Recommended Follow-Up Activities

Intensive sampling is underway using the established protocols to better understand the succession of bacterial species during fish maturation, and the relationship of bacterial succession in the intestines with the surrounding water environment.

Technical Update

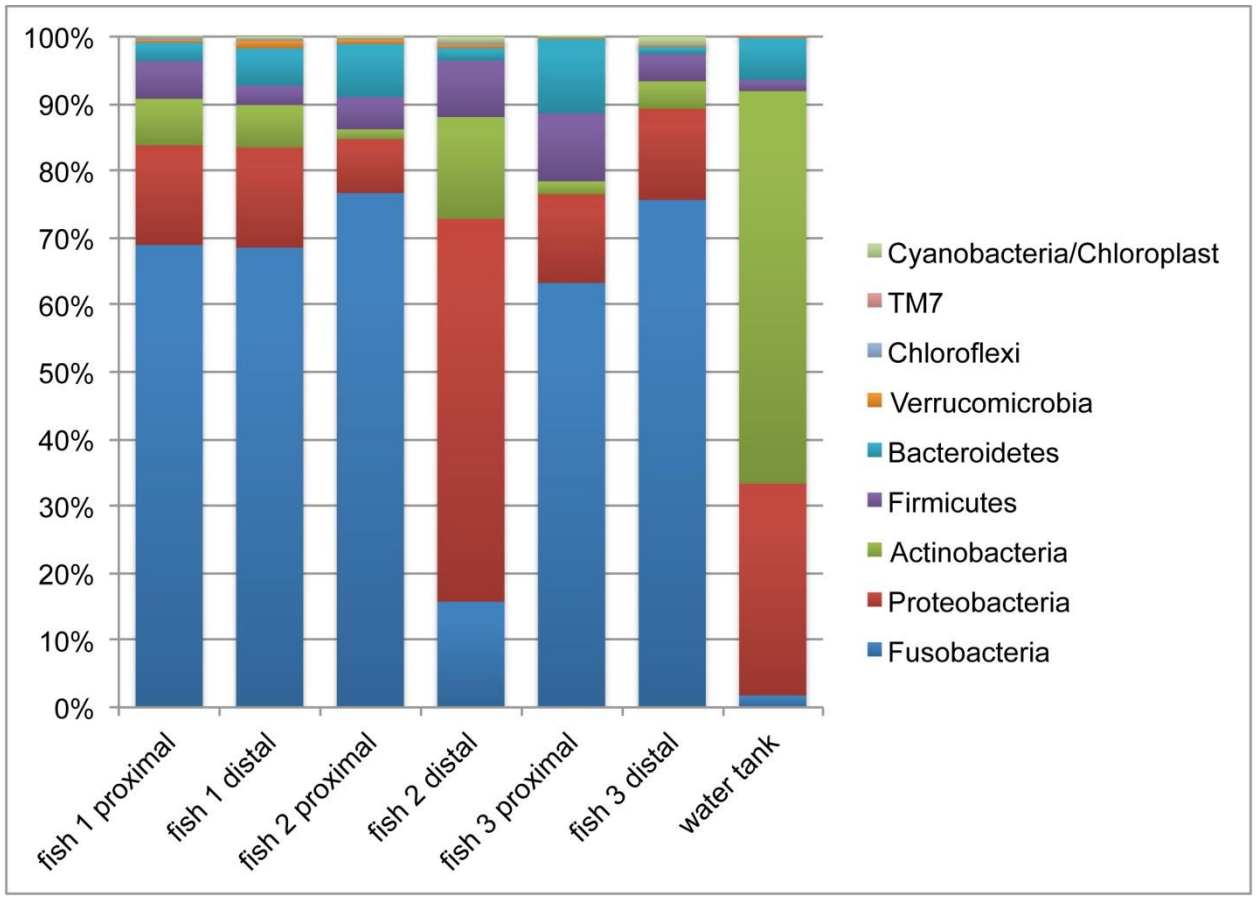

Figure. 1 Identification of microbiota based on amplicon sequencing of 16S fragment from intestinal content from farmed yellow perch and the water from the tank the fish were in.

