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PREVALENCE AND CONTROL OF *SALMONELLA SPP*. AND SANITARY INDICATOR MICROORGANISMS IN WILD CAUGHT AND FARM RAISED CATFISH (*ICTALURUS PUNCTATUS)*

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Nutrition and Food Sciences

by
Bilan Costley Jessie
B.S., Dillard University, 2005
December 2019

To God my faithful Father,

Shirley and Benny Costley Jr., John Costley, my angels

&

My treasured daughters, Kennedy and Sydney

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ABSTRACT

Catfish inspections have transitioned from the U.S. Food and Drug Administration (FDA) to the United States Department of Agriculture (USDA). The USDA Food Safety and Inspection Service (FSIS) and Food Emergency Response Network (FERN) consider it important to assess the food safety risk associated with consuming catfish in the United States. Surveillance of farm-raised and wild-caught catfish for pathogens is relevant as a public safety protocol. The purpose of this study was to detect the prevalence of *Salmonella* spp., identify and examine isolates for antibiotic resistance to clinical treatments, evaluate the effectiveness of antimicrobial chemical treatments against *Salmonella enterica,* and assess the presence of sanitary indicator organisms in raw wild-caught (WC) and farm-raised (FR) retail channel catfish carcasses. Catfish samples (*n*=240) were collected from retail markets in the southeast region of Louisiana for 24 months. *Salmonella spp.* detection was conducted using the USDA Microbiology Laboratory Guidebook (MLG) methods MLG 4.08 and MLG 4C.07. Presumptive positives were confirmed using biochemical assay and serological testing. Confirmed *Salmonella* positives were examined by VITEK®2 AST-GN69 in vitro for antibiotic resistance of 16 clinical antibiotic agents. Retail channel catfish inoculated with a combination of *S. enterica* strains (*S. typhimurium, S. senftenberg*, *S. concord, S. infantis*) (~ 5.5 log CFU ml/g) were tested with 12 antimicrobial chemical treatments and sterile ice water at 4°C for 8 days to examine the effectiveness of the treatments. Retail market channel catfish samples (*n*=120) were studied for the enumeration of aerobic plate count (APC), coliforms, and *Escherichia coli* (*E. coli*), *Staphylococcus aureus* and *Salmonella spp*. using appropriate selective and differential media. *Salmonella* spp. was detected and confirmed positive of 6 wild caught and 0 farm raised catfish. *Salmonella* isolates (*n*=6) expressed resistance to cefazolin, gentamicin, tobramycin out of 16 applied

antibiotics. WC catfish has larger sanitation indicator microorganism load than FR catfish. There is a significant sanitation indicator bacteria difference among retail markets. Antimicrobial chemical treatments were effective at reducing *S. enterica.* Organic acid treatments were most influential in the eradication of *S. enterica.*

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Federal Regulations

The United States Congress mandated a change in regulating and inspecting domestic and imported fish production of the order Siluriformes. A Food, Conservation, and Energy Act of 2008, Farm Bill, transfers the responsibility of inspecting Siluriformes and its by-products from the United States Food and Drug Administration (FDA) to the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) under the Federal Meat Inspection Act (FMIA).

Since its proposal, the rule has sparked an international controversy (Nixon 2013) because it delegates regulatory responsibility for the inspection of Siluriformes, also termed catfish, to the FSIS, an office housed within the USDA. With the passage of the amendment to the FMIA in the 2008 Farm Bill and the subsequent shift in regulatory oversight from the FDA to the FSIS, catfish will become the first and only seafood product to be subject to the FSIS's system of mandatory and continuous inspection under the USDA (Fernandez 2015). This will differentiate catfish inspections from all other seafood inspections, which the FDA handles (Hemingway 2014).

The transition in regulation will subject catfish to more stringent, continuous, and mandatory inspections and will require nations that export catfish to the United States to establish inspection systems equivalent to those in place in the United States. The final rule published by FSIS in 2015 is titled "Mandatory Inspection of Fish of the Order Siluriformes and Products Derived from Such Fish" (FSIS 2015). While the domestic catfish industry supports the rule as necessary to ensure food safety and the economic security of their industry, foreign

exporters find it to be arbitrary, subjective, and protectionist in nature (Lowery 2011). Foreign catfish producers contend that the rule is in direct violation of the World Trade Organization (WTO) Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) (Binh Minh 2013). The SPS Agreement is a set of binding rules and disciplines for all relevant laws, regulations, and procedures directly related to food safety in the Member countries (McNiel 1998).

