PROJECT TITLE: Evaluation of Probiotic and Prebiotic Supplements with Catfish, Golden Shiners, Hybrid Striped Bass and Tilapia under Conditions of Commercial Production

PROJECT DURATION: 2 years

FUNDING REQUESTED: $300,000

	Year 1	Year 2	Total
Texas A&M University Delbert Gatlin	$35,912	$30,183	$66,095
Texas A&M University Todd Sink	$4,270	$4,344	$8,614
Auburn University Allen Davis and Jeff Terhune	$27,959	$52,081	$80,040
USDA/ARS WARU Brian Peterson	$31,902	$0	$31,902
USDA/ NCMAC Brian Peterson	$0	$27,999	$27,999
University of Arkansas at Pine Bluff Rebecca Lochmann	$37,733	$21,925	$59,658
TOTAL	$137,776	$136,532	$274,308

OBJECTIVES:

1) Evaluate commercially available prebiotics and probiotics under conditions simulating commercial production by assessing the additive's effects on:
   a) production characteristics such as growth, feed efficiency, survival, and other responses as deemed appropriate and insightful; and;
   b) resistance of the fish to relevant diseases when exposed to pathogens using methods that simulate natural routes of infection.
2) Evaluate the economy of the additives based on observed improvements in fish production and disease resistance relative to feed costs associated with including the additives.

ANTICIPATED BENEFITS:

This project is designed to evaluate a commercially available prebiotic and probiotic under conditions simulating commercial production with prominent fish cultures in the southern region including hybrid catfish, golden shiners, and hybrid striped bass in ponds and tilapia in recirculating aquaculture systems. It is anticipated that these functional feed additives can serve as alternative disease prevention and treatment strategies compared to more traditional uses of vaccines and drugs which are expensive, subject to regulatory constraints and/or associated with inconvenient administration options. This project will provide a thorough evaluation including economic assessment of these functional feed additives.

PROGRESS AND PRINCIPAL ACCOMPLISHMENTS:

Objective 1. Evaluate commercially available prebiotics and probiotics under conditions simulating commercial production by assessing the additive's effects on:
Sub-Objective 1a. Production characteristics such as growth, feed efficiency, survival, and other responses as deemed appropriate and insightful.

Four different dietary treatments have been applied in separate production trials with the four different fish species. The dietary treatments have consisted of: 1) a nutritionally complete basal diet, 2) the basal diet supplemented with 2% Grobiotic®-A (International Ingredient Corporation, St. Louis, MO), 3) the basal diet supplemented with the probiotic Aquablend® (BIO-CAT, Troy, VA), and 4) the basal diet supplemented with both Grobiotic®-A and Aquablend®. The same formulation consisting of a 28% crude protein practical diet was used for the hybrid catfish, golden shiner and tilapia production trials. The basal diet and basal diet supplemented with 2% Grobiotic®-A were prepared by extrusion processing at the Delta Western experimental feed mill in Indianola, MS and shipped to project participants in Alabama, Arkansas and Mississippi without oil top-coating. At each research facility, the four dietary treatments were achieved by periodically top-coating the basal diet and basal + Grobiotic®-A diet with either fish oil at 1% or fish oil at 1% with Aquablend® added to achieve a dietary concentration of 500 million *Bacillus* cells per lb of diet (Stacy Ingram, BIO-CAT, Inc., personal communication). A similar approach was taken with the hybrid striped bass diets except a 43% crude protein basal diet in meal form was provided by Rangen, Inc. of Angleton, TX and the four experimental diets were extruded and prepared at the Texas A&M Food Protein R&D Center.

