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PRO-INFLAMMATORY PROPERTIES OF FLAVOBACTERIUM COLUMNARE CELLS AND PRODUCTS ON CHANNEL CATFISH (ICTALURUS PUNCTATUS) NEUTROPHILS

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PRO-INFLAMMATORY PROPERTIES OF FLAVOBACTERIUM COLUMNARE
CELLS AND PRODUCTS ON CHANNEL CATFISH (ICTALURUS
PUNCTATUS) NEUTROPHILS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Biological Sciences

by
Shannon Michelle Billings
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Accepted by:
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Dr. Tom R. Scott
Dr. Thomas E. Schwedler

ABSTRACT

Flavobacterium columnare and *Edwardsiella ictaluri* are the leading causes for losses in the aquaculture industry. The losses from the diseases caused by these pathogens total \$2.5 million for catfish and \$100 million for total aquaculture. *F. columnare*'s infection rates and stressors associated with outbreaks have been well-characterized, but the specific inflammatory responses of catfish neutrophils to the bacteria have not been studied. The objective, therefore, was to investigate the pro-inflammatory effects of the outer membrane proteins, extracellular products, and whole live bacteria, heat-killed (HK), and formalin-killed (FK) *F. columnare* and *E. ictaluri* to determine the effects on channel catfish phagocyte physiology. Phorbol myristate acetate-stimulated hydrogen peroxide production, inducible nitric oxide production, and phagocytosis of yeast particles, and the expression of CYP1A and lysozyme protein were measured in phagocytes after exposure to *F. columnare* and *E. ictaluri* bacteria or product. *F. columnare*-treated cells did not produce hydrogen peroxide or nitric oxide, nor was phagocytosis affected. *E. ictaluri* increased hydrogen peroxide production, nitric oxide production, and enhanced phagocytosis in phagocytes when given as ECPs, OMPs, HK bacteria, and FK bacteria. Both OMPs increased lysozyme protein expression, and ECPs and OMPs of both bacteria suppressed CYP1A expression.

DEDICATION

I dedicate this work to my family and friends. I could not have done any of this without their love and support.

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LITERATURE REVIEW

Aquaculture

Aquaculture is an ancient practice that dates back to 2000 BC. It may occur with fresh or salt water species, and the majority of production is of fin and shellfish (Stickney, 2005). Worldwide, more than thirty percent of consumed fish are farmed fish, and as many as 297 different species are cultivated each year (Pillay, 2005). In the United States, 90% of the fish production is of crawfish, catfish, trout, and salmon, where the rest is made up mostly of striped bass, tilapia, eel, and alligator (Parker, 1995). Other popularly farmed fish are halibut, perch, carp, clams, mussels, oysters, shrimp, and crab (Stickney, 2005).

In 1995, the estimated value of aquaculture worldwide was approximately \$42.3 billion US (Dudley-Cash, 1998). In order to put this into perspective and realize the economic impact of aquaculture, the US gross national product (GNP) is approximately 12 trillion dollars (OECD, 2004). The worldwide value of aquaculture is 0.4 % of the US GNP.

The main drawback to aquaculture is the susceptibility to diseases - namely, bacterial, viral, fungal, and parasitic. Fungal and/or parasitic infections commonly occur annually, bacterial infections multiple times annually, and viral infections are harder to predict (Parker, 1995). Bacterial diseases are usually non-specific to a particular species where viruses are very specific to groups or species of organisms. Viruses, like the catfish channel virus (CCV) does not affect salmonids such as trout or salmon, and shrimp viruses do not attack other decapods. Fungal infections of *Saprolegnia* are most common in aquaculture,

whereas the most common bacterial diseases are caused by *Aeromonas salmonicida*, *Vibrio*, and *Escherichia coli*. Trematodes, cestodes, nematodes, sea lice, *Icthyophthirius multifiliis*, *Costia*, and *Amyodinium* are most common of parasitic infections. Viral infections such as CCV in channel catfish, Infectious Hematopoietic Necrosis Virus (IHVN) in salmonids, Viral Hemorrhagic Septicemia (VHS) in trout, and Infectious Salmon Anemia (ISA) in American Salmon are common in aquaculture (Stickney, 2005).

Disease can decrease harvest yields quality, with reduced growth and malnutrition of the fish (Thoratinnsson and Powell, 2006). Increased density of animals and increased stress exposure all lead to greater incidence of disease. Environmental changes such as water temperature alterations, low dissolved oxygen, pH, or salinity changes all increase the susceptibility of disease outbreaks. For some pathogens, overcrowding and handling stress are important stressors to avoid (Stickney, 2005). According to the World Bank in 1997, global estimates of the losses from white spot syndrome of shrimp were approximately \$3 billion per year, and the epizootic ulcerative syndrome cost the industry \$100 million (Pillay and Knutty, 2005). These are extreme cases of disease losses, but the Food and Agriculture Organization (FAO) of the United Nations estimates that disease outbreaks cost \$3 billion annually in the United States (Stickney, 2005). An estimate of international losses from disease outbreaks and the costs of treatments (vaccine development and commercialization, antibiotic treatments) is \$30 million (Ray, 2005).

Bacterial infections are the leading cause of losses in the commercial production of channel catfish. The losses from columnaris disease are second only to enteric septicemia of catfish (ESC) caused by *Edwardsiella ictaluri*, which account for 60% of all reported fish diseases from 1996 to 2001 (Bader et al, 2003). Columnaris is a known problem in hatcheries, pen, and cage and pond cultures and is responsible for serious fish kills the world over (Eimers and Cardella, 1990). From producer reports in Arkansas, Alabama, Louisiana, and Mississippi, 78.1% of all operations and 42.1% of all ponds in these areas had problems with ESC/columnaris. In a majority of these operations, these bacteria were the causes of the greatest economic losses from 1999 to 2002. ESC and columnaris caused almost half of the mortality of channel catfish these states in 1996 (Bader, et. al, 2003; Wagner, et al, 2002).

Flavobacterium columnare

The genus *Flavobacterium* has been difficult to classify phenotypically due the nutritional inconsistencies that vary within strains with incubation temperatures averaging around 20°C (Woo and Bruno, 1999). Variation is also observed among different hosts and geographical regions. The optimal media and temperature conditions, color, and colony margins differ, though the biochemical properties generally remain the same (Thomas-Jino and Goodwin, 2004).

The causative agent in columnaris disease is *Flavobacterium columnare*, which causes phenomenal losses in aquaculture each year. *F. columnare* is a

Gram-negative bacteria, with long, slender rods, and gliding mobility. It was first discovered in 1917 in 16 warm water fish in the Mississippi River and has since been found worldwide in a large variety of fish (Pillay and Knutty, 2005; Figueiredo, et al, 2005; Grabowski, et. al. 2004; Woo and Bruno, 1999; Altinok, 2004). In 1922, this caused a fish kill in the Mississippi River and was named *Bacillus columnaris* at that time (Thomas-Jinu and Goodwin, 2004; Woo and Bruno, 1999). Since then, the bacteria has been named and re-named several times (Table 1). Isolates from around the world are phenotypically similar, though genetically different and have varying optimal growth temperatures (Thomas-Jinu and Good win, 2004).

Name	Year
<i>Bacillus columnaris</i>	1922
<i>Cytophaga columnaris</i>	1945
<i>Chondrococcus columnaris</i>	1944
<i>Flexibacter columnaris</i>	1974,1989
Flavobacterium columnare	1996

Table 1. Names of *Flavobacterium columnare* since its discovery in 1917

(Thomas-Jinu and Goodwin, 2004; Suomalainen, et. al., 2005).

Columnaris can occur in cold or warm water fishes. It can be a chronic or acute infection with the virulence varying from high to low, depending on the strain, and it has been found in at least 36 species worldwide (Pillay and Knutty, 2005; Bader, et al, 2003). Although virulence has been defined to the time in which it takes the bacteria to cause the disease and to kill the fish, it always

depends on the species of fish infected. Highly virulent strains of *F. columnare* will cause the disease and kill within 24 hours (some strains can kill within 8 hours) and less virulent strains may take 48 hours to weeks after exposure, though many less virulent strains may kill the host between 48 and 96 hours (Pillay and Knutty, 2005; Thomas-Jinu and Goodwin, 2004; Soltani, et al, 1996).

This bacteria is only found in freshwater although there is a marine counterpart of columnaris disease caused by *Flexibacter maritimus* (Figueiredo, 2005). The most common fish for *F. columnare* to infect are channel catfish, sunfish, carp, koi, walleye, striped bass, eels, goldfish, tilapia, sturgeon, and most salmonids (Grabowski, et. al., 2004; Altinok, 2004). The primary sites for columnaris infection are the gills, mouth, fins, and any places on the body where there are abrasions (Woo and Bruno, 1999). The bacteria usually stay in these sites of infection to replicate and destroy the tissues to which it is attached (Altinok, 2004). One of the first symptoms of columnaris is mucus increase on the head which usually leads to a growth that is yellowish in color and cottony ("cotton-wool patches"), and the gills will get white spots and continue to overgrow on the filaments (Woo and Bruno, 1999; Soltani, et al, 1996).

Signs of the disease occurs in the dorsal fin area with discolored, often gray patches on the skin, often called saddleback lesions (Bader, et al, 2003). Highly virulent strains, however, attack gill tissues and causes internal, systemic infection commonly referred to as gill rot. Sometimes, the infection never shows externally and the fish can die before any outward signs of disease (Grabowski, et. al, 2004). Less virulent forms are responsible for the cutaneous lesions and

more external presentation of the disease. If left untreated, the lesions in gill tissue or on the skin will continue to grow and expose the underlying muscle. Columnaris was first thought to be caused by a fungus because of the filamentous bacteria that grows from the gills, causing fusion of the gill filaments and excess mucous (Pillay and Knutty, 2005; Figueiredo, et. al., 2005). Other symptoms of columnaris include shedding of scales and discoloration of skin patches. Along with fin damage, there can be mouth and barbell erosion, tail loss, and gill necrosis (Parker, 1995). As the disease progresses, there may be red, inflamed areas which lead to widespread necrosis, bacteraemia, and ultimately death (Woo and Bruno, 1999).

Death is generally caused by obstructed oxygen uptake by the gills, but it may not be the only factor in lethality. It is suggested by Foscarini (1989) that the problems in gill circulation caused by the fusion and degeneration of the gill lamellae and hyperplasia of the gill epithelium causes heart performance changes. Heart rate changes correlate with the degree of infection and impairment of the gill blood circulation, and the degeneration of the gill lamellae led to edema, blood congestion, and tachycardia. The interaction between the gill blood circulation and the heart performance may be what ultimately causes the death of the fish (Foscarini, 1989).

A specific diagnosis of *F. columnare* as the cause of the disease is to take a scraping or smear from the infected gill tissue or mucus and observe the bacteria under the microscope (Woo and Bruno, 1999, Pillay and Knutty, 2005). These bacteria spread or glide with a different flagellar movement, have no

resting cells, fruiting bodies, spores, or microcysts. Further, all *Flavobacterium* bacteria generally have oxidases, phosphatases, and ribonucleases responsible for the lesions where the bacteria attach and release these protein- and cartilage-degrading enzymes (Woo and Bruno, 1999; Altinok, 2004). Under the microscope, the bacteria appear like haystack columns in yellow-orange colonies, hence the name columnaris. Further tests should look for adherence to agar (Hsu-Shotts), a color change from yellow to pink in 3% sodium hydroxide (NaOH), and the production of chondroitinase (Woo and Bruno, 1999).

Healthy fish are found to not be affected by *F. columnare* and may even serve as carriers of the disease in some populations (Suomalainen, et al, 2005; Woo and Bruno, 1999). Although there are a number of factors that increase the possibility of columnaris infection, stress is well known as the leading cause of outbreak. The stress may be environmental, such as high temperatures, or physical, overcrowding or rough handling (Pillay and Knutty, 2005; Parker, 1995; Woo and Bruno, 1999; Suomalainen, et al, 2002; Bader, Shoemaker, and Klesius, 2003; Altinok, 2004; Eimers and Cardella, 1990). Fish with any type of lesions can be a source of infection to an entire tank, and it is uncertain if carriers of *F. columnare* can be stimulated to become infectious to other fish though it has been shown to be inducible with other *Flavobacterium* species (Pillay and Knutty, 2005; Suomalainen, et al, 2005). Fish that survive a columnaris outbreak may show melanosis, tiny hemorrhagic spots, and many display a rippling of the fins and body, hovering, nipping, and flashing swimming behavior (Soltani et al, 1996). Some of the leading causes of columnaris outbreaks, besides

overcrowding and handling stress, are high water temperatures (optimal temperatures are 22-24°C, for virulent strains, 28°C), poor water quality, and extreme pH (below 6 or above 9), and malnutrition (Wagner, et al, 2002; Suomalainen, et al, 2005; Parker, 1995; Suomalainen, et al, 2005).