According to the 2008 Farm Bill, the regulatory shift would not apply until the FSIS issued implementing regulations. In February 2011, the FSIS began applying processes previously used for meat, poultry, and egg products to catfish and catfish by-products. The proposed rule was designated as a "major regulation" due to economic impact under the Federal Crop Insurance Reform and Department of Agriculture Reorganization Act of 1994, which required the proposal to be supported by a risk assessment promulgated by the FSIS. In accordance with the mandate, assessing the potential risks posed by catfish for its required risk assessment, the FSIS searched for susceptibilities related to microbial pathogens, bacterial contaminants, heavy metals, unapproved antimicrobials, and pesticides, drawing on data from the FDA, the Center for Disease Control (CDC), state public health agencies, and the WHO (FSIS 2015). As a result of extensive research into various vulnerabilities, the FSIS's risk assessment ultimately focused on the potential risks associated with *Salmonella*, identifying the need to protect catfish consumers from this target pathogen as the primary scientific justification for the rule (USDA, FSIS 2011).

The USDA published the final rule in the Federal Register on December 2, 2015, first in December 2014 and later in April 2015 (Brasher 2015). The rule applies the need to investigate

both domestically and internationally farmed fish of the order Siluriformes, which became effective in March 2016.

1.2 Order Silurifomes

Siluriformes are a diverse range of whiskered fish commonly known as catfish. Catfish is one of the largest orders of teleosts containing ~4100 species, representing ~12% of all teleosts and ~6.3% of all vertebrates (Eschmeyer and Fong 2014; Wilson and Reeder 2005). Worldwide catfish has over 477 genera, and 36 families (Ferraris 2007). Catfish are named because of their feline resembled whisker barbells, which are located on the nose, each side of the mouth, and on the chin. Most catfish have leading spines in their dorsal and pectoral fins. Characteristically, catfish are distinguished from most teleost fish because of their scaleless skin (Arce et al. 2013, Armbruster 2004, Burgess 1989, Ferraris 2007). Catfish have a cylindrical body with a flattened ventral to allow for benthic feeding (Bruton 1996).

Over half of the order Siluriformes are found primarily in North America and are of the family Icataluridae. In 2009, the United States imported more than 129 million pounds (59 million kg) of catfish from several countries, including Cambodia, Canada, China, Indonesia, Mexico, Panama, Peru, Thailand, and Vietnam (NASS 2010). In recent years, other species of catfish within the order Siluriformes have been produced in Vietnam and imported into the United States in large quantities. Imports of basa (*Pangasius bocourti*), tra (*Pangasius hypophthalmus*), and swai (*Pangasius micronemus*) from Vietnam increased by about 800% from 1997 to 2002 (Hargreaves 2004).

In the US, the production of ictalurid catfish is the core of the aquaculture industry, and catfish are produced primarily in Mississippi, Arkansas, Alabama, Texas and Louisiana (NASS

2010). In the southern region of the United States (US) there are 3 common catfish species, blue catfish *(Ictalurus furcatus)*, flathead catfish *(Ictalurus catus),* and channel catfish *(Ictalurus punctatus)*. Channel catfish were originally found only in the Gulf States and the Mississippi Valley north to the prairie provinces of Canada and Mexico but were not found in the Atlantic coastal plain or west of the Rocky Mountains. Since then channel catfish have been widely introduced throughout the United States and the world (Texas Agricultural Extension Service 1988). As a result of their worldwide distribution and diversity, catfish are interesting models to ecologist and evolutionary biologists, and are important for biogeographical studies (Sullivan et al. 2006).

1.2.1 *Ictalurus punctatus* aquaculture impact

Channel catfish (*I. punctatus*) is the most important aquaculture species in the United States, accounting for more than 60% of all aquaculture production (USDA 2001). It is also an important game fish with a broad geographic range encompassing a variety of habitats (Dunham 1984). Channel catfish is the top farm-raised fish in the US with a production of more than 750 million pounds per year (Tucker 1990, Hargreaves 2004), generating approximately half of the freshwater aquaculture value in 2014 (NOAA 2016b). In 2014, according to the US Catfish Database, sales of domestic catfish and catfish products were approximately $352 million. This is a 1.4% decrease from 2013 of $357 million in sales. The US catfish industry has been on a contracting course since a high mark in 2003 when 662 million pounds of round weight catfish were processed. In 2014, 301 million pounds were processed, down 32 million pounds (-10%) from 334 million pounds processed in 2013; and a 54% decrease since the 2003 peak (Hanson 2015).