USDA-ARS Stoneville, MS
A 6-month hybrid catfish feeding study was initiated in May of 2015 and completed at the end of 2016. Fish in all treatments gained closed to 1 pound (454 g) during the production period, and those fed the combination diet had the greatest weight gain of 509 g; however, differences among treatments were not statistically significant (Insert Table 1 here). The most efficient feed conversion ratio (FCR) values and survival also was achieved by catfish fed the combination diet compared to the other treatments, although statistical differences were not evident. Representative fish from each treatment were processed to compute carcass yield, fillet yield and nugget yield, and proximate composition of fillets was also determined (Insert Table 2 here). The various dietary treatments did not have any significant effects on processing characteristics or composition.

University of Arkansas at Pine Bluff
On June 25, 2015, ninety golden shiners averaging 0.77$\pm$ 0.01 g in weight were stocked into each of four floating netpens (5-ft.-diameter=1.5 m, with lids) placed in four different aerated 0.10-acre (0.04-ha) ponds. This is equivalent to a stocking density of 200,000 fish/acre, which is in the range of commercial stocking densities. The four different diets were prepared in the UAPB wet lab according to the protocol given for the project. Each of the four experimental diets was fed to one net pen per pond in a randomized complete block design. Due to the difficulty in seeing the fish during feeding, and the fact that the feed was finely ground to accommodate their small gape, it was not possible to feed accurately to satiation. Therefore, fish were fed 4% of their body weight daily. This rate was adequate for good growth based on results of previous trials with

similarly sized golden shiners in outdoor systems. Water quality characteristics including dissolved oxygen (DO) and temperature were monitored daily and other parameters weekly. Fish were not sampled to determine growth prior to harvest due to concerns over escapement. However, very few mortalities were observed.
On March 17, 2016 all fish were counted and bulk-weighed by netpen. Overall percent increase in growth was 849.3 $\pm$ 76.6 (SE). Mean individual weight gain ranged from 6.1 - 6.9 g/fish, and there were no differences among treatments (P = 0.9569). Survival (with one outlier removed) ranged from 89.3 - 93.3% and there were no differences among treatments (P=0.8991). Subsamples of fish from each netpen were frozen for proximate analysis. Of the remaining live fish, 50 fish per netpen were reserved for the disease challenge (next section).

Auburn University
Two trials were conducted to compare the effects of the probiotic, pre-biotic and combination probiotic/prebiotic in the feed on Nile tilapia (*Oreochromis niloticus*). The trials were conducted at the E. W. Shell Research Station of the School of Fisheries, Aquaculture, and Aquatic Sciences in Auburn, AL. Prior to the beginning of the experiment, fish were acclimated to laboratory conditions for 2 weeks in a recirculating system. A total of 880 juvenile Nile tilapia were sorted for size uniformity and stocked into the culture tanks. Fish of initially averaging 0.01 lb. (5.14 g) were divided randomly into the four dietary treatments with 11 replicates of 20 fish per tank. Fish were fed twice daily with daily feed rations calculated based on % body weight. The rations (5% to 2.5%) were adjusted based on growth and observation of the feeding response. The growth trial was conducted in a recirculating system with 44, ~20-gallon (76-L) aquaria culture tanks, water pump, and supplemental aeration (with central line, regenerative blower and air diffusers) in addition to mechanical and biological filtration to maintain adequate water quality conditions.

At the end of the 10-week experimental period, the fish were counted and weighted, and fish samples were taken for proximate analysis. Results showed that there were no notable effects of the dietary treatments on weight gain or survival (Insert Table 3 here). Because no significant mortalities ensued during the initial 10-week feeding trial, water flow to the tanks was discontinued from recirculating flow and switched to flow through from an outside pond. During this time period, water temperatures decreased from 21 C to 17 C. Again no appreciable mortalities ensued due to pathogens (protozoan nor bacterial). Results showed that there were no notable effects of the dietary treatments on weight gain or survival.