Generally, the water quality conditions necessary to maintain healthy fish are as follows: dissolved oxygen of 5 ppm, pH range of 6.7-8.6, total free CO₂ of 3 ppm or less, ammonia of 0.02 ppm or less, and alkalinity of at least 20 ppm (as CaCO₃) (Pillay and Knutty, 2005). In order to reduce the risk of developing bacterial infections, especially columnaris, increasing the salinity slightly (0.5% will inhibit *F. columnare* survival), keeping a pH of 6, soft water (10 ppm CaCO₃), along with a decrease of population density will significantly decrease the likelihood of columnaris outbreaks (Woo and Bruno, 1999). Outbreaks are known to most occur in spring and autumn and the disease is common in warm and summer months which is helpful to use prophylactic treatment to attempt to prevent outbreaks at these times (Grabowski, et al, 2004; Pillay and Knutty, 2005).

Few studies have been dedicated to the mechanisms of *F. columnare* virulence and transmission; however, the capsular outer glycocalyx of mucopolysaccharide is known to be used in the attachment to the host, and an increase in iron may increase the virulence of some strains (Suomalainen, et al, 2005; Woo and Bruno, 1999). It was found there is a correlation between the virulence of *F. columnare* and the ability for the bacteria to adhere to the gill tissue (Thomas-Jinu and Goodwin, 2004). Although it is known that adherins

play a role in columnaris infection, there are still questions as to which adhesins (cohesion factors) are present (Dalsgaard, 1993). Also, even after the bacteria attach to the gill tissues, the bacteria have a thin slim layer that encompasses the cells that attach and this layer allows further movement of the bacteria (Dalsgaard, 1993). Other components such as proteases (e.g. hemolysin), lipopolysaccharides, and extracellular products and proteins (ECPs) are thought to also be important in the virulence and pathogenicity of *F. columnare* (Dalsgaard, 1993).

Mortality is certain if columnaris is left untreated and large fish kills can take a matter of two to three days with virulent strains depending on the conditions (death usually occurs very quickly in overcrowded situations [Altinok, 2004; Woo and Bruno, 1999]). Srisopaporn (2002) found two additional markers of distinguishing between ATCC, Auburn, South Carolina, and Clemson isolates of *F. columnare*: presence of a flexirubin-type pigments and a glycocalyx. Srisopaporn found that a 30 kDa protein was found to be unique to the channel catfish which indicates that it should be studied for use in vaccines against *F. columnare* (2002).

Immune system response to bacterial products

In fish, LPS is known to stimulate various responses in different fish such as macrophage and lymphocyte proliferation (Acosta, et al, 2004). In channel catfish, LPS stimulates expression of cytokines (e.g. IL-1), and in rainbow trout, LPS causes the expression of Tumor Necrosis Factor- α (Steine, et al, 2001).

Earlier studies with Gram-negative bacteria and LPS demonstrated that they induce Type-I Interferon (INF) responses in higher vertebrates. Bacterial DNA is also known to activate innate immune responses from unmethylated CpG motifs, and some synthetic structures containing these unmethylated motifs have induced IFN-like responses and Mx expression in Atlantic salmon (Acosta, et al, 2004). The Mx expression from the injected bacterin could be from either LPS or DNA content, but DNA responses are faster and peak quicker than the LPS response. Both responses were seen to disappear around the same time, and it was concluded that the LPS of *Listonella anguillarum* has different properties than previously realized and contains concentrated CpG motifs (Acosta, et al, 2004). In humans, LPS is a T-independent antigen, but it is unknown for fish. In some fish species, the presence of surface Ig-negative lymphocytes and T-like lymphocytes has been identified. For salmon, T-cell receptor genes have also been found and it has been shown that vaccination with LPS induces low protection (Steine, et al, 2001).

Toll-like receptors (TLRs) in mammals recognize lipopeptides (TLR2), dsRNA (TLR3), lipopolysaccharides (TLR4), flagellin (TLR5), or nucleic acid and heme motifs (TLR7-9). Although many TLRs have been identified, there are still many without known specific ligands. TLR2 is best known for recognizing conserved components of Gram-positive bacteria and known to interact with zymosan (from yeast), glycosylphosphatidyinositols (from protozoan parasites), and LPS (from some Gram-negative bacteria and zoonotic pathogens). TLR4 is traditionally responsible for binding Gram-negative bacteria; however, recent

studies show that it may also function with TLR4, complex with TLR1 or 6, or work independently.

Recent studies with Baopraserkul, et al (2006) indicated that TLR2's function in *Edwardsiella ictaluri* may be broader than originally considered. In catfish, studies with *E. ictaluri* showed classic TLR3 expression with dsRNA (2006) although viral DNA is the mammalian ligand for TLR3. In mammals, toll-like receptors (TLRs) have been found to be involved in innate and adaptive immunity, and to date, there have been 14 TLRs identified in teleosts (*I. punctatus*, *Danio rerio*, *Fugu rebripes*, *Carrassius auratus*, *Paralichthys olivaceus*, and *Oncorhynchus mykiss*). Although many fish TLRs have high homology to the mammalian TLRs, there is still little data available on the functional role in fish. TLR3 and TLR5 have both been identified in channel catfish, and TLR5 expression was higher than TLR3 (Bilodeau, et al, 2006). It is known that TLR3 binds dsRNA and TLR5 binds flagellen, as found in human and mouse models; however, in catfish, the ligands for these TLRs have not been found although they are known to be involved in innate immunity. TLR5 is highly expressed in the liver and kidney in catfish, while TLR3 is expressed more in the stomach in catfish. It was also found that in the presence of bacteria, TLR3 was more highly expressed than TLR5. This led Bilodeau, et al (2006) to believe that bacterial uptake occurs in the stomach and TLR3 has a widespread role in innate immunity more so than previously thought (2006).

INTRODUCTION

The Channel Catfish, *Ictalurus punctatus*, is one of the most intensely farmed fish in the USA (Dudley-Cash, 1998). Losses to channel catfish aquaculture are primarily due to disease resulting from viral, parasitic, and bacterial infections (Parker, 1995). Prophylactic treatments with antibiotics and chemicals can be expensive and ineffective; therefore, aquaculture professionals are working toward better preventative measures such as vaccines.

Development of vaccines can only begin when the full etiology of a disease is understood. Bacterial infections are generally nonspecific infections that can affect a wide range of fish species, and these infections are the leading cause of losses in channel catfish production. The two most prominent bacteria that cause widespread losses, both in production and profit, are *Flavobacterium columnare* and *Edwardsiella ictaluri*. These bacteria are the causative agents of columnaris disease and enteric septicemia of catfish, respectively (Stickney, 2005). It is estimated that these two diseases alone are responsible for \$2.5 million in catfish losses and approximately \$100 million in general aquaculture (Iwama, et al, 1997).

Flavobacterium columnare is a Gram-negative rod bacteria that can grow under varying conditions depending on the strain, with a range of incubation temperatures between 20°C and 28°C (Woo and Bruno, 1999). *F. columnare* causes a disease known in the aquaculture industry as columnaris, named for the columnar, haystack appearance of the bacteria under the microscope, and it

has been found in at least 36 freshwater species around the world (Pillay and Knutty, 2005). The losses from columnaris are second only to enteric septicemia of catfish (ESC) caused by *E. ictaluri*. Many strains of *F. columnare* are virulent enough to infect and kill without any external signs of infection (Bader et al, 2003; Grabowski, et al, 2004).

Little is known about the pro-inflammatory potential of *F. columnare*. In contrast, a fairly extensive literature base exists on the pro-inflammatory effects of *E. ictaluri* (Iwama, et al, 1997; Paul, 2003). *E. ictaluri* is a Gram-negative bacteria with flagellated rods that is responsible for enteric septicemia of catfish (ESC) and other intestinal diseases in fish (Woo and Bruno, 1999). In channel catfish phagocytes, nitric oxide synthase activity was detected after a single injection with *E. ictaluri*. The cells involved in the production of iNOS activity were not identified in this experiment; however, phagocytes were most likely the source (Iwama, et al, 1997). *E. ictaluri* is internalized in macrophages and is known to spread through the phagocytes. It is believed to be an intracellular pathogen which increases phagocytosis of the host (Shoemaker, 1997). The predominant leukocytes found in lesions caused by *E. ictaluri* infection are macrophages. Macrophages and neutrophils were found to decrease superoxide anion production after infection of *E. ictaluri* (Askoy, et al, 2004).

The effects of *F. columnare* on fish phagocytes are unknown. Therefore, the primary objective of this study was to examine the pro-inflammatory properties of *F. columnare* and compare these properties to *E. ictaluri*. This was done by observing the effects of the outer membrane proteins (OMPs), secreted

extracellular products (ECPs), live bacteria, heat-killed bacteria, and formalin-killed bacteria on catfish neutrophils *in vitro*, and measuring the reactive oxygen intermediate production, the production of nitric oxide, and phagocytosis in lymphoid phagocytes after 24 hours of exposure. An ancillary objective of this study was to quantify the expression of key pro-inflammatory proteins in phagocytes exposed to ECPs and OMPS from *F. columnare* and *E. ictaluri*.

Ultimately, this study should provide some understanding of how ESC and columnaris differ in terms of their disease etiology and host phagocytic responses to the two bacteria. Moreover, this information will be useful in formulating a vaccine or adjuvant for columnaris that plagues the aquaculture industry, especially against the more virulent strains of *F. columnare* that do not present external symptoms of disease (Bader, et al, 2003). The results from this study will be helpful in indicating whether or not there is an inflammatory response to *F. columnare* in Channel Catfish.

Materials and Methods

Media and Growth Conditions

The Clemson strain of *F. columnare* was grown in Hsu-Shotts medium, while *E. ictaluri* was grown in Heart-Brain Infusion medium. Both were incubated for 48 hours at 26°C under constant shaking conditions. Once the cultures were confluent, bacteria in media were centrifuged to collect pellets. Cell pellets were either used directly to obtain outer membrane proteins (OMP), or they were resuspended in media containing 20% glycerol and stored at -80°C for further use. The supernatant containing the extracellular products (ECP) was stored at -20°C until further use.

Outer Membrane Preparation

Outer membrane proteins were isolated as described previously by Srisopaporn (2002). Bacterial cell pellets were resuspended in 10 ml of 10 mM HEPES buffer (pH 7.4) supplemented with the following three protease inhibitors – pepstatin (12.5 µg/ml), aprotinin (80 µl/ml), and PMSF (Phenyl methyl sulfonyl fluoride 10 µM). Samples were then sonicated for 15 seconds and allowed to rest for 15 seconds. This process was repeated 3 more times. Broken cells were collected by centrifugation for 20 minutes at 1750 x gravity (g) and centrifuged again at 100,000 x g for 1 hour at 4°C. Cell pellets were then treated with equal volumes of 2% (w/v) lauryl sulfonate (Sodium Dodecyl Sulfate, SDS) and 10 mM HEPES buffer (pH 7.4) before incubating for 30 minutes at room

temperature, followed by another 100,000 x g centrifugation for 1 hour at 4°C.

The pellet of insoluble outer membrane proteins was collected and resuspended in deionized water and stored at -20°C.

Extracellular Protein Preparation

Dialysis tubing (10 kD MW cut-off) was filled with the supernatants from centrifuged *F. columnare* and *E. ictaluri* cultures. Dialysis tubes containing samples were then laid on paper towels and covered with Polyethylene glycol (PEG) 8000, molecular grade (Sigma chemicals). As the PEG dampened, it was removed and replaced with dry PEG. This process was continued for approximately two hours until the bags were nearly flat. The contents were then dialyzed against PBS, filter sterilized, transferred to a 15 ml tube, and the protein concentration determined.

Live, heat-killed, and formalin-killed bacteria

Twenty-four hour cultures of bacteria were centrifuged at 1300 x g for 10 minutes and washed twice with catfish Hanks balance salt solution (HBSS). The pellets were then resuspended in 10 ml of HBSS and the OD recorded. Two ml of bacteria suspension were added to 2 mls of 3% formalin and placed in ice for 1 hour. Two ml of bacteria suspension were also placed in a 70°C water bath for 1 hour, and two ml of live bacteria suspension were placed in ice for an hour. Heat-killed (HK) and formalin-killed (FK) were then centrifuged at 1500 x g for 10 min, washed twice with HBSS, and resuspended in HBSS to O.D. reading of

0.155 at 600 nm. One hundred-fold dilutions of each bacterial prep were made in AL-5 media, from which 20 μ l were used to treat catfish phagocytes (macrophages and neutrophils) *in vitro*. Twenty μ l of 0.155 OD stock of bacteria preparation was assigned a value of 1 Unit (U); therefore, bacterial dilutions were as follows: 1 U, 0.01 U, and 0.0001 U.