In contrast to the decrease in sales, channel catfish producers profited in spite the loss of retail revenue. Economic theory indicates that maximum profits will occur at a level of production that is less than maximum yield (Baumol 1991). Total producer income in 2014 was $358 million, a 10% increase over 2014 producer income of $325 million, due primarily to the increase in price paid by processors to producers as overall quantity sold decreased (NASS 2010). The catfish industry provides employment opportunities for tens of thousands of producers, processors, service providers, marketers, retailers, and restaurant owners (Jin et al 2016).

1.2.2 Hatchery and natural practices

Producers engage in the farm raising of catfish for food or raw material. Processors purchase catfish from producers (farmers) and/or fishermen, processors prepare the harvest (farm raised catfish) or catch (wild caught catfish) by skinning, eviscerating, and storing the product for retail distribution. Aquaculture products are sourced or referenced in two ways: natural capture (wild caught) or controlled hatchery-raised (farm raised). Hatchery-cultured fish typically encounter conditions very different from those encountered by their wild counterparts. Consequently, cultured fish may exhibit behavioral, morphological, and physiological differences due to a variety in learning, expression of phenotypic traits, and genotypic selection (Weber 2003, Huntingford 2004, Thorpe 2004).

Wild caught fish is sourced from seas, rivers, and other natural bodies of water. Farm raised fish are raised in controlled tanks, irrigation ditches, and ponds using special formulated feeds based on natural grains. According to the National Academy of Sciences, the ability of fish populations

to reproduce and replenish themselves is declining across the globe. This discovery affects the recreational and commercial natural capture of catfish along the Gulf Coast region.

The production of farm raised channel catfish is the major source of retail catfish sales in comparison to wild caught channel catfish. However, wild caught catfish sales peaked at $2.3 million in 2011 according to the Gulf Seafood Institute. The rapid depletion of feral stocks of fish and shell-fish in recent years has resulted in high-density farming conditions required to maximize biological yields and to satisfy growing market demands which allow the widespread infection of species reared in earthen ponds and other unprotected facilities that are continuously exposed to environmental contamination (Ferraris 2007).

The use of raw meat scrapes and offals, soil potentially contaminated with typhoid and paratyphoid salmonellae, and of *Salmonella*-contaminated animal feeds and feces is not uncommon in fish production, processing, and natural environments. Clearly, such husbandry practices favor widespread bacterial contamination during rearing (D'Aoust 1994).

1.3 *Salmonella spp.*

Salmonella spp. are gram-negative, non-spore forming, rod-shaped facultative anaerobes characterized by O, H, and Vi antigens (usually 0.7-1.5 x 2-5 µm in dimensions) belonging to the family of *Enterobacteriaceae* (Blackburn 2004, USDA 2012a). Members of this genus are motile by flagella with the exception of *Salmonella* serovar Pullorum and *Salmonella* serovar Gallinarum. The genus *Salmonella* consists of two species, *Salmonella bongori* and *Salmonella enterica* (Reeves 1989) both contain multiple serovars (Table 1). *S. bongori* was formerly known as subspecies V of *S. enterica*. *S. enterica* a type species with six subspecies *S. enterica* is

expressed by Roman numerals and subspecies names. *S. enterica* is differentiated by biochemical traits and genomic relatedness (Agbor 2011).

Table 1. Species within the *Salmonella* genusa.

Salmonella species and subspecies	No. of serovars
S. enterica subsp. *enterica* (I)	1,504
S. enterica subsp. *salamae* (II)	502
S. enterica subsp. *arizonae* (IIIa)	95
S. enterica subsp. *diarizonae* (IIIb)	333
S. enterica subsp. *houtenae* (IV)	72
S. enterica subsp. *indica* (VI)	13
S. bongori (V)	22
Total	2,541

[a] From reference Agbor 2011.

Salmonellae have the ability to metabolize nutrients by respiratory and fermentative pathways. This chemoorganotrophic characteristic allows *Salmonella* to grow at 37°C and digest D-glucose and polyhydroxy aldehydes, producing acid and gas (Gurtler 2015). These traits form the basis for presumptive biochemical identification of *Salmonella* isolates. There are more than 2500 serovars and are considered potential pathogens in animal and human (Wan Norhana 2010).

