EVALUATION OF THE COMMERCIALLY-AVAILABLE PROBIOTIC

LYMNOZYME® AS AN EFFECTIVE CONTROL OF BACTERIAL

INFECTIONS IN CHANNEL CATFISH

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EVALUATION OF THE COMMERCIALLY-AVAILABLE PROBIOTIC LYMNOZYME® AS AN EFFECTIVE CONTROL OF BACTERIAL INFECTIONS IN CHANNEL CATFISH

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EVALUATION OF THE COMMERCIALLY-AVAILABLE PROBIOTIC LYMNOZYME® AS AN EFFECTIVE CONTROL OF BACTERIAL INFECTIONS IN CHANNEL CATFISH

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Daniel Larbi Aboagye, son of Samuel Awua Aboagye and Ellen Adubea Abusu, was born on June 12, 1980, in Sekondi, Ghana. In 2004, he graduated from Kwame Nkrumah University of Science and Technology, Kumasi, Ghana, with a Bachelor of Science in Natural Resource Management. He then spent a year at the Ghana Ministry of Food and Agriculture for his national service. In January 2006, he entered Auburn University and worked in the Department of Fisheries and Allied Aquacultures as a Graduate Research Assistant.

THESIS ABSTRACT

EVALUATION OF THE COMMERCIALLY-AVAILABLE PROBIOTIC LYMNOZYME® AS AN EFFECTIVE CONTROL OF BACTERIAL INFECTIONS IN CHANNEL CATFISH

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A major problem of commercially-cultured channel catfish (*Ictalurus punctatus*) in the S. E. United States is disease-related mortality. Probiotics may reduce diseases. A series of pond and challenge studies were conducted to determine the effects of the commercially-available probiotic LymnoZyme® on production characteristics and survival of channel catfish exposed to *Edwardsiella ictaluri*, causative agent of enteric septicemia of catfish (ESC). Two pond studies were conducted in six 0.04-ha earthen ponds beginning on April 11, 2006 and on April 13, 2007 with channel catfish of mean weights of 9.8 g and 6.6 g stocked at densities of 12,350 and 4,942 fish/ha, respectively. Fish were grown for 210 and 43 days, respectively, with or without LymnoZyme®. Fish

(32-d post-stocking) from the second study were used in the first challenge study. They were stocked into 18 57-L aquaria (15 fish/aquarium; n=3) with 32 L of flow-through water and exposed to 1.2 x 10^6 CFU/ml of *E. ictaluri* for 2 h under static conditions 6-h post stocking. A second challenge study (18 aquaria; 14 fish/aquarium) was conducted 43 days post-stocking using remaining fish from two control ponds. LymnoZyme® was added daily at 5 ml/aquaria (8-hr static exposure) to 12 aquaria (six from first stocking and six at the onset of mortality), with the remaining six as controls. Fish were exposed to 1.3 x 10^6 CFU/ml of *E. ictaluri* for 2 h. Both challenge studies lasted two weeks. The first study showed significant differences in mean mortalities between treatment (85%) and control (99%). In the second study, mortality rates in the continuously exposed LymnoZyme® treatment (45%) were significantly reduced compared to those from the control (80%) or those under application of LymnoZyme® after onset of ESC (75%).

In the third study using specific pathogen free (SPF) channel catfish, all fish (15/aquarium; n=5) were maintained for 3 d prior to challenge with three treatments receiving LymnoZyme® daily and a control receiving none. The following treatments occurred post-challenge: control-no LymnoZyme®; 1-received no LymnoZyme®; 2-LymnoZyme® daily for the next 7 d; and 3- LymnoZyme® throughout study. Fish were challenged with 2 X 10^6 CFU/ml of *E. ictaluri*. Control fish had a mean mortality of 80%, while treatment 1, 2 and 3 had mean mortalities of 80%, 47% and 40%, respectively. A forth study using SPF fish (control) and surviving fish from treatment 3 from the third study was conducted to determine if fish had developed resistance to ESC. All fish (10 fish/aquaria; 10 aquaria; n=5) were challenged with 2 x 10^6 CFU/ ml of *E. ictaluri*. Control and LymnoZyme®-treated fish had 94% and 14% mortalities, respectively.

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I. INTRODUCTION

Status of the U.S. catfish industry

Catfish farming is the largest and most well developed segment of the U.S. aquaculture industry contributing about 46% of the value of aquaculture production in the country. In 2005, production levels were worth a market value of $461,885,000 (USDA 2006). About 396,554,000 food-size (~0.7 kg) catfish with a total weight and sales of 275,753,769 kg and $429,245,000, respectively, were produced (USDA 2006). About 94% of the farm-raised catfish in the U.S. is produced in Alabama, Mississippi, Louisiana and Arkansas. The catfish industry is critical to the economic viability of these states, providing about 14,142 on-farm jobs and paying about $168,724,000 in salaries (USDA 2006). The primary catfish species cultured in these states is the channel catfish, *Ictalurus punctatus* (Rafinesque), contributing about 70% of all U.S. aquaculture production (Goldburg et al. 2001). The production of channel catfish increased over 100% from 1986 to 1996 with a total production of about 264 million kilograms in 2006 (USDA 2008).

This growth of the industry has been threatened by importation of catfish from countries such as China and Vietnam, creating competition that has resulted in the market value of catfish falling. For example, the production value of catfish fell from 480 million dollars in 2004 to 462 million dollars in 2005 (USDA 2006). Vietnam started to export

frozen Vietnamese catfish (basa) to the U.S. in 1996 following normalization of relationships between these two countries. According to the U.S. International Trade Commission, between 1998 and 2002 the import volume of frozen basa fillet increased 20-fold, reaching 21,000 tons in 2002, accounting for over 26% of the total frozen catfish fillet on the U.S. market in 2002.

The competition created by imports from Vietnam, China and other countries has resulted in price cuts in the catfish raised in the USA. The low prices received by local producers have caused economic hardships not only for them but to the whole economy of the dependent states. The following is a statement by the president of the Catfish Farmers of America at the regional farm bill hearing of the Agriculture, Nutrition and Forestry Commission of the U.S. Senate in Lubbock, Texas, and summarizes the frustrations of the U.S. catfish industry:

> "…As of 1998, total imports of frozen basa and tra fillets from Vietnam were approximately 2 million pounds. By bootstrapping their product into the U.S. catfish industry's successful marketing program, the Vietnamese exporters and seafood importers were able to take a significant share of the U.S. market and drive down prices of processed catfish and the price available to farmers. By specifically marketing their product as cheaper substitutes for U.S. farm-raised catfish fillets, total imports from Vietnam increased dramatically to 19 million pounds in 2000, 30 million pounds in 2001, and 46 million pounds in 2002. As the total imports increased, the prices per pound significantly decreased to levels that were below the production costs of our very efficient farmers and processors. Consequently,

imports took a large share of the U.S. market for frozen catfish fillets…"

There is a strong agreement among catfish stakeholders that considering the fact that about 70% of seafood consumed in the US is imported, with a seafood trade deficit of $7.8 billion in 2003 (USDA, 2005), domestic catfish production is critical to maintaining food security and protecting the industry. Production has to be more efficient, competitive, sustainable and profitable.

Catfish Diseases

According to the 2003 United States Department of Agriculture National Animal Health Monitoring System (USDA-NAHMS) report (USDA 2003), bacterial diseases are responsible for about 70% of all diseases affecting channel catfish in the Southeastern U.S. with enteric septicemia of catfish (ESC) and columnaris (caused by *Edwardsiella ictaluri* and *Flavobacterium columnare*, respectively) being the main diseases. These diseases cost the U.S. catfish industry $50-$70 million annually (USDA 2006).

Enteric Septicemia of catfish

Enteric septicemia of catfish (ESC), caused by the gram negative bacterium, *E. ictaluri*, is considered to be one of the two most prevalent diseases affecting farm-raised channel catfish and is responsible for about 30% of total losses per year costing farmers millions of dollars (Plump 1999). ESC is a chronic to sub-acute disease which affects all ages of channel catfish (Hawke 1979; Newton et al. 1989). The group of fish hit hardest by ESC seems to be the young of year fingerlings going into their fall season when they are naive.

ESC accounts for about 30% of all disease cases submitted to fish diagnostic laboratories in the southeastern United States.

ESC was first recognized as a new infectious bacterial disease of pond-raised channel catfish in 1976 through the examination of diseased specimens from Alabama and Georgia submitted to the Southeastern Cooperative Fish Disease Laboratory (SECFDL) at Auburn University (Hawke 1979).The causative agent was later named *Edwardsiella ictaluri* (Hawke et al. 1981). ESC can occur when a susceptible channel catfish host encounters the bacterium under conditions conducive to the pathogen's proliferation and that are stressful to the host (Wise et al. 1993; Ciembar et al. 1995). Stress factors, such as handling, close confinement, improper diet, low water chlorides, poor water quality, and water temperature fluctuations, all lead to increased susceptibility to infection. Pathogenesis studies have shown that *E. ictaluri* can enter catfish through the gut, the nares (nasal openings) and possibly the gills (Miyazaki and Plump 1985; Shotts et al. 1986). Transmission probably occurs from fish to fish via the water by organisms shed via the feces, by cannibalism of infected fish, or by feeding on dead, infected carcasses (Hawke et al. 1981; Shotts et al. 1986; Klesius 1994). Another way ESC can be transmitted is by birds picking up dead fish from one pond, flying to another pond and dropping the infected carcasses (Taylor 1992). *E. ictaluri* can also be transferred from pond to pond on wet nets and equipment.

ESC occurs within a specific temperature range sometimes referred to as the "ESC window". The disease is seasonal with outbreaks typically occurring in the spring and fall when water temperatures are between 22 and 28°C. Mortalities slow and usually

stop outside this temperature range. Catfish affected with ESC often are seen swimming in tight circles chasing their tails. This head-chasing-tail, whirling behavior is due to the presence of the *E. ictaluri* in their brain. Affected fish also hang in the water column with the head up and tail down. In addition, catfish with ESC tend to stop eating shortly after becoming infected (Hawke 1979).

Externally, ESC-affected catfish frequently have skin ulcerations, ranging from pinhead size to about half the size of a dime, covering their skin. Petechial hemorrhages are found under their heads and in the ventral region (Hawke et al. 1981; Jarboe et al. 1984; Miyazaki and Plump 1985). There are also ulcerations at the cranial foramen between the eyes that can progress into what is commonly called the "hole-in-head" condition (Hawke 1979; Hawke et al. 1981). Internal build-up of fluid can lead to a swollen abdomen and exophthalmia.

Internally, clear, straw-colored or bloody fluid is often present in the fish's body cavity. The liver typically has necrotic areas and may appear mottled (Areechon and Plump 1983). Petechial hemorrhages can be found in the muscles, intestines and fat of the fish. The stomach and intestine are frequently distended and often filled with body fluid. Intestinal gas production may contribute to distension of the gut (Francis-Floyd et al. 1987). Other intestinal structures, such as the spleen, anterior kidney, or posterior kidney, may be soft, pale in color and have bloody spots (Hawke 1979; Areechon and Plump 1983).

Columnaris Disease

Columnaris, one of the oldest known diseases of warmwater fish, was first described by Davis (1922) but the causative organism was isolated and characterized 22 years later (Ordal and Rucker 1944). It is caused by the gram-negative rod bacteria, *F. columnare,* that has been referred to by several names, including *Bacillus columnaris* (Davis 1922), *Chondrococcus columnaris* (Ordal and Rucker 1944) *Cytophaga columnaris* (Garnjobst 1945) and *Flexibacter columnaris* (Leadbetter 1974; Bernardet and Grimont 1989). It was later re-described and named by Bernardet et al. (1996) as *Flavobacterium columnare*. Recent diagnostic cases show that columnaris is the most prevalent disease of farm-raised catfish in the Southeastern U.S. This disease is often referred to as "fin rot", "cotton wool", "saddleback" or "cotton mouth" disease (Decostere et al. 1998).

F. columnare is ubiquitous in freshwater aquaculture environments and often cause disease when the fish is stressed from handling, high water temperatures, high ammonia, low dissolved oxygen, high nitrite levels, crowding or in the presence of another disease (Meyer 1970; Hanson and Grizzle 1985; Chowdhury and Wakabayashi 1989; Hawke and Thune 1992). In channel catfish, columnaris occurs when temperatures are between 15 to 30^{o}C in the spring, summer and fall (Plump 1999). Once the disease has been established, the infection can spread via the water in the holding or culture facility, leading to high mortalities and sometimes leading to secondary infections like winter saprolegniosis in pond environments.

Fish affected with columnaris usually have yellowish to brown lesions on their gills, skin and sometimes the fins. When skin lesions occur, there is usually a necrosis of

the dermis and the underlying musculature (Bootsma and Clerx 1976).The bacteria can attach themselves to the gill tissue (Decostere et al. 1999a; Decostere et al. 1999b), multiply in spreading patches and finally cover individual gill filaments in the process, releasing protein and cartilage-degrading enzymes that erode the gills and result in cell death (Hawke and Thune 1992). Lesions caused by columnaris are usually in the nature of pale white bands encircling the body (Davis 1922). This condition is called "saddleback" and often leads to columnaris being mistaken for a fungal infection. In certain situations, there is a yellowish-brown, mucus-like growth of the bacteria inside the fish's mouth. When grown in vitro, the bacterium produces a yellow pigment. On a wet mount, the bacteria are identified as long, thin rods, 7-10 millimeters and approximately 10-20 times longer than wide, moving by flexing and gliding on the surface and forming "haystacks" (Bernadet et al. 1996).

F. Columnare grow on media with low nutrient content, such as Ordal's medium, and can be isolated from sites with mixed infections by the use of selective media, such as Selective Cytophaga Agar (SCA) (Anacker and Ordal 1959) and Hsu Shotts (HS) (Shotts 1991) medium. The bacterium can grow in the presence of neomycin (5 mg/l) and polymyxin B (200 units/ ml) when mixed into these media; whereas, most other fish pathogens and aquatic bacteria are inhibited (Fijan 1969).

In most situations, the occurrence of columnaris can be greatly minimized by reducing stress on the fish (Rottmann et al. 1992). Potassium permanganate ($KMnO_4$) is often used as a therapy for external infections, although Terramycin®, specifically labeled for treatment of *Aeromonas hydrophilia,* is sometimes used in medicated feed for

both internal and external infections (Amend 1970).

The use of antimicrobial agents as a preventive measure against both ESC and columnaris has been questioned given their indiscriminate use leading to antimicrobial resistance among pathogenic bacteria (FAO 2006; McEwen and Fedorka-Cray 2002; Toranzo et al. 1984). In addition, the problem of tissue residues has raised trade issues on the international market (USDA 2003). To prevent the above mentioned problems, several alternative approaches, including the use of prebiotics and probiotics, have been proposed.

Prebiotics

Prebiotics are described by Gibson and Roberfroid (1995) as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or more limited number of bacteria in the gut. These bacteria are believed to improve the host resistance to diseases, thereby, improving its performance. In aquaculture, the use of prebiotics as a food supplement has been generating a lot of interest, given the potential of these compounds to enhance the existing natural microbial community without introducing a foreign organism whose efficiency could be affected by feed processing (Burr et al. 2005).

Prebiotics that feed the beneficial bacteria in the gut are mostly oligosaccharides. Since these carbohydrates cannot be digested, they remain in the digestive tract and stimulate the growth of beneficial bacteria, particularly bifidobacteria. Examples of oligosaccharides classified as prebiotics are fructo-oligosaccharides (FOS), ismalto-

oligosacharides, inulins, lactilol, lactulose, lactosucrose, soy oligosaccharides, pyrodextrins, transgalacto-oligosaccharides and xylo-oligosaccharides (Grizard and Barthomeuf 1999; Macfarlane and Cummings 1999). The use of inulin and oligofructose has been demonstrated to improve growth in the Siberian sturgeon (*Acipenser baerii*) and African catfish (*Clarias gariepinus*) (Mahious 2005).

According to Gibson et al. (2004), for any food substance to be considered a prebiotic it should have the following characteristics:

1) Ability to resist gastric acidity, hydrolysis by digestive enzymes and gastrointestinal absorption.
2) Selective fermentation by beneficial intestinal bacteria
3) Selective stimulation of the growth and the activity of intestinal bacteria associated with health.

The use of prebiotics in aquaculture has, however, received very little attention due to the problem of the stability of feed pellets in the culture medium. Further investigation of the use of prebiotics in the use of disease prevention and control is needed.

Probiotics

Probiotics are naturally occurring microorganisms that provide beneficial effects to the host or the environment by displacing pathogens. The word probiotic comes from the Greek “pro bios” meaning “for life” (Vine 2004). The first widely accepted definition of probiotics was given by Fuller (1989) as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance”. According to

Verschuere et al. (2000), the interaction between the probiotics and the host is, however, not limited to the intestinal tract. Probiotics can be active on the gills or skin of the host and also in its ambient environment. Also, the intensive interaction between the culture environment and the host in aquaculture implies that a lot of probiotics are obtained from the culture environment and not directly from feed as stipulated by the definition given by Fuller (Verschuere et al. 2000). In contrast to the definition by Fuller, where the emphasis was placed on live cells, feed and intestinal balance, Salminen et al. (1999) defined a probiotic as any microbial (dead or alive) preparation or the components of microbial cells with a beneficial effect on the health of the host. Taking this broad interaction of probiotics and the host into consideration, a group of experts put together by the United Nations' Food and Agriculture Organization (2001) gave the definition of probiotics as "live microorganisms administered in adequate amounts which confer a beneficial health effect on the host".

This variation in definitions is an indication of the differences in approach taken by various scientists in the emerging field of probiotics. The most specific definition of probiotics is, however, given by Verschuere et al. (2000) as "a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment".

Most probiotic products on the market contain bacteria from the genera *Lactobacillus* or *Bifidobacterium*, although other genera, such as *Escherichia*,

Enterococcus, and *Bacillus* are also marketed as probiotics (pro basics). Historically, lactobacilli and bifidobacteria associated with food have been considered to be safe (Adams and Marteau 1995).

Mode of action of probiotics

Although the exact mode of action of probiotics is not fully understood (Balcazar et al. 2006; Irianto and Austin 2002), it is widely accepted that probiotics generally work by preventing pathogens from proliferating in the intestinal tract, on the superficial structures or in the culture environment; securing optimal use of the feed by aiding in its digestion; improving water quality; or stimulating the immune system of the host. Several studies suggest that the administration of probiotics to the aquaculture environment can competitively exclude pathogenic bacteria from the culture environment (Moriarty 1998; Gomez-Gil et al. 2000; Balcazar 2003; Balcazar et al. 2006; Vine et al. 2004). This exclusion feature of probiotics has been attributed to the production of chemical substances, such as organic acids or hydrogen peroxide (Ringø and Gatesoupe 1998), siderophores (Yoshida et al. 2002; Braun and Braun 2002) or bacteriocins and antibiotics (Brock and Madigan 1997) that are toxic and, therefore, cause antagonistic activity towards other microorganisms (Vine 2004; Fjellheim 2006).

Probiotics have also been shown to act as a source of nutrients and enzymatic contribution to digestion (Balcazar et al. 2006; Burr et al. 2005). *Bacteroides* and *Clostridium* sp. have been reported to supply fatty acids and vitamins to the host fish, thereby, improving overall nutrition (Sakata 1990). Some microorganisms like

Agrobacterium sp., *Pseudomonas* sp., *Brevibacterium* sp., and *Staphylococcus* sp. are believed to contribute to the nutritional process of the Arctic charr, *Salvelinus alpines* L. (Ringø et al. 1995). Some bacteria have also been reported to contribute to the digestive process of bivalves by producing extracellular enzymes, such as proteases and lipases, as well as providing the necessary growth factors (Balcazar et al. 2006; Prieur et al. 1990).

The non-specific immune system can be stimulated by probiotics (Balcazar et al. 2006). According to Sakai et al. (1995), oral administration of the bacteria, *Clostridium butyricum*, increased the resistance of rainbow trout (*Oncorhynchus mykiss*) to vibriosis by increasing the phagocytic activity of leucocytes. Balcazar (2003) also demonstrated that the administration of a mixture of bacterial strains (*Bacillus* and *Vibrio* sp.) to juveniles of the white shrimp (*Litopenaeus vannamei*) improved their growth and survival and protected them against, *Vibrio harveyi* infections and White Spot Syndrome Baculovirus Complex, by stimulating the immune system through increased phagocytosis and antibacterial activity. Mortalities caused by Edwardsiellosis in the European eel (*Anguilla Anguilla*) were reduced by applying *Enterococcus facium* SF 68 (Chang and Liu 2002). Other notable examples where probiotics have demonstrated some level of efficiency in combating fish diseases are by Smith and Davey (1993), Bly et al. (1997), Gram et al. (1999), Douillet and Langdon (1994), Andlid et al. (1995).

Probiotics have also been shown to improve the quality of the culture water (Dalmin et al. 2001). This influence on water quality has been associated with *Bacillus* sp. and the rationale is that gram positive bacteria are better converters of organic matter back to carbon dioxide than gram negative bacteria (Balcazar et al. 2006). Thus, high

levels of gram positive bacteria during the production cycle can minimize the build up of dissolved and particulate organic carbon (Balcazar et al. 2006). For the probiotic to have such an influence on the water quality it has to be applied directly to the culture water and the application has to be done regularly to maintain the probiotic in the water (Burr et al. 2005). This mode of application has been demonstrated by Austin et al. (1995); Hjelm et al. (2004); Moriarty (1998); Ringø and Vadstein (1998); Ringø and Birkbeck, (1999), Gram et al. (1999). For maximum benefit, the probiotic should not be harmful to the host (Salminen et al. 1999) and, most importantly, be effective over a range of temperatures and salinities (Fuller 1989).

Application of probiotics in the culture water as a biological conditioning and control agent is based on the premise that both environmental conditions and chance influence the emergence of microbial communities (Verschuere et al. 2000). Instead of allowing spontaneous primary colonization of the rearing water by bacteria already present, the water could be pre-emptively colonized by the addition of probiotic bacteria since it is generally recognized that pre-emptive colonization may extend the reign of pioneer organisms (Atlas and Bartha 1997). According to Verschuere et al. (2000), in a new aquaculture environment where the microbial communities are yet to develop, adding just one culture of a probiotic may be enough to achieve this colonization, especially when the probiotic is well adapted to the existing environmental conditions. On the other hand, when there is already an existing stable microbial community, the probiotic culture will have to be added on a regular basis to achieve and maintain its artificial dominance.

Despite several studies showing varying degrees of efficiency involving the use of bacterial inocula in aquaculture production involving various fish species (Gullian et al. 2004; Gibson et al. 1998; Ruiz-Ponte et al. 1999; Hjelm et al. 2004 and Gram et al. 1999), little research has been reported in catfish production. The few reported catfish production studies that have been performed in this area have failed to show convincing differences in terms of fish production and water quality between treated and control ponds (Queiroz and Boyd 1998; Boyd et al. 1984; Tucker and Lloyd 1985; Chiayvareesajja and Boyd 1993). Much of the testimonials and the advertisement of probiotics in channel catfish are from satisfied users and are difficult to substantiate; therefore, more studies need to be conducted to determine whether the potential benefit of probiotic to aquaculture, especially channel catfish production, is perceived or real (Queiroz and Boyd 1998; Irianto and Austin 2002).

The objective of this research was to test the hypothesis that the use of the commercially-available probiotic, LymnoZyme®- Liquid (Keetons Industries Inc., Wellington, Colarado, USA) in channel catfish ponds will affect the following:

1. Aquatic bacterial community;
2. Number of disease incidences;
3. Mortalities associated with E. *ictaluri* and *F. columnare;* and
4. Fish production characteristics.

To further evaluate its effects on *E. ictaluri* in channel catfish and its potential use in fish health management, a series of challenge studies were also conducted.

II. MATERIALS AND METHODS

Two related pond experiments were conducted in different years to assess the effective of LymnoZyme® to improve production and control bacterial infections in channel catfish.

Experiment 1

Experimental Fish

Channel catfish fingerlings with a mean weight of 9.8 g were purchased from a commercial farm and held for two days in flow-through raceways receiving reservoir water prior to stocking into each of six 0.04-ha earthen ponds at the E. W. Shell Fisheries Research Station, Auburn, Alabama, at a density of 12,350/ha on April 11, 2006. Fish were counted in lots of 100 into aerated buckets and quickly transported to ponds. Fish were reared for one growing season (210 days), with three ponds being used as controls (no LymnoZyme®) and three as treatments, and harvested on November 11, 2006. Because fish were apparently stressed during handling in transport or at stocking, some mortality was experienced in the first week after stocking. Dead and moribund fish tested at the Auburn University Fish Diagnostic Laboratory showed that fish were infected with *F. columnare*. Each pond was treated with potassium permanganate ($KMnO_4$) at a rate of 2mg/l based on the potassium permanganate demand test (Boyd 1990).

Description of Culture Ponds

Six 0.04-ha earthen ponds with width, length and maximum depth approximately 13.8 m, 29.2 m and 1.5 m, respectively, were used. Each of the ponds was filled about 75% full two weeks before stocking with water from a watershed reservoir located on the property. Each pond was equipped with a standing drain pipe fitted to the bottom of the pond, and water levels were maintained at approximately 15 cm from the top of these drain pipes to prevent water overflow after a rainfall. All water inlets to each pond were covered with fine mesh (1 mm) saran netting "sock" strainer to prevent the entry of wild fish.

Feeding

Fish were fed twice daily with 60% of total estimated daily feed amount given in the morning and 40% late in the afternoon seven days a week with a commercially-available 32% protein, (16% fat and 0.2 cm pellet size) extruded diet (Aquafeed, Cargill Animal Nutrition, Minneapolis, MN, USA). Fish were fed to satiation by giving feed in smaller quantities every fifteen minutes until fish stopped feeding, using 3% body weight as a guideline. The amount of feed fed each day was recorded. Daily feed allotment was adjusted weekly based upon an estimated feed conversion ratio (FCR) of 1.5 and known mortality. Final FCR was calculated by dividing the total feed input (kg) by the wet weight gain (kg) derived from the harvest data.

Sampling

A sample of fish was collected two months after stocking and every month thereafter.

Fish were sampled by seining the whole pond with nets of mesh sizes ranging from 6 to 19 mm and all fish counted by hand and weighed in bulk on an electronic balance (XWS150MS, Mettler Toledo Inc., Columbus, Ohio, USA) to determine the average weight and total biomass in each pond based on observed mortalities. Fish were then returned to the ponds. At harvest, fish were removed by seining, counted and weighed in bulk. These weights were added to those remaining after total drainage to calculate the net production and feed conversion ratios.

Pond Water Quality Monitoring and Management

LymnoZyme® was applied at the end of the third week, after pond stocking. LymnoZyme® was spread evenly on each pond at manufacturer's recommended rates of 20 L/ha (4.9 L/pond) every three days in the first nine days and then 20 L/ha/wk for maintenance.

Dissolved oxygen (DO), water temperature, and pH in each of the ponds was measured with a handheld YSI 556 MPS multiparameter instrument (YSI Incorporated, Yellow Springs, OH, USA) at an average depth of 0.3 m, twice daily, at approximately 0600 h and 1730 h. Ponds were aerated using 0.33- kW vertical pump aerators (Air-o-Lator Corporation, Kansas City, Missouri, USA) connected to a timer, during the last nine days of the study when daily morning and nighttime DOs were falling below 4 mg/l. Agricultural lime was applied at the rate of 4 MT/ha to each pond a month after stocking to increase alkalinity and buffer pH. Ammonia-nitrogen, nitrite-nitrogen, total alkalinity and total hardness levels were measured once weekly using the AQ-2 model of the Octa-

Slide comparator test kit (LaMotte Company, Chestertown, Maryland, USA). Unionized ammonia (NH_3) was calculated from recorded total ammonia-nitrogen using pH and temperature tables given by Boyd (2000).

Reservoir water was added periodically to compensate for evaporative losses and seepage. On the 178th day after stocking, ammonia levels in one control pond rose to 1.1 mg/L at a pH of 8.6, and, so, water was added to the pond as a top off to dilute the ammonia concentration.

Experiment 2

Description of Culture Ponds

Ponds used in experiment 2 were of similar size to those used in experiment 1. Ponds were given similar preparations as in experiment 1 except that agricultural lime was applied at a rate of 4 MT/ha a week before stocking.

Stocking

On April 13, 2007, two hundred channel catfish fingerlings (average weight of 6.6 g) purchased from FarmFresh Fingerling Farm, Newbern, Alabama, were counted by hand, weighed in bulk on an electronic balance (XWS150MS, Mettler Toledo, Inc., Columbus, Ohio, USA), transported in lots of 100 in aerated buckets and stocked into each of six 0.04-ha ponds at a density of 4,940 fish/ha. Two treatments were assigned randomly to the six ponds. LymnoZyme® was applied to three ponds and the remaining ponds used as controls. Fish were fed with the same feed as in experiment 1 using a similar feeding protocol and grown for 43 days.

Pond Water Quality Monitoring and Management

Water quality monitoring and management were similar to experiment 1 except that ponds were never mechanically aerated because daily morning and afternoon DOs were above 4 mg/L throughout the study, which is the level considered stressful (Chapman 1992). LymnoZyme®, applied at the beginning of the second week, followed the same application rates and methodology as in experiment 1.

Sampling and Harvesting

A month after stocking, each pond was partially harvested by seining (mesh size of 19 mm) to remove fish for the first challenge study. Fish were counted by hand and weighed in bulk on an electronic balance (XWS150MS, Mettler Toledo Inc., Columbus, Ohio, USA) to determine the average weight. Two ponds (one treatment pond and one control pond) were, however, completely harvested because aquatic weeds and filamentous algae prohibited collection by seining. The remaining ponds were harvested 11 days later. Some fish from the final harvest were used in the second challenge study. Total weights from first harvest were added to the final harvest weights to calculate the gross yield, net production, and feed conversion ratio.

DISEASE CHALLENGE

To determine the virulence of the isolate and the dosage for challenge, a series of pre-challenge studies were conducted with the same fish used in the second pond study for the first two challenge studies. For the third and fourth aquaria studies; however, specific disease-free fish were acquired from the School of Veterinary Medicine, Auburn University, Auburn, Alabama, for each pre-challenge study. The stock, S97-773, of *E. ictaluri* used was a virulent form originally isolated from wild fish diagnosed at the Fish Disease Diagnostic Laboratory at Auburn University and maintained for challenge studies.

In each pre-challenge study, six aquaria were grouped into sets of two and then stocked with 10 naïve fish each. Different volumes of *E. ictaluri* were cultured in brain

heart infusion broth (Difco, Baltimore, MD, USA) between 24-48 hours in a shaker incubator at 30^{o}C and the number of colony forming units (CFU) determined by the dilution plate counting method. These were applied to each aquarium with same volume of *E. ictaluri* for each set. After two weeks of observation, the volume that caused 50% mortality was used in each subsequent challenge. In each challenge study, water temperature was kept at 27^{o}C throughout the study. Dissolved oxygen levels were measured twice daily with a YSI oxygen meter –Model 55 (Yellow Springs Instruments, Yellow Springs, OH, USA) while total alkalinity and total ammonia were measured as previously described.

FIRST CHALLENGE STUDY

Eighteen 57-L flow-through aquaria were filled with 32 L of water from a well located on the property. Nine aquaria were used as treatment and the other nine as controls. Fish from the second pond study were used to stock the aquaria (15 fish/aquarium) with each pond represented by three aquaria (n=3, 18 aquaria total). Six hours after stocking into the aquaria, a 15 ml Brain Heart Infusion (BHI) culture containing 1.2 x 10^{6} CFU/ml of *E. ictaluri* (pre-determined from the pre-challenge study conducted a week earlier) was applied to each of the aquaria after the water had been turned off, with aeration still continuing. The fish were given a 2-hour static exposure after which the water (15 ml per second flow rate) was turned back on. Starting on the second day, each morning fish in each aquarium were fed 4.5 g (3% of biomass, 150g) of a floating catfish diet, Aquamax Grower 400 (PMI Nutrition International, LLC, Brentwood, Missouri, USA) containing

45% crude protein, once every two days. Each aquarium was equipped with an airstone to keep the water aerated at all times. Using a siphon, waste from feed and feces were drawn out every Monday and Thursday morning to keep aquaria clean. The duration of the study was 18 days. Mortalities were recorded daily (0700h and 1600h), moribund or dead fish collected and then subjected to a necropsy; whereby, bacteriological examinations of the kidney and the spleen were done to verify the presence of *E. ictaluri* or other pathogens.

SECOND CHALLENGE STUDY

Fish from the two remaining control ponds (no pre-exposure to LymnoZyme®) in experiment 2 were stocked at a rate of 14 fish per aquarium into eighteen 57-L aquaria, (similar to those used in first aquaria study), filled with 32 L of water. LymnoZyme® was added daily to six aquaria at a rate of 5 ml/ aquaria (treatment 1), while the remaining twelve aquaria received no LymnoZyme® (control and treatment 2). Water to both treatment and control aquaria was cut off for 8 hours when LymnoZyme® was added to the treatment aquaria, but continuous aeration was provided. After the 8-hour exposure, water was turned on and fish fed. Thirty-two hours after initial LymnoZyme® addition, a 15 ml BHI culture containing 1.3 $x10^6$ CFU/ml of *E. ictaluri* was applied to each of the aquaria after the water had been turned off. After a 2-hour static exposure, the water was turned back on. At the first sign of mortality in treatment 2, LymnoZyme® was added to these six aquaria at a rate of 5 ml/aquaria. The same management practices, including necropsy, used in the first study were employed in managing all the aquaria. The duration of the study was 17 days.

THIRD CHALLENGE STUDY

A third aquaria study using specific pathogen free (SPF) channel catfish was conducted for 17 days under similar conditions to the second study to confirm those results and to evaluate the timing of the application of LymnoZyme®. All fish (15 fish/aquarium; 24 aquaria total; n=6) were maintained for 3 days prior to challenge with three treatments receiving LymnoZyme® daily as previously described and a control receiving none. Post-challenge, the following treatments occurred: control-no LymnoZyme®; 1-received no more LymnoZyme®; 2-LymnoZyme® daily for the next 7 days; and 3-LymnoZyme® throughout the study period. Both control and treatment fish were given a 2-hour static exposure to 2 x 10^6 CFU/ ml of *E.ictaluri*. After 17 days, LymnoZyme® application to treatment 3 was stopped and mortalities were observed for a week.

FOURTH CHALLENGE STUDY

A fourth aquaria study was organized using fish from the third study to determine if fish has developed immunological resistance to ESC. Fish left from those treated with LymnoZyme® throughout the third challenge study were stocked into five aquaria (10 fish/ aquaria; n=5). SPF fish from the same stock as those used in the third challenge study were used as controls. Fish were given a 2-hour static exposure to 2 x 10^9 CFU/ ml of *E.ictaluri*. Similar management practices as used in the previous three studies were employed in this study. The duration of this study was 14 days.

E. ictaluri Plate Culture

A BHI agar was made with 0.03% LymnoZyme® by adding 15 ml of filtered (using a 0.45 µm syringe filter) LymnoZyme® to 500 ml of autoclaved BHI. LymnoZyme® was first centrifuged at 2,200 *g* for 10 minutes and then filtered using a vacuum filter (Whatman #2). This was then added to BHI agar using a 0.45 µm syringe filter. *E. ictaluri* was added to two control plates (no LymnoZyme®) and two LymnoZyme® plates and then put into a 30°C incubator for 48 hours to observe for growth of *E. ictaluri.*

Growth Rates

Average growth rate of fish from each pond was calculated using the equation:

$$\text{Average growth rate (g/day)} = (W_f - W_i)/t$$

Where, W_f and W_i are the individual harvest and stocking weights, respectively; whereas, t is time (days) from stocking to harvesting.

Specific growth rate was calculated using the equation below.

$$\text{Specific growth rate} = (\ln W_f - \ln W_i) \times 100/t$$

Where, $\ln W_f$ and $\ln W_i$ are the natural logarithm of the individual harvest and stocking weights, respectively; where, t is the time (days) between stocking and harvest dates.

All growth rates were used to assess the performance of fish in terms of growth for each treatment.

Statistical Analysis

Data generated from both pond and aquaria studies were subjected to an analysis of variance (ANOVA) and general linear model using SAS (version 9.1, SAS Institute Inc., Cary, NC, USA). T-test was used to compare the difference between treatments for both pond studies. For pond studies, growth and survival characteristics were compared; whereas, for challenge studies, cumulative mortalities were compared to identify significant differences ($p<0.05$) among treatment means with Tukey's multiple range test. The first challenge study was analyzed as a nested design where the aquaria were treated as nested in the ponds. The rest of the challenge studies were analyzed as completely randomized designs. Where no differences were found, the overall means are presented.

III. RESULTS AND DISCUSSION

Experiment 1

Water Quality

There were no significant differences between treatment and control ponds in terms of water quality. Mean overall weekly morning and afternoon water temperatures of the catfish ponds are illustrated in Figure 1. Optimal channel catfish growth is known to occur from 27^{o}C to 30^{o}C. This range is indicated by two dotted lines on Figure 1. There were about four months when the overall mean water temperatures fell into this range. Although catfish can survive at temperatures ranging from just above freezing to about 37^{o}C, growth is slow at water temperatures below and above 20 and 35 ^{o}C, respectively (Tucker et al. 2004). Figure 1 shows that water temperatures for most of the study were above this level.

The mean number of times the concentration of dissolved oxygen levels in the ponds were recorded below 4.0 and pH went above 9.0 were compared between treatments and controls and are shown in Table 1. The mean number of times that dissolved oxygen levels fell below 4.0 mg/L in the treatment and control ponds was not significantly different ($p=0.143$) and had an overall mean of 1.3 (SE ± 0.56). The mean number of times that pH rose above 9.0 was also not significantly different ($p=1.000$) with an overall mean of 0.8 (SE ± 0.3). The highest and lowest recorded dissolved

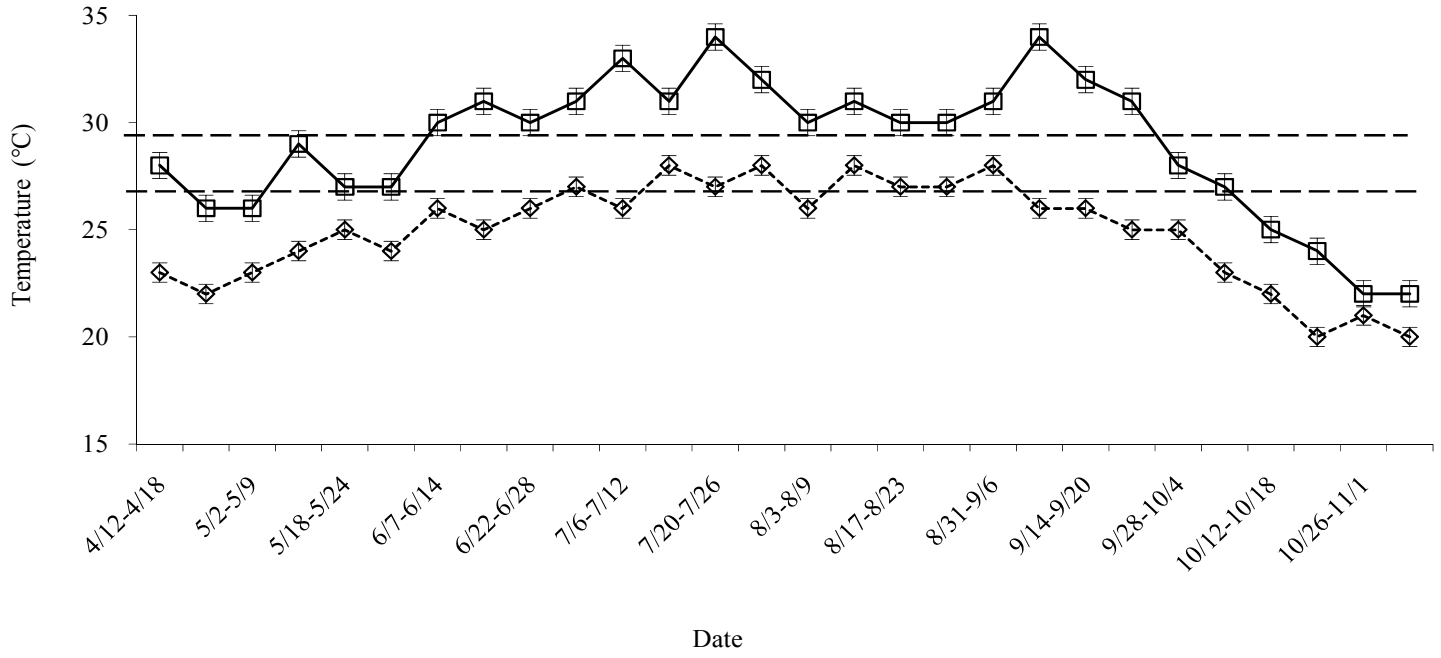

FIGURE 1. Mean overall weekly morning Δ and afternoon $\square$ water temperatures (^{o}C) in channel catfish ponds stocked at 12,350 fish/ha and treated with or without LymnoZyme®**.** Dotted lines indicate optimal temperature range for channel catfish growth.

TABLE 1. Mean (±SE[1]) number of times the recorded dissolved oxygen fell below 4.0 mg/L or pH rose above 9.0 in channel catfish ponds stocked at 12,350 fish/ha and treated with or without LymnoZyme®.

	n	# of Times DO < 4.0 mg/L	# of Times pH > 9.0
LymnoZyme®	3	2 ±0.2	3 ±0.3
Control	3	6 ±0.4	2 ±0.3

[1]SE= Standard Error

oxygen levels were 14.4 and 3.1, respectively.

There were no significant differences for mean weekly morning and afternoon DO (Figure 2). There were also no significant differences ($p = 0.7247$) detected for mean weekly pH between the two treatments. Overall, weekly means for pH in the morning and afternoon (ranging from 6.5 to 9) for the whole growing season are shown in Figure 3. For optimal catfish growth in ponds, optimum levels of dissolved oxygen and pH have to be maintained. Fish may become stressed, eat less, and become increasingly susceptible to diseases if optimal dissolved oxygen levels are not maintained (Stickney 1994). According to Chapman (1992), an oxygen concentration of not less than 4.0 mg/L is needed for good catfish growth. Figure 2 shows that this level of dissolved oxygen concentration was maintained for most of the study although there were some daily fluctuations in both treatment and control ponds throughout the study. Although daily fluctuations in dissolved oxygen are common in channel catfish ponds, there is little knowledge about their effect on channel catfish (Tucker and Boyd 1985).

The pH of water is one of the most important water quality parameters in an aquatic ecosystem because it directly or indirectly affects almost all other chemical and biological parameters (Tucker and Hargreaves 2004). The most important effect of pH is on ammonia toxicity. It has been shown that a high pH level in the afternoon will stress fish and cause a large proportion of toxic, unionized ammonia to be produced in the water, thereby slowing fish growth (Tucker and Boyd 1985). The optimal pH for channel catfish ponds is 6.5-9.0; thus, pH was considered optimal throughout most of the study.

Table 2 shows the overall changes in mean monthly alkalinity, total hardness,

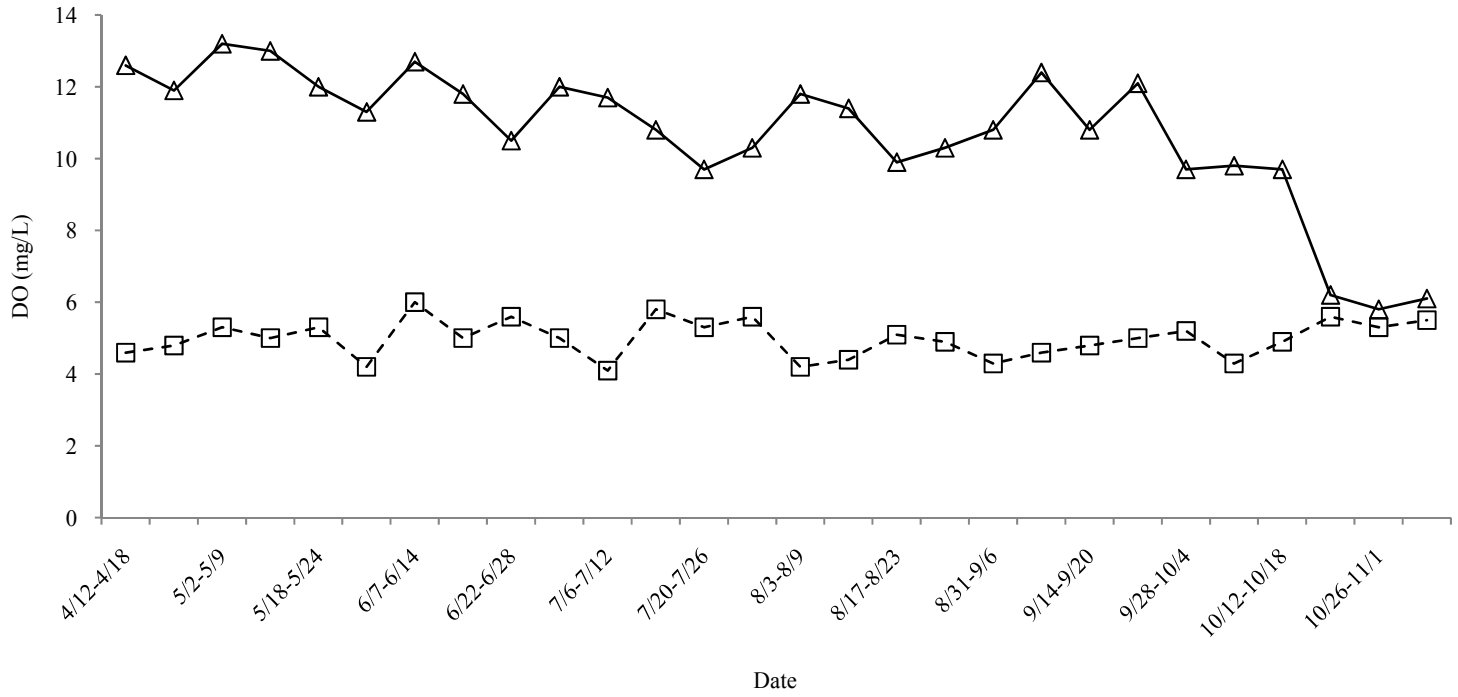

FIGURE 2. Overall mean weekly dissolved oxygen (DO, mg/L) in the morning □ and afternoon Δ in channel catfish ponds stocked at 12,350 fish/ha and treated with or without LymnoZyme®.

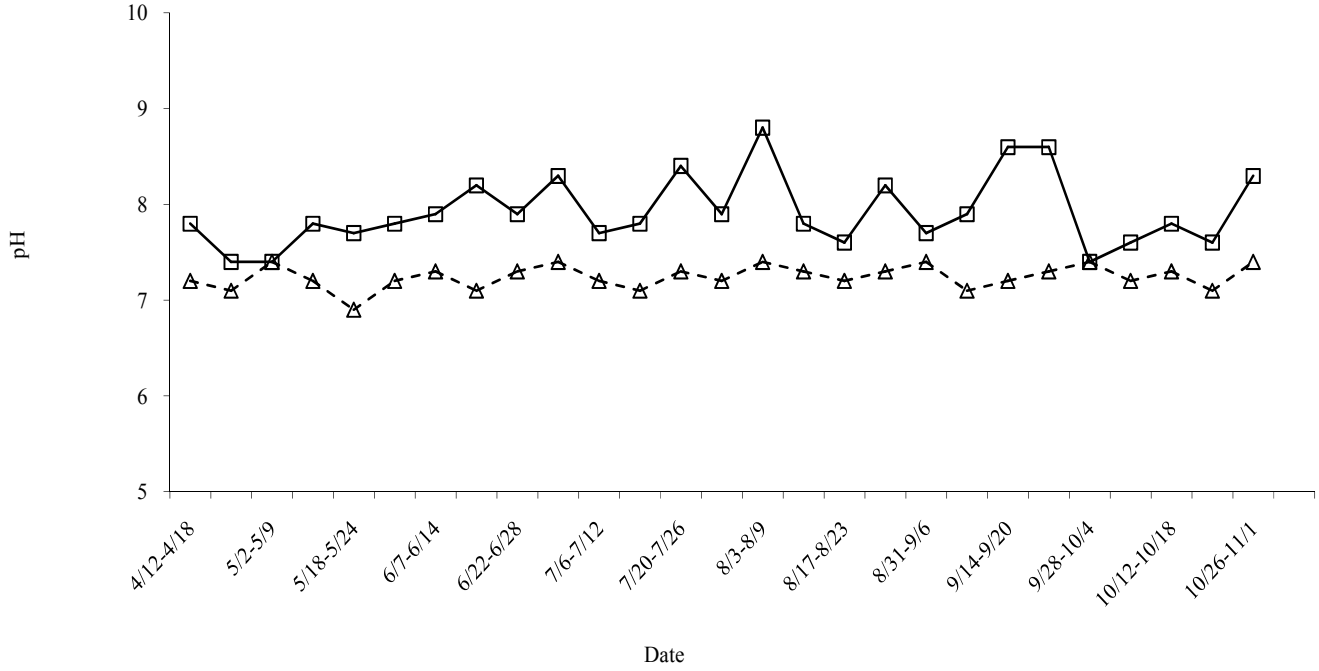

FIGURE 3. Overall mean weekly pH in the morning Δ and afternoon □ of catfish ponds stocked at 12,350 fish/ha and treated with or without LymnoZyme®.

TABLE 2. Overall mean (±SE[1]) monthly alkalinity, total hardness, TAN (total ammonia nitrogen), unionized ammonia and nitrite of channel catfish ponds stocked at 12,350 fish/ha and treated with or without LymnoZyme®.

Month	Alkalinity mg/L	Total Hardness mg/L	TAN mg N/L	NH_3 mg N/L	Nitrite mg N/L
April	58	54	0.07 ±0.02	0.01 ±0.01	0.02 ±0.02
May	67	57	0.11 ±0.04	0.01 ±0.04	0.08 ±0.02
June	82	52	0.13 ±0.07	0.03 ±0.01	0.11 ±0.03
July	86	66	0.18 ±0.08	0.03 ±0.01	0.13 ±0.04
August	89	68	0.8 ±0.14	0.04 ±0.02	0.31 ±0.01
September	84	62	0.9 ±0.13	0.10 ±0.03	0.35 ±0.04
October	86	65	0.92 ±0.16	0.14 ±0.01	0.39 ±0.02
November	86	63	0.98 ±0.19	0.14 ±0.04	0.39 ±0.04

[1]SE= Standard Error

TAN (total ammonia nitrogen), unionized ammonia (NH_3) and nitrite. Alkalinity and total hardness levels between the ranges of 50-100 mg/L are recommended in catfish ponds for optimal production (Chapman, 1992). There were no differences between treatment and control ponds for alkalinity (p=0.70), total hardness (p=0.64), TAN (p=0.69), unionized ammonia (p=0.423) and nitrite (p=0.46). For a successful aquaculture operation in a freshwater environment, the ideal alkalinity should generally be between 30 and 200 mg/L as $CaCO_3$ (Stickney 1994), although levels as low as 5 mg/L and high as 300 mg/L are recorded in catfish ponds (Tucker and Hargreaves 2004). The range of total alkalinities (58 to 86 mg/L as $CaCO_3$) recorded in this study fall within the range (50 to 250 mg/L) considered normal in catfish ponds (Tucker and Hargreaves 2004). Usually hardness and alkalinity levels are positively correlated and levels around 50 mg/L to 100 mg/L are needed to provide a good buffer to pH swings in ponds (Chapman 1992). As mentioned earlier, unionized ammonia is the stressful portion of TAN and it changes with changes in water temperature and pH. In channel catfish ponds, concentration of unionized ammonia usually ranges from 0 to 1 mg and in certain situations more (Tucker and Boyd 1985). The mean overall unionized ammonia for this study was 0.14 mg N/L ranging from 0.005 to 0.38 mg N/L. Nitrite concentration levels in commercial catfish ponds usually range from 0 to 0.25 mg N/L (Tucker and Boyd 1985) although it can sometimes exceed 4 mg/L (Tucker 1996). In this study, the overall nitrite levels ranged from 0.01 to 0.14 mg N/L.

Survival

Table 3 shows the stocking and harvest data for the pond study. There was no significant difference in mean survival between treatment and control ($p = 0.869$). Treatment ponds had a mean survival of 35.9% while the controls were 34.1%. The low percent survival is believed to be due to a columnaris infection at the beginning of the study leading to mortalities in all ponds.

Due to infection and the small size of fish relative to the feed particles offered, feeding activity was very poor in the first three weeks. As a result, they were weak and more susceptible to columnaris disease. All fish sampled and sent to the Auburn University Fish Diagnostic Laboratory for microbial tests during that period showed the presence of *F. columnare.* Dead fish collected from ponds also showed clinical signs of columnaris. Because potassium permanganate was used to treat the ponds for columnaris, the application of LymnoZyme® was delayed. Since no other mortalities and associated outbreaks were recorded after this initial event, it is assumed that the mortalities occurred prior to application of LymnoZyme®.

Growth Rates

There was no significant difference in mean stocking weights (Table 3) between the treatment and control ($p = 0.635$); therefore, specific and average growth rates (Table 3) were calculated and used to compare growth differences. Mean harvest weights (Table 3) between the two groups also showed no significant difference ($p = 0.857$). There were no

TABLE 3. Mean (±SE[1]) number of fish stocked and mean individual stocking weight, individual harvest weight, survival (%), average growth rate and specific growth rate of channel catfish stocked at 12,350 fish/ha in earthen ponds and treated with or without LymnoZyme® over 210 days.

	Mean # of fish stocked	Mean Stocking weight (g)	Mean harvest weight (g)	Mean survival (%)	Average growth rate[2] (g/day)	Specific growth rate[3] (g/day)
LymnoZyme®	500	9.8±0.3	553 ±15.4	35.9 ±7.5	2.7 ±0.18	1.9 ±0.05
Control	500	9.7 ±0.4	570 ±11.4	34.1 ±7.2	2.7 ±0.32	1.9 ±0.05

[1]Standard Error

[2]Average growth rate (g/day) = $(W_f - W_i)/t$, where W_f and W_i are the individual harvest and stocking weights respectively, whereas t, is time (days) from stocking to harvesting.

[3]Specific growth rate = $(\ln W_f - \ln W_i) \times 100/t$, where In W_f and In W_i, are the natural logarithm of the individual harvest and stocking weights respectively; where t is the time (days) between stocking and harvest dates.

significant differences between treatment and control for mean specific growth rate ($p = 0.836$) or mean growth rate ($p = 0.857$). Mean net production (Table 4) also showed no significant difference ($p = 0.400$) between treatment and control ponds. Because recorded mortalities in production occurred early in the study prior to application of LymnoZyme®, growth may not have been affected by treatment. The relatively low final standing crops did not negatively impact water quality, thus potentially masking any potential treatment effect. In the study by Queiroz and Boyd (1998), where fish with an average size of 10.7g were stocked in similar ponds and conditions at the same facilities at a rate of 15,000 fish/ ha and fed with a 32% crude protein diet and raised from May to October within a temperature of 18-29^{o}C, fish treated with a commercial bacterial inoculum known as Biostart™ showed a net production greater than control fish by 718 kg/ha. Survival was also greater in the treatment ponds (86.7%) than the control ponds (56.2%) and this could be the reason for the greater net fish production in the treatment ponds.

TABLE 4. Mean (±SE[1]) initial and final standing crops and net production of channel catfish stocked at 12,350 fish/ha in earthen ponds and treated with or without LymnoZyme® over 210 days.

	Initial standing crop (kg/ha)	Final standing crop (kg/ha)	Net production[2] (kg/ha)
LymnoZyme®	122.5 ±2	2,480 ±354	2357.5 ±337
Control	121.3 ±3	2,429 ±346	2307.7 ±330

[1] Standard Error

[2] Net Production (kg/ha) = Final Standing Crop (kg/ha) – Initial Standing Crop (kg/ha).

Experiment 2

This study was conducted to determine if LymnoZyme® application after *E. ictaluri* infection will reduce mortalities in channel catfish. Fish harvested from this study were used to stock aquaria for the first and second challenge studies. Overall mean daily morning and afternoon water temperatures, DO and pH are shown in Table 5. Overall mean daily dissolved oxygen, pH, alkalinity and total ammonia levels were 8.6±0.4 mg/L, 7.6 ±0.15, 89 ±0.27 mg/L and 0.2 ±0.11 mg/L, respectively. Although fish were raised for only a little over one month, the temperature conditions were mostly within the recommended optimum temperature range of 27-30^{o} C (Figure 4). The number of times the DO concentration levels in the ponds were recorded below 4.0 and pH went above 9.0 were compared between treatment and control and shown in Table 5. Also shown in Table 5 are overall mean alkalinity, total hardness, total ammonia nitrogen (TAN) and unionized ammonia (NH_3) for the growing period. There were no significant differences for both dissolved oxygen below 4.0 mg/L and pH above 9.0. The recommended optimum oxygen concentration of not less than 4.0 mg/L and pH of 6.5-9.0 were maintained for most of the study. These are also shown in Figures 5 and 6, respectively.

Production parameters (Table 6) showed no significant differences. Mean stocking weights were 6.6 g and did not differ significantly. Overall mean harvest weight, survival, average growth rate and specific growth rate were 68.3 g, 97.8 %, 1 g/day and 5.7 g/day, respectively.

TABLE 5. Mean (± SE[1]) times of dissolved oxygen (DO) below 4.0 mg/L, pH above 9.5, alkalinity, total hardness, total ammonia nitrogen (TAN) and unionized ammonia (NH_3) in channel catfish ponds stocked at 4,940 fish/ha in earthen ponds and treated with or without LymnoZyme®

Average Times of DO < 4.0 mg/L	2 ±0.2
Average Times of pH > 9.5	1 ±0.1
Mean Daily Morning DOs (mg/L)	5.2 ±0.7
Mean Daily Afternoon DOs (mg/L)	9.7 ±0.3
Mean Daily Morning Temperature (°C)	24 ±0.6
Mean Daily Afternoon Temperature (°C)	29 ±0.8
Mean Alkalinity (mg/L)	82 ±3
Total Hardness (mg/L)	66 ±2.4
TAN (mg N/L)	0.12 ±0.01
NH_3 (mg N/L)	0.02 ±0.01

[1]SE= Standard Error

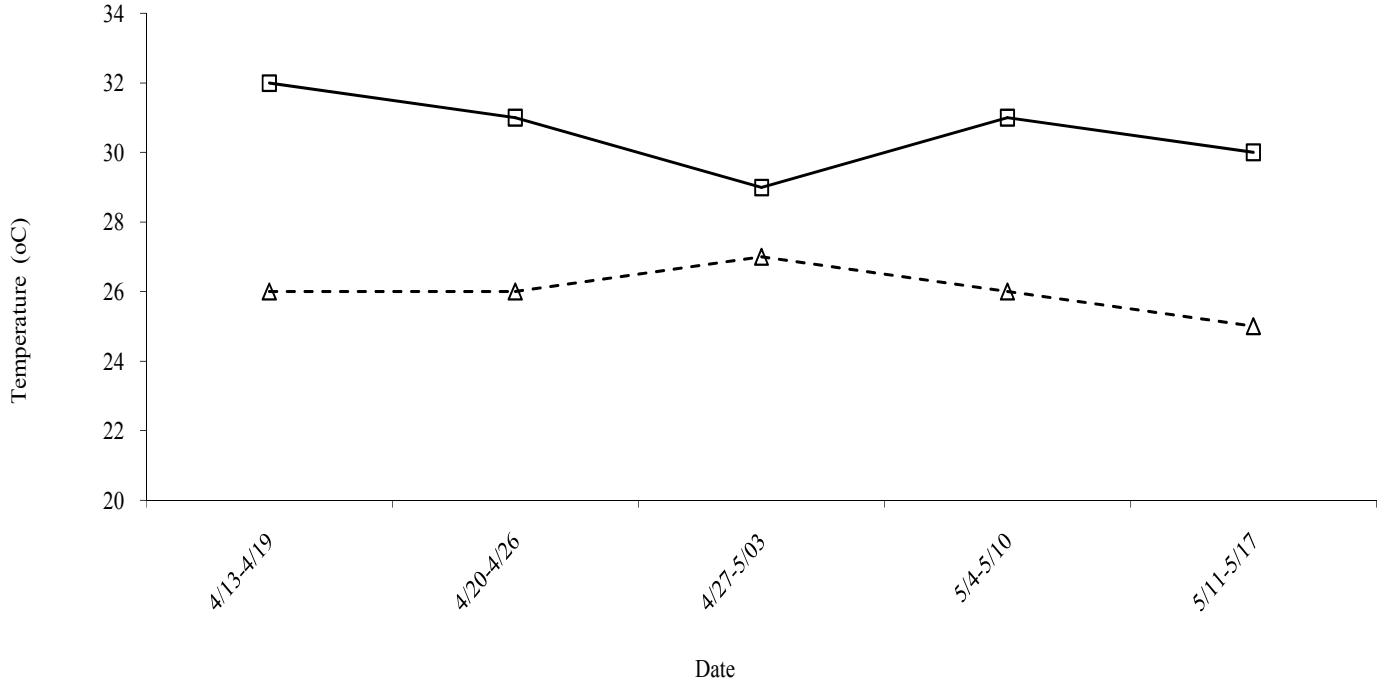

FIGURE 4. Mean overall weekly morning Δ and afternoon □ water temperatures (°C) of catfish ponds stocked at 4,940 fish/ha and treated with or without LymnoZyme® and grown for 41 days.

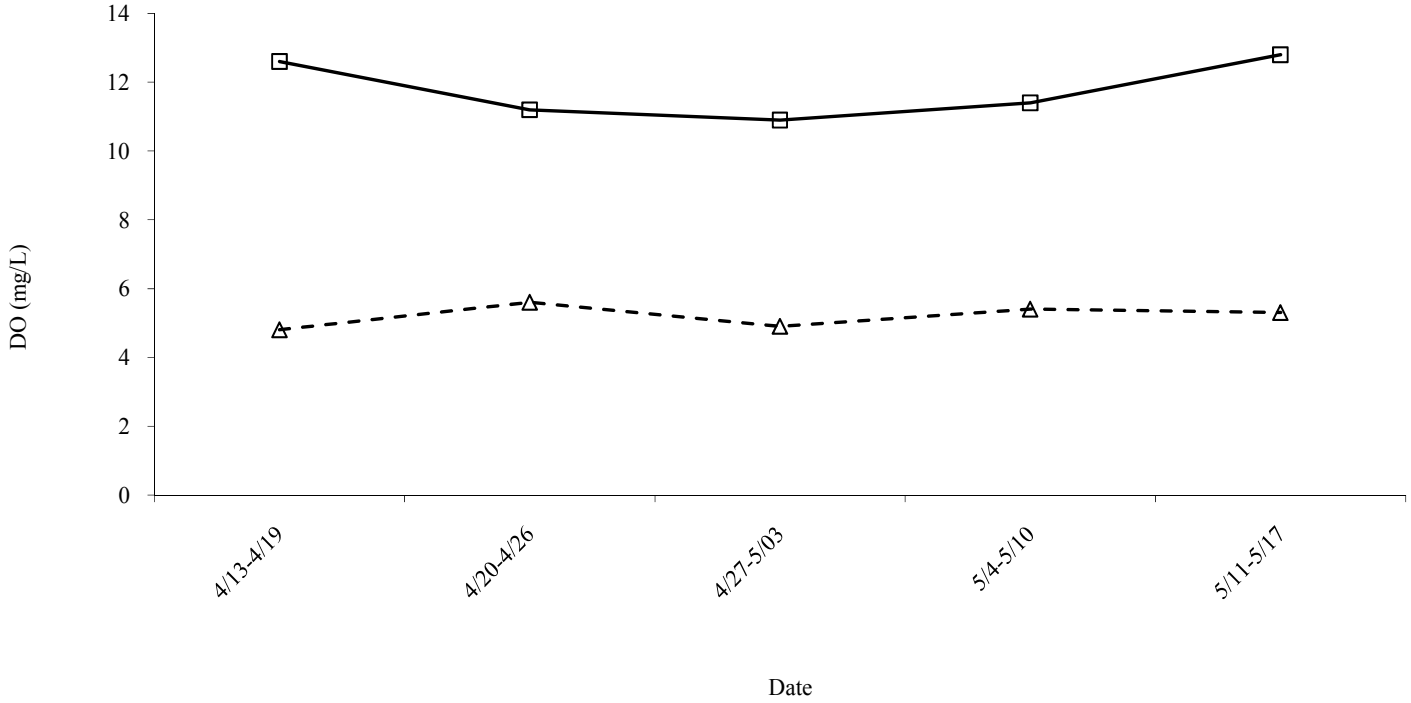

FIGURE 5. Overall mean weekly dissolved oxygen (mg/L) in the morning Δ and afternoon □ of catfish ponds stocked at 4,940 fish/ha and treated with or without LymnoZyme® and grown for 41 days.

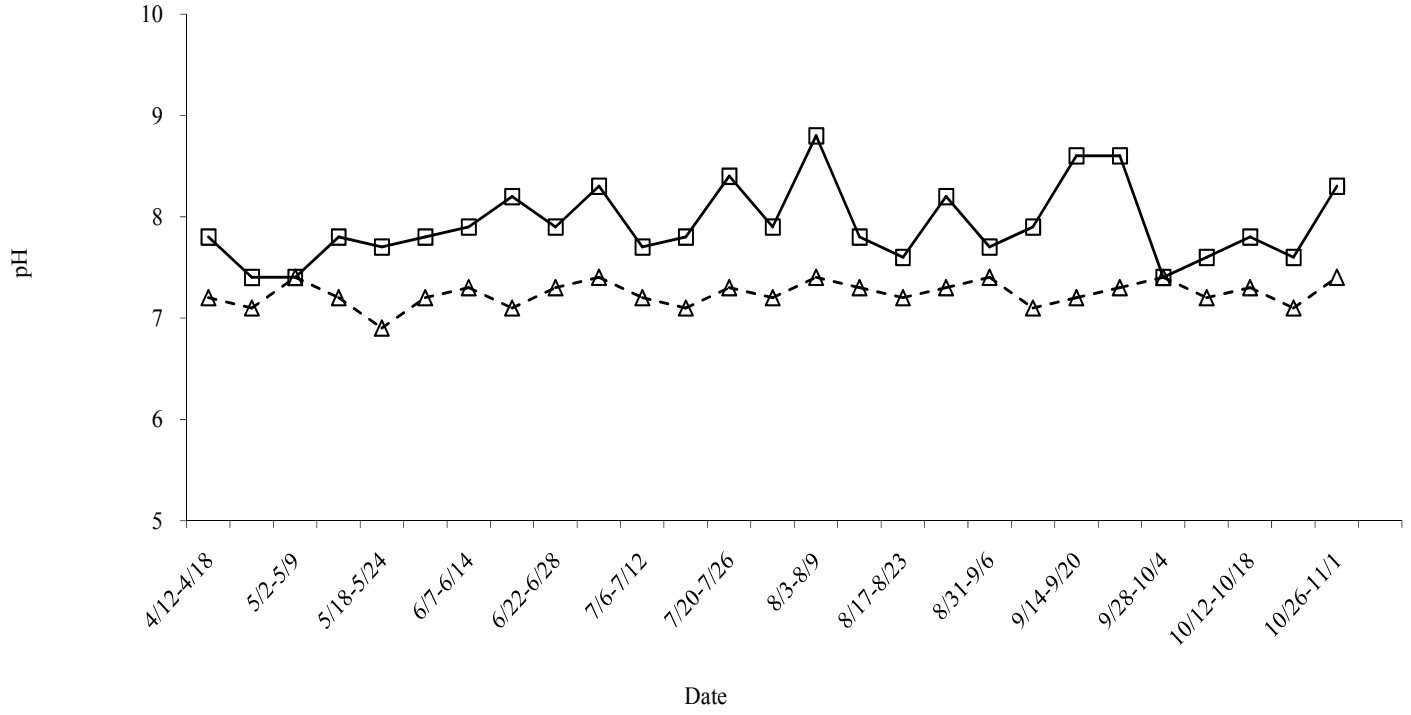

FIGURE 6. Overall mean weekly pH in the morning Δ and afternoon □ of catfish ponds stocked at 4,940 fish/ha and treated with or without LymnoZyme® and grown for 41 days.

TABLE 6. Mean (±SE) number of fish stocked, individual stocking weights, individual harvest weights, survival (%), average growth rates and specific growth rates of channel catfish grown in earthen ponds and treated with or without LymnoZyme® over 41 days.

	Mean # of fish stocked	Mean Stocking weight (g)	Mean harvest weight (g)	Mean survival (%)	Average growth rate[2] (g/day)	Specific growth rate[3] (g/day)
LymnoZyme®	200	6.6 ±0.2	65.59 ±0.01	98.5 ±0.03	1.4 ±0.03	5.59 ±0.04
Control	200	6.6 ±0.2	70.93 ±0.05	97 ±0.01	2.6 ±0.01	5.78 ±0.06

[1]SE= Standard Error

1 Average growth rate (g/day) = $(W_f - W_i)/t$, where W_f and W_i are the individual harvest and stocking weights respectively, whereas t, is time (days) from stocking to harvesting.

2 Specific growth rate = $(\ln W_f - \ln W_i) \times 100/t$, where In W_f and In W_i, are the natural logarithm of the individual harvest and stocking weights respectively; where t is the time (days) between stocking and harvest dates.

Challenge Studies

Figures 7 and 8 illustrate cumulative percent mortalities after challenge with ESC for first and second challenge studies, respectively. Mortalities for each study represent deaths recorded at least 3 days after fish were challenged with *E. ictaluri*. Channel catfish mortalities for both study one and two began on day 6. While the first study showed significant differences (p=0.035) in mean cumulative mortalities (Figure 7) between LymnoZyme® (85% ± 1.18) and control (99% ±0.58), mortalities were still high. Mortalities were similar for both control and LymnoZyme®-treated fish for the first two days of mortalities (days 6-7), then mortalities for control fish started increasing rapidly thereby increasing the difference between them from days 9 to 19.

In the second study, mean final cumulative mortality (Figure 8) in the continuously exposed LymnoZyme® treatment (45% ±1.43) was significantly reduced (p=0.002) compared to those from the control (80% ± 2.15) or those under application of LymnoZyme® after onset of ESC (75% ± 1.21). Mortalities began on day 6 as in the first challenge study. On the next day, the only significant difference was between those treated with LymnoZyme® after onset of mortalities and the control, with the latter having a higher mortality. From the third day of mortalities (day 8 of study), mortalities for fish treated with LymnoZyme® after onset of mortalities and the control were almost the same until the end of the study. Fish treated with LymnoZyme® from the beginning, however, showed significantly lower mean cumulative mortalities between them and the other two treatments from day 8 of study to the end.

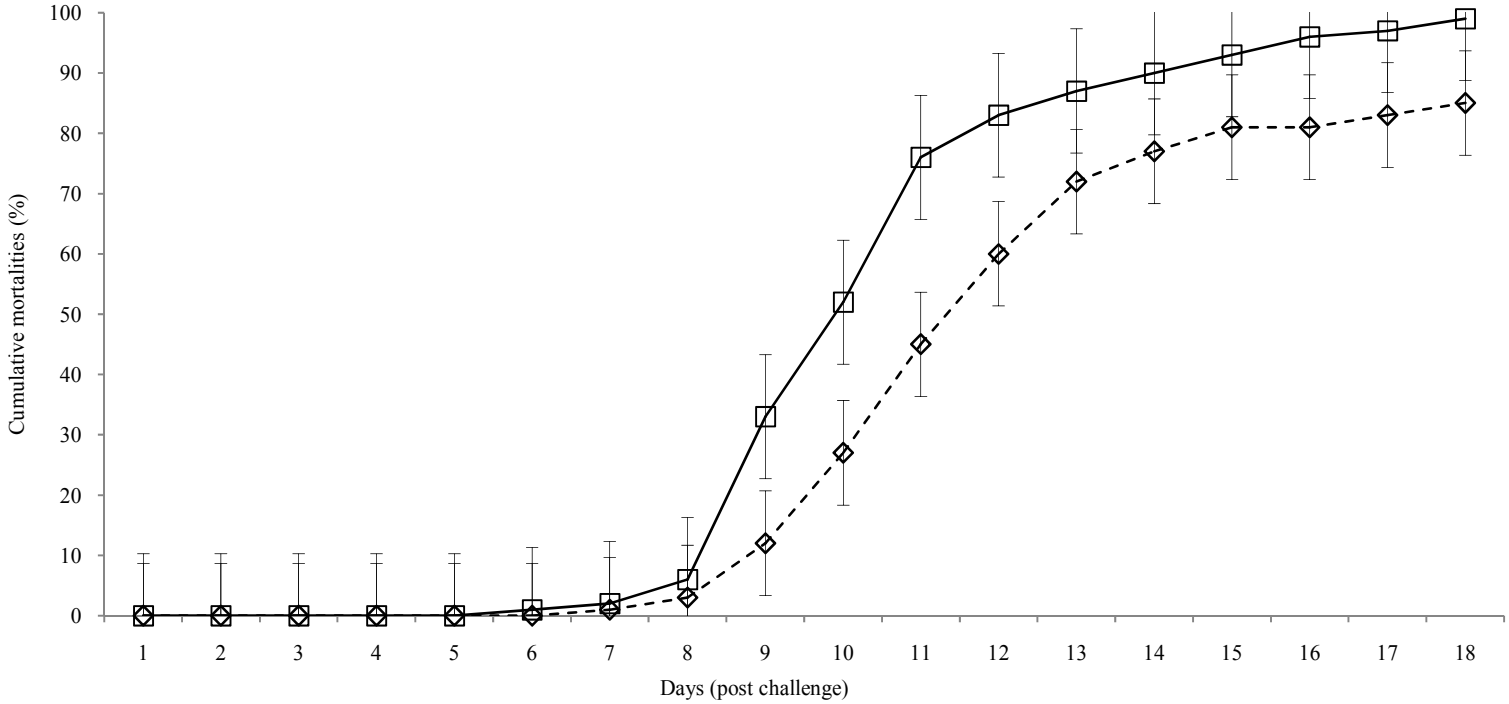

FIGURE 7. Average cumulative mortalities expressed as a percentage of stocking number (15 fish/ aquaria) for channel catfish grown for one month in earthen ponds with ◊ or without LymnoZyme® □ and then challenged with 1.2 X 10^6 CFU/ml of *E. ictaluri* (S97-773) in 57-L flow-through aquaria. There was a significance difference between treatments.

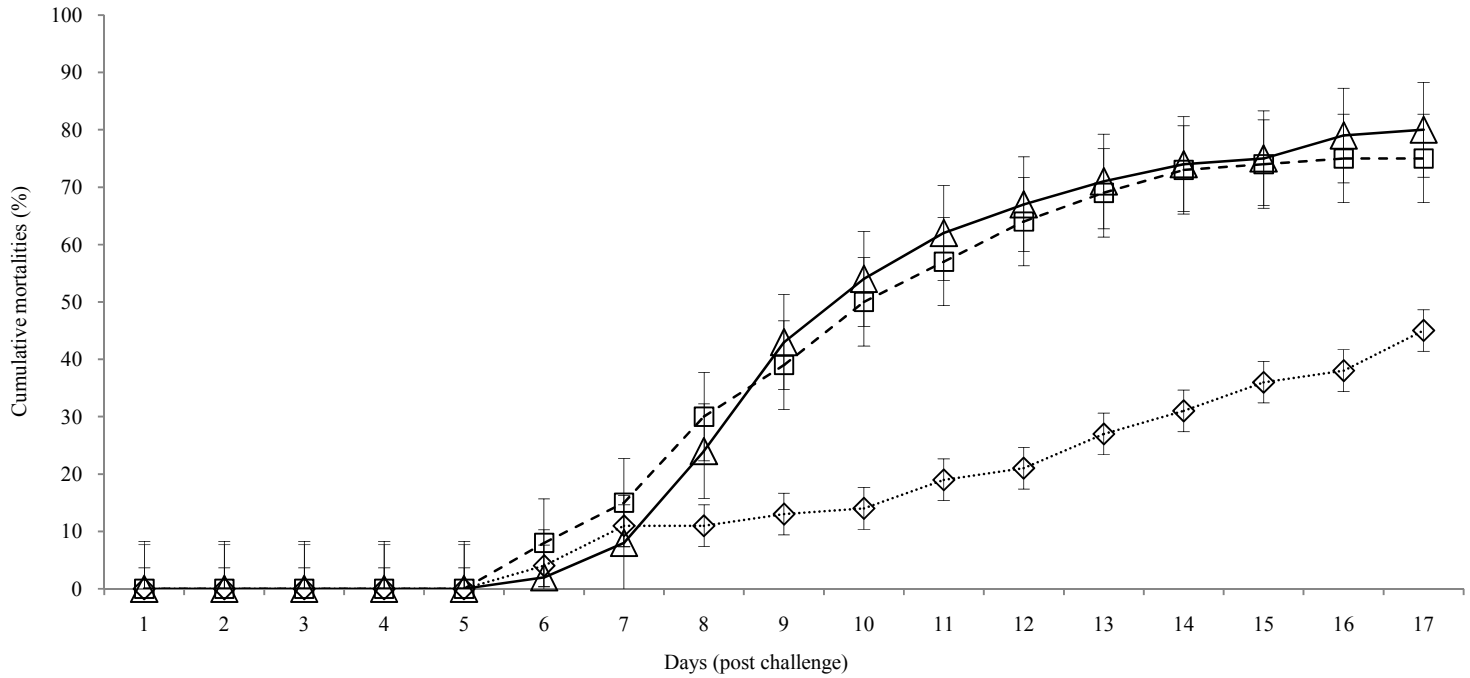

FIGURE 8. Average cumulative mortalities expressed as a percentage of stocking number (14 fish/ aquaria) for channel catfish grown for about 6 weeks in earthen ponds with or without LymnoZyme® and then challenged with 1.3 X 10^6 CFU/ml of *E. ictaluri* (S97-773) in a 57-L flow through aquaria. ◊ Represents fish treated with LymnoZyme® throughout the study (a). □ Represents fish treated with LymnoZyme® at the onset of first mortality (b). Δ Represents fish with no LymnoZyme® treatment (control) (c).There was a significant difference between a and b; a and c.

All fish necropsied from the first challenge study showed clinical signs characteristic of ESC. For the second study, 74% showed signs of ESC only, whilst 26% showed signs of both ESC and columnaris. The columnaris is believed to have been caused by an outbreak in the aquaria system at the start of the study caused by stress of the fish due to handling during harvesting. Both ESC and columnaris are endemic to the pond facility.

In challenge study 3, all mortalities were caused by ESC as determined by cultures made from the kidney and spleen. As illustrated in Figure 9, there was no significant difference ($p= 0.976$) in mean cumulative mortality between fish with no exposure to LymnoZyme® after *E. ictaluri* challenge (80% ±2.46) and control (80% ±2.33). Although the increase in mean cumulative mortalities for the former was initially slower after challenge, it increased very rapidly for the next seven days. Mortalities for this study also began on day 6. There were significant differences ($p= 0.0001$) in mean cumulative mortalities between fish exposed to LymnoZyme® for seven days after challenge (47% ±1.48) or exposed daily (40% ±1.26) and those for the control (80% ±2.33). Fish exposed to LymnoZyme® daily for 17 days (40% ±1.26) experienced no mortality when LymnoZyme® application was stopped and fish kept in the aquaria for 7 extra days.

Mortalities in challenge study 1 were higher as compared to those in experiments 2 and 3 possibly because fish were stressed from being moved from the ponds directly to the aquaria and then challenged with *E. ictaluri* six hours later. In challenge study 2, fish were challenged 32 h after they have been moved from the ponds. Thus, there was extra

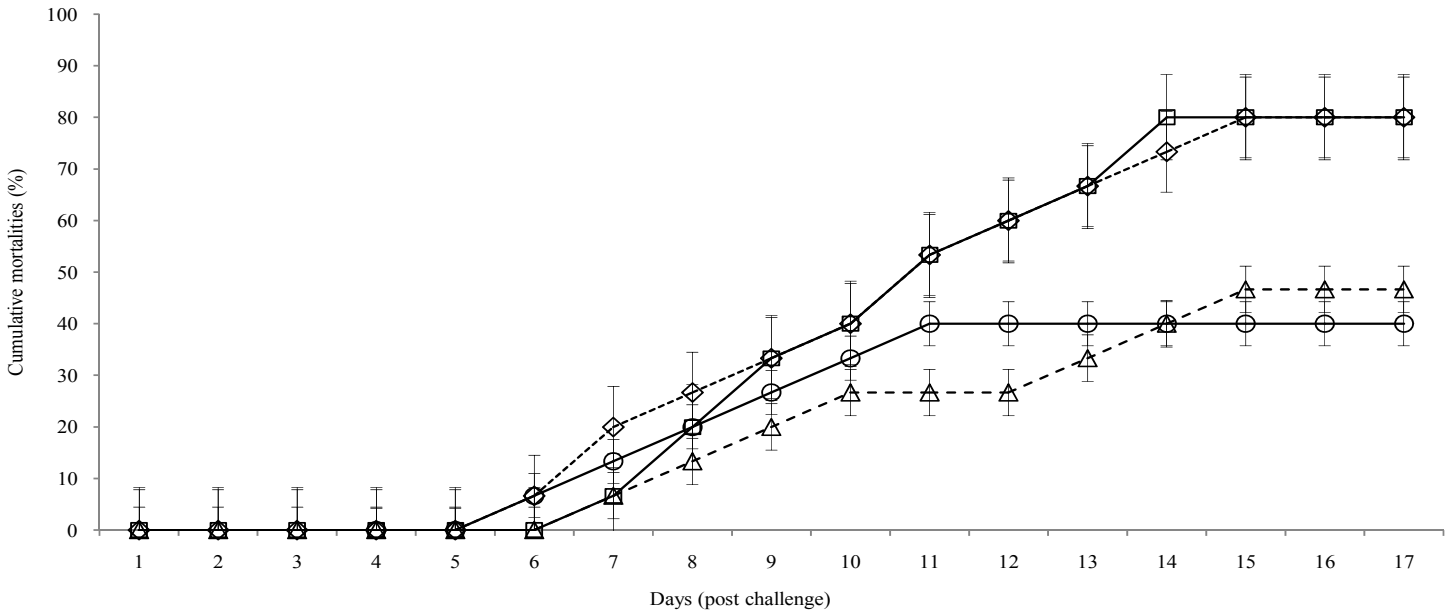

FIGURE 9. Average cumulative mortalities expressed as a percentage of specific pathogen-free fish stocked at a density of 15 fish/ aquaria and challenged with 1.3 X 10^6 CFU/ml of *E. ictaluri* (S97-773)
3- day exposure to LymnoZyme®, then no exposure after ESC treatment (a)□
3-day exposure to LymnoZyme®, then LymnoZyme® for next 7 days (b) Δ
3-day exposure to LymnoZyme®, then continues till the end (c) ○
Control- No exposure to LymnoZyme® throughout the study (d) ◊
There was a significant difference between c and a; c and b; c and d; b and a; b and d.

time for the fish to acclimatize to the aquaria conditions. Fish in the third aquaria study were maintained for 3 days before *E. ictaluri* challenge. The mortality patterns between fish exposed to LymnoZyme® for seven days after challenge and those exposed to LymnoZyme® continuously were very similar for the first 7 days after *E. ictaluri* challenge with the former showing a lower cumulative mortality for days 8 and 9. However, after day 9, the mean cumulative mortality for the seven-day LymnoZyme®-exposed fish started increasing until it surpassed that of the daily LymnoZyme®-exposed fish in the last few days of the study. No mortalities occurred in those continuously exposed to LymnoZyme® after day 8. Fish only treated with LymnoZyme® for 7 days may have become re-infected after day 9. This may indicate that although fish exposure to LymnoZyme® before and for 7 days after *E. ictaluri* challenge significantly reduces the rate of mortality, additional treatment is needed to prevent re-exposure to *E. ictaluri* and resultant mortalities.

This raises the question of what would be the ideal time for LymnoZyme® treatment in order to achieve the maximum reduction in mortalities. There is also the question of whether the mortality for the various treatments would have been less if fish had been exposed to LymnoZyme® for a longer period than three days prior to *E. ictaluri* challenge. This question arises because in study 3, although mortalities between fish exposed to LymnoZyme® after *E. ictaluri* challenge were significantly reduced, 40% and 47% were still very high and this could be due to the heavy doses (relative to typical exposure in culture) of *E. ictaluri* used in the challenges. The doses were higher than what is normally seen in fish ponds. It is not clear what the mode of action of the

LymnoZyme® is, but the results suggest that the probiotics in the water colonize the fish skin, guts and other parts of the body leading to one of two things (or both) happening:

1. Beneficial bacteria present in the LymnoZyme® mixture are out-competing the *E. ictaluri*, thus rendering it ineffective in killing the channel catfish. Probiotic bacteria in the LymnoZyme®-treated water may have entered the gut of the fish orally and colonized the intestinal tract of the fish. By colonizing the intestinal mucus layer, the LymnoZyme® bacteria inhibited the colonization by *E. ictaluri*. The complex microbial ecology of the gut of the fish is known to provide nutritional benefits as well as protection against pathogens (Balcazar et al. 2006; Vandenbergh 1993) and the ability of the fish to develop immune responses to a pathogen may depend on the ability of the beneficial bacteria present to prevent the domination of the intestinal flora by the pathogenic bacteria.

   The ability of both aerobic and anaerobic intestinal bacteria with probiotic properties to adhere to the fish intestinal mucus and inhibit the growth of pathogenic bacteria has been demonstrated to occur in freshwater fishes. Sugita et al. (1996) isolated intestinal bacteria from 7 freshwater fish species, ayu *(Plecoglossus altivelis; 25-38 g* body weight), common carp ***(****Cyprinus carpio; 21-56 g),* goldfish *(Curussius aurutus;* 1 l-41 g), Japanese eel (*Anguilla japonica; 20-45 g),* rainbow trout *(Oncorhynchus mykiss; 76-88 g),* tilapia *(Oreochromis niloticus****;*** 19-460 g), and channel catfish *(Ictalurus punctutus; 4-5 g)*. These fish isolates were tested against 18 pathogenic strains: *Aeromonas caviae* ATCC 15468; *A. enteropelogenes* ATCC 49803; *A. eucrenophila* ATCC 23309;

A. hydrophila ATCC 7966; *A. ichthiosmia* ATCC 49804; *A. junduei* ATCC 49568; *A. media* ATCC 33907; *A. sulmonicida* ATCC 33658; *A. schubertii* ATCC 43700; *A. sobria* ATCC 43979; *A. trota* ATCC 49657; *A. veronii* ATCC 35624; *Enterococcus faeculis* ATCC 29212; *Escherichia coli* IAM 1264; *Micrococcus luteus* ATCC 4698; *Plesiomonas shigelloides* ATCC 14029; *Pseudomonas aeruginosu* ATCC 27853; *Staphylococcus aureus* ATCC 25923. Fish isolates with antibacterial effects were detected from each of the seven fish species.

Lactic acid bacteria like Lactobacilli and Bifidobacteria which are found in LymnoZyme® are considered the most important bacteria that effectively compete with pathogenic bacteria in the intestine of fish by producing bacteriocins (Bruno and Montville 1993; Vandenbergh 1993). These bacteria are known to resist gastric acid and pancreatic enzymes, adhere to intestinal mucosa and readily colonize the intestinal tract (Rolfe 2000). Although these bacteria have been found not to be dominant in the normal intestinal microbial community of fish (Ringo and Gatesoupe 1998), several studies have been done to show the possibility of inducing the artificial dominance of lactic acid bacteria and other Gram positive bacteria in the intestine. A non-virulent strain of *Carnobacterium* sp. (strain K1), a Gram positive rod has been found to inhibit the growth of *A. salmonicida.* This strain was examined *in vitro* for characteristics necessary for the colonization of the gastrointestinal tract of the rainbow trout *(Oncorhynchus mykiss*) and *in vivo* for persistence in the tract of the fish after oral dosing by Joborn et al. (1997). The results showed that the K1 strain adhered non-

specifically to the intestinal mucus of the rainbow trout and multiplied in the intestinal mucus. Freshly collected fecal extracts showed relatively high densities of the K1 strain (10^5 CFU/ g), indicating that bacterium cells remained viable in the intestinal tract of the fish.

Nicoskelainen et al. (2001) further demonstrated this probiotic property of lactic acid bacteria by administering two doses (10^9 and 10^{12} CFU/ g feed) of a commercial dry feed mixed with *Lactobacillus rhamnosus* (ATCC 53103) suspended in oil to rainbow trout *(Oncorhynchus mykiss*) for 51 days. Sixteen days after the start of the *Lactobacillus* feeding, fish were challenged with *Aeromonas salmonicida*. At the end of the trial, *L. rhamnosus* was found to reduce the mortality of the fish significantly from 52.6% in the control (which was fed the dry feed without the *L. rhamnosus* bacteria) to 18.9% and 46.3% in the 10^9 CFU/ g and 10^{12} CFU/ g feed groups, respectively.

The LymnoZyme®-ESC plate culture study showed growth in both control and LymnoZyme® plates. This supports the above assertion that LymnoZyme® bacteria did not kill *E. ictaluri* but possibly colonized the intestinal mucus layer, thus reducing the pathogenic effect of *E. ictaluri*.

2. The second possible explanation is that LymnoZyme® bacteria ingested by fish boosted the non-specific immune response of the channel catfish such that it was able to withstand the *E. ictaluri* infection. Bacteria in the LymnoZyme® may have first served as a defensive barrier on the skin, gills and gut of the channel catfish

against the invading *E. ictaluri*. The bacteria may have also induced various non-specific immune responses in the fish. Peptidoglycan, which is a major component of bacterial cell walls and present in both gram-positive and gram-negative bacteria, is known to induce a variety of non-specific immune responses (Erikson and Hubbard 2000).

Nikoskelainen et al. (2003) showed this immune enhancement ability of probiotic bacteria by administering *L. rhamnosus* (ATCC 53103) through feed to rainbow trout (*O. mykiss*) for two weeks and then changing the feed to un-supplemented diet. By the end of the first week, fish samples taken 24 h after the last feeding showed a dose-related amount of *L. rhamnosus* on the intestine and skin mucus, which shows that *L. rhamnosus* colonized these surfaces. At the end of the second week, the number of viable lactobacilli had increased from below detection limit levels of less than 10 CFU/g at the beginning of the study to levels between 3.6×10^5 and 9.0×10^{10} CFU/ ml. At the end of the second week, some experimental fish showed a significant increase in respiratory burst activity. *L. rhamnosus*, however, disappeared from the intestine, skin mucus and tank water within just a week after the switch to the non-supplemented feed.

A different strain (JCM 1136) of *L. rhamnosus* was also used by Panigrahi (2005) to test this immune response induced by the bacteria. Rainbow trout (*O. mykiss*) was fed a diet incorporated with different forms (heat-killed, live-sprayed or freeze-dried) of *L. rhamnosus* twice daily for 30 days, after which the diet was withdrawn and replaced with diet used for the control fish for up to 45 days. At

the end of the study, fish fed the viable form (live-sprayed) of *L. rhamnosus* showed better phagocytic and complement activities. The plasma immunoglobulin levels also showed an increasing trend in those fish fed the live-sprayed diet. However, as has already been shown in previous examples, the lactic acid bacteria disappeared from the intestines upon the withdrawal of the probiotic diet.

The reduction in the number of bacteria in these studies after fish had been switched to non-supplemented feed may be representative of what happened in the LymnoZyme® aquaria study 3 where fish treated with LymnoZyme® for longer periods during the study showed significant differences in survival from those treated with LymnoZyme® for shorter periods of time. The reduction in probiotic bacteria cell numbers in fish treated with LymnoZyme® for 7 days post-challenge, allowed for re-infection of the channel catfish through the multiplication of *E. ictaluri* cells excreted into the water. Therefore, to ensure a continuous protection of the fish by the probiotic bacteria against *E. ictaluri*, LymnoZyme® may have to be applied on a continuous basis as long as *E. ictaluri* is in the system.

Results from the fourth aquaria study (Figure 10), where control and LymnoZyme®-treated fish had 94% and 14% mortalities, respectively, showed that fish had developed some form of immunological response to ESC. Fish developing immunological responses after primary exposure to ESC has been demonstrated by various studies. Petrie-Hanson and Ainsworth (1999)

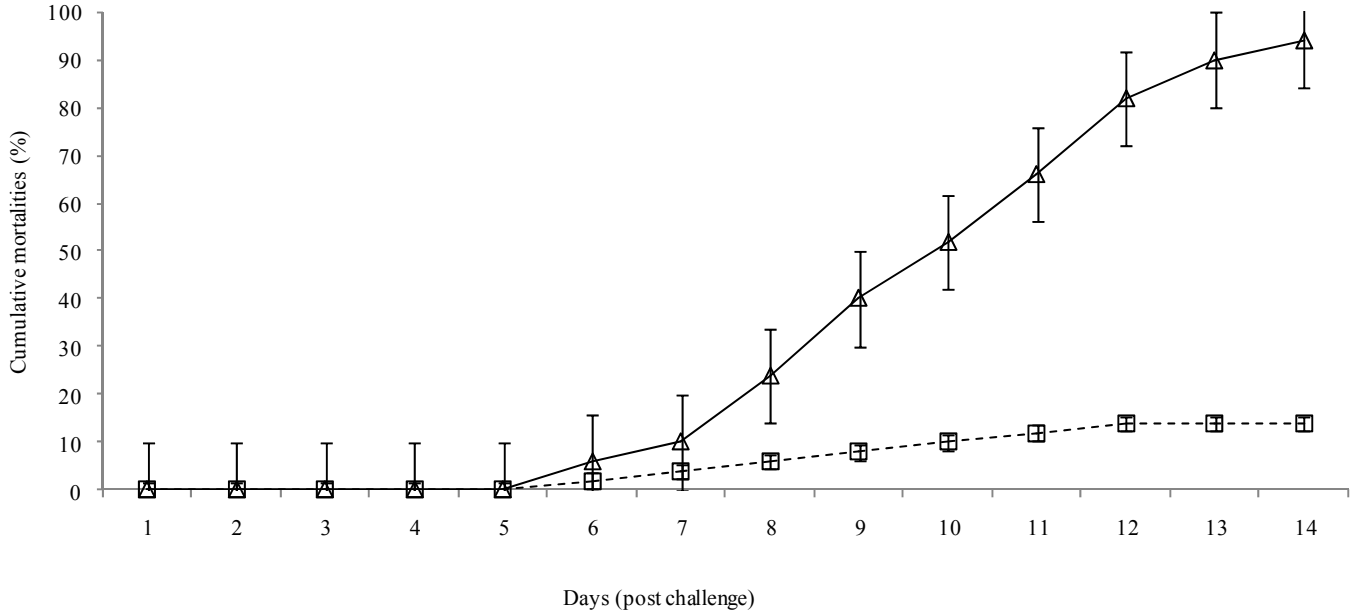

FIGURE 10. Average cumulative mortalities expressed as a percentage of specific pathogen free fish (control) Δ and fish previously exposed to LymnoZyme® (and challenged with 1.3 X 10^6 CFU/ml of *E. ictaluri*) □ stocked at a density of 10 fish/ aquaria and then challenged with 2 X 10^6 CFU/ml of *E. ictaluri* (S97-773).There was a significant difference between treatments.

demonstrated this by giving specific pathogen free catfish groups of ages from 1 week old and above a primary immersion exposure to *E. ictaluri* (6.4×10^4 CFU/ml). Each age group of fish was given a second immersion exposure 4 weeks after the first exposure and was sampled 2 weeks after each exposure. Results from antibody titer tests showed that fish age four weeks and above acquired varying degrees of immune response with those at ages 8 weeks and above showing stronger responses (corresponding to an increase in maturity of the lymphoid system).

It is difficult to determine how long and to what extent fish might survive a continuous exposure to ESC. If answers to these key questions are found and they prove positive, then one may know whether a farmer can limit fish exposure to LymnoZyme® only to the hatchery phase and thus eliminate the cost of LymnoZyme® application in the pond phase. LymnoZyme® was applied to the aquaria at approximately ten times the concentration used in ponds. A higher dose was used in order to increase the chances of more bacteria being ingested by the fish before the LymnoZyme® is washed off by the flow through system. One potential problem in adding probiotics to catfish ponds is the differences in bacterial communities between ponds. According to Arias et al. (2006), bacterial communities in a catfish aquaculture setting can vary widely from pond to pond at any given location. This implies that different concentrations of bacteria cells may be needed in different ponds to ensure an effective colonization of the different communities in each pond.

IV. CONCLUSIONS

Based on the present study, the following conclusions can be drawn:

1) The first pond study did not demonstrate any benefit from application of LymnoZyme®, but this may be the result of the low biomass caused by heavy mortalities prior to treatment.
2) The second pond study did not demonstrate any benefit in production during its short duration.
3) Exposure of fish to LymnoZyme® in aquaria before and after *E. ictaluri* challenge further reduces mortality from ESC.
4) Fish challenged with *E. ictaluri* under presence of LymnoZyme® develop a resistance to ESC under subsequent challenge. The duration of this resistance was not determined. However, LymnoZyme® application did reduce mortalities from ESC.

Further studies are required to determine how best to improve the efficiency of LymnoZyme® and the economics of its application. To make LymnoZyme® application in ponds cheaper for farmers, the company has developed a technology to produce it on site. The mode of action of LymnoZyme® and the beneficial components of the mixture are yet to be determined.

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