Preparation of TRITC-labeled Yeast Cells

Flieschmann's Active Dry Yeast was resuspended in Phosphate Buffered Saline (PBS) at 0.1 g/ml and boiled for 30 minutes. The yeast were then washed by centrifugation and resuspended in 50 mM Na₂HPO₄, pH 9.3, at 10⁹ yeast particles per ml. TRITC (Molecular Probes, Eugene, OR USA) was added at 0.1 mg/ml in anhydrous DMSO (Sigma) and incubated for 30 minutes with rocking at 37°C. The yeast-TRITC mixture was washed 3 times for 5 minutes by centrifugation at 110 x g and resuspended at a concentration of 8 x 10⁸ particles per ml in PBS. On the day of the assay the prepared TRITC-labeled yeast cells were centrifuged at 110 x g for 20 minutes and resuspended in 5 ml of HBSS, then counted and diluted for *in vitro* experiments (Peracino, et al, 1998; Spring and Rice, 2006).

Phagocyte Collection and Preparation

Three channel catfish (600-800 grams) per experiment were anesthetized using buffered tricane (MS-222) and bled from the caudal sinus into vacutainers. Anterior kidneys and spleens were removed from each fish, homogenized in 15

ml of HBSS, and placed on ice to allow debris to settle out by gravity. Cells were transferred to a sterile 15 ml tube and washed twice with cold HBSS by centrifugation. Cells were then layered over percoll gradients of 1.055 and 1.080 g/ml and centrifuged for 25 min at 350 x g (Braun-Nesje, et al, 1981; Buchanan, et. al, 2005). Cells residing at the percoll interface, primarily neutrophils (Waterstrat, et al, 1991) were removed and washed twice in cold HBSS, then resuspended in culture medium consisting of 50% L-15 media and 50 % AIM-5 medium (both from Gibco) supplemented with sodium bicarbonate, HEPES, sodium pyruvate, L-glutamine, and Pen-Strep (Hereafter referred to as AL-5 medium; Miller, et al, 2001). Cells were then counted using a hemocytometer. Viability was determined using trypan blue exclusion staining. Blood samples were allowed to clot at room temperature for 1 hr, then centrifuged to collect serum, which was pooled and filter sterilized through a 0.45 μm filter and then with a 0.22 μm filter, then placed on ice to be used for cell culture.

Stimulation of catfish phagocytes

E. ictaluri and *F. columare* ECPs, OMPs, live bacteria, heat-killed bacteria, and formalin-killed bacteria were used in 24 hr *in vitro* assays to determine their effects on Phorbol myristate acetate (PMA)-stimulated hydrogen peroxide production, to induce nitric oxide synthase (iNOS), and ability of neutrophils to phagocytose yeast particles. One hundred μl of catfish cells (5×10^5) in AL-5 media containing 5% catfish serum were added to each well of six 96-well, black-walled microtiter plates per experiment. Plates were pre-labeled for *F.*

columnare and *E. ictaluri* products, and for the endpoints; hydrogen peroxide production, nitric oxide production, and phagocytosis of yeast cells. Eighty μ l of Catfish AL-5 were then added to all wells, followed by the addition of 20 μ l of each treatment, including 20 μ l of catfish AL-5 medium in the control wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Control						0.01 U live					
B	ECP: 10 μ g/ml						0.0001 U live					
C	ECP: 1 μ g/ml						1 U HK					
D	ECP: 0.01 μ g/ml						0.01 U HK					
E	OMP: 10 μ g/ml						0.0001 U HK					
F	OMP: 1 μ g/ml						1 U FK					
G	OMP: 0.01 μ g/ml						0.01 U FK					
H	1 U live						0.0001 U FK					

Table 2. The experimental set-up used for each plate. Each well contained 100 μ l of catfish phagocytes with 5% pooled serum, 80 μ l of Catfish AL-5 medium, and 20 μ l of the specific treatment. Row A received 20 μ l of AL-5 instead of treatment for the control.

The final concentration of ECPs and OMPs were 10, 1, and 0.1 μ g/ml. Based on a common O.D. reading, followed by 100-fold dilutions of live, heat-killed, and formalin-killed bacterial, cells were treated with 1, 0.01, 0.0001 Units of bacteria. Specific assays were initiated as follows:

PMA-Induced H₂O₂ Production: Twenty μ l of a 10⁻⁴ M solution of 2,7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) in HBSS were loaded into each

well and left for 30 minutes. After 30 minutes, 20 μl of media were removed from plate columns 4-6 and 10-11, and replaced with 20 μl of a 10^{-5} M solution PMA in HBSS. Thirty min later the fluorescence of each well was measured at an excitation/emission ratio of 485/528 λ using a fluorescence plate reader.

H₂DCFDA is a useful probe for hydrogen peroxide production since it is non-fluorescent until the acetate groups are removed by intracellular esterases and oxidized in the presence of reactive oxygen intermediates to the fluorescent form. The data were recorded as stimulation indices determined as a ratio of PMA-stimulated to unstimulated (control) cells. The data for the 6 replicated wells per treatment were averaged.

Nitric Oxide Production: Twenty μl of medium were removed from each well and replaced with 20 μl of a 10^{-4} M solution of 4,5-diaminofluorescein (DAF-2DA) (Alexis Biochemicals) in HBSS. Thirty min later the fluorescence of each well was measured at an excitation/emission ratio of 485/528 λ using a fluorescence plate reader. DAF-2DA is a nonfluorescent molecule which is cleaved by esterases, and thus remains within the cell. Nitric oxide oxidizes the DAF-2 molecule and the molecule becomes the fluorescent DAF-2T. The data were recorded as stimulation indices determined as a ratio of treated (products or bacteria) wells to un-treated wells (AL-5 medium only). The data for the 6 replicated wells per treatment were averaged.

Phagocytosis Assay: Twenty μl of medium were removed from each well and replaced with 20 μl of TRITC-labeled yeast (6:1 ratio yeast:cells). The plates were incubated for 90 min at which time 100 μl of overlying medium were

removed from each well and replaced with 100 μ l of a 0.4 % solution of trypan blue in HBSS to quench the fluorescence of unphagocytosed yeast. The fluorescence of each well was then measured at an excitation/emission ratio of 485/528 λ using a fluorescence plate reader. The data were recorded as stimulation indices determined as a ratio of treated wells to un-treated wells. The data for the 6 replicated wells per treatment were averaged.

Protein Expression In Neutrophils Following Treatment With ECP and OMP

Phagocytes were isolated from the anterior kidneys and spleens of 3 adult channel catfish as described above. Cells were pooled, washed and counted, then used to determine the expression profiles of select proteins indicative of pro-inflammatory processes. Twelve T-75 flasks were seeded with 10^8 cells in AL-5 medium containing 2.5% catfish serum and 10 μ g/ml of *E. ictaluri* ECP or OMP, and 10 μ g/ml *F. columnare* ECP or OMP, medium only as a treatment control. Flasks were treated for 24 hr at which time the cells were scraped from their respective flask, washed twice by centrifugation in HBSS, and frozen as dry pellets at -20°C . Cells were then re-suspended in 500 μ l lysis buffer (20 mM Tris HCl pH 8.0, 150 mM NaCl, 1 mM MgCl_2 , 1mM KCl, 50 mM EDTA, 1% IPEGAL, and a 1X solution of protease inhibitor cocktail). The solution was vortexed lightly and placed on ice for 20 min, transferred to a microcentrifuge tube and centrifuged at 1300 x g for 20 min at 4°C . The supernatants containing cell lysates were transferred to another tube and the protein content determined.

Lysates were then diluted to a common protein concentration, diluted in 4X reducing sample buffer, and boiled for 5 min.

Ten μg of cell lysate protein were diluted then applied to 4-20 % acrylamide gels and subjected to SDS-PAGE. Following electrophoresis, the proteins were transferred overnight onto 0.45 μM Immulon membranes and blocked for 1 hr with 3 % BSA in PBS. The membrane was then probed for 1 hr with either M24-2 (specific for fish lysozyme), or mAb C10-7 (specific for fish CYP1A). Following three 10 min washes with PBS containing tween-20 (PBS-TW-20), the membranes were incubated with alkaline-phosphatase-conjugated goat anti-mouse IgG. After another three 10 min washes with PBS-TW-20, alkaline phosphatase activity was visualized with BCIP/NBT in AP buffer, and the results were scanned and recorded.

Results

In three independent experiments, pools of channel catfish lymphoid phagocytes were stimulated for 24 hr with ECPs, OMPs, and whole cells as formalin-killed, heat killed, or live cultures of *Edwardsiella ictaluri* and *Flavobacterium columnare*. Twenty-four hours later, phagocytes were then examined for their ability to 1. produce hydrogen peroxide radicals following stimulation with Phorbol ester, 2. produce nitric oxide radicals, and 3. to phagocytose yeast particles.

The effect of ECPs from *E. ictaluri* and *F. columnare* on PMA-stimulated hydrogen peroxide production was highly variable between experiments, resulting in high variability and no significant difference between treatment groups when examined collectively (Figure 1).

Extracellular proteins of *E. ictaluri* induced nitric oxide synthase activity in neutrophils, as indicated by activation of the fluorescence nitric oxide probe DAF-2DA, whereas *F. columnare* ECPs failed to significantly induce the production of nitric oxide radicals (Figure 2). A positive effect of *E. ictaluri* ECPs was observed but only in the highest dose (10 ug/ml), which resulted in nearly a 3-fold increase over untreated cells in all three experiments. Phagocytosis of TRITC-labeled yeast was increased in cells treated with *E. ictaluri*-derived ECPs, but only at the highest concentration (Figure 3).

There were no effects on PMA-stimulated hydrogen peroxide production and nitric oxide production due to OMPs from either bacteria (Figure 4; Figure 5).

However phagocytosis was increased in cells treated with OMPs isolated from *E. ictaluri* (Figure 6), but only in cells treated with the highest dose.

PMA-induced hydrogen peroxide production following treatment with live *F. columnare* and *E. ictaluri* was highly variable between experiments; therefore, no differences due to treatment could be detected (Figure 7). Likewise, there were no differences in nitric oxide production due to treatment with live bacteria (Figure 8). Phagocytosis of yeast was unaffected by treatment with live bacteria (Figure 9).

Heat-killed bacteria were examined for their effects of PMA-induced hydrogen peroxide production, and the results were highly variable, resulting in no detectable significance when the treatment was examined over the 3 independent experiments (Figure 10). Likewise, nitric oxide production in phagocytes was unaffected by treatment with heat-killed bacteria (Figure 11). However, heat-killed *E. ictaluri* increased phagocytosis of yeast in the two lowest levels of treatment (Figure 12).

The effects of formalin-killed bacteria on PMA-stimulated hydrogen peroxide production in phagocytes were highly variable, with no overall difference in treatment effects (Figure 13). Likewise, there were no differences in nitric oxide production in phagocytes following treatment (Figure 14). However, as observed in cells treated with heat-killed *E. ictaluri*, phagocytosis of yeast was enhanced in formalin-killed *E. ictaluri* at all three levels of treatment (Figure 15).

Lysozyme expression was decreased by ECP treatment with *E. ictaluri*, and CYP1A was decreased after treatment with 10 µg/ml of both OMPs and ECPs of each bacteria over a 24 hour period (Figure 16 and 17).