Salmonellae can be present in various environmental conditions and is frequently found in the intestinal tract of numerous animals including humans, birds, and fish. *Salmonella spp*. consists of resilient microorganisms that readily adapt to extreme environmental conditions. Some strains can thrive at heightened temperatures ≤54°C, while others express psychrotrophic characteristics in their ability to grow at 2°C to 4°C (D'Aoust 1991). The physiological

adaptability of *Salmonella spp*. is further demonstrated by their ability to proliferate at pH values ranging from 4.5 to 9.5, with an optimum pH for growth of 6.5 to 7.5 (D'Aoust 1992). This demonstration of affability in a broad range of environments causes *Salmonella spp*. to be a public health concern.

1.4 *Salmonella* - public health concern

1.4.1 Clinical symptoms

Salmonellosis is an infection caused by *Salmonella* and can lead to various clinical conditions. In the U.S., it is estimated 1.4 million non-typhoidal *Salmonella* infections are reported annually resulting in 168,000 visits to physicians, 15,000 hospitalizations and 580 deaths. (WHO 2003). Human *Salmonella* infections can result in various clinical conditions, including but not limited to enteric typhoid fever, uncomplicated enterocolitis, and systemic infections by nontyphoid microorganisms (D'Aoust 1989). *Salmonella* symptomatic infective dosage is 10^7- 10^8 cells (Li 2013). These symptoms develop within 12-14 hours of exposure with duration of 2-3 days. Patients typically recover without treatment. However, immuno-compromised individuals may become *Salmonella* carriers upon recovery (CIDRAP 2009), developing extra intestinal focal infections (Hohmann 2001).

1.4.2 Virulence characteristics

Within a given serovar, different strains of S*almonella spp*. vary in their virulence. Virulence is partially determined by the ability of *Salmonella spp*. to invade nonphagocytic host cells (Bean 1997). The invasion gene *(invA)* on the *Salmonella* chromosome encodes an invasion protein InvA (Galan 1992). InvA protein assists *Salmonella* in penetrating host small intestine epithelium cells. The location and persistence of *Salmonella* in the intestinal epithelium and the

lymph nodes accounts for protracted shedding which lasts for 3 to 6 weeks. Fecal shedding is continuous for the first week but then becomes intermittent (Tsolis 1999). Phagocytic cells in the intestinal lymph nodes, liver, or spleen may harbor organisms persistently, even in the absence of fecal shedding.

Succeeding invasion, *Salmonella* enters the submucosa via local macrophages. The survival of *Salmonella* is dependent on a variety of factors such as nutrient availability and the avoidance of antibacterial mediums (Ibarra 2009). *Salmonella* will then spread throughout the blood stream and collect in mesenteric lymph nodes and spleen, causing inflammation which leads to salmonellosis (Salcedo 2001). *Salmonella* can produce enterotoxins and cytotoxins in intestinal tracts which have minor effects on the infection (Jay 2005). Consequently, *Salmonella* causes typical foodborne infections instead of intoxication.

1.5 *Salmonella* in food

Salmonella is the second leading cause of foodborne outbreaks. However, it is the most common organism associate with foodborne hospitalization According to the CDC *Salmonella* food contamination attributed to 23% of all foodborne outbreaks and 60% of foodborne pathogenic hospitalizations (Figure 1).