In a parallel study, the effects of the chosen commercial probiotic, prebiotic, and combination of the two also were evaluated with tilapia in 20, 50-gallon (190-L) tanks. Water supply for the tanks was pumped from an adjoining commercial-scale heterotrophic culture unit [~27,000-gallon (102,600-L) tank with a standing crop of approximately 24,000 pounds (10,896 kg) of fish] with a return system in place. The experimental tanks were flushed for 1.5 h, twice per day with the average flow rate into each tank at 1.6 gallons/min (6.1 L/min). Each tank was stocked with 40 Nile tilapia at an average weight of 0.25 pound (113.5 g) per fish. Tanks (N=20; 5 tanks per treatment group) were randomly assigned one of the four dietary treatments described previously. The fish were fed to approximate satiation twice per day. Fish were initially stocked and allowed to acclimate 4 days prior to receiving the treatment diets. Water temperature and oxygen levels were monitored twice per day and total ammonia, nitrite, total alkalinity, total

hardness, and pH recorded at least once per week. All fish were removed and weighed every 2 weeks through week 8 and final harvest occurred at week 12. Mortalities were recorded and were necropsied if suitable for cause of death. Similar to the aquaria trial above, no significant differences in initial weight, weight gain, FCR, or survival were observed between any of the treatments (Insert Table 4 here).

Texas A&M University

The production trial with hybrid striped bass began in June 2016 with juvenile hybrid striped bass averaging approximately 0.15 lb. (71 g) being stocked at a density of 325 fish per pond in 0.1-acre (0.04-ha) ponds. Each of the four experimental diets were fed to fish in three replicate ponds at a rate approaching apparent satiation at least once per day and twice per day when feeding activity was greatest. Fish were fed as much feed as they would consume over a 20-minute period, and feed intake per pond was quantified on a weekly basis.

After 4 months of feeding the experimental diets, a sub-sample of 50 fish were sampled. Average weight was determined for each sample of 50 fish, and five fish per pond were sampled for various tissues. Intestinal samples were collected for microbial composition, and blood as well as head kidney samples were removed for macrophage isolation and measurement of various innate immune responses. An additional 25 fish per pond were relocated to the wet laboratory and subjected to a controlled exposure to *Streptococcus iniae* (described under Sub-Objective 1b).

The gastrointestinal tract of fish fed the Combination diet had a distinct microbial composition compared to the other treatments based on denaturing gradient gel electrophoresis (Insert Fig. 1 here).

The feeding trial was terminated in May of 2016 at which time fish in all treatments were approaching 1 1 pound (454 g). Weight gain, feed conversion ratio and survival of fish fed the various diets were not significantly influenced by the dietary treatments (insert Table 5 here). Although only five dead fish were recovered from all of the ponds throughout the feeding trial, survival at the end of the trial was only around 70%. Bird depredation was observed during the feeding trial, and although efforts were made to limit this, it obviously impacted survival over the 11-month trial period.

At the final sampling, fish were collected for measuring body condition indices, proximate composition of whole body and muscle tissues, as well as intestinal samples for microbial composition. In addition, blood as well as head kidney samples were removed for macrophage isolation and measurement of various innate immune responses.

Hybrid striped bass fed the diet supplemented with Grobiotic®-A showed a significant immunostimulation with regard to neutrophil oxidative radical production as well as plasma lysozyme activity compared to fish fed the Control diet (insert Table 6 here). Body composition analysis was completed and showed no differences related to the various dietary treatment.

Sub-Objective 1b. Resistance of the fish to relevant diseases when exposed to pathogens using methods that simulate natural routes of infection.

USDA-ARS Stoneville, MS

At the end of the production trial, hybrid catfish averaging 1.11 lb. (505.2 g) were subjected to a controlled disease challenge in which they were exposed to fish previously infected with *Edwardsiella ictaluri.* The hybrid catfish showed limited susceptibility to *Edwardsiella ictaluri* under these conditions regardless of dietary treatment. Survival at 21 days after challenge ranged from 94 to 97%.

University of Arkansas at Pine Bluff

After collecting harvest data from the feeding trial, 50 of the remaining live golden shiners per netpen were stressed by holding them out of the water in a net for 30 seconds. Immediately afterward, they were returned to the same netpens they occupied during the feeding trial. For 2 weeks, fish were fed daily to satiation as described for the feeding trial, and inspected for signs of *Flexobacter columnare* disease at each feeding. During this time, only one sick fish was observed, and it had no clinical signs of columnaris. Survival (one outlier removed) at the end of the 2-week period ranged from 96 - 99%, and there were no treatment differences (P=0.6736). It is suspected that the high survival was due to the relatively low water temperature (17ºC) at harvest, which may have reduced the impact of handling and net stress.

Because of the negative results and difficulty in attempting a "natural" challenge in ponds, a controlled laboratory columnaris challenge was performed in indoor tanks. Golden shiners were stocked as groups of 20 fish per tank (0.001 lb. or 0.5 g initial weight) in each of eight replicate 30-gal (110-L) tanks per treatment and fed the same four diets used in the pond study twice daily to satiation for 6 weeks. Fish were then exposed to a virulent strain of columnaris (based on an LC50 determined previously). Mortalities were tracked for 2 weeks post-exposure. Survival ranged from 95.0 - 98.6% and did not differ among treatments (P=0.5017). Although we attempted to maintain a target temperature of 28ºC during the challenge, the actual range was 25 - 26ºC. Also, the bacterial virulence was apparently lower than expected, because survival was uniformly high - even in the control tanks.

In 2017 several attempts were made to repeat the feeding trial and *Columnaris* challenge in aquaria. However, we could not get any young-of-the year fish, and the “off season” fish we got succumbed to viral or bacterial challenges before we could get feeding trials established. The final feeding trial of 2017 ran for 6 weeks, but survival of the fish before challenge only averaged 65%. We did expose the fish to *Columnaris* using our previous methods, but due primarily to the poor quality of the fish we did not obtain reliable results from this trial. Overall, results from this study did not support the use of the prebiotic or probiotic tested in golden shiners relative to the control diet.

Auburn University

Two trials were initiated with the objective of comparing the effects of two probiotics, a prebiotic and a combination probiotic/prebiotic feed in Nile tilapia *Oreochromis niloticus* and their susceptibility to an emerging, highly virulent strain of *Aeromonas hydrophila*. The trials were conducted at the E. W. Shell Research Station of the School of Fisheries, Aquaculture, and Aquatic Sciences in Auburn, AL. In the first trial, two different probiotics, Aquablend® by BIO-CAT or *Bacillus amyloliquefaciens* strain AP193, were formulated with a basal control diet either by themselves or in combination with the prebiotic GroBiotic-A according to

manufacturer's recommendation or, for AP193, 10^7 cfu spores/g of feed. The spores for *Bacillus* strain AP193, were isolated and obtained from stock supplies located at Auburn University. The tilapia with known disease histories used in these studies were spawned and reared at the E.W Shell Fisheries Research Station, Auburn, AL. In the first trial, fish averaging 10 ± 0.15 g each were held in 20-gallon (75 L) aquaria with 20 fish per tank. Water in tanks was maintained under static conditions with aeration and with approximately 50% of the water flushed twice daily. Water temperatures in tanks were maintained between 25-28 C. Fish were randomly assigned to each tank with three replicate tanks per treatment group. The fish were fed one of six experiment diets two-times daily for 4 weeks, to approximate satiation. The treatment diets were categorized as follows:

Diet 1 - Basal diet; Diet 2 - GroBiotic-A; Diet 3 - Aquablend®; Diet 4 - Aquablend® + GroBiotic-A; Diet 5 - AP193; Diet 6 - AP193 + GroBiotic-A

After 4 weeks of feeding trial, a challenge test was conducted on each treatment group with *A. hydrophila*. (an isolated colony of ML09-119 strain). The bacterial suspension was prepared by overnight culture from single colony forming units (CFU) in multiple 25 ml TSB cultures and incubated at 30 C. After 16 h of shaking-culture, all cultures were transferred to new TSB to make a larger batch for immersion procedure and incubated for another 4 h. The turbidity of cultured media was measured to give an OD600 nm value of 1.2 which expected to be $1x10^9$ CFU mL-1 based on standard plate count procedures and previous bacterial growth curves.

The fish were removed from the tanks and an approximately 0.5-cm skin scrape was performed removing the scales and the outer epidermal skin layer. This procedure more closely represents how fish contract *Aeromonas* infections (due to breaks in the skin during handling events). The scrape was made dorsally between the dorsal and caudal fins. Fish were challenged by immersion with *A. hydrophila* at concentration of $1x10^7$ CFU mL-1 for 60 min, and the systems flushed for 24 h at a flow rate of 0.1 L per minute. Once the challenge test was performed, no feed was provided until 24 h after challenge. Feeding and water exchanges were resumed as prior to the challenge.

The cumulative percentage mortalities ranged from 55% in the control group to 17% in the AP193 only treatment group (insert Table 7 here). Due to failure of assumptions for ANOVA analysis, nonparametric tests (NPAR1WAY) were utilized. No significant difference was confirmed among the treatments (P = 0.633) compared to the control group (Diet 1). However, the results showed a reduced mortality trend with AP193 was incorporated into the diets.

Following the results of the above experiment a second feed/challenge experiment was performed with the AP193 and GroBiotic-A only. Tilapia with an average weight of 0.06 lb (25 ± 3.9 g) were held in 20-gallon (75 L aquaria). Twenty fish were randomly assigned to each tank with five replicate tanks per treatment group. Similar to the previous experiment, the fish were fed with the experimental diets two times daily for 4 weeks to approximate satiation. The treatment diets were either basal diet or diet containing 10^7 AP193 spores/g of feed and the manufacturer's recommended amount of GroBiotic-A per g feed. Fish were fed different lengths of time before challenge and then only the basal diet beginning the day after challenge. The four treatment groups were as followed:

Diet 1 - fed 4-weeks with basal diet; Diet 2 -fed 3-weeks with basal diet and 1 week with AP193 and GroBiotic-A; Diet 3 - fed 2 weeks with basal and 2 weeks with AP193 and GroBiotic-A; Diet 4 -fed 4-weeks with AP193 and GroBiotic-A.

After 4 weeks of feeding, a challenge was conducted with *A. hydrophilia* (strain ML09-119). Bacterial preparation, growth and challenge procedures were conducted as described in the previous experiment. The percent mortality in each group were determined after 10 d. The percent mortalities were significant (p-value = 0.004) and ranged between 18% and 54% with significant differences being observed between the basal diet only and diets fed the probiotic/prebiotic combination for 2 and 4 weeks (Insert Table 8 here). No difference was observed among any of the three diets fed the probiotic/prebiotic combination.

Texas A&M University
Hybrid striped bass in each experimental pond were sampled in October 2016 and 25 fish from each pond were moved into a separate tank in a recirculating system consisting of 12, 1200-L fiberglass tanks in an isolated laboratory. A separate group of hybrid striped bass not in the feeding trial are currently infected with *Streptococcus iniae* by injection and introduced into the tanks containing fish fed the experimental diets to allow a natural disease challenge via co-habitation. This type of challenge resulted in very few mortalities regardless of dietary treatment.

In 2017, a challenge with *Mycobacterium marinum* was attempted in a recirculating system after hybrid striped bass had been fed the four experimental diets for 8 weeks. This natural exposure to *Mycobacterium marinum* did not result in greater than 10% mortality and thus dietary differences were not discerned.

Objective 2. Evaluate the economy of the additives based on observed improvements in fish production and disease resistance relative to feed costs associated with including the additives.

Auburn University
The commercial suppliers of Grobiotic®-A and Aqua-blend® were contacted earlier this year to obtain current pricing information on the diet supplements. International Ingredient Corporation indicated Grobiotic®-A is selling for $0.75 per pound, and Bio-Cat quoted a price for Aqua-blend® of $7.27 per pound. This information was provided to Terry Hansen along with production data from the trials with various species. Growth trial analyses reported no significant differences among treatments for all treatments and all species in the project. Thus, the economic analysis did not result in any significant differences due to the dietary treatments.

IMPACTS:

Specific impacts of this project have been limited to date because of the inability to execute effective disease challenges using natural means of infection with the various fish species. However, dramatic improvements in handling stress were observed with hybrid striped bass fed Grobioic®-A. In addition, feeding tilapia a diet with both *Bacillus amyloliquefaciens* strain AP193 and Grobioic®-A for 2 to 4 weeks prior to challenge with *A. hydrophila* resulted in significantly reduced mortality (from 54 to 18%) compared to fish the diet without supplements.

PUBLICATIONS, MANUSCRIPTS OR PAPERS PRESENTED:

A) Oral Presentations:

Ju, M. and D. M. Gatlin III. 2017. Effects of dietary prebiotic and probiotic supplementation on growth performance and disease resistance of hybrid striped bass *Morone chrysops* x *Morone saxatilis* in ponds. Aquaculture America 2017, San Antonio, TX.

Ju, M. and D. M. Gatlin III. 2018. Physiological responses and mortality of hybrid striped bass *Morone chrysops* x *Morone saxatilis* subjected to acute stress after being fed dietary prebiotic and probiotic supplements. Aquaculture America 2018, Las Vegas, NV.

Peterson, B. C., B. G. Bosworth and M. H. Li. 2018. Growth performance, survival and processing characteristics of hybrid catfish fed pre and probiotics. Aquaculture America 2018, Las Vegas, NV.

Lochmann, R., E. Thompson, R. Chen, R. Graham, and A. Porterfield. 2016. Evaluation of prebiotic and probiotic dietary supplements in golden shiners in ponds. UAPB Aquaculture/Fisheries Field Day, Oct. 6, Pine Bluff, Arkansas.

B) Extension/Outreach Products

Peterson, B. C., Bosworth, B. G., Li, M. H. 2018. Growth performance, survival, and processing characteristics of hybrid catfish fed pre- and probiotics. Global Aquaculture Advocate June p. 1-6.

C) Peer-reviewed

Ju, M. and D. M. Gatlin III (in review) Effects of dietary prebiotic and probiotic supplementation on growth performance, innate immunity, intestinal microbiota and histology of hybrid striped bass *Morone chrysops* x *M. saxatilis* in ponds. Aquaculture.

Ju, M. and D. M. Gatlin III (in review) Physiological responses and mortality of hybrid striped bass *Morone chrysops* × *M. saxatilis* subjected to acute stress after being fed dietary prebiotic and probiotic supplements. Fish. Physiol. and Biochem.

Ju, M. and D. M. Gatlin III (in review) Evaluation of temporal effects of a commercial prebiotic and probiotic on growth and gut microbiota of hybrid striped bass *Morone chrysops* × *M. saxatilis* in a recirculating system and survival after a water quality challenge. North American Journal of Aquaculture.

RESULTS AT A GLANCE:

The commercial probiotic and prebiotic supplements did not negatively affect production of hybrid catfish, golden shiner, tilapia and hybrid striped bass under conditions simulating commercial production. However, limited positive effects were observed due to the inability to

execute effective disease challenges using natural means of infection with the various fish species. However, dramatic improvements in handling stress were observed with hybrid striped bass fed Grobiotic®-A. In addition, feeding tilapia a diet with both *Bacillus amyloliquefaciens* strain AP193 and Grobiotic®-A for 2 to 4 weeks prior to challenge with *Aeromonas hydrophila* resulted in significantly reduced mortality (from 54 to 18%) compared to fish the diet without supplements.