Figure 1

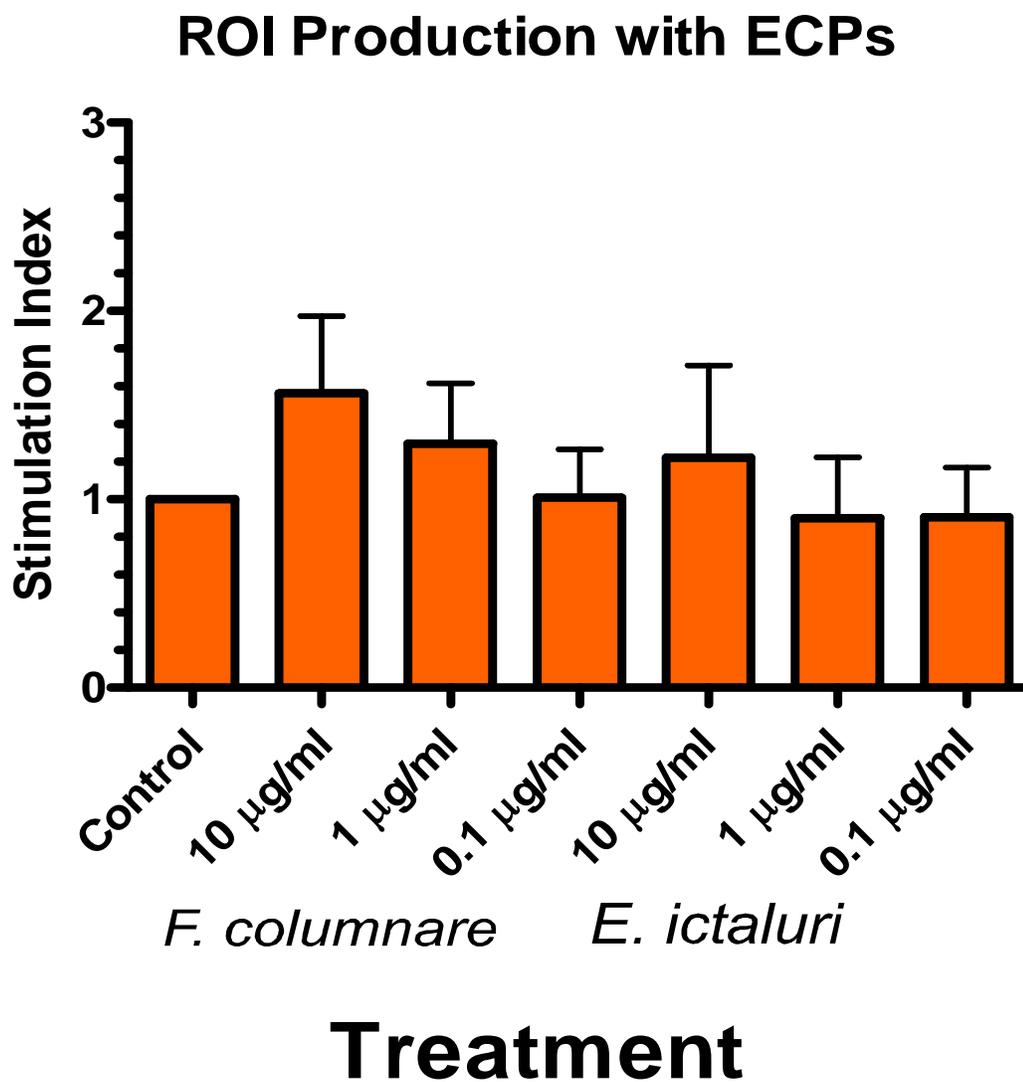


Figure 1. PMA-stimulated Reactive oxygen intermediate production in channel catfish neutrophils after 24 hour stimulation with ECPs.

Figure 2

Effect of ECPs on Nitric Oxide Production

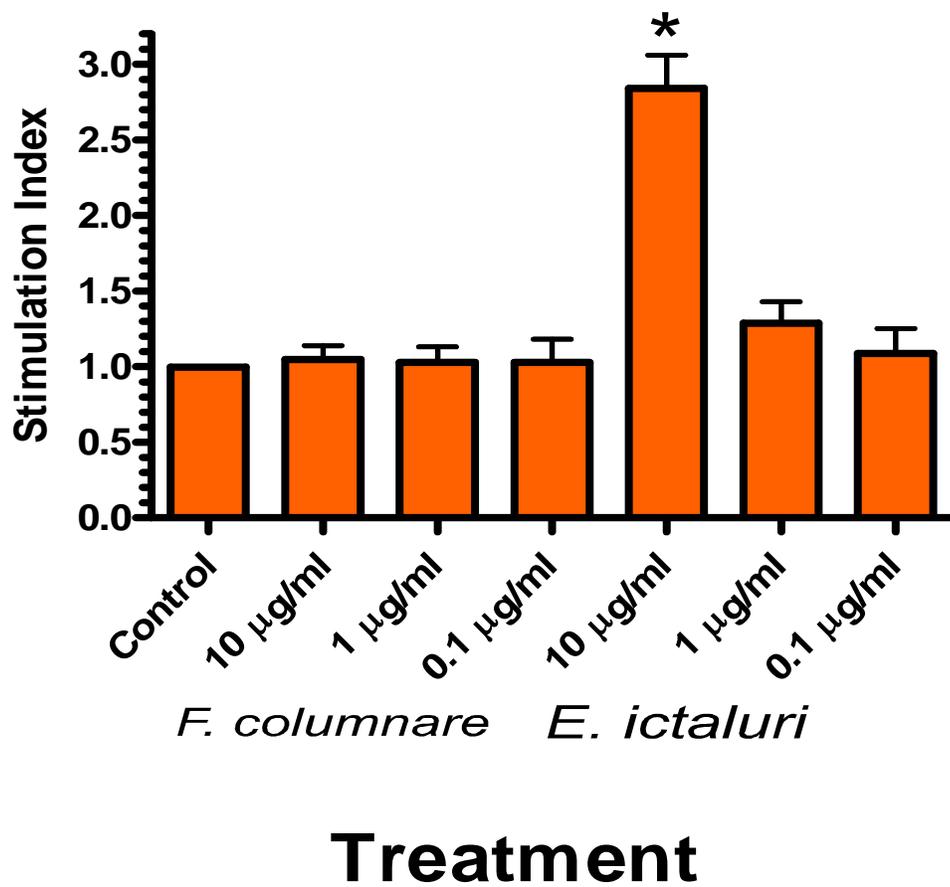


Figure 2. Nitric oxide production in channel catfish neutrophils after 24 hour stimulation with ECPs.

Figure 3

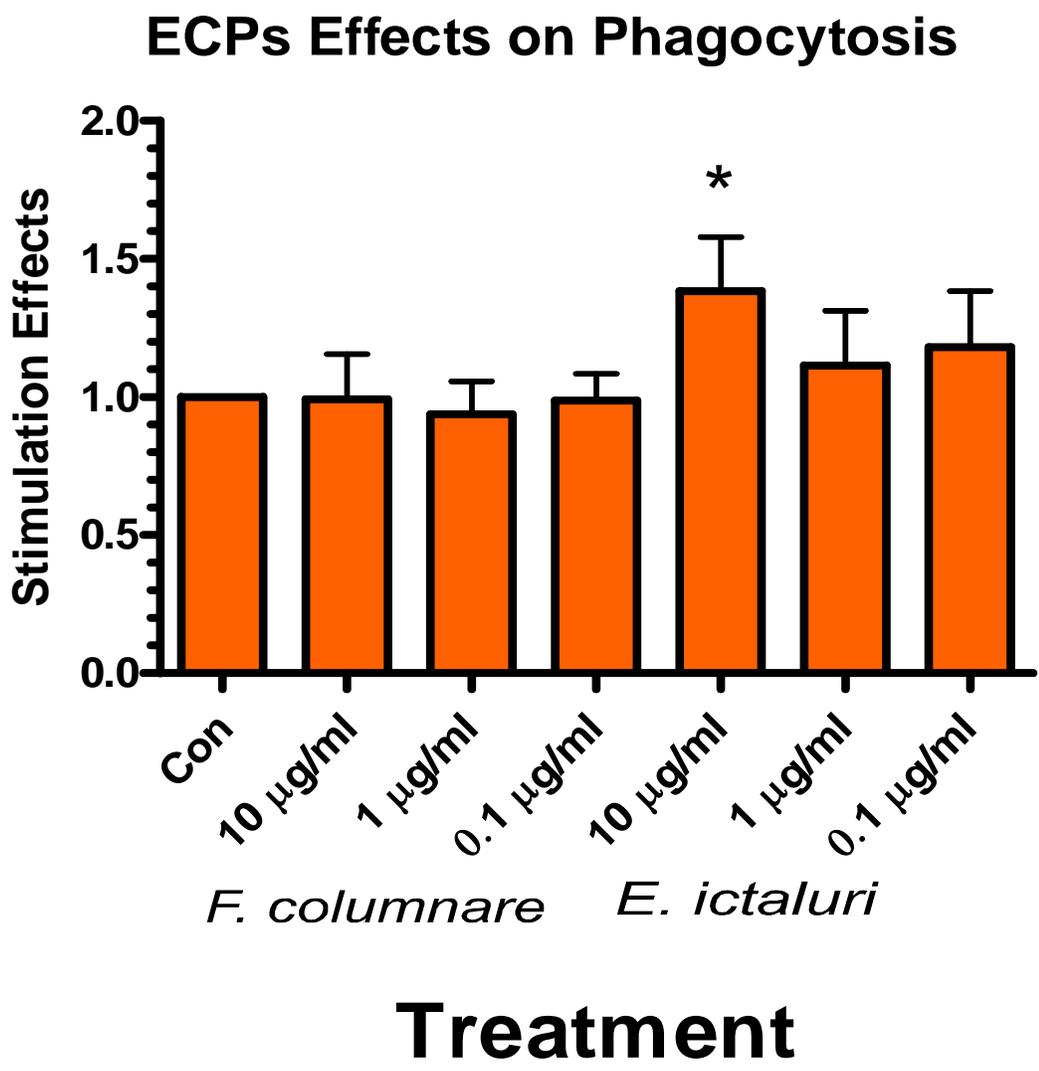


Figure 3. Phagocytosis of yeast in channel catfish neutrophils after a 24 hour stimulation with ECPs.

Figure 4

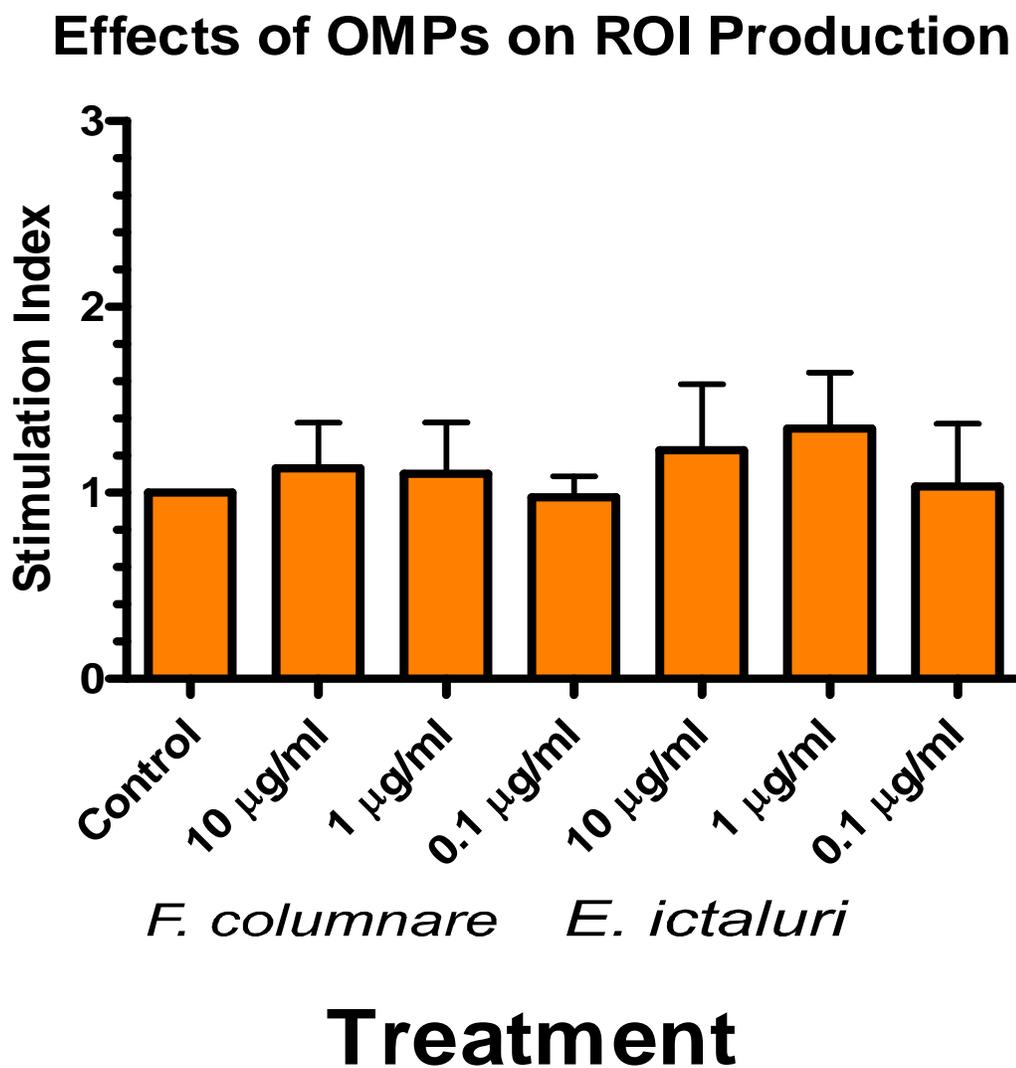


Figure 4. Effects of OMPs on reactive oxygen production in channel catfish neutrophils after 24 hour stimulation.