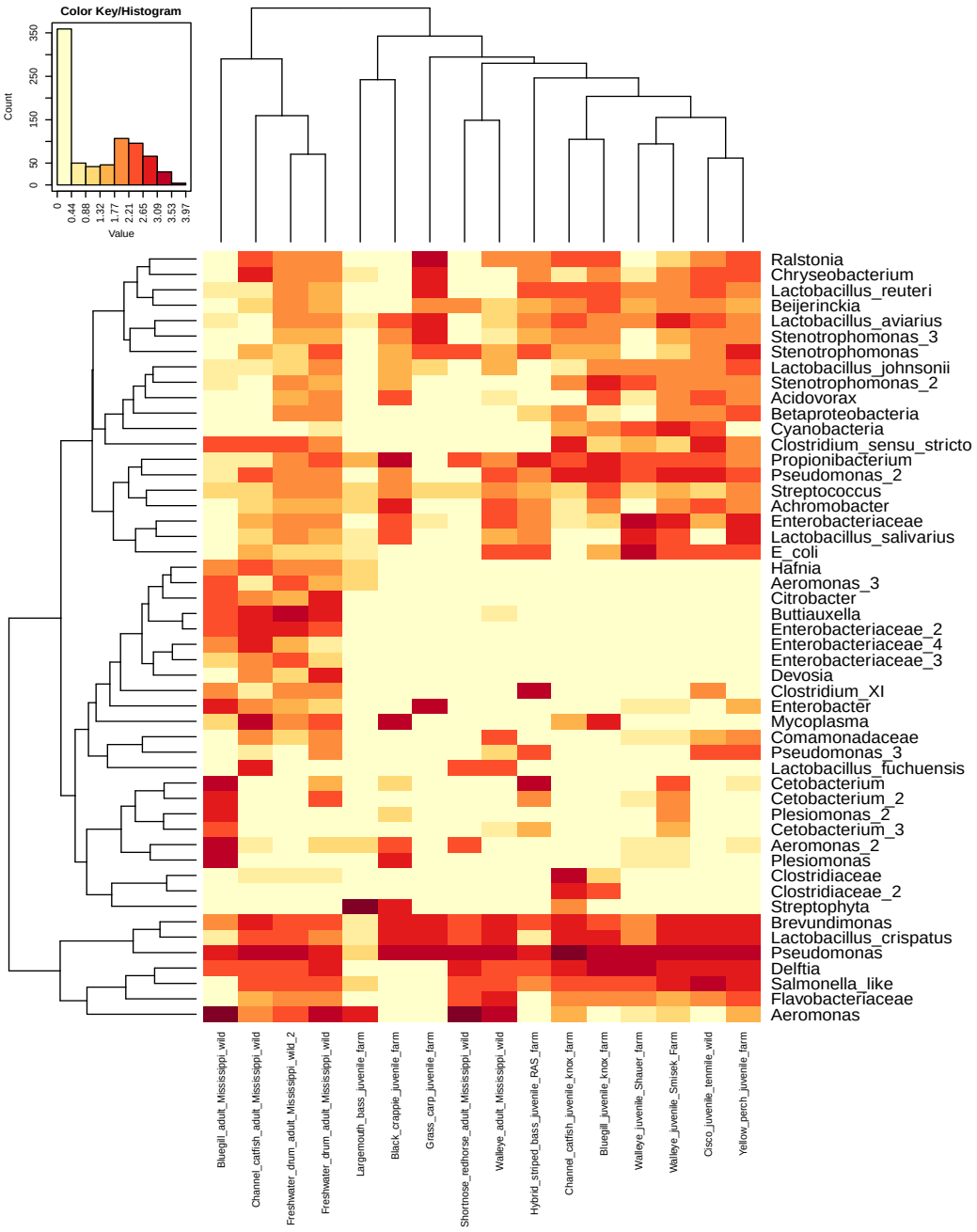

Figure 2 Bacterial community profiling of wild and farmed fish in the upper Midwest. The plot is generated using the presence or absence of all OTUs among all samples tested, and samples that are more closely related to one another are closer to each other on the plot. Some trends were observed. For example, bluegill separated from the rest of the fish, which were mostly intermingled. In particular wild bluegill were responsible for this separation while farm bluegill clustered with other fish types. Except for bluegill, it was difficult to distinguish between species of fish, location of fish, or even farm versus wild fish.

Figure 3. Axenic apparatus after modifications that followed preliminary experiments with zebrafish larvae. A stainless steel “air-stone” was added to each water reservoir (not shown) so that oxygenation with pure oxygen gas is more efficient. Also, AV fistula with 16 gauge syringe needles were added to the rubber septa in order to limit punctures when feeding (zooplankton suspension) or injecting embryos. This reduces the risk of damaging the septa caps due to the use of a large syringe for multiple injections per day. A water pump was added to the outflow manifold to suck water from the system. It was observed that gravity did not provide enough force to remove water from each chamber. Adding a pump, increases the flow rate so that water changes are more efficient and the system is easier to operate.