Idaho Incubation Fund Program

Final Report Form

Proposal No.	**IF13-002**
Name:	**Kenneth Cain**
Name of Institution:	**University of Idaho**
Project Title:	**Commercializing specific "naturally occurring" probiotic bacterial strains as feed additives to improve fish health and aid in disease management for aquaculture**

Information to be reported in your final report is as follows:

1. Provide a summary of overall project accomplishments to include goals/milestones met, any barriers encountered, and how the barriers were overcome:

Objective 1: Complete remaining objectives from FY12 funding

This project had a primary focus on prevention or control of coldwater disease (CWD), caused by *Flavobacterium psychrophilum*, in trout and salmon using beneficial bacteria originally isolated from the gastrointestinal tract of fish. An important aspect of this project was to optimize growth and storage conditions for probiotic bacteria to address manufacturing needs. We determined that C6-6 can be grown and preserved with relative ease under laboratory conditions and scale up is feasible. Using common and relatively inexpensive growth media C6-6 can reach concentrations suitable for harvest within 5-6 hours of inoculation. This rapid growth is ideally suited for large scale fermentor production needed for product commercialization. The bacterium is also well suited to preservation and storage using lyophilization (freeze drying) technology. This process only required the use of food safe non-fat dry milk as a cryoprotectant making the preserved product ideal for direct addition (top coating) to fish feed.

Objective 2: Initiate laboratory and field trials to determine effectiveness of utilizing probiotics in a combined strategy (probiotics followed by vaccination)

Since our laboratory has been actively pursuing methods to control or prevent CWD for over a decade, we were interested in determining if probiotics could be combined with a recently patented CWD vaccine. It was hypothesized that such a combined approach may be a feasible strategy for aquaculture facilities to reduce impacts due to CWD.

Laboratory trials:

The ability of the probiotic *Enterobacter* C6-6 and C6-8, or a combination of the two

probiotics to work in conjunction with an attenuated *F. psychrophilum* vaccine was investigated. This study consisted of two disease challenges with a virulent *F. psychrophilum* strain. In the first challenge rainbow trout (*Oncorhynchus mykiss*) fry were fed a diet coated with menhaden oil (negative control), C6-6, C6-8, or a combination of the two, from the time of first feeding. At a size of approximately 1 gram (54 days post hatch) the fry were challenged with *F. psychrophilum*. Mortalities in all groups were high and there were no differences in the cumulative percent morality (CPM) or log rank survival curves between any of the treatments. However, we were able to reisolate all probiotics from the gastrointestinal tract for at least 36 days following completion of probiotic feeding. In the second challenge experiment a subset of fish, fed the same probiotic treatments as above but not challenged, were then vaccinated with the *F. psychrophilum* vaccine at approximately 1 gram (54 days post hatch). Fish were then booster vaccinated at 2 weeks and challenged with the virulent *F. psychrophilum* 4 weeks after the booster. In this second challenge relative percent survival (RPS) values were much lower than expected, but fish fed C6-6 + vaccination had significantly lower CPM and survival rates when compared to the control fish fed oil coated feed and mock vaccinated. Results from this study showed that C6-6 and C6-8 are able to colonize the gut of rainbow trout for at least 36 days after feeding probiotics. Despite low RPS values we did see significant changes in survival of fish fed C6-6 and vaccinated. This indicates that there is potential for such an approach to provide an alternative management strategy for *F. psychrophilum* infections using a combination of probiotic feeding and vaccine administration.

Field trial:

A large parallel field trial was conducted in collaboration with the Utah Division of Wildlife Resources in one of their state rainbow trout hatcheries. Unfortunately, the trial had mixed results. With the exception of one tank of fish, there was no natural CWD outbreak during the 30 day feeding of probiotics prior to vaccination. This was good for the hatchery, but not what we hoped for the trial. At about 1.5 months following vaccination, CWD was confirmed in fish. However, no distinct trends in mortality could be observed except that all fish receiving the vaccine had delayed mortality due to CWD. Two groups of vaccinates (with pre treatments of probiotics) showed distinct reduction in mortality over corresponding controls. Nevertheless, antibiotic treatments had to be applied due to the need of the hatchery to meet production targets for stocking. By the end of the trial (following antibiotic treatments), the two groups showing the lowest mortality were fish fed C6-6 only and the fish fed C6-6 followed by vaccination.

Taken together the data generated from the lab and field trial suggests that a 30 day feeding of probiotics prior to vaccination may have some benefit, but did not provide the level of impact we expected. This should be interpreted cautiously as we do not fully understand the mechanism associated with protection conferred by the probiotics. Additional trials were conducted to partially address the role of the immune system (see below) and how it may or may not be stimulated following

probiotic delivery. If the immune system is stimulated, it is possible that prolonged feeding could suppress this affect in some way and would therefore better explain the observed results.

Objective 3: Determine if C6-6 and/or C6-8 are effective at reducing mortality due to columnaris in warmwater aquaculture species.

Earlier trials demonstrated that the C6-8 strain was capable of inhibiting or reducing growth of a virulent channel catfish isolate of *Flavobacterium columnare*, the causative agent of columnaris disease. Based on these *in vitro* results, a feeding trial was performed to determine if delivery of the probiotic C6-8 via feed reduces mortality following *F. columnare* challenge in channel catfish. In the trial, two groups of channel catfish (~ 3.0 g) were fed for 10 days with either the treatment, consisting of standard feed top dressed with menhaden oil containing the probiotic, or the control, consisting of standard feed top dressed with menhaden oil (without probiotic). After the 10 day feeding period, triplicate groups of 25 fish from treatment and control groups were challenged with a high and low dose of *F. columnare*, and mortalities were monitored for 11 days. At the high challenge dose, the mean mortality ± SEM of the control group was 68 ± 15 %, while the mortality of the treatment group was 55 ± 24%. At the low challenge dose, the mean mortality ± SEM of the control group was 23 ± 11 %, while the mortality of the treatment group was 11 ± 4%.

These results indicate a potential beneficial effect (i.e. reduced mortality) of feeding the C6-8 probiotic strain prior to *F. columnare* challenge, but it should be realized that there were no significant differences between the treatment and control groups in either of the challenge experiments described above. Further studies to determine if altering the dose or duration of probiotic feeding increases the effects for columnaris control are needed.

Additional trial:

Although somewhat outside of the direct objectives of this project, we felt it was important to gain insight on the potential mechanisms that may be involved with the observed benefits of the probiotics. Therefore, we conducted a study that delivered *Enterobacter* strain C6-6 (including culture supernatant and killed bacteria) to fish by injection. Earlier attempts to determine if feeding resulted in stimulation of immunity in fish were inconclusive. This may have been due to our assay limitations and an inability to detect subtle changes in antibody responses following feed delivery. From past studies on the immune system in fish we know that injection delivery typically provides a solid indication of how a treatment may stimulate a protective immune response. Although not necessarily a practical strategy for disease control for small fish in a hatchery, we wanted to assess the immune response following injection delivery of C6-6. We hypothesized that direct injection of C6-6 would result in reduced mortality following challenge of fish with *F. psychrophilum*, and such protection would be due to stimulation of immunity.

An experiment was designed and rainbow trout fry (1.3 ± 0.1g fish^{-1}) received an intraperitoneal injection of phosphate buffered saline (PBS) (negative control), supernatant from a C6-6 culture, formalin killed C6-6, or live C6-6. Fish were then challenged with *F. psychrophilum* 7 days (trial one) and 28 days (trial two) post probiotic injection. In trial one, CPM was significantly reduced ($P < 0.05$) in fish injected with formalin killed (62.7 ± 7.4%) and live C6-6 (48 ± 6.9%) compared to the negative control (92 ± 2.3%) and survival curves analysis showed significant differences in all treatments when compared to the negative control (Fig 1). In trial two (28 days post injection), significant survival improvements were observed in fish injected with formalin killed and live C6-6 (Fig 2). One of the most interesting aspects of this study was that antibody titers (against *F. psychrophilum*) were detected following C6-6 treatments. At 7 days post injection of the probiotic treatments, antibody titers were below the detectable limit. However, low level titers were observed in the supernatant, formalin killed and live C6-6 injected groups, 50 ± 0, 200 ± 55 and 400 ± 0, respectively at 28 days post injection. There were significant differences ($P < 0.05$) between all groups except the between the PBS and supernatant injected groups. Since fish are cold blooded, adaptive immunity develops slowly, so the detection of cross-reactive antibodies at 28 days was surprising. This demonstrates that the *Enterobacter* strain C6-6 stimulates important innate immune responses early on (7 days post injection) and a potential adaptive antibody response (by 28 days) that appears to be cross-reactive to the pathogen causing CWD. This provides some evidence of the potential mechanisms of protection observed by both feed and injection delivery. If immunity does play a primary role in cross-protection to CWD, then the probiotics utilized in this project may be more representative of an “alternate” type of vaccine. Such a finding has significant implications on how such a product would be licensed through the various regulatory agencies.

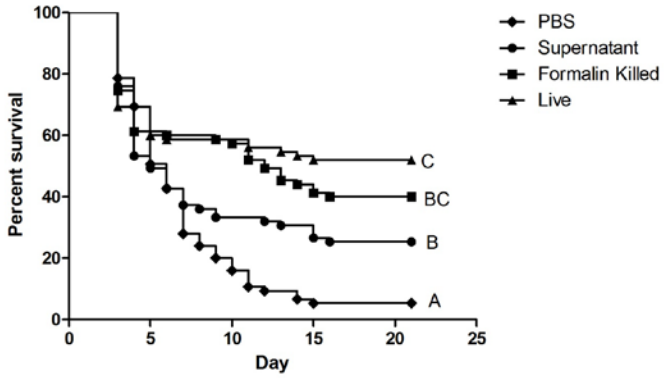

Figure 1. Survival curves of rainbow trout fry challenged with *F. psychrophilum* 7 days after i.p. injection with PBS, supernatant from C6-6, formalin killed C6-6, or live C6-6. Different letters indicate significant differences ($P < 0.05$) in survival curves.

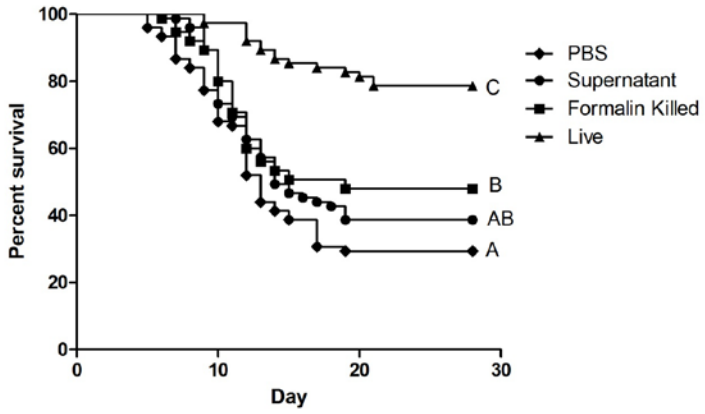

Figure 2. Survival curves of rainbow trout fry challenged with *F. psychrophilum* 28 days after i.p. injection with PBS, supernatant from C6-6, formalin killed C6-6, or live C6-6. Different letters indicate significant differences ($P < 0.05$) in survival curves.

2. Describe the current state of the technology and related product/service:

   The "probiotics" described in this project are relatively well defined at this point and represent a viable tool for controlling some fish diseases at aquaculture facilities. The work described above indicates that production limitations would not impair commercialization potential and that delivery strategies could be by feed or possibly by injection. The *Enterobacter* strain C6-6 is furthest along in testing and appears to have the greatest potential as a useful product. All indications are that it would be well suited for immediate commercialization.

3. List the number of faculty and student participants as a result of funding:

   Faculty: 1

   Students: Graduate: 1

   Undergraduates: 4

4. What are the potential economic benefits:

   There are a number of potential economic benefits that commercialization of this product could provide. In the private sector, aquaculture represents a major industry in Idaho and supplies approximately 80% of the commercially produced rainbow trout in the US. Disease related impacts affect over 30% of production on average, and if such impacts could be reduced, even by a small margin, it would translate to direct economic benefits and greater revenue for Idaho companies. In addition, this product would be widely used by public sector

(State, Federal, and Tribal) aquaculture facilities rearing trout and salmon for sportfishing and/or recovery of endangered/threatened stocks. Again, reduced cost of production for state hatcheries and increased opportunities of sportfishing harvest of trout and salmon would have direct benefit for Idaho and its citizens. Since public and private sector aquaculture in Idaho loses over $10 million annually to CWD, a substantial market for this product exists within the state and beyond.

5. Description future plans for project continuation or expansion:

   We had hoped that our listed commercial partner (Aquatic Life Sciences) would be a licensee for this technology; however, to date they have not signed an agreement with the University of Idaho in this context. We will continue to work with them but are currently seeking other potential companies with Aquatic Animal Health interests as potential commercial partners.

6. Please provide a final expenditure report (attached) and include any comments here:

   See attached form

7. List invention disclosures, patent, copyright and PVP applications filed, technology licenses/options signed, start-up businesses created, and industry involvement:

   United States Patent **Cain et al. (US 8,518,413 B2)** “Probiotic bacterial strains for use to decrease mortality in fish due to bacterial disease” was issued on August 27th, 2013.

8. Any other pertinent information:

FINAL EXPENDITURE REPORT

A. FACULTY AND STAFF		
Name/Title	$ Amount Requested	Actual $ Spent
Kenneth Cain/Professor	$9,200	5,834.02
B. VISITING PROFESSORS		
Name/Title	$ Amount Requested	Actual $ Spent
C. POST DOCTORAL ASSOCIATES/OTHER PROFESSIONALS		
Name/Title	$ Amount Requested	Actual $ Spent
D. GRADUATE/UNDERGRADUATE STUDENTS		
Name/Title	$ Amount Requested	Actual $ Spent
Tyson Fehringer		13,143.50
Amy Long		10,737.07
Josh Grondolski		1,565.89
Total:	**(22,400.00)**	**(25,446.46)**
E. FRINGE BENEFITS		
Rate of Fringe (%)	$ Amount Requested	Actual $ Spent
	6,800.00	2931.00
PERSONNEL SUBTOTAL:		
F. EQUIPMENT: (List each item with a cost in excess of $1000)		
Item/Description	$ Amount Requested	Actual $ Spent
1.		
2.		
3.		
4.		
EQUIPMENT SUBTOTAL:		
G. TRAVEL		
Description	$ Amount Requested	Actual $ Spent
1.Meetings/conferences	2,900.00	2,083.79
2.		
3		
TRAVEL SUBTOTAL:	2,900.00	2,083.79
H. PARTICIPANT SUPPORT COSTS:		

Description	$ Amount Requested	Actual $ Spent
1.tuition	3,100	0
2.		
3		
PARTICIPANT SUPPORT COSTS SUBTOTAL:	3,100	

I. OTHER DIRECT COSTS:

Description		$ Amount Requested	Actual $ Spent
1.Opperating expenses		5,600	13,704.74
2.			
3.			
OTHER DIRECT COSTS SUBTOTAL:		5,600	13,704.74
TOTAL COSTS (Add Subtotals):		50,000	50,000.01
TOTAL AMOUNT REQUESTED:			50,000
TOTAL AMOUNT SPENT:			50,000.01