Figure 5

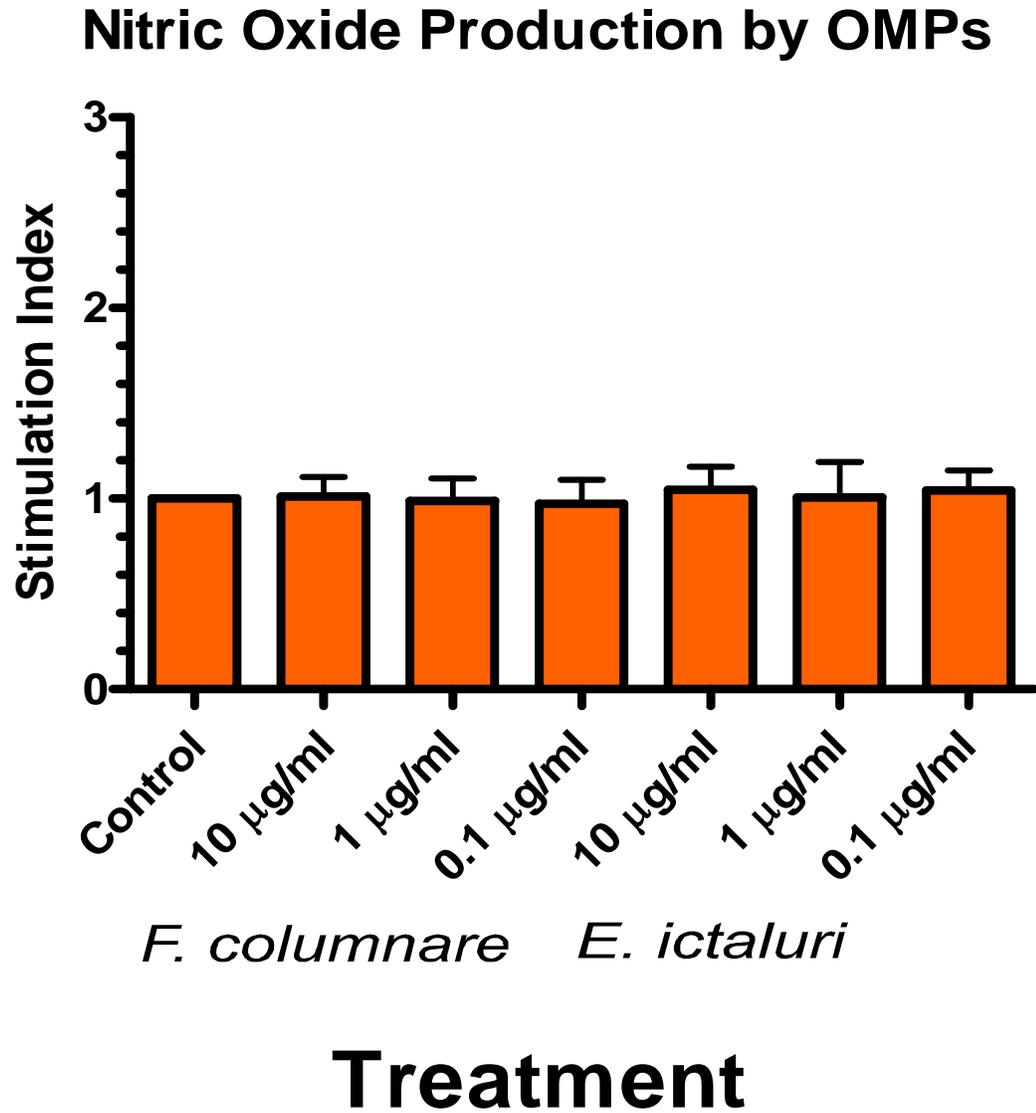


Figure 5. Effect of OMPs on nitric oxide production in channel catfish neutrophils after 24 hour stimulation.

Figure 6

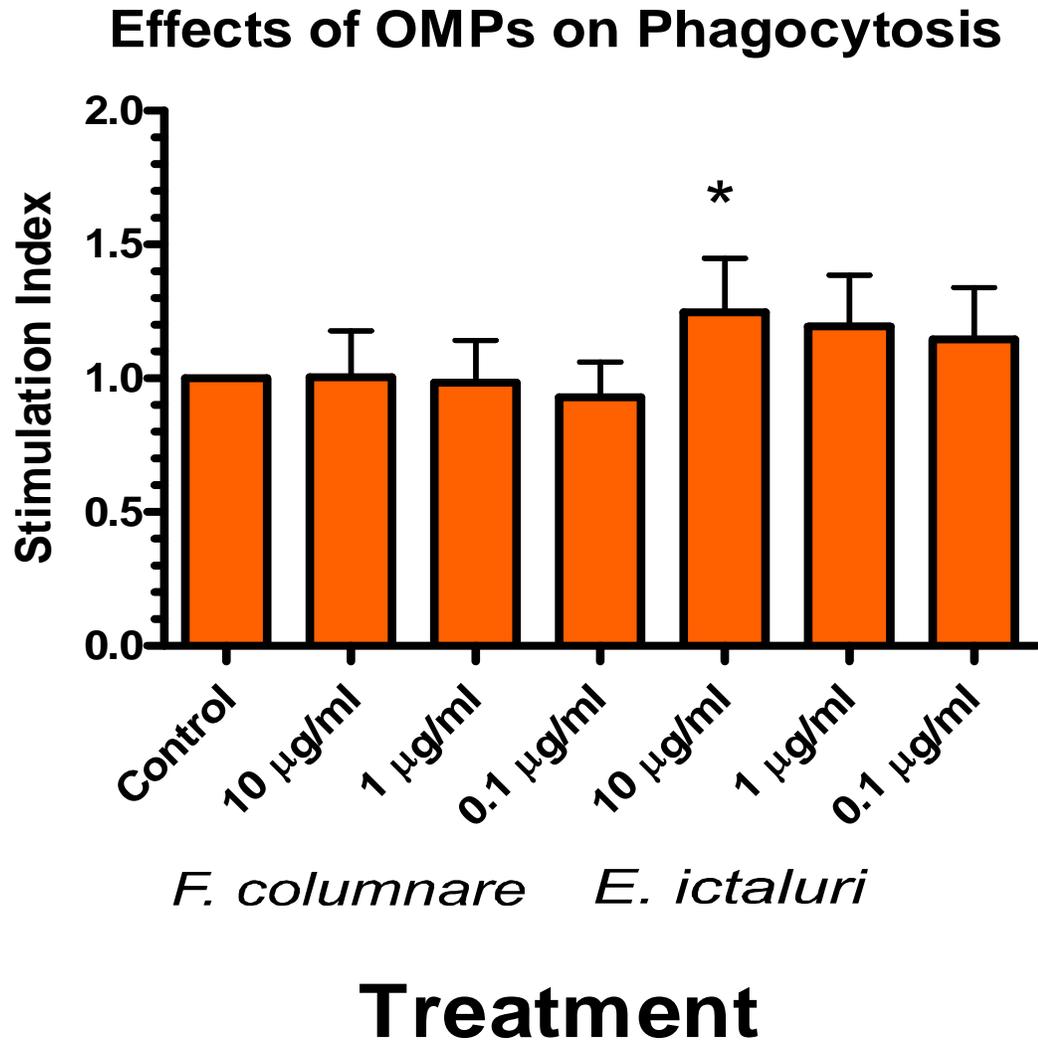


Figure 6. Effects of OMPs on phagocytosis in channel catfish neutrophils after 24 hour stimulation.

Figure 7

Effects of Live Bacteria on ROI Production

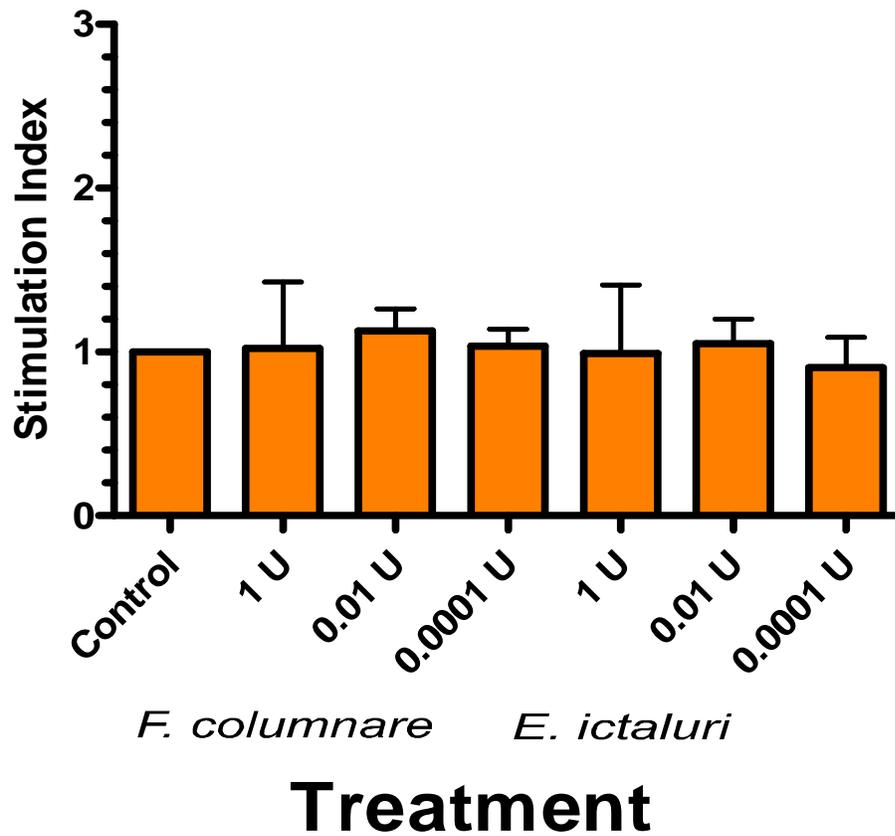


Figure 7. Effect of live bacteria on reactive oxygen intermediate production in channel catfish neutrophils after 24 hour stimulation.

Figure 8

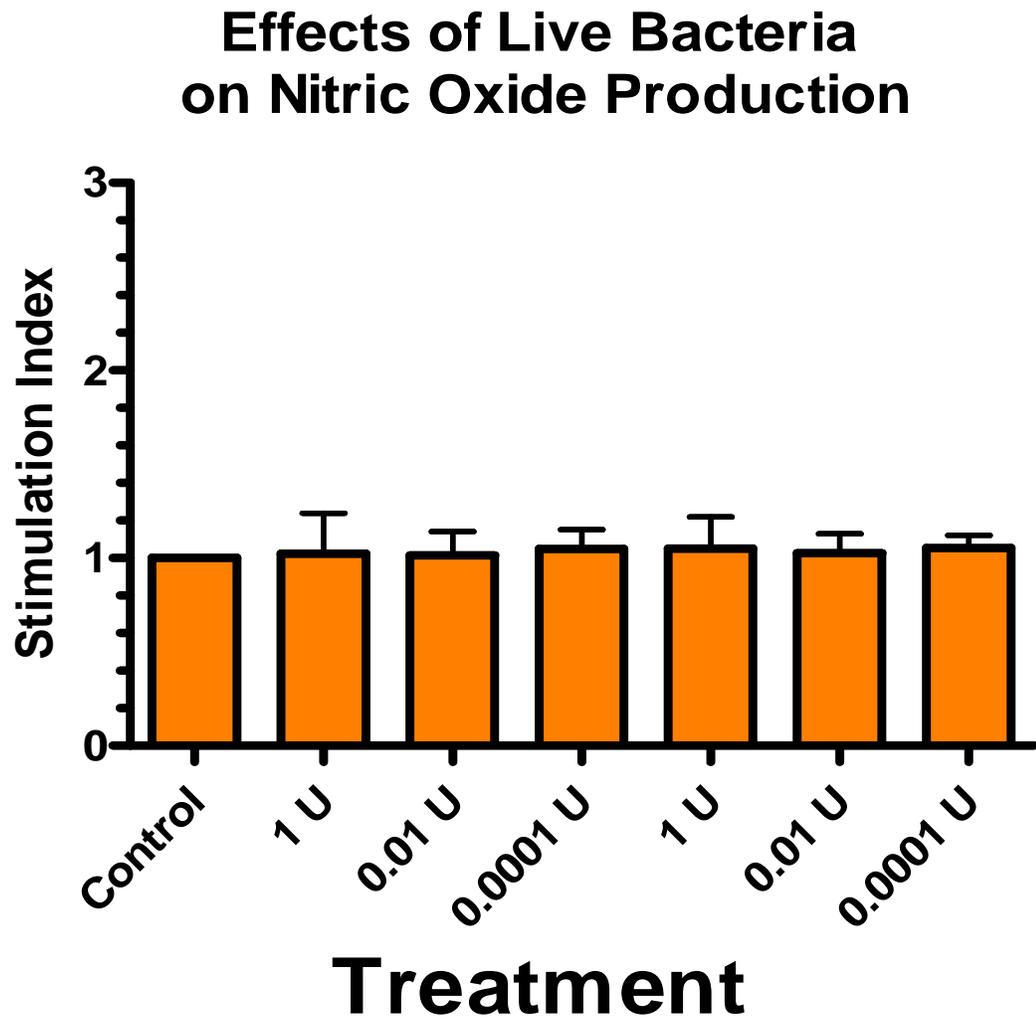


Figure 8. Effects of live bacteria on nitric oxide production in channel catfish neutrophils after 24 hour stimulation.