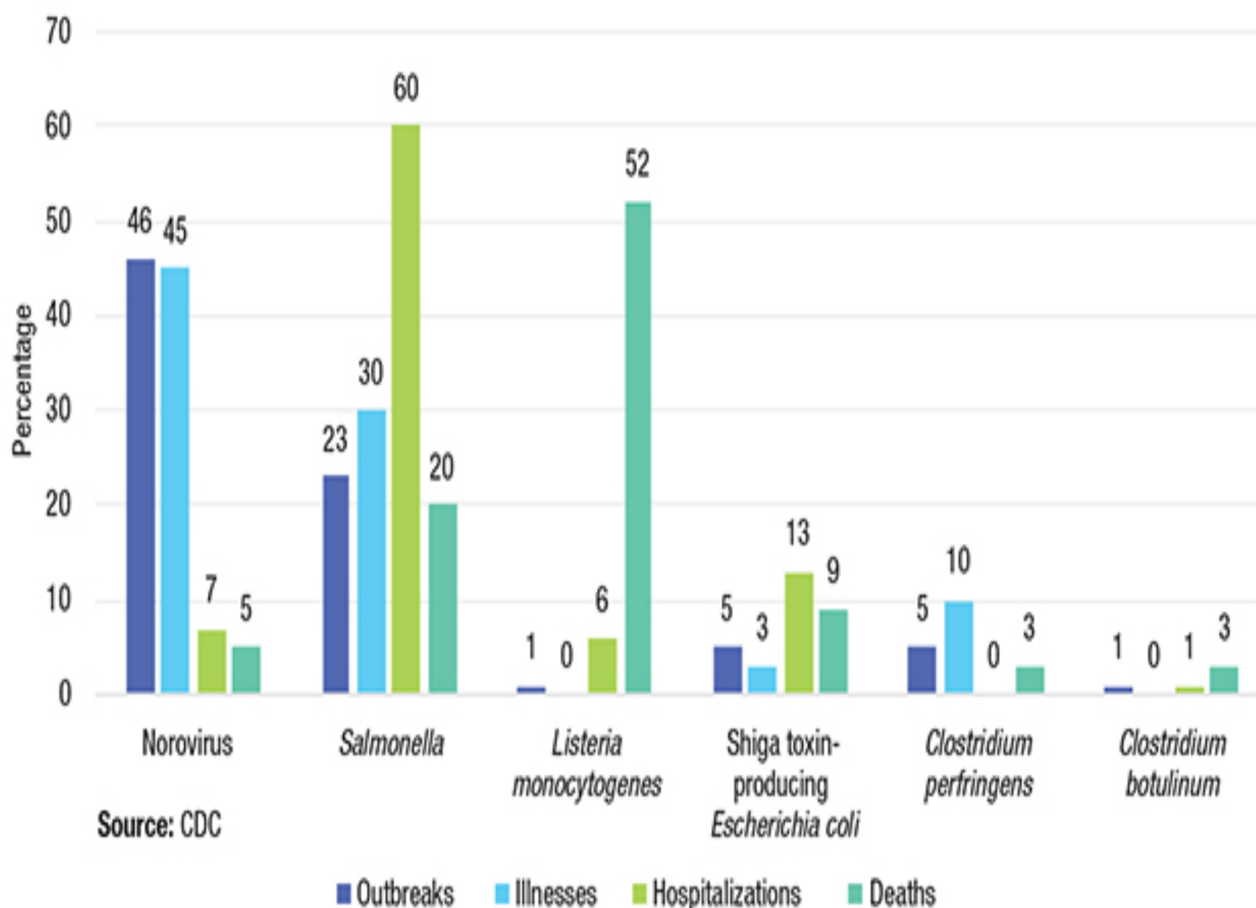


Figure 1. Most common organisms associated with foodborne disease outbreaks, illnesses, hospitalizations and deaths (confirmed and suspected, 2009-2015, CDC) (Scallen 2011

The CDC estimates of all known foodborne pathogens in the U.S., approximately 1.4 million foodborne illness cases, were caused by *Salmonella* annually. *Salmonella* accounts for 11% of bacterial contamination associated with food (Scallan 2011). In the U.S., *Salmonella* was the most frequent cause of outbreaks of seafood illnesses from 1998 to 2004 (National Advisory Committee on Microbiological Criteria for Food (NACMCF), 2008). Contrary to the fact that seafood is not considered the natural host for *Salmonella* and further, it is always transported at low temperatures, the incidences of *Salmonella* in seafood is in increasing order (Heinitz 2000,

Amagliani 2012). As previously discussed, the growth and multiplication of *Salmonella* is primarily dependent on temperature, pH, and the availability of essential nutrients.

1.5.1 *Salmonella* prevalence in catfish

A food matrix such as seafood provides a collection of elements such vital nutrients, pertinent salts and sufficient amounts of water necessary to support the growth of *Salmonella*. Despite the fact that numerous attempts have been made to understand the growth of *Salmonella* in beef, pork and chicken (Nissen 2001, Ingham 2005) limited reports are available on the dynamics of *Salmonella* in seafood.

