

PROBIOTICS IN YELLOW PERCH AND TILAPIA CULTURE

SUMMARY OVERVIEW

Chairperson: Konrad Dabrowski, Ohio State University

Industry Advisory Council Liaison: William Lynch, Marysville, Ohio

Extension Liaison: Nicholas Phelps

Funding request: \$240,000

Duration: 2 Years (September 1, 2012 - August 31, 2014)

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.

Proposed Budgets:

Institution	Principal Investigator	Objectives	Year 1	Year 2	Total
The Ohio State University (OSU)	Konrad Dabrowski Zhongtang Yu	2-4	\$90,000	\$90,000	\$180,000
University of Minnesota (UM)	Timothy Johnson Nicolas Phelps	1	\$30,000	\$30,000	\$60,000
TOTALS			\$120,000	\$120,000	\$240,000

Non-funded Collaborators:

Facility	Collaborator
Millcreek Perch Farm, Marysville, Ohio	William Lynch
University of Ghent, Ghent, Belgium	Peter Bossier
Brehm Perch Farm, West Liberty, Ohio	Jim Brehm
Oswald Fisheries, Ellendale, Minnesota	Greg Oswald
Scales to Tails Seafood, Wooster, Ohio	Dave Lemke

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JUSTIFICATION

Diseases are a major impediment to the further development and domestication of aquaculture species and expansion of the aquaculture industry. Diseases occur in all life stages of fish. However, newly domesticated fish, such as yellow perch or walleye, are particularly vulnerable to disease. In such fish, the proliferation of pathogenic and opportunistic microorganisms, protozoa, fungi, and viruses leads to decreased growth and food utilization, and in many instances, massive mortalities. Conventionally bacterial diseases in aquaculture operation are prevented and treated through application of antibiotics and other pharmacologically active substances. However, the use of chemotherapeutic drugs, even if effective in the short term, may have severe negative impacts on fish, and most importantly leads to development of drug-resistant pathogens. Antibiotics in aquaculture are a direct threat to the health of humans and terrestrial animals because antibiotic resistance can be horizontally transferred to other pathogenic bacteria (Alcaide et al. 2005; Defoirdt et al. 2007; Apun-Molina et al. 2009). Further, the use of chemotherapeutic drugs in pond culture, common to the North Central Region (NCR), is not economically feasible.

Probiotics are preparations of live microbial species, which, after ingestion, inhabit the digestive system of the host and confer direct and indirect health benefits. Therefore, probiotics may reduce or eliminate the use of chemotherapeutic drugs, thus contributing to sustainable development of the aquaculture industry and helping to secure an organic produce status for fish. Disease prevention is the most cost-effective way to decrease losses and increase resistance to infection via the inclusion of prebiotics, substances that selectively promote or inhibit the growth of bacteria (e.g. poly-hydroxybutyrate), or phytochemicals that might disrupt the quorum sensing system of pathogens. While products such as these have been identified for their beneficial effects, their precise mechanisms of action are poorly understood.

In order to better understand the mechanisms by which alteration of microbial communities affect host-fish growth and diet utilization, an integrated project focused on defining the microbial communities involved in fish health is proposed. First, the core microbial communities present in laboratory versus commercial fish, and healthy versus diseased fish will be defined. This work will define fish microbial species associated with optimal health. A model for raising fish species in axenic conditions (all microorganisms are excluded) will then be developed. Prior experience with axenic fishes includes early work by Shaw and Aronson (1954) with tilapia; indeed a species that can flourish in confined conditions and has been suggested as a model for freshwater, commercially-important fish.

Egg disinfection is a method proven to produce axenic fish. By raising axenic tilapia, a fish with a relatively short life cycle, the colonization of these fish will be pursued with specific microbial species or complex consortia, and their pathogenic properties and means of preventing infections, increasing resistance, etc. will be examined. The definition of microbial communities associated with healthy fish is a

critical step towards identifying the most appropriate approaches to optimal probiotic administration. The assessment of the effects of probiotics on the microbial communities, gut development, and resulting growth characteristics needs to be elucidated if optimal fish growth, development, and health are to be realized.

RELATED CURRENT AND PREVIOUS WORK

Focus on fish species important to the NCR

Yellow perch *Perca flavescens* is a species raised in ponds and increasingly in intensive recirculation systems in the region. Recent developments have revealed important diseases and pathological problems that lead to mass mortality due to bacterial diseases. Early on, 108 strains of *Aeromonas hydrophila* were identified and isolated in wild yellow perch. Although no relation was established with lake pollution, several strains appeared to be pathogenic to yellow perch (Vezina and Desrochers 1970).

Tilapia is also farmed in the NCR. This fish has a relatively long intestinal tract, with its length being 11- to 13-fold longer than the body length (Smith et al. 2000). It lives in a warm water habitat, and it displays omnivorous feeding habits. Because these traits result in an intestinal bacterial community that plays an important role in nutrient acquisition, tilapia is an ideal model for understanding host-microbe interactions in health and diseases (Marques et al. 2006). Indeed, tilapia and its gut microbes have been studied extensively (see reviews: Dabrowski and Portella, 2005; Portella and Dabrowski 2008).

Evidence and conditions of yellow perch microbial community

Scientific information on the microbiota in perch originates exclusively from studies on its European sister species, *Perca fluviatilis*, which has been domesticated for several generations and subjected to intensive farming (Doux fils et al. 2011). Wahli et al. (2005) described isolation of *Aeromonas sobria* from farmed European perch and provided evidence based on experimental infection that it was a primary pathogen responsible for disease-associated mortality due to skin lesions and fin rot. However, the inability of some of the *Aeromonas* strains to cause disease in wild and domesticated perch (Doux fils et al. 2011) also indicates the complexity of bacteria-host interactions. Probiotic use in fish shows a potential to reduce disease and improve growth although such therapy is in its infancy and requires improvement. For example, Mandiki et al. (2011) demonstrated a measurable effect of commercial probiotics composed of three species of *Bacillus* on growth and survival of European perch during the first 28 days of life. The effect was dose-dependent and live *Artemia* nauplii were the carriers of bacteria. Probiotics provided through water had no effect on growth or survival of the fish. The authors argued that bacterial preparations had influenced lysozyme activity and total immunoglobulin (Ig) that correlated with fish performance.

The efficacy of dietary probiotics towards a stable fish microbiota is paramount to their success, including their ability to enhance response to pathogens. The only evidence thus far that perch pathogenic *Aeromonas* infection can be controlled by inoculation of probiotic *Pseudomonas chlororaphis* was

recently documented by Gobeli et al. (2009). To our knowledge, no in-depth studies have examined the baseline microbiota of freshwater percid fish or the microbiota compositions of percids relative to health status. Collectively, this underscores a need for better definition of the core fish microbiota, optimization of probiotic usage in fish, and additional studies focusing on the potential benefits of probiotics.

Axenic and gnotobiotic fish The first visionary study on axenic (sterile) fish, *Tilapia macrocephala*, was performed by Shaw and Aronson (1954). The authors were able to sterilize embryos of tilapia in formaldehyde solution and provided evidence of no bacterial (agar plate) or fungus (corn meal agar plate) growth. Embryos remained in sterile medium during hatching and survived until their yolk sac was absorbed (day 24 after fertilization). Some progress has been made with axenic ovoviviparous fish, *Lebistes reticulatus* (Lesel and Dubourgent 1979), however, major impetus in development of fish sterilization technology has only recently taken place (Pham et al. 2008; Rekecki et al. 2009). Rawls et al. (2004) have made a major effort to elucidate the importance of microbiota in the ontogenetic development of the digestive tract of zebrafish, a frequently used model for vertebrates by molecular biologists. The authors claimed that 212 genes related to gut functions are regulated by microbiota (such as epithelium proliferation or promotion of nutrient absorption). Based on the bacterial species identified (from sequencing 16S rDNA amplicons) the authors examined the host-bacterial interactions and argued that bacterial inoculation is essential to development and metamorphosis during early life. Under the experimental conditions of this study when germ-free (GF), sterile zebrafish were fed an autoclaved commercial diet, mortality was 100% by 20 days post-fertilization. These conclusions may be far-fetched in light of the authors' statement that "the organization of the zebrafish gut is similar to that of mammals."

Rawls et al. (2004) designed experiments that included larvae raised with rotifers, *Artemia nauplii*, the commercial feed TetraMin (CONR), GF- larvae, and, as they were called then, conventionalized fish larvae. In the latest treatment, fish were provided with water from a non-sterile culture of zebrafish at day 3 or 6 after fertilization. Females were sterilized in 10% polyvinyl-pyrrolidone. Embryos were raised in a solution of antibiotics. Autoclaved (sterilized) feed (ZM000) was provided. No evidence was given that this feed can support zebrafish growth when given as an exclusive diet. Therefore, this study had its limitations: (1) no control group was included that was fed with an "autoclaved" diet in a conventional rearing system; (2) no fasting control group was included to account for "microbial and protozoan" food presence in the conventional rearing system; (3) GF fish "rescued" 6 days after (post) fertilization (dpf) and fed autoclaved food are not appropriate controls as no record of the accompanying live food (or fasting) was provided; (4) epithelium proliferation studies concentrated on 6 dpf (only 1 day after commencement of feeding), so the difference may be simply related to feed acceptance (delay in formulated diets acceptance is frequently observed); and (5) "autoclaved" food had several vitamins destroyed, lipids were oxidized, and amino acid availability diminished. Therefore, the conclusions drawn by Rawls et al. (2004) suggesting that zebrafish transcriptional responses were most likely related to the presence/absence of microbiota but not the bioavailability of nutrients appear to be unjustified.

If this objection is correct, then coupling a gnotobiotic fish model with functional genomics is, as-of-yet, scientifically unproven. The host-microbial relationship other than pathogenic in larval fish is highly unlikely if someone considers that the food evacuation rate in the intestine frequently only takes 30-40 min (Kaushik and Dabrowski 1983). In conclusion, it is highlighted here that experiments on axenic/gnotobiotic fish are challenging and several confounding factors need to be eliminated in order for them to be effective.

Bates et al. (2006) used zebrafish larvae sterilized with antibiotics (GF confirmed) which were then compared with conventionally raised zebrafish “mono-inoculated” with one of the bacterial strains (*Aeromonas* or *Pseudomonas*). The authors reported an arrest in the gut epithelium differentiation and the lack of brush border intestinal alkaline phosphatase activity. Heat-killed bacteria inoculation restored only partial functionality of the intestine. We argue that these experiments also lacked appropriate control groups, offered nutritionally complete (sterilized) feed, and, therefore, the results might not be conclusive. It is well documented that fish growth can be supported by bacterial biomass (Dabrowski and Portella 2005) whereas heat-killed bacteria are most likely an inadequate source of nutrients.

Until recently there was no evidence that zebrafish can be reared solely on formulated, artificial diets. Carvalho et al. (2006) documented that zebrafish larvae did not perform well when offered a diet based on casein-gelatin and hydrolyzates (semi-purified) and commercial diets in comparison to live food. Zebrafish larvae grown until the age of 21 days (1.43 cm; total length) resulted in 86% survival when fed exclusively live *Artemia* nauplii, suggesting for the first time that live rotifers or *Paramecium* are not needed as the starter food in this species. Kaushik et al. (2011) reported further progress with larval fish diet formulation and claimed that they were able to grow zebrafish larvae using a compound, dry, microparticulate diet (Gemma micro, Skretting, Stavanger, Norway) with an excellent result of 89% survival and good growth (2.3 cm) in 9 weeks. This would certainly be a major breakthrough in freshwater larvae nutrition and extremely useful in the development of sterile feeds for axenic fish. Tilapia early development appears to provide less challenge in respect to rearing conditions and feed acceptance (Hussein et al. 2012). Therefore, tilapia is a better suited model for establishing technologies using a commercially aquacultured fish species.

ANTICIPATED BENEFITS

Fish culture operations in the 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.

The proposed studies include comprehensive characterization of the microbiota of the yellow perch digestive tract and surrounding water in production facilities of the 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. 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. We propose to raise one species in axenic conditions (all microorganisms are excluded). If we are able to raise axenic tilapia, a fish species with relatively short life cycle, colonization of these fish with specific microbial species or complex consortia will be pursued, and their pathogenic properties and efficacy of preventing infections, increasing resistance, etc. will be examined. The effect of probiotics on the microbial communities and gut development and resulting growth characteristics need to be elucidated if optimal fish growth, development, and health are to be realized.

OBJECTIVES

1. Characterize the microbial community of early ontogeny of yellow perch and tilapia during growout phase in a 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.
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4. Establish culture of axenic fish model to evaluate probiotics and inoculants which possess disease inhibition.

PROCEDURES

Characterization of the Microbial Communities in Yellow Perch Culture Facilities in Ohio and Minnesota (Objective 1)

The microbial communities in both the intestines and water environment of early ontogeny of both yellow perch and tilapia will be characterized with respect to bacterial species present and their relative abundance using metagenomics empowered by the Roche 454 DNA pyrosequencing technology, the state-of-the-art technology for this type of analysis. The determined microbial communities will then be compared among different samples: fish reared in a laboratory setting versus fish raised in farms and healthy fish versus fish with external symptoms of diseases. The unique species or group(s) of bacteria indicative of each condition will be identified and correlated to major parameters of each environment or condition. These biomarkers will ultimately aid in the identification of a candidate microbial species to be used in Objective 2 and development of diagnostic markers of fish predisposition to diseases caused by *Aeromonas* spp. and *Vibrio* spp. It is also anticipated that new knowledge will be obtained on the microbial communities that play an important role in the health and disease of yellow perch and tilapia.

Locations in Minnesota and Ohio were selected which were willing to cooperate and represent typical facilities and conditions in the NCR, such as no previous use of antibiotics. Yellow perch will be provided by Oswald Fisheries, Ellendale, Minnesota; Millcreek Perch Farm, Marysville, Ohio; and Brehm Perch Farm, West Liberty, Ohio. Tilapia commercial sites include Ray Barber, St. Mary's, Ohio, and Dave Lemke, Wooster, Ohio.

The overall goal of Objective 1 is to identify intestinal bacteria indicative of a "healthy" state versus that associated with disease caused by bacterial pathogens. Intestinal sections will be collected from fish from the following groups: (1) ten fish each from two laboratory populations for each fish species studied; (2) ten fish each from two aquaculture production populations for each species studied (20 fish each for tilapia and yellow perch, 40 fish total); and (3) ten fish each from laboratory populations of each fish species challenged with *Aeromonas hydrophila* (challenge protocol described in Objective 3; 20 fish each for tilapia and yellow perch, 40 fish total). That is, two different populations ($N = 10$) of yellow perch and tilapia from laboratory stocks ($N = 40$) and aquaculture production ($N = 40$) will be used for the total of 80 healthy fish analyzed; two replicates of challenged fish ($N = 10$) will be performed for the total of 40 diseased fish analyzed. In total, samples from 120 individual fish will be obtained. Some of these samples will be analyzed individually, and all will be analyzed pooled by group as described below. Temporal analysis of microbiota of the different ontogenetic stages, juveniles, and yearlings (growouts) will be included if data collected in Objective 2 is strongly indicative of the benefits of this additional information.

Intestinal sections will be dissected and gut contents will be scraped off and placed into sterile tubes. Ten individual samples from each of the above groups and fish species will be studied to assess fish-to-fish variation ($N = 60$), and the pooled samples representing each group of ten will also be used ($N = 12$). Total DNA will be isolated using an established well-tested method. The V1-V3 region of the 16S rRNA gene will be amplified and tagged for each fish/group. The 72 purified amplicons will be pyrosequenced using a GS FLX Titanium system on a single plate, resulting in over 15,000 reads per sample and 100,000 reads per group. Reads will be quality assessed, trimmed, and analyzed using taxonomic-based and operational taxonomic unit (OTU)-based approaches (Schloss et al. 2009). Class prediction will be used to identify OTUs that are most strongly correlated with the gut microbiota of “healthy” fish, compared to those affected by disease (Golub et al. 1999). The result of this work will (1) comprehensively characterize the gut microbial communities of laboratory, commercial, and disease-affected fish; and (2) identify microbial biomarkers of “healthy” versus “diseased” fish. These biomarkers may ultimately aid in the identification of microbial species to be used in later objectives and for enhanced diagnostic markers of fish predisposition to diseases caused by *Aeromonas* and *Vibrio* spp.

The goal of this research is to identify microbial markers that correlate with a healthy versus diseased state. Class Prediction will be used to identify correlations between positive health and gut microbiome composition based upon both group-level comparisons. With these datasets, the microbial species most strongly associated with positive health that are observed inter-individual and across groups examined (4) will be defined. Each microbial species whose presence or absence is strongly correlated with positive health (i.e., fish with no disease) will be represented by a vector $v(g) = (e_1, e_2, \dots, e_n)$, where e_i denotes the presence of species g (+1 or 0) in the i th isolate out of n total isolates. The class grouping is represented by a vector $c = (c_1, c_2, \dots, c_n)$, where $c_i = +1$ or 0 according to whether the i th isolate belongs to the “optimal” or “diseased” group. The correlation between a microbial species and “healthy” versus “diseased” group can be measured in a variety of ways, including standard Pearson correlation coefficients or Euclidean distances. These methods will be utilized as well as a correlation strategy especially developed by Golub et al. (1999?) (4). These analyses will be performed using an S+ ArrayAnalyzer (Insightful, Seattle, Washington). Once a description of the correlation between each microbial species and class (healthy versus diseased) is obtained, a set of microbial species that are most useful for classifying a fish into the appropriate class will be identified. Each informative microbial species will be “weighted” based on its degree of correlation with a class. These weighted values will be summed into a “prediction strength” that ranges from 0 to 1. By varying the cut-off for the prediction strength, the set of microbial species that most accurately place a fish into the appropriate class will be determined. This set of microbial species will then be termed the class predictor. The expected result from this Class Prediction analysis is a set of microbial species that are most strongly correlated with optimal fish health or diseased fish state. The goal is to identify a set of ten or fewer species that are predictive of each state.

The outcomes of this objective will be (1) the definition of the intestine microbiomes of tilapia and yellow perch, (2) identification of differences in microbial composition and diversity between laboratory-raised and commercially-raised tilapia and yellow perch, (3) identification of changes in the intestine microbiome in response to *A. hydrophila* challenge, and (4) identification of microbial biomarkers of optimal fish health.

We do not anticipate any technical difficulties with the metagenomic techniques as they have been previously optimized and used extensively by the PI for both turkey and chicken intestinal samples. It is possible that we will encounter difficulties identifying distinguishing microbial species that can be used as markers of gut health. However, we have already identified significant and predictable microbial differences in our previous projects in poultry, so this is unlikely to present a problem.

Identify and Isolate Bacteria that Possess the Characteristics Inhibitory towards Pathogenic *Aeromonas* and *Vibrio* Species (Objective 2)

Potential probiotic bacterial strains will be isolated from the intestines of healthy yellow perch identified and characterized; tested for their ability to inhibit growth of *Aeromonas hydrophila*, *Aeromonas sobria*, and *Vibrio anguillarum*; and evaluated for their ability to colonize the intestine of yellow perch.

Sampling and Isolation of Bacteria:

Healthy yellow perch will be selected from outdoor ponds (Marysville, Ohio) and one indoor facility (Ohio State University [OSU] Aquaculture Laboratory). The digestive tract of perch will be divided into three sections: pyloric caeca, anterior, and posterior (distal or rectum) intestine. The content and mucosa of each section will be collected aseptically. The water from these ponds or recirculation system will also be sampled because the rearing water environment of fish may also contain probiotic bacteria (Lauzon et al. 2008). Multiple ponds or aquaria will be sampled. The intestinal content, mucosa, and water samples will be serially diluted in a phosphate-buffered saline (PBS) and plated onto genetic nonselective agar plates. Two different kinds of agar plates will be used: tryptone soya agar (TSA; Sigma-Aldrich) supplemented with 0.3% (w/v) yeast extract (=TSAY) and de Man, Rogosa, and Sharpe (MRS) agar (Merck Chemical). The agar plates will be incubated at 20°C (68°F) for up to 4 days.

In-vitro Evaluation of Antagonistic Activities:

Two different methods, an agar plate overlay method (Ouweland et al. 2003) and the spot-on-lawn method (Hur et al. 2000) will be used. Briefly, each of the agar plates will be replicated using the same media (either TSAY or MRS). The master plates will be kept for recovery of colonies, while the replica plates will be incubated for colony development. Mid-log culture of each of the three pathogenic bacteria will be mixed into the top agar and overlaid over the replica plates.

Following incubation, inhibition against each of the pathogenic bacteria will be assessed by any halo (zone of growth inhibition), which is measured from the edge of the colony of isolates to the area of the growth of the pathogens. Colonies that produce a surrounding halo have the ability to inhibit the pathogens. If this agar overlay method causes streaking of some colonies, a “pour-plate-spread-plate” method will be used.

In the spot-on-lawn method, the isolated bacteria will be cultured in either a tryptone soy broth (TSB) or an MRS broth (depending on the agar plate from which the isolates are isolated) until the culture reaches the stationary phase. Then the bacterial cells will be removed by centrifugation at 4 °C (39 °F). The supernatant of each culture will be concentrated 10 fold in a lyophilizer. Ten µl of each concentrated supernatant will be spot-applied to freshly inoculated lawns of each of the pathogenic bacteria on nutrient agar plates (Sigma-Aldrich). Each agar plate (15 cm; 5.9 in plates) will be spotted with concentrated culture supernatants from multiple isolates (at least 100). Following incubation at 20 °C (68 °F) for 24 h or longer (depending on the growth of colonies), the inhibition zone surrounding each spot will be recorded. Inhibition zones with a diameter of 0.5 cm (0.196 in) or larger will be considered positive.

The inhibitory colonies identified using either of the two methods above will be further evaluated using cross-streaking (Machan et al. 1991) to confirm their ability to inhibit *A. hydrophila*, and *A. sobria*. Briefly, each of the three pathogenic bacteria will be separately streaked across the middle of a TSAY or an MRS plate.

Then, each of five candidate bacterial isolates will be streaked across the inoculated pathogenic bacterium. Multiple plates will be cross streaked to test all the candidate probiotic isolates. The plates will be incubated at 20 °C (68°F) for 24 h or longer (depending on the growth of colonies). Inhibition of the isolate against the pathogenic bacterium will be judged from a lack of growth of the pathogen at their intersection with the candidate bacterial strains. All the confirmed candidate probiotic strains will be identified phylogenetically by sequencing their 16S rRNA genes (Yu et al. 2000). For those isolates that have identical 16S rRNA genes, only one representative will be chosen for further analysis, reducing analysis of redundant isolates.

Determination of Possible Harmful Effects of the Candidate Bacteria on Yellow Perch:

The candidate probiotic isolates will be tested on yellow perch to examine if they can harm yellow perch prior to testing their probiotic effect. The method used by Kim and Austin (2006) will be used. Briefly, each candidate bacterium will be grown in TSB or MRS broth (depending on the agar plate from which the isolate is recovered) at 20 °C (68°F) for 24 h. The bacterial cells will be harvested by centrifugation at 4 °C (39 °F), washed twice, and re-suspended in PBS.

The cell concentration will be determined microscopically and approximately 10^7 cells (in less than 0.1 ml) of each isolate will be injected intraperitoneally (i.p.) and intramuscularly (i.m.) into separate groups of 10-20 yellow perch. Another group of 10 fish will be injected with PBS, as controls. Separately, 10^9 - 10^{10} cells will be mixed thoroughly into 30 g (0.96 ounces) of the yellow perch feed to achieve 10^7 bacterial cells/g feed. Groups of 10 yellow perch will then be fed three times a day for 7 days. Controls will be fed with a normal diet supplemented with the same volume of PBS. After 7 days, survivors will be killed and examined for evidence of infection. The isolates that cause any infection or disease will be excluded from further evaluation.

Expected Results:

It is expected that a large number of bacterial isolates will be obtained from either the intestinal content (108 strains of *Aeromonas* were collected in European perch by Goldschmidt-Clermont et al. 2008) or the rearing water of yellow perch. Some of the isolates are expected to be antagonistic toward one or more of the two selected pathogens and harmless to yellow perch. These isolates will serve as putative probiotic strains and used in the *in-vivo* test (Objective 3).

Pitfalls and Alternative Approaches:

The agar plate overlay method (Ouwehand et al. 2003) is proposed to isolate *Aeromonas*- or *Vibrio*-inhibiting probiotic strains because this method allows rapid screening of large numbers of isolates. Some of the colonies may cause "streaking" that may make it difficult to identify the colonies that produce a halo. In such a case, a "pour-plate-spread-plate" method will be used. Briefly, each of the three pathogens will be mixed into a generic agar and poured into plates. After the agar sets, a mid-log liquid culture of the streaking colonies will be spread on the poured plates. These plates will be incubated and antagonistic isolates will inhibit the growth of pathogens inside the agar producing a surrounding halo.

An i.p. (IP) challenge will be used to make sure there is a model to test the efficacy of the probiotic strains. Again, this model has been successfully used with European perch (see references in the project). If the first results of such challenges prove to be of little value, the research plan will be revised by eliminating the i.p challenge.

Compare Commercial Probiotics to those Isolates Identified in Objective 2

The putative probiotic strains identified in Objective 2 will be evaluated and compared using yellow perch. It is difficult to predict how many putative probiotic strains will be obtained. However, we will test multiple strains (at least five of the most effective strains) using 50 juveniles (1-2 months old) of yellow perch for each strain. Two different methods will be used: in-feed application and in-rearing water application. In the in-feed application, mid-log cells of each putative probiotic strain will be mixed into the feed at a level of 10^7 cells/g feed (Taoka et al. 2006).

The control diet contains no live or dead probiotic strains. Both feeds will be fed to the yellow perch fish in separate, replicated ($N = 3-4$) aquaria for 14 days. Then each fish will be challenged by i.p. injection (approx. 0.1 ml) with $2 \times LD_{80}$ of each of the three pathogens (the lethal dose, LD, will be predetermined using yellow perch and mid-log culture of each pathogenic species, *Aeromonas salmonicida*, *A. sobria*, *Vibrio* spp, tested separately). The probiotic-fed and control fish will be fed their respective diet for another two weeks. The fish will be visually examined twice daily (early morning and late afternoon). At the end of the four weeks, all fish will be weighed.

Dead and moribund fish will be removed, and mortality will be calculated for each tank and then computed per treatment or control. The dead or moribund fish will be examined pathologically to determine if death was caused by the pathogenic challenge. Pathological symptoms described by Wahli et al. (2005) and observed in OSU facilities include large white to reddish foci on the skin, often overgrown with fungus (*Saprolegnia* sp.). These will be used to identify diseased fish. Extensive erosion and hemorrhages of fins, particularly pectoral and caudal, have also been observed. Specific real-time PCR assay will be used to detect and quantify the pathogens challenged to the fish (Fukushima et al. 2007; Trakhna et al. 2009). Colonization of the intestine of the fish by the putative probiotic strain will also be determined using specific real-time PCR assays, which will be developed based on the 16s rRNA gene sequence of each putative probiotic strains. To that end, the entire gut of fish in each treatment will be combined and subjected to DNA extraction (Yu and Morrison 2004). Then the number of putative probiotic cells in the DNA will be quantified using the specific real-time PCR and compared to the control that received no probiotics.

The putative probiotic strains will also be evaluated by inoculating them into the rearing water. Briefly, each of the putative probiotic strains will be cultured in a liter-volume broth medium. At mid-log phase, the cells will be harvested by centrifugation at 4 °C (39 °F) and resuspended in PBS to a concentration of 10^{10} cells/ml. The cells will be inoculated into aquaria containing 50 fingerlings of yellow perch fish to a final cell concentration of 10^6 cells/ml aquarium water. For each putative probiotic strain, 9 aquaria will be used, with 3 of the aquaria being mock inoculated with an equal volume of PBS. The survival of the inoculated putative probiotic strains in the aquaria will be monitored by specific real-time PCR assays. Three of the inoculated aquaria will be inoculated again with the same number of cells of the putative probiotic strain one week after the first inoculation. One week after the second inoculation, the fish will be challenged with each of the three pathogens by inoculating each pathogen into the aquaria water to a final concentration of 10^5 cells/ml of aquarium water. The probiotic-protected and the control fish will be fed the same diet during the entire study. The fish will be visually examined twice daily (early morning and late afternoon). Three weeks after the pathogen challenge, the fish body weight will be recorded for each treatment and control. The mortality and pathogenicity will be determined as described for the in-feed application.

Two commercial probiotic products (e.g., BioPlus 2B, Chr. Hansen A/S, Horsholm, Denmark and SANOLIFE MIC-F, INVE Aquaculture, Belgium) will be compared to the putative probiotic strains selected during this research. The two methods mentioned above will be used to determine the efficacy of the commercial products. The body weight gain and mortality from the commercial probiotic products will be compared to those of the probiotic strains isolated from this study.

Statistical analysis will be performed using SPSS for Windows (version 11.5). Univariate analysis of variance (ANOVA) and Least Significant Difference (LSD) test will also be used to determine significant differences in mortality, body weight gain, colonization of putative probiotic strains, and pathogen load.

Expected Results:

To date, no commercial probiotic strains are available for yellow perch. The two commercial probiotics products chosen to compare in this study were developed for other fish species. Thus, this is probably the first study that seeks to develop probiotics for yellow perch. Several probiotic strains that protect yellow perch fingerlings from infections by the three common pathogens without other negative effects on the host fish are expected to be identified. The *in-vivo* evaluation and the comparison with the commercial probiotics will provide opportunities to determine if and to what extent the probiotics can improve fish growth.

Pitfalls and Alternative Approaches:

The Principal Investigators have extensive experience in rearing yellow perch (Dabrowski et al. 1996; Rinchar et al. 2008) and conducting microbiological analysis of intestinal and environmental bacteria. No major difficulties in accomplishing this objective are anticipated. However, because no previous study has been reported that examined the effect of probiotics on yellow perch, the study has been designed based on studies on other fish species. The concentrations of probiotics and/or pathogens will be adjusted if needed after the initial experiment.

Establish culture of axenic fish model (Objective 4)

Tissue culture flasks will be used with replicated groups; the system has been successfully tested with invertebrates and marine fish larvae (Rekecki et al. 2009). Recently the same system was tested with Nile tilapia (*Oreochromis niloticus*) at the University of Ghent, Belgium. The germ-free tilapia embryos will be obtained at the eyed stage by immersion in a series of disinfectants, hydrogen peroxide, potassium permanganate, glutaraldehyde, or formaldehyde (Shaw and Aronson 1954; Rasowo et al. 2007). Other investigators argue in favor of a culture of gnotobiotic zebrafish, inoculated with one strain of bacteria or defined microbial community (Pham et al. 2008).

Both options will be tested, initially with tilapia larvae obtained from manually expressed gametes for *in vitro* fertilization and immersed in antibiotic (ampicilin and rifampicin). When appropriate, freshly hatched GF brine shrimp nauplii (Marques et al. 2006) will be added to culture bottles every day. Embryos, larvae, and juveniles will be aseptically removed from incubation jars and homogenates tested by placing aliquots in Difco broths. Control culture of fish in conventional conditions will be carried out in parallel. Formulated starter diets will be tested for suitability to autoclaving procedures and tested along with conventional and axenic tilapia larvae.

Expected results:

To date, no standardized conditions have been described for culturing axenic tilapia larvae. No probiotic strains are available for inoculating gnotobiotic tilapia. The two commercial probiotics products chosen to be compared in this study with yellow perch can be also used as controls in gnotobiotic tilapia culture. Thus, this is probably the first study that seeks to develop probiotics for tilapia that has been reared axenic from the embryonic stage. A few probiotic strains that protect tilapia during larval development and differentiation of the digestive tract are expected to be identified. The *in vivo* evaluation of isolates from commercial tilapia culture and the comparison with the commercial probiotics will provide opportunities to determine if and to what extent the probiotics can improve fish survival and growth.

Pitfalls and alternative approaches

The Principal Investigators have extensive experience in rearing tilapia larvae and juveniles (Rodriguez-Montes de Oca et al. 2009) and in conducting nutritional studies (Mbahinzireki et al. 2001). No major difficulties in accomplishing the high density rearing of tilapia in small containers are anticipated. However, because no previous study has been reported that examined the effect of sterile environment and live food, the study has been designed that would gradually integrate the level of replacement of live feeds with formulated diets based on results from studies on other fish species (Rinchard et al. 2008). Several species of inoculates will be tested and the concentrations of probiotics will be adjusted as needed after the initial experiment.

EXTENSION PLAN

The results of the experiments, where appropriate, will be presented at scientific meetings and extension workshops and may be published in scientific journals, extension bulletins, or North Central Regional Aquaculture Center (NCRAC) fact sheets. The research results will also be disseminated through the NCRAC Annual Progress Reports available on the NCRAC Web site (<http://www.ncrac.org>).

FACILITIES

OSU OSU is a world-renowned institution with a proven research history in aquaculture and fisheries. OSU's state-of-the art research facility includes three research laboratories (analytical, histology/histochemistry, and radioactive laboratories) and two experimental fish facilities for temperate and tropical fishes. The facilities are equipped with technology to support fish rearing and exposures of fish to compounds during complete life cycle.

UM Work will be conducted at the UM College of Veterinary Medicine and within a newly renovated 750 sq ft laboratory space in the Veterinary Science Building on the St. Paul campus. This space is designed and equipped to accommodate a molecular pathogenesis laboratory. It is a Biosafety Level 2 facility containing a wide variety of equipment suited for studies involving bacterial pathogenesis. Computer stations are present for day-to-day work, bioinformatical analysis, and database development.

PERSONNEL

OSU

Dr. Yu has served as a principal investigator on numerous research projects funded by federal and state agencies and private companies. His research budget totaled over several million dollars over the past five years. His research focuses on the gastrointestinal microbiota in food animals and humans with an emphasis on understanding the interrelationship among host, feed, and intestinal microbiota and improved feed efficiency and health. His laboratory is well equipped with capabilities in microbiology, molecular biology, and metagenomics, and these capabilities were already applied to studies on intestinal microbiota in tilapia fed bacterial polyhydroxybutyrate. Traditional cultivation-based and contemporary molecular biology, metagenomic, phylogenetic, and bioinformatic techniques and technologies are routinely used in various researches on individual bacteria (including probiotic bacteria such as *Bifidobacterium* and *Lactobacillus*) or microbial communities in gastrointestinal tract of animals and humans, and environments.

UM Dr. Johnson's work focuses on bacterial pathogenomics and microbial horizontal gene transfer. He is a key member of the metagenomics core group in the College of Veterinary Medicine, which has established the methods for physical metagenomic sequencing of gut and skin samples in chickens, turkeys, pigs, dogs, and mice. His group has established an in-house pipeline for the analysis of these metagenomic data and use of the data towards class prediction of microbial species associated with a particular condition of interest. Johnson's technician, Jessica Danzeisen, is well versed in all of the above methods and will participate in the study.

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PROJECT LEADERS

<u>State</u>	<u>Name/Institution</u>	<u>Area of Specialization</u>
Ohio	Konrad Dabrowski The Ohio State University	Aquaculture/Aquatic Toxicology
	Zhongtang Yu Ohio State University	Animal microbiology
Minnesota	Timothy Johnson University of Minnesota	Molecular biology, medical microbiology
	Nicolas Phelps University of Minnesota	Veterinary sciences, fish diseases

PARTICIPATING INSTITUTIONS AND CO-PRINCIPAL INVESTIGATORS

Ohio State University

Konrad Dabrowski
Zhongtang Yu

University of Minnesota

Timothy Johnson
Nicolas Phelps

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE
BUDGET

ORGANIZATION AND ADDRESS The Ohio State University 2021 Coffey Rd. Columbus, OH 43210			Year 1: Objectives: 2-4			
			Duration Proposed Months: <u>12</u>	Duration Proposed Months: _____	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/ Matching Funds Approved by CSREES (If Different)
PROJECT DIRECTOR(S) Konrad Dabrowski Zhongtang Yu			Funds Requested by Proposer		Funds Approved by CSREES (If different)	
A. Salaries and Wages			CSREES FUNDED WORK MONTHS			
1. No. of Senior Personnel			Calenda	Academi	Summer	
a. ___ (Co)-PD(s)						
b. ___ Senior Associates						
2. No. of Other Personnel (Non-Faculty)			# months			
a. _1_ Research Associates-Postdoctorates . . .				\$27,000		
b. ___ Other Professionals						
c. ___ Paraprofessionals						
d. _1_ Graduate Students				\$19,200		
e. _1_ Prebaccalaureate Students				\$3,200		
f. ___ Secretarial-Clerical						
g. ___ Technical, Shop and Other						
Total Salaries and Wages <input type="checkbox"/>				\$49,400		
B. Fringe Benefits (If charged as Direct Costs)				\$12,147		
C. Total Salaries, Wages, and Fringe Benefits (A plus B) <input type="checkbox"/>				\$ 61,547		
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)						
E. Materials and Supplies				\$27,453		
F. Travel				\$1,000		
G. Publication Costs/Page Charges						
H. Computer (ADPE) Costs						
I. Student Assistance/Support (Scholarships/fellowships, stipends/tuition, cost of education, etc. Attach list of items and dollar amounts for each item.)						
J. All Other Direct Costs (In budget narrative, list items and dollar amounts and provide supporting data for each item.)						
K. Total Direct Costs (C through I) <input type="checkbox"/>				\$90,000		
L. F&A/Indirect Costs. (If applicable, specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs in on/off campus bases.)						
M. Total Direct and F&A/Indirect Costs (J plus K) <input type="checkbox"/>						
N. Other <input type="checkbox"/>						
O. Total Amount of This Request <input type="checkbox"/>				\$90,000		
P. Carryover -- (If Applicable)			Federal Funds: \$	Non-Federal funds: \$	Total	
Q. Cost Sharing/Matching (Breakdown of total amounts shown in line O)						
Cash (both Applicant and Third Party) <input type="checkbox"/>						
Non-Cash Contributions (both Applicant and Third Party) <input type="checkbox"/>						
NAME AND TITLE (Type or print)	SIGNATURE (required for revised budget only)			DATE		
Project Director						
Authorized Organizational Representative						
Signature (for optional use)						

According to the Paperwork Reduction Act of 1995, an agency may not conduct or sponsor, and a person is not required to respond to a collection of information unless it displays a valid OMB control number. The valid OMB control number for this information collection is 0524-0039. The time required to complete this information collection is estimated to average 1.00 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing the reviewing the collection of information.

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE
BUDGET

ORGANIZATION AND ADDRESS The Ohio State University 2021 Coffey Rd. Columbus, OH 43210			Year 2: Objectives: 2-4			
PROJECT DIRECTOR(S) Konrad Dabrowski Zhongtang Yu			Duration Proposed Months: <u>12</u> Year 2 Funds Requested by Proposer	Duration Proposed Months: _____ Funds Approved by CSREES (If different)	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/ Matching Funds Approved by CSREES (If Different)
A. Salaries and Wages 1. No. of Senior Personnel			CSREES FUNDED WORK			
			Calenda	Academi	Summer	
a. ___ (Co)-PD(s)						
b. ___ Senior Associates						
2. No. of Other Personnel (Non-Faculty) a. <u>1</u> Research Associates-Postdoctorates . . .			#			
			months		\$28,350	
b. ___ Other Professionals						
c. ___ Paraprofessionals						
d. <u>1</u> Graduate Students.....					\$19,200	
e. <u>1</u> Prebaccalaureate Students					\$3,200	
f. ___ Secretarial-Clerical						
g. ___ Technical, Shop and Other						
Total Salaries and Wages					\$50,750	
B. Fringe Benefits (If charged as Direct Costs)					\$13,142	
C. Total Salaries, Wages, and Fringe Benefits (A plus B)					\$63,892	
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)						
E. Materials and Supplies					\$25,108	
F. Travel					\$1,000	
G. Publication Costs/Page Charges						
H. Computer (ADPE) Costs						
I. Student Assistance/Support (Scholarships/fellowships, stipends/tuition, cost of education, etc. Attach list of items and dollar amounts for each item.)						
J. All Other Direct Costs (In budget narrative, list items and dollar amounts and provide supporting data for each item.)						
K. Total Direct Costs (C through I)					\$90,000	
L. F&A/Indirect Costs. (If applicable, specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs in on/off campus bases.)						
M. Total Direct and F&A/Indirect Costs (J plus K)						
N. Other						
O. Total Amount of This Request					\$90,000	
P. Carryover -- (If Applicable)			Federal Funds: \$	Non-Federal funds: \$	Total	
Q. Cost Sharing/Matching (Breakdown of total amounts shown in line O)						
Cash (both Applicant and Third Party)						
Non-Cash Contributions (both Applicant and Third Party)						
NAME AND TITLE (Type or print)	SIGNATURE (required for revised budget only)				DATE	
Project Director						
Authorized Organizational Representative						
Signature (for optional use)						

According to the Paperwork Reduction Act of 1995, an agency may not conduct or sponsor, and a person is not required to respond to a collection of information unless it displays a valid OMB control number. The valid OMB control number for this information collection is 0524-0039. The time required to complete this information collection is estimated to average 1.00 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing the reviewing the collection of information.
Form CSREES-2004 (12/2000)

BUDGET EXPLANATION FOR OSU

(Dabrowski)

Objectives 2-4

- A. Salaries and Wages.** Year 1: Salary (\$19,200) is requested for one graduate student and (\$3,200) for one prebaccalaureate student to complete literature review, collect rearing pond water chemistry data and conduct laboratory toxicity studies. Salary (\$27,000) is requested for one postdoctoral fellow to analyze samples, calculate results, perform statistical evaluations and prepare description of the results in the context of defined objectives. Year 2: Salary (\$19,200) is requested for one graduate student and (\$3,200) for one prebaccalaureate student to conduct field toxicity studies and assist in final study reports preparation. Salary (\$28,350) is requested for one postdoctoral fellow to calculate results with statistical evaluations and prepare description of the results and final reports.
- B. Fringe Benefits.** \$12,147 year 1 (10% for graduate students and 36.1% for postdoctoral fellow and 15% for undergraduate student); \$13,142 year 2 ((11% for graduate students and 37.1% for postdoctoral fellow and 11% for undergraduate student).
- E. Materials and Supplies.** Year 1: General wet laboratory supplies (\$11,000); general analytical laboratory supplies such as denaturing gradient gel electrophoresis and sequencing services (\$11,703); respirometer for O₂ analysis in axenic cultures (\$3,750); office and study automation and record keeping supplies (\$1,000). Year 2: General wet laboratory supplies (\$11,000); general analytical laboratory supplies (\$13,108); office and study automation and record keeping supplies (\$1,000). Total for year 1 will be \$27,453 and for year 2, \$25,108.
- F. Travel.** Year 1: \$1,000 is requested for transportation, lodging, and meal expenses to conduct pond study at Upper Midwest Environmental Sciences Center, La Crosse, Wisconsin. Year 2: \$1,000 is requested for transportation, lodging, and meal expenses to conduct natural rearing pond experimental exposures.

July 5, 2012

Dr. Joseph E. Morris, Director
North Central Regional Aquaculture Center
Iowa State University
339 Science II
Ames, Iowa 50011-3221
jemorris@iastate.edu

Re: Collaborative **NCRAC** Proposal titled: ***"Probiotics in Yellow Perch and Tilapia culture."***

Dear Colleague:

The Ohio State University (Office of Sponsored Programs) is pleased to indicate our institution's willingness to participate with Michigan State University on the above referenced **North Central Regional Aquaculture Center (NCRAC)** research proposal. The budget has been reviewed and is approved in the amount of **\$180,000**. The portion of the project to be conducted at this institution will be under the direction of Dr. Konrad Dabrowski, Professor in the School of Environment and Natural Resources at The Ohio State University.

The Ohio State University (Office of Sponsored Programs) administers all grants and contracts on behalf of the faculty of the University. Therefore, any awards should be addressed to The Ohio State University (tax I.D. #31-6025986; DUNS# 832127323, Congressional District: OH-015th and current federal negotiated rate agreement: http://rf.osu.edu/documents/FA_rate.pdf) and directed to my attention at the following address:

The Ohio State University Office of Sponsored Programs
1960 Kenny Rd.
Columbus, Ohio 43210-1016

We look forward to a productive collaboration in this program. Please contact Dr. Dabrowski at (614) 292-4555 or e-mail: dabrowski.1@osu.edu for technical information regarding the proposal. Questions administrative in nature should be directed to the undersigned at (614) 292-3721, or e-mail: finch.24@osu.edu.

Respectfully,

THE OHIO STATE UNIVERSITY
Office of Sponsored Programs



ORGANIZATION AND ADDRESS
 University of Minnesota (UMN),
 College of Veterinary Medicine
 1971 Commonwealth Ave, St. Paul, MN 55108

PROJECT DIRECTOR(S)
 Timothy J. Johnson

Year 1: Objectives: 1

Duration Proposed Months: <u>12</u>	Duration Proposed Months: _____	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/ Matching Funds Approved by CSREES (If Different)
Funds Requested by Proposer	Funds Approved by CSREES (If different)		

A. Salaries and Wages	CSREES FUNDED WORK		
	Calenda	Academi	Summer
1. No. of Senior Personnel			
a. ___ (Co)-PD(s)			
b. ___ Senior Associates			
2. No. of Other Personnel (Non-Faculty)	4.8		
a. _1_ Research Associates-Postdoctorates . . .			
b. ___ Other Professionals			
c. ___ Paraprofessionals			
d. ___ Graduate Students.....			
e. ___ Prebaccalaureate Students			
f. ___ Secretarial-Clerical			
g. ___ Technical, Shop and Other.....			
Total Salaries and Wages <input type="checkbox"/>			

\$15,284			
\$15,284			
\$6,312			
\$21,596			
\$8,404			
\$30,000			

B. Fringe Benefits (If charged as Direct Costs)	
C. Total Salaries, Wages, and Fringe Benefits (A plus B) <input type="checkbox"/>	
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)	
E. Materials and Supplies	
F. Travel	
G. Publication Costs/Page Charges	
H. Computer (ADPE) Costs	
I. Student Assistance/Support (Scholarships/fellowships, stipends/tuition, cost of education, etc. Attach list of items and dollar amounts for each item.)	
J. All Other Direct Costs (In budget narrative, list items and dollar amounts and provide supporting data for each item.)	
K. Total Direct Costs (C through I) <input type="checkbox"/>	
L. F&A/Indirect Costs. (If applicable, specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs in on/off campus bases.)	
M. Total Direct and F&A/Indirect Costs (J plus K) <input type="checkbox"/>	
N. Other <input type="checkbox"/>	
O. Total Amount of This Request <input type="checkbox"/>	

\$15,284			
\$6,312			
\$21,596			
\$8,404			
\$30,000			
\$30,000			

P. Carryover -- (If Applicable) Federal Funds: \$ Non-Federal funds: \$ Total \$30,000

Q. Cost Sharing/Matching (Breakdown of total amounts shown in line O)	\$11,000
Cash (both Applicant and Third Party)	
Non-Cash Contributions (both Applicant and Third Party) <input type="checkbox"/>	

NAME AND TITLE (Type or print)	SIGNATURE (required for revised budget only)	DATE
Project Director		
Authorized Organizational Representative		
Signature (for optional use)		

ORGANIZATION AND ADDRESS University of Minnesota (UMN), College of Veterinary Medicine 1971 Commonwealth Ave, St. Paul, MN 55108				Year 2: Objectives: 1					
				Duration Proposed Months: <u>12</u> Year 2 Funds Requested by Proposer	Duration Proposed Months: _____ Funds Approved by CSREES (If different)	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/ Matching Funds Approved by CSREES (If Different)		
PROJECT DIRECTOR(S) Timothy J. Johnson									
A. Salaries and Wages 1. No. of Senior Personnel		CSREES FUNDED WORK							
		Calenda	Academ	Summe					
a. ___ (Co)-PD(s)									
b. ___ Senior Associates									
2. No. of Other Personnel (Non-Faculty) a. <u>1</u> Research Associates-Postdoctorates . . .					\$15,284				
b. ___ Other Professionals									
c. ___ Paraprofessionals.....									
d. <u>1</u> Graduate Students									
e. ___ Prebaccalaureate Students									
f. ___ Secretarial-Clerical									
g. ___ Technical, Shop and Other									
Total Salaries and Wages <input type="checkbox"/>					\$15,284				
B. Fringe Benefits (If charged as Direct Costs)					\$6,312				
C. Total Salaries, Wages, and Fringe Benefits (A plus B) <input type="checkbox"/>					\$21,596				
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)									
E. Materials and Supplies					\$8,404				
F. Travel									
G. Publication Costs/Page Charges									
H. Computer (ADPE) Costs									
I. Student Assistance/Support (Scholarships/fellowships, stipends/tuition, cost of education, etc. Attach list of items and dollar amounts for each item.)									
J. All Other Direct Costs (In budget narrative, list items and dollar amounts and provide supporting data for each item.)									
K. Total Direct Costs (C through I) <input type="checkbox"/>					\$30,000				
L. F&A/Indirect Costs. (If applicable, specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs in on/off campus bases.)									
M. Total Direct and F&A/Indirect Costs (J plus K) <input type="checkbox"/>									
N. Other <input type="checkbox"/>									
O. Total Amount of This Request <input type="checkbox"/>					\$30,000				
P. Carryover -- (If Applicable) Federal Funds: \$				Non-Federal funds: \$		Total \$30,000			
Q. Cost Sharing/Matching (Breakdown of total amounts shown in line O)									
Cash (both Applicant and Third Party) <input type="checkbox"/>									
Non-Cash Contributions (both Applicant and Third Party) <input type="checkbox"/>									
NAME AND TITLE (Type or print)			SIGNATURE (required for revised budget only)				DATE		
Project Director									
Authorized Organizational Representative									
Signature (for optional use)									

BUDGET EXPLANATION FOR UM

(Johnson)

Objective 1

- A. Salaries and Wages.** Years 1 and 2: Salary (\$15,284/year) is requested for 40% of an Assistant Scientist's time to complete sample collections, challenge studies, sample processing, sequencing, and data analysis.
- B. Fringe Benefits.** Years 1 and 2: The fringe benefit rate is 41.3%.
- E. Materials and Supplies.** Years 1 and 2: Funds are requested for sequencing (\$4,500); amplicon QC (\$125); titration (\$1,250); sample preparation (\$900); fish challenge experiments and sample collection (\$1,629).

UNIVERSITY OF MINNESOTA

Twin Cities Campus

Sponsored Projects Administration

*450 McNamara Alumni Center
200 Oak Street S.E.
Minneapolis, MN 55455
Office: 612-624-5599
Fax: 612-624-4843*

July 17, 2012

Dr. Joseph E. Morris, Director
North Central Regional Aquaculture Center
Iowa State University
339 Science II
Ames, Iowa 50011-3221

SUBJECT: Project entitled "Probiotics in Yellow Perch and Tilapia Culture"

Dear Dr. Morris:

As the Authorized Organizational Representative (AOR) I would like to inform you the Regents of the University of Minnesota (U of M) wishes to participate in the above referenced project as a subcontractor to Ohio State University. Dr. Timothy Johnson will serve as the Principal Investigator of the subcontract and he has access to all of the necessary equipment, laboratory, and office space to successfully undertake this project. I also approve the budget as submitted for Dr. Johnson's involvement in this project. Upon issuance of approval to the North Central Regional Aquaculture Center for this project the Regents of the University of Minnesota (U of M) will enter into a formal agreement with your institution.

Sincerely,



April Coon
Assistant Director

BUDGET SUMMARY FOR EACH PARTICIPATING INSTITUTION

Year 1

	UMN	OSU	Totals
Salaries and Wages	\$15,284	\$56,400	\$71,684
Fringe Benefits	\$6,312	\$12,956	\$19,268
Total Salaries, Wages, and Fringe Benefits	\$21,596	\$69,356	\$90,952
Nonexpendable Equipment			
Materials and Supplies	\$8,404	\$18,644	\$27,048
Travel		\$2,000	\$2,000
All Other Direct Costs			
TOTAL PROJECT COSTS	\$30,000	\$90,000	\$120,000

Year 2

	UMN	OSU	Totals
Salaries and Wages	\$15,284	\$56,800	\$72,084
Fringe Benefits	\$6,312	\$13,478	\$19,790
Total Salaries, Wages, and Fringe Benefits	\$21,596	\$70,278	\$91,874
Nonexpendable Equipment			
Materials and Supplies	\$8,404	\$17,722	\$26,126
Travel		\$2,000	\$2,000
All Other Direct Costs			
TOTAL PROJECT COSTS	\$30,000	\$90,000	\$120,000

SCHEDULE FOR COMPLETION OF OBJECTIVES

Objective 1: Initiated in Year 1 completed in Year 1.

Objective 2: Initiated in Year 1 completed in Year 2.

Objective 3: Initiated in Year 1 completed in Year 2.

Objective 4: Initiated in Year 2 completed in Year 2.

PRINCIPAL INVESTIGATORS

Konrad Dabrowski, Ohio State University
Timothy Johnson, University of Minnesota
Nicholas Phelps, University of Minnesota
Zhongtang Yu, Ohio State University

VITA

Konrad Dabrowski
The Ohio State University, School of Environment
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EDUCATION

M.S. Inland Fisheries; Agriculture University, Olsztyn, Poland, 1972
Ph.D. Fisheries; Agriculture University, Olsztyn, Poland, 1976
D.Sc. Fish Physiology, Agricultural University, Szczecin, Poland, 1984

POSITIONS

Professor, School of Natural Resources, The Ohio State University, Columbus, Ohio (1989-present)
Visiting Professor, University of Ghent, Belgium (2009)
Visiting Professor, Institute of Zoology, University of Innsbruck, Innsbruck, Austria (1987-1989)
Visiting Professor, Department of Biology, University of Paris VII, Orsey, France (1985)
Visiting Professor Department of Aquaculture, University of Fisheries, Tokyo (1984-1985)
Assistant/Associate Professor, Inland Fisheries and Water Protection, Agriculture University, Olsztyn,
Poland (1972-1987)

SCIENTIFIC AND PROFESSIONAL ORGANIZATIONS

American Fisheries Society
World Aquaculture Society

SELECTED PUBLICATIONS

- Jaroszewska, M., Lee, B.J., Dabrowski, K., Czesny, S., Rinchar, J., Trzeciak, P., Wilczyńska, B.
2009. Effects of vitamin B₁ (thiamine) deficiency in lake trout (*Salvelinus namaycush*) alevins at
hatching stage. *Comparative Physiology and Biochemistry* 154A: 255-262.
- Dabrowski, K., Ware, K., Jaroszewska, M., Kwasek, K., 2009: Evaluation of walleye embryo survival
and larval viability following iodine treatment. *North American Journal of Aquaculture* 71:122-129.
- Czesny, S., Dettmers, J.M., Rinchar, J., Dabrowski, K. 2009. Linking egg thiamine and fatty acid
concentrations of Lake Michigan lake trout with early life stage mortality. *Journal of Aquatic
Animal Health* 21:262-271.
- Jaroszewska, M., Dabrowski, K. 2009: The nature of exocytosis in the yolk trophoblastic layer of
silver arowana (*Osteoglossum bicirrhosum*) juvenile, the representative of ancient teleost fishes.
Anatomical Record-Advances in Integrative Anatomy and Evolutionary Biology 292:1745-1755.
- Rodriguez de Oca, G.A.R.M., Dabrowski, K., Park, K., Lee, K.J., Abiado, M.A., 2009. Interaction of
phytochemical-quercetin with the other antioxidant, ascorbic acid and their protective effect in
tilapia after ultraviolet irradiation. *Journal of the World Aquaculture Society* 40:586-600.
- Dabrowski, K., M. Arslan, J. Rinchar, M.E. Palacios. 2008. Growth, maturation, induced spawning,
and production of the first generation of South American catfish (*Pseudoplatystoma* sp.) in the
North America. *Journal of the World Aquaculture Society*. 39: 174-183.

VITA

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The Ohio State University, Department of Animal Sciences
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Columbus, OH 43210

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Fax: (614) 292-2929
E-mail: yu.226@osu.edu

EDUCATION:

M.S. New Mexico State University, 1995, Biology (minor in Environmental Toxicology)
Ph.D. New Mexico State University, 1997, Molecular Biology

POSITIONS:

Assistant Professor, 10/2006-present, Dept. of Animal Sciences, and Ohio Agricultural Research Development Center, The Ohio State University, Columbus, Ohio.
Visiting professor, 9/2007-present, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences
Research Scientist, 2003 to 06/2005. Dept. of Animal Sciences, The Ohio State University.
Senior Associate Research Scientist, 2001 to 2002. Dept. of Animal Sciences, The Ohio State University.
Postdoctoral Researcher, 1997 to 2000, Dept of Microbiol & Immunol, University of British Columbia, Canada.

SCIENTIFIC AND PROFESSIONAL ORGANIZATIONS:

American Association for Microbiology (ASM)
International Society for Microbial Ecology (ISME)

SELECTED PUBLICATIONS:

- Kim, M.S., M. Morrison, and Z. Yu .2011. Evaluation of different partial 16S rRNA gene sequence regions for phylogenetic analysis of microbiomes. *J. Microbiol Methods* 84(1): 81-87.
- Kongmun, P., M. Wanapat, P. Pakdee, C. Navanukraw and Z. Yu (2011). Manipulation of rumen fermentation and ecology of swamp buffalo by coconut oil and garlic powder supplementation. *Livestock Sciences* 135(1): 84-92.
- Cressman, M.D., Z. Yu, M.S. Lilburn, M.C. Nelson, S.J. Moeller, and H.N. Zerby (2010). Interrelations between the microbiotas in the litter and the intestines of commercial broiler chickens. *Applied and Environmental Microbiology* 76(19): 6572-6582.
- Kim, M.S., M. Morrison, and Z. Yu (2010). Status of the phylogenetic diversity census of ruminal microbiomes. *FEMS Microbiology Ecology*, in press.
- Zhao, S., D. Bu, J. Wang, K. Liu, Y. Zhu, Z. Dong, and Z. Yu (2010). Novel glycoside hydrolases from a metagenome library of the rumen of Chinese Holstein dairy cows. *Applied and Environmental Microbiology* 76(19):6701-6705.
- Kang, S., S.E. Denman, M. Morrison, Z. Yu, J. Dore, M. Leclerc, and C.S. McSweeney (2010). Dysbiosis of fecal microbiota in Crohn's disease patients as revealed by a custom phylogenetic microarray. *Inflammatory Bowel Disease* 16(12):2034-2042.

VITA

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EDUCATION

B.S. Microbiology, 2004, North Dakota State University
Ph.D. Molecular Pathogenesis, 2004, North Dakota State University

POSITIONS

Assistant Professor, Department of Veterinary and Biomedical Sciences, University of Minnesota, Saint Paul, MN, 2007-present

SELECTED PUBLICATIONS

Li, G., Tivendale, K.A., Liu, P., Feng, Y., Wannemuehler, Y.M., Cai, W., Mangiamele, P., Johnson, T.J., Penn, C.W., and Nolan, L.K. In Press. Transcriptome analysis of avian pathogenic *Escherichia coli* O1:K1:H7 in chicken serum reveals adaptive responses to systemic infection. *Infection and Immunity*.

Johnson, T.J., Thorsness, J.L., Anderson, C.P., Lynne, A.M., Foley, S.L., Han, J., Fricke, W.F., McDermott, P.F., White, D.G., Khatri, M., Stell, A.L., Flores, C., and Singer R.S. 2010. Horizontal gene transfer of a ColV plasmid has resulted in a dominant avian clonal type of *Salmonella enterica* serovar Kentucky. *PLoS One* 22:e15524.

Johnson, T.J., Jordan, D., Kariyawasam, S., Stell, A.L., Bell, N.P., Wannemuehler, Y.M., Fernandez-Alarcon, C., Li, G., Tivendale, K.A., Logue, C.M., and Nolan, L.K. 2010. Sequence Analysis and Characterization of a Transferrable Hybrid Plasmid Encoding Multidrug Resistance and Enabling Zoonotic Potential for Extraintestinal *Escherichia coli*. *Infection and Immunity* 78:1931-1942.

Johnson, T.J., and Nolan, L.K. 2009. Pathogenomics of the virulence plasmids of *Escherichia coli*. *Microbiology and Molecular Biology Reviews* 73:750-774.

VITA

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University of Minnesota
Veterinary Diagnostic Laboratory
1333 Gortner Avenue
St Paul, MN 55108

Phone: (612) 624-7450
Fax: (612) 62 8707
E-mail: phelp083@umn.edu

EDUCATION

B.S. Aquatic Biology, Bemidji State University, 2005
M.S. Aquaculture/Fisheries, University of Arkansas at Pine Bluff, 2007
Ph.D. Veterinary Medicine – Comparative Medicine and Pathology, University of Minnesota,
in progress

POSITIONS

Instructor/Aquaculture Extension Specialist, Department of Veterinary Population Medicine, University of Minnesota, Saint Paul, MN, 2011-present

Aquaculture Specialist, Veterinary Diagnostic Laboratory, University of Minnesota, St. Paul, MN
2007-2011

SCIENTIFIC AND PROFESSIONAL ORGANIZATIONS

American Fisheries Society, Sections: Fish health, genetics, culture
American Association of Veterinary Laboratory Diagnosticians, Committee: Aquaculture

SELECTED PUBLICATIONS

Jarnot, C., D. G. Cloutman, and N. B. D. Phelps. In review. Standard size-based mortality caps for northern pike, yellow perch, and walleye. *North American Journal of Fisheries Management*.

Phelps, N. B. D., and A. E. Goodwin. 2008. Vertical transmission of *Ovipleistophora ovariae* (Microspora) within the eggs of the Golden Shiner. *Journal of Aquatic Animal Health* 20:45-53.

Phelps, N. B. D., and A. E. Goodwin. 2007. Validation of a quantitative PCR diagnostic method for detection of the microsporidian *Ovipleistophora ovariae* in the cyprinid fish *Notemigonus crysoleucas*. *Diseases of Aquatic Organisms* 76: 215-221.

VITA

Peter Bossier,
Ghent University, Belgium

EDUCATION

B.S. Secondary education; Sint Franciscus Xaverius Institute, Belgium, 1976
M.S. Bioscience Engineering; Ghent University (UGent), Belgium, 1981
Ph.D. Applied Biological Science; Ghent University (UGent), Belgium, 1985.

POSITIONS

Assistant Professor, Lab. Aquaculture and Artemia Ref. Center, Ghent University, (2002-present)
Ministry of Small Traders and Agriculture, Sea Fisheries Department, Oostende, Belgium (1997-2002)

Laboratory of Microbial Ecology, Ghent University, Gent, Belgium (1994-1997)

Laboratório Genética Molecular, Instituto Gulbenkian de Ciência, Apartado, Portugal (1990-1994)
Biological Laboratory, University of Kent at Canterbury, Kent, GB (1987-1990)

Microbial Ecology, Faculty of Bioscience Engineering, Ghent University, Gent, Belgium (1981-1987)

SCIENTIFIC AND PROFESSIONAL ORGANIZATIONS

ICES "Working Group on the Application of Genetics in Fisheries and Mariculture"
European Society of Marine Biotechnology
VLIZ (Vlaams Instituut voor de Zee, Oostende)
KVIV (Royal Society of Flemish Engineers)
EAS (European Aquaculture Society)

SELECTED PUBLICATIONS

Defoirdt, T., Darshanee H.A. Ruwandeeepika, Indrani Karunasagar, Boon, N. and Bossier, P. 2010. Quorum sensing negatively regulates chitinase in *Vibrio harveyi*. Environmental Microbiology Reports, 2: 44-49.

Beristain, P. Gajardo, G., Bossier, Peter (2010) Species-specific RFLP pattern in the Heat Shock Protein26 gene (Hsp26): a single-locus tool for species identification and experimental testing of habitat-induced isolation in the New World *Artemia* species. Molecular Ecology Resources, 10: 229-231.

Han, Y.X., Li, Z. Qi, X.-H. Zhang, P. Bossier 2010 Detection of different quorum-sensing signal molecules in a virulent *Edwardsiella tarda* strain LTB-4 Journal of Applied Microbiology, 108: 139-147.

Qi, Z., Dierckens, K., Defoirdt, T., Patrick Sorgeloos, Nico Boon, Zhenmin Bao, Peter Bossier (2009) Analysis of the evolution of microbial communities associated with different cultures of rotifer strains belonging to different cryptic species of the *Brachionus plicatilis* species complex. Aquaculture 292: 23 -29.