Figure 9

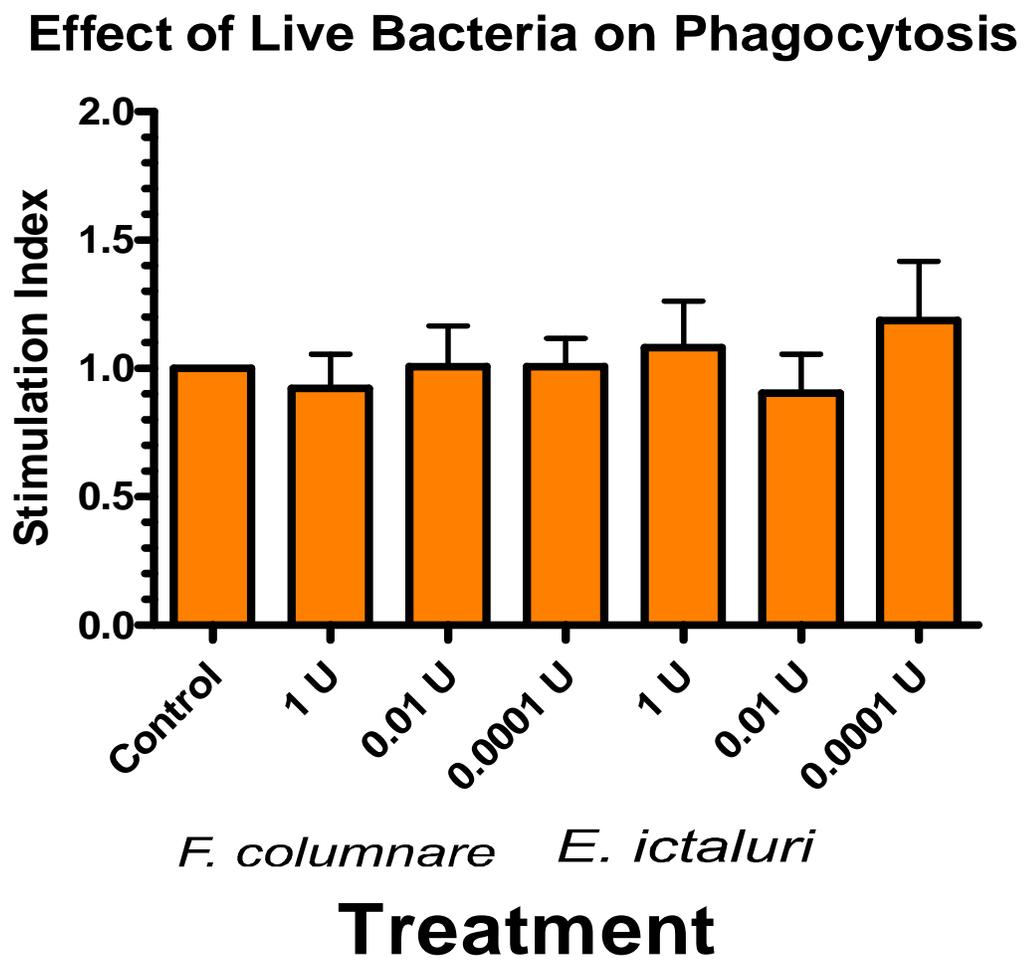


Figure 9. Effect of live bacteria on phagocytosis in catfish phagocytes after 24 hour stimulation.

Figure 10

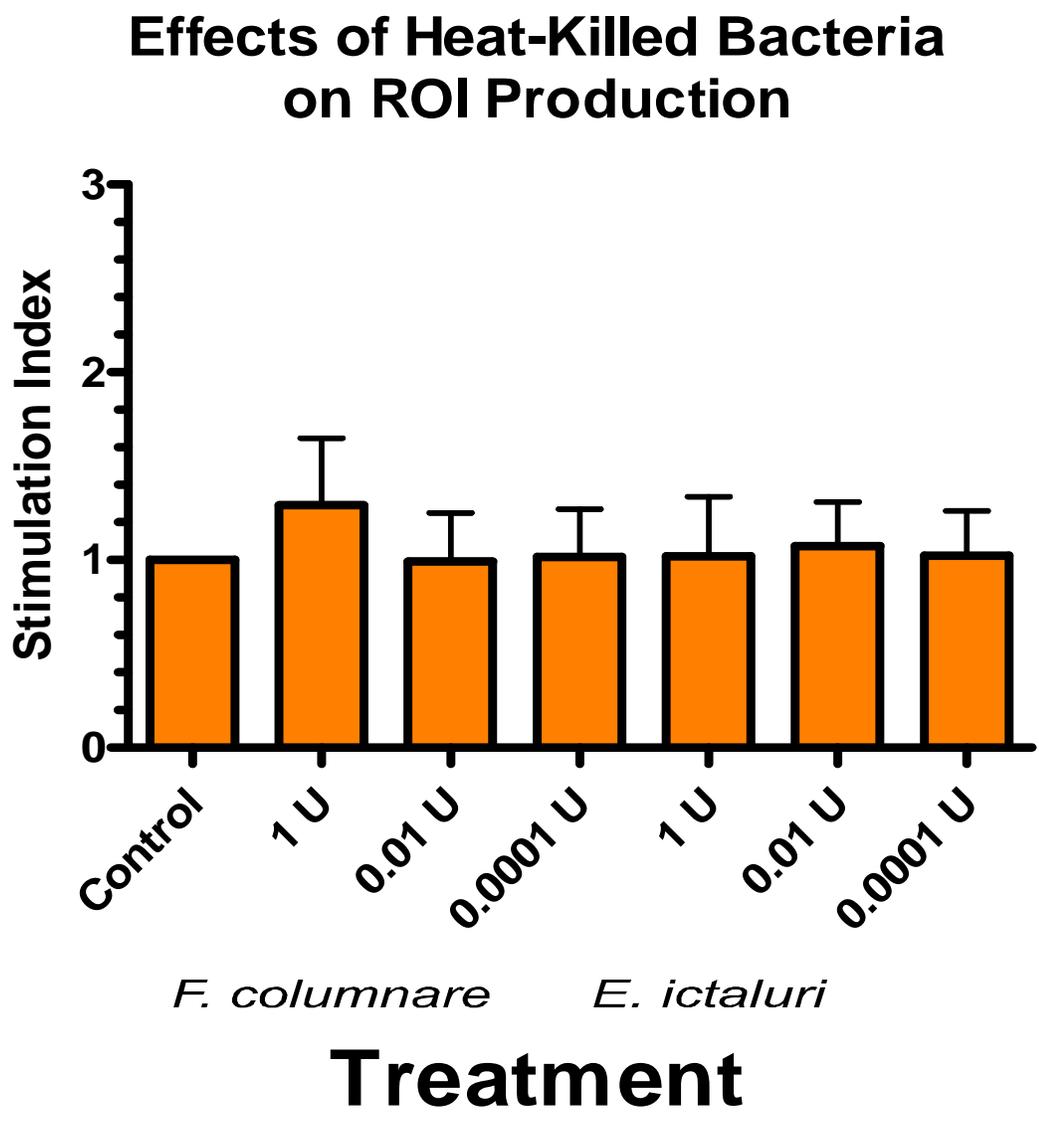


Figure 10. Effect of heat-killed bacteria on reactive oxygen production in channel catfish phagocytes after 24 hour stimulation.

Figure 11

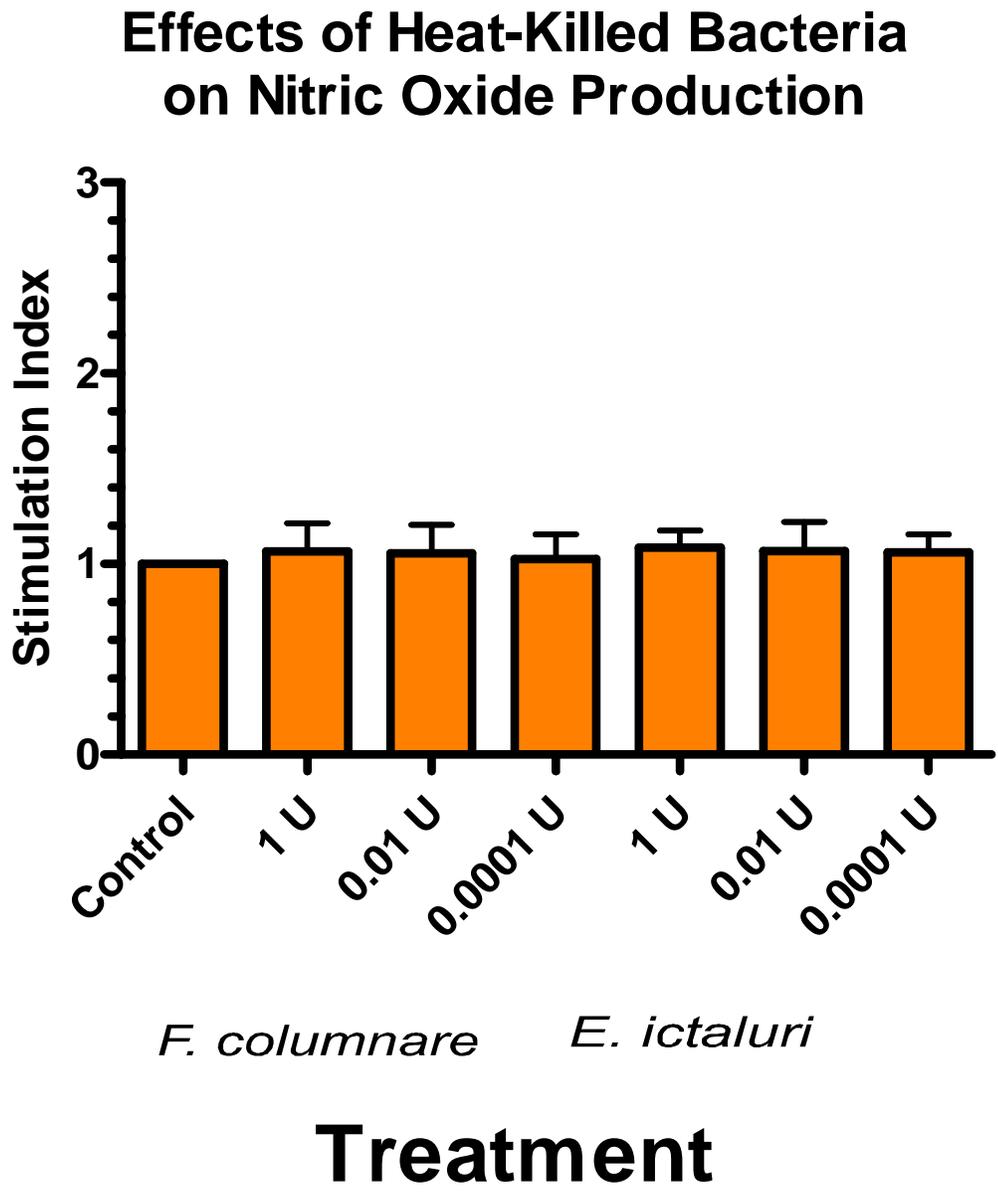


Figure 11. Effect of heat-killed bacteria on nitric oxide production in channel catfish neutrophils after 24 hour stimulation.

Figure 12

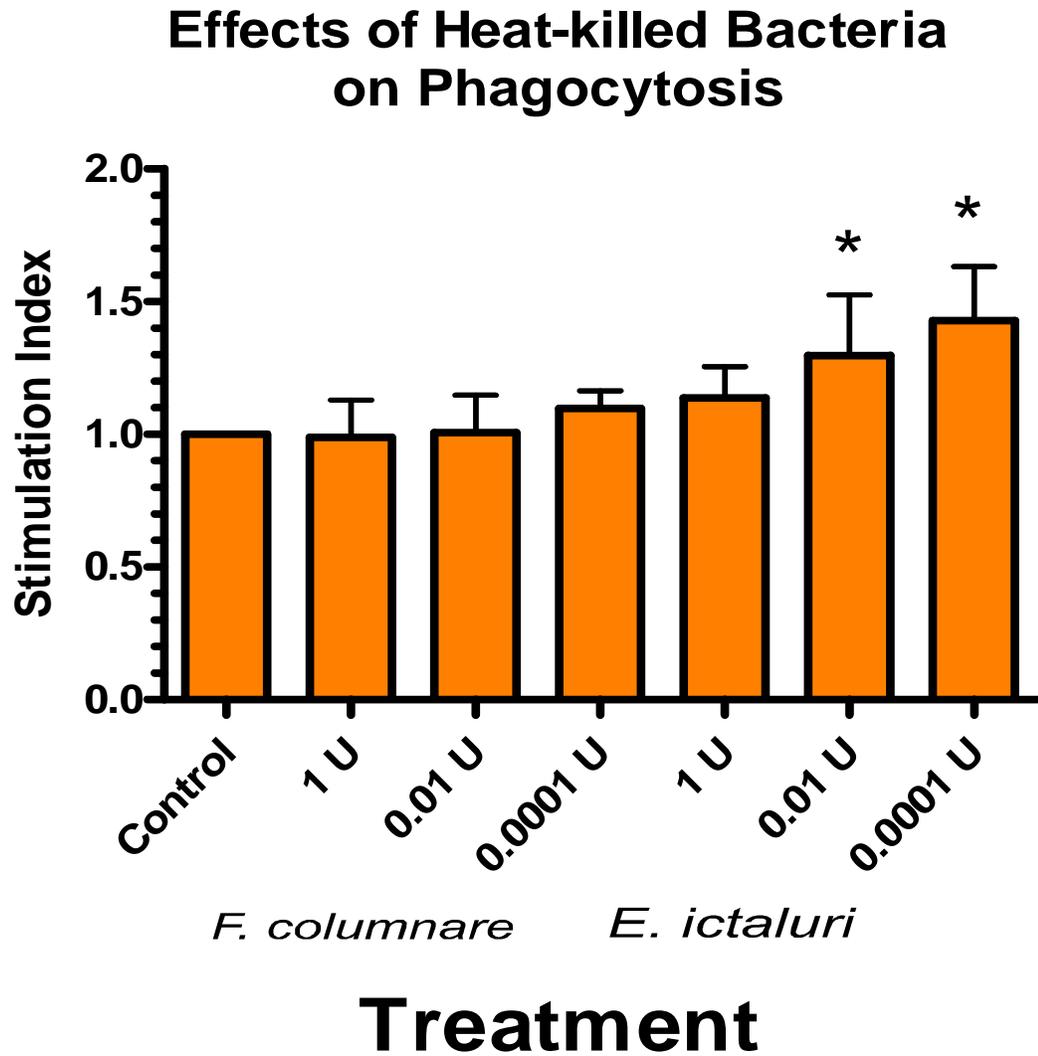


Figure 12. Effect of heat-killed bacteria on phagocytosis in channel catfish neutrophils after 24 hour stimulation.

Figure 13

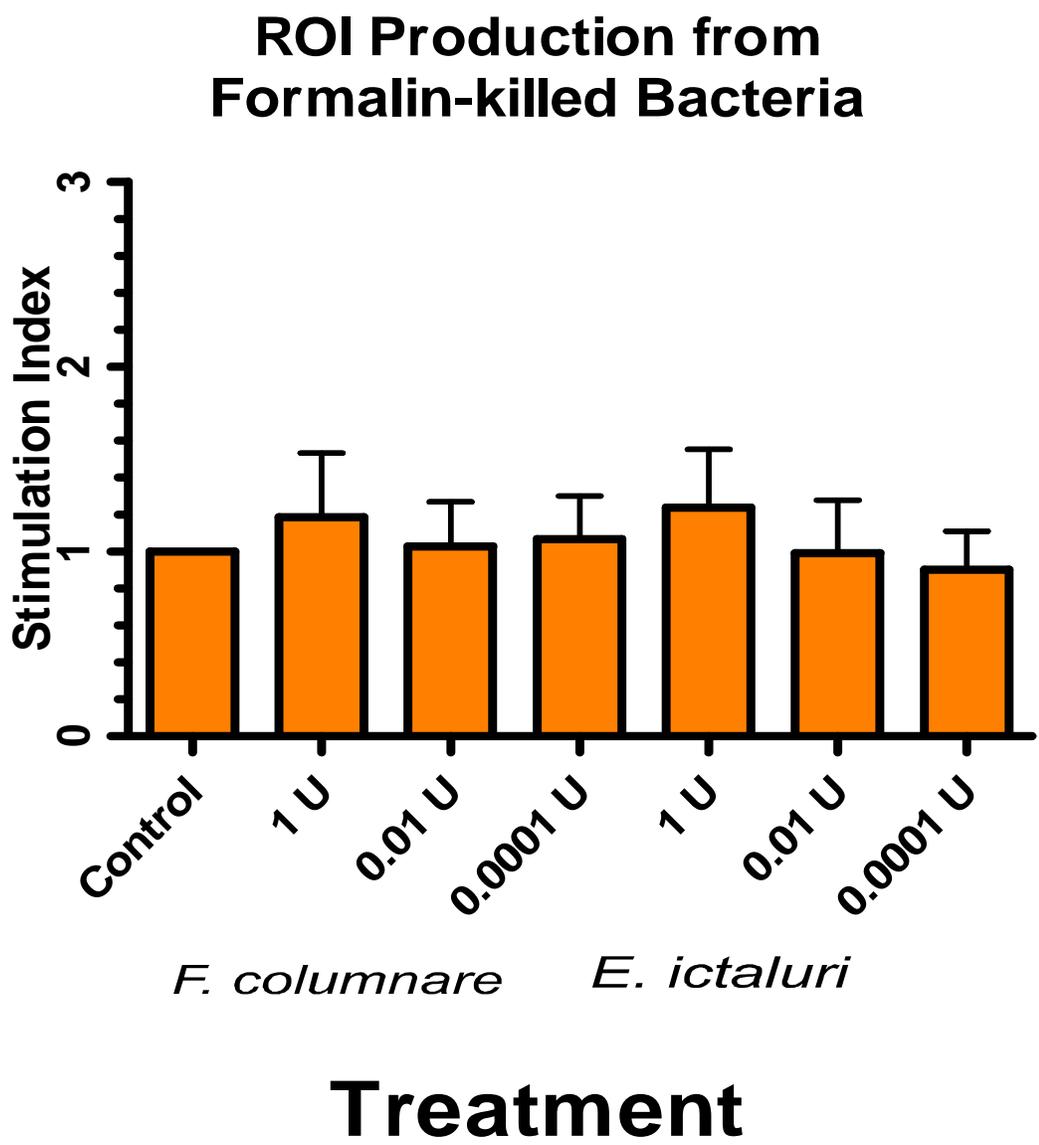


Figure 13. Effect of formalin-killed bacteria on reactive oxygen intermediate production in channel catfish neutrophils after 24 hour stimulation.

Figure 14

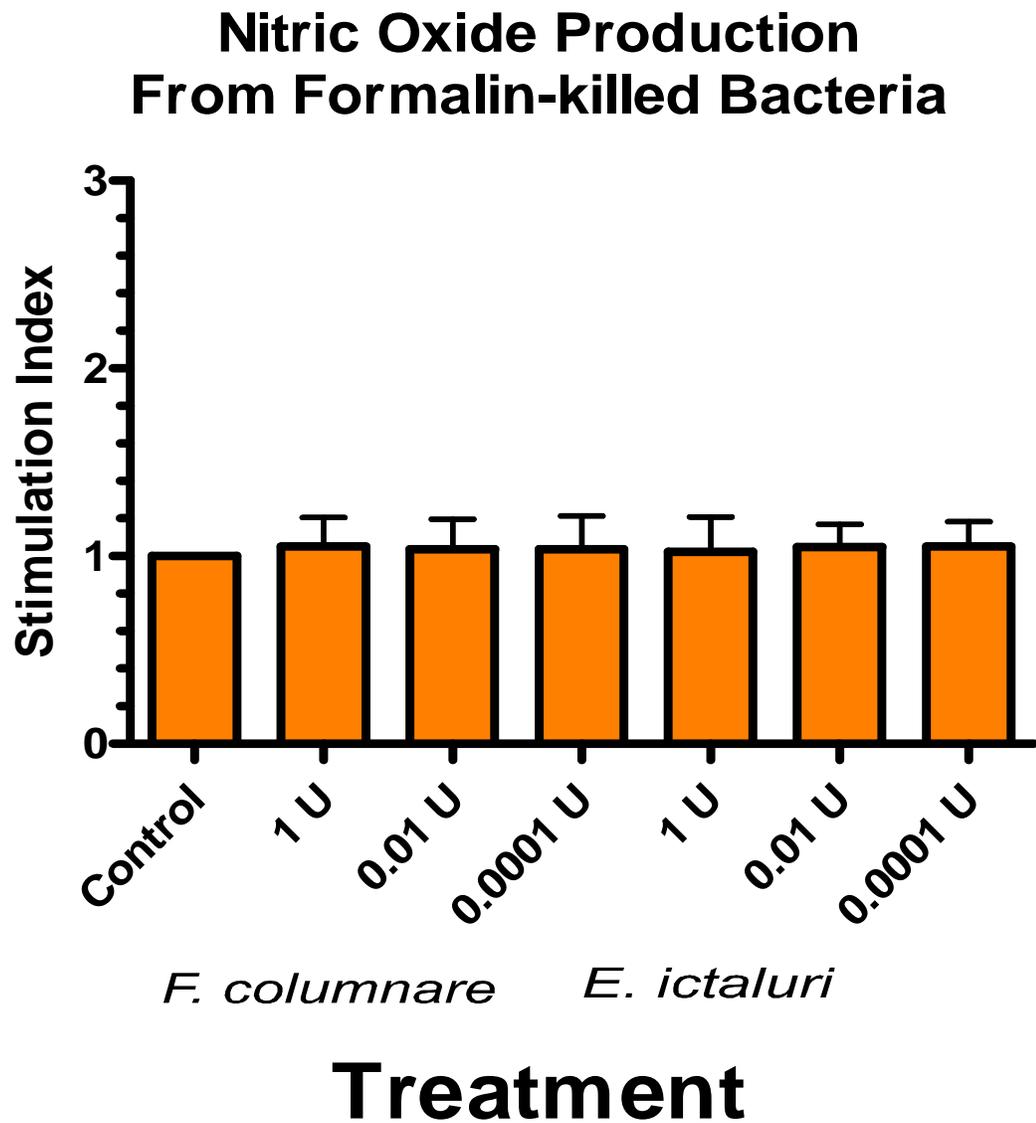


Figure 14. Effect of formalin-killed bacteria on nitric oxide production in channel catfish neutrophils after 24 hour stimulation.

Figure 15

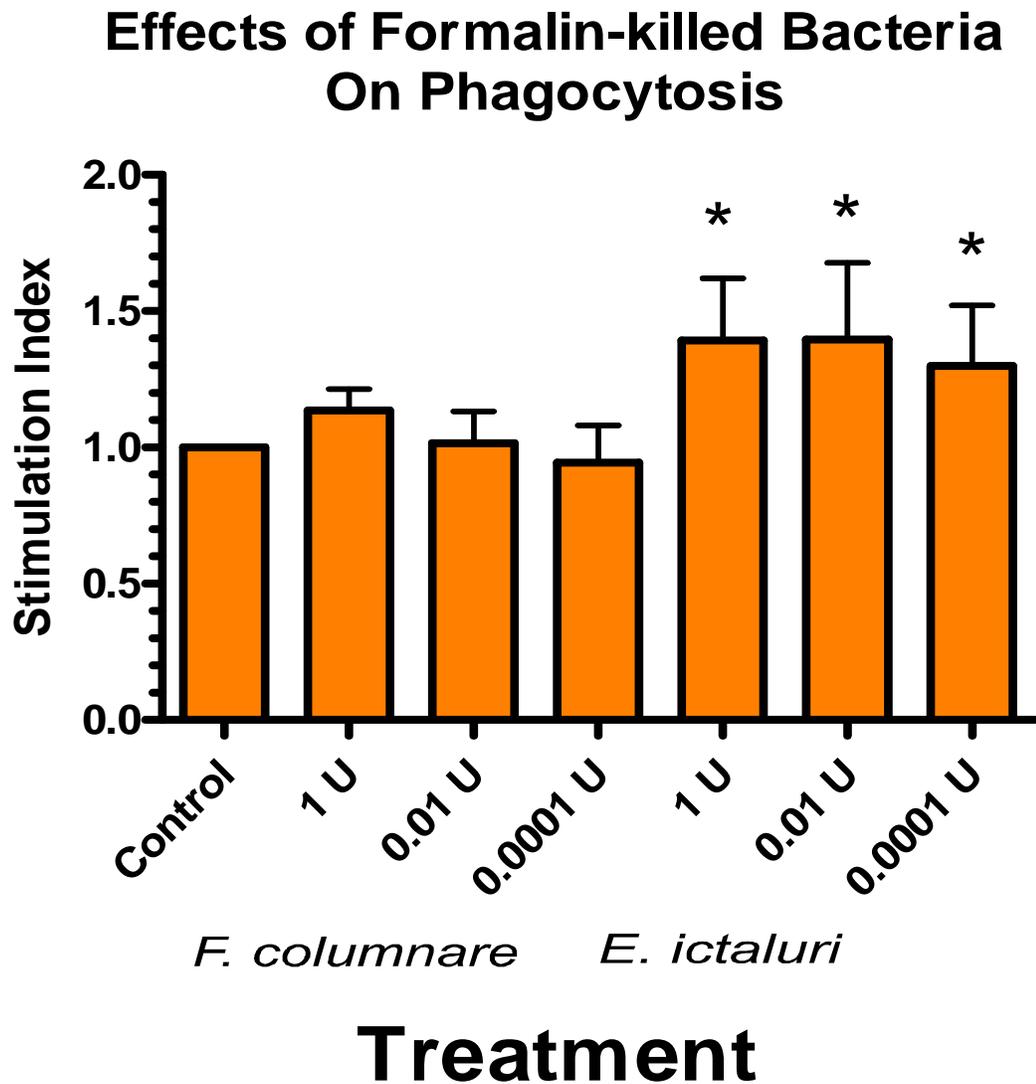


Figure 15. Effects of formalin-killed bacteria on phagocytosis in channel catfish neutrophils after 24 hour stimulation.

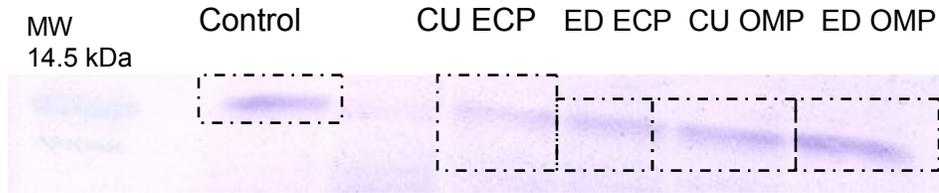


Figure 16. Lysozyme protein expression after 24 hour stimulation with *F. columnare* and *E. ictaluri* ECPs and OMPs. The bars are the same molecular weight; however, as the immulon membranes dried, there was a shift in band positions.

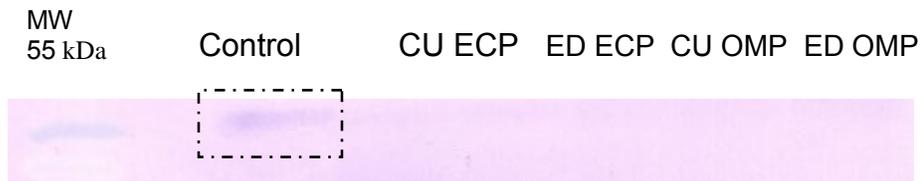


Figure 17. CYP1A expression after 24 hour stimulation with *F. columnare* and *E. ictaluri* ECPs and OMPs.

Discussion

Since the discovery of *F. columnare* in 1917 from a large fish kill, only the biology of the bacteria and the stressors leading to outbreaks have been studied in detail (Figueiredo, et al, 2005; Thomas-Jinu and Goodwin, 2004; Parker, 1995). The specific immune response of fish to *F. columnare* has not been characterized, which makes it difficult to develop preventative treatments against this pathogen. In order to advance the study of more effective treatments, the immune response, specifically, the inflammatory responses to *F. columnare*, must be better understood.

In contrast to the lack of information regarding phagocyte responses to *F. columnare*, the pro-inflammatory properties of *E. ictaluri* are fairly well described (Camp, et al, 2000). *E. ictaluri* is an intracellular Gram-negative pathogen that spreads via activated phagocytes, namely neutrophils and macrophages. One of the hallmark features of *E. ictaluri* is its ability to inhibit the respiratory burst (superoxide production) in phagocytes, which allows them to further the infection of the host (Paul, 2003). Because the inflammatory response of *E. ictaluri* in channel catfish is well understood and characterized, *E. ictaluri* can be used as a positive control in efforts to better understand the interactions between *F. columnare* and fish phagocytes (Iwama, et al, 1997; Paul, 2003).

The use of extracellular products and outer membrane proteins of bacteria have been used extensively in the investigation of adjuvant and vaccine development in aquaculture (Kwon, et al, 2006; Vazquez-Juarez, et al, 2005; Bader, et al, 2004; Pasnik, 2006; Kawakami, et al, 1997). ECPs contain several

toxins that are not only responsible for many of the symptoms of a particular disease, but are generally pro-inflammatory. It has been suggested that bacterial ECPs are more important than the whole bacterial cell to the inflammatory reaction (Mutoloki et al, 2006). In the study described herein, ECPs did not affect PMA-stimulated hydrogen peroxide production. Others have shown that whole *E. ictaluri* inhibit the respiratory burst of phagocytes (Paul, 2003); therefore, based on these results it is unlikely that inhibition of ROI production is due to ECPs. However, the ECPs of *E. ictaluri*, but not *F. columnare* ECPs, significantly increased nitric oxide production. Others (Iwama, et al, 1997; Yano, 1996) have shown that catfish head kidney cells produce nitric oxide following infection with *E. ictaluri*, and based on the results herein, it is likely that neutrophils were the cellular source and that ECPs play a major role.

Bacterial OMPs should, theoretically, be useful in developing protective vaccines because the cell surface makes contact with cells of the immune systems, thereby initiating the immune responses. Some OMPs are immunogenic, while others are not (Kwon, et al, 2006; Vazquez-Juarez, et al, 2005). Vaccination with OMPs against *Aeromonas salmonicida* and *A. hydrophila* were successful (Vazquez-Juarez, 2005), although OMPs from a virulent isolate of *E. ictaluri* proved to be fractionally successful (Bader, et al, 2004). In the study described herein, *E. ictaluri* OMPs increased PMA-stimulated hydrogen peroxide production but not nitric oxide production. Interestingly, *E. ictaluri* OMPs increased the phagocytic capability of neutrophils, while those of *F.*

columnare did not. Overall, it appears that OMPs of *E. ictaluri* are more potent in activating phagocytes of channel catfish than are OMPs of *F. columnare*.

Attenuated live bacteria have been tested as a vaccine for both *E. ictaluri* and *F. columnare*, but without success (Kawakami, et al, 1997). Experiments described herein show that live bacteria do not affect phagocyte activation and ability to phagocytose yeast particles, further supporting the futility of using live bacteria as a vaccine. On the other hand, heat killed *F. columnare* immunized orally were successful against columnaris in coho salmon but not channel catfish (Grabowski, et al, 2004; Eimers and Cardella, 1990). Heat-killing and formalin-killing can alter the physio-chemical properties of the surface antigens of bacteria or lead to a blocked antigen from the cross-linking of the chemicals, which can cause inadequate protection against the live bacteria (Kwon, et al, 2006). In the study at hand, both bacteria failed to affect hydrogen peroxide production when either heat-killed or formalin-killed. Neither bacteria when heat-killed affected nitric oxide production. However, exposure to heat-killed *E. ictaluri* increased phagocytosis of yeast by neutrophils, suggesting that the process of heat-killing changes a character of the bacteria, thereby leading to phagocyte activation.

Investigators have shown that formalin-killed bacteria induce higher defenses than do heat-killed cells of some bacteria (Pasnik, et al, 2006). Formalin-killed *F. psychrophilum* provided low to moderate protection against rainbow trout and ayu (Rahman et al, 2002). In this study with channel catfish, there was no effect of formalin-killing of either bacteria on PMA-stimulated hydrogen peroxide or on nitric oxide production. However, formalin-killed *E.*

ictaluri at all three levels increased phagocytic capability after exposure to neutrophils. Formalin-killed *F. columnare* did not affect phagocytosis, suggesting yet another significant difference between *E. ictaluri* and *F. columnare* in terms of their ability to initiate pro-inflammatory responses.

Lysozyme is an enzyme that breaks the $\beta(1\rightarrow4)$ glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the cell wall of Gram-positive bacteria, and is one of several humoral and cellular factors associated with front line, innate immunity in all vertebrates (Yano, 1996). In higher vertebrates, mature and fully differentiated phagocytes are thought to be the primary source of lysozyme (Cross et al., 1988), thus this enzyme is a key bioindicator of innate immunity in aquatic organisms. Pertinent to this study, *E. ictaluri* though may use lysozyme expression in order to facilitate its phagocytosis into the phagocyte (Paul, 2003). This study shows that lysozyme expression is not affected by treatment with OMPs and ECPs from *F. columnare*, nor OMPs from *E. ictaluri*.

Cytochrome P4501A is an inducible enzyme system that is involved in one-electron oxidation-reduction reactions associated with the metabolism of a variety of endogenous and xenobiotic substrates. It is now well known that pro-inflammatory signals down regulate both CYP1A expression and enzymatic activity (Reynaud et al., 2002, 2004, 2006), and may be a useful maker of inflammation in lymphoid cells (Nicholson and Renton, 2002). Interestingly, CYP1A expression was reduced after 24 hour stimulation with ECPs and OMPs from both bacteria, suggesting that either *F. columnare* products are more pro-

inflammatory than shown by the other endpoints used in this study, or that CYP1A may not be a reliable marker of inflammation after all, at least in fish.

From this study, it has been demonstrated that, overall, *F. columnare* does not have the same pro-inflammatory properties as *E. ictaluri* in channel catfish phagocytes. It is possible that *F. columnare* may shut down its capsule synthesis (OMP and ECP production) until it has reached the deeper tissues of the epithelium, where it resumes the production of the capsule and initiates the symptoms indicative of the disease. Virulent strains of Gram-negative bacteria such as *F. columnare* contain high concentrations of sialic acid which mimics eukaryotic levels of sialic acid. Because of this similarity, the bacteria is able to further infection without being recognized by the immune system. Sialic acid has also been found to cause the bacteria to become negatively charged and to inhibit C3b of the alternative complement pathway. Without C3b, there is no further activation of the complement pathway (Iwama, et al, 1997).

In mammals, TLR4 is responsible for the recognition of LPS from Gram-negative bacteria. However, TLR4 has not been identified in channel catfish (Iliev, et al, 2005). It was hypothesized by Iliev, et al (2005) that since most fish lack the accessory proteins of the TLR4 complex, this may be the reason why there seems to be no recognition of LPS in bacteria. It is possible that the LPS binding protein (LBP) in fish may act like mammalian bacterialcidal permeability increasing (BPI) protein which bypasses CD14 of TLR4 complex. CD14 is responsible for activating LY96 (MD2) of TLR4 which leads to the full activation and signaling cascade of TLR4. BPI is known in mammals to neutralize LPS, but

its function is not known in fish. Also, CD14, LY96, and TICAM2 (an accessory molecule of TLR4) have not been identified in any fish genomes. Without such important accessory molecules present, TLR4, if present, cannot be activated (Iliev, et al, 2005).

Further research is needed to investigate the specific mode of infection of *F. columnare* and understand how the bacteria is able to destroy tissues of the fish without activating the immune system. Which, if any, Toll-like receptors are important in the pathogenesis of *F. columnare* need to be elucidated. The components of OMPs and ECPs of *F. columnare* are still unknown and therefore should be investigated as well.

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