As part of seafood commodities, catfish are considered a low-risk carrier of microbial foodborne illness due to its short shelf life and methods of preparation that typically destroy pathogens. However, some outbreak data suggest catfish consumption may contribute to illness. The CDC foodborne disease outbreak database recorded seven catfish associated illnesses, from 1991 to 2007 it was estimated that 66 catfish related illnesses occurred (CDC 2007). In contrast, only one reported farm raised catfish related outbreak in 1991 of 10 cases of salmonellosis associated with a New Jersey restaurant. It is challenging to attribute *Salmonella* infection to specific food sources such as catfish due to the small percentage of salmonellosis cases reported (Mead 1999). The isolated farm raised catfish related *Salmonella* outbreak is the only microbial pathogen dilemma linked to catfish consumption alone (CDC 1991). However, *Salmonella* has been detected in catfish ponds, processing plants, and retail products (Cotton 1998, Dalsgaard 1998). Over several decades, investigations at these points have produced various results for *Salmonella* prevalence. Sampling and testing methods, storage and production practices may play a role in the different results in the studies of the prevalence of *Salmonella* in catfish

products, ponds, and processing plants. There are no reported cases of *Salmonella* infection caused by wild caught catfish.

Possible factors, such as pond temperature, stocking density, organic matter content, and the size of the fish, have been documented to affect *Salmonella* levels in aquaculture ponds. A 1979 study found *Salmonella* only in densely stocked ponds with large fish (0.5 to 1.4 kg) and warm pond temperatures (26 to 29°C) (Wyatt 1979). Future research is necessary to explore all avenues by which *Salmonella* contamination might be introduced to catfish at production and processing levels.

Prevalence of *Salmonella* in catfish varies at retail markets. Former retail investigations discovered that 4.5% of fresh processed farm raised catfish and 1.5% of frozen processed farm raised catfish were positive for *Salmonella* (Andrews 1977). However, in another study of retail catfish fillets purchased from local Virginia markets and internet retailers none of the fillets were positive for *Salmonella* (Pao 2008). Catfish samples were taken from both retail and processing facilities to compare fresh farm-raised and fresh commercially wild-caught catfish and catfish imported from Mexico and Brazil. Farm-raised fish resulted in 21% *Salmonella* positives and the wild caught fish 5% positive for *Salmonella*. The imported catfish samples were negative for *Salmonella* (Wyatt 1979). An increase of *Salmonella* positive farm raised catfish samples collected from July through September was observed in comparison with farm raised catfish samples collected from January through March (Andrews 1977). In contrast, another survey of *Salmonella* contamination of catfish samples collected from a production line at various processing plants showed no seasonal difference on *Salmonella* prevalence (McCaskey 1998). Fluctuating outdated studies on *Salmonella* prevalence of farm raised and wild caught catfish

within habitat and processing, retail markets, and seasonal effects demonstrate a need for more investigations to determine its pervasiveness.

1.6 Antibiotic resistance

Antibiotic resistance is a world anomaly that causes the rise of pathogens with resistance to important antibiotics, resulting in the need for new treatment strategies (Bell 2014). Antibiotic-resistant bacteria cause life-threatening illness in humans and pose a significant threat to health and well-being. It is estimated that antibiotic-resistant pathogens cause ~2 million illnesses and 23,000 deaths annually in the U.S. These illnesses cause an additional healthcare cost of $20 billion and a productivity loss of $35 billion to the U.S. economy.

1.6.1 Clinical resistance of *Salmonella*

Antibiotic resistance in foodborne pathogens such as *Salmonella* is a major concern for public health safety (CDC 2013). Food animals are usually a repository of pathogens that are difficult to destroy such as *Salmonella.* Non-typhoidal *Salmonella* (NTS) causes the highest number of illnesses, hospitalizations, and deaths associated with foodborne illness (Scallan 2011). It is associated with more than 1,200,000 illnesses annually, and among these at least 100,000 infections are due to antibiotic-resistant *Salmonella*, including those that are resistant to clinically-important drugs such as ceftriaxone (36,000 illnesses/year) and ciprofloxacin (33,000 illnesses/year) (CDC 2013). In fact, *Salmonella* isolates conferring resistance to ≥5 antibiotics accounted for more than 66,000 illnesses from 2009 to 2011 in the U.S. (CDC 2013). The selectivity of antibiotic drugs against invading bacteria ensures minimal harm to the patients and at the same time guarantees maximum eradication of the target bacteria (Nami 2015). NTS infections do not usually require treatment with antibiotic drugs; however, complications such as

meningitis and septicaemia do occur and require treatment with antibiotic drugs, including ciprofloxacin, ceftriaxone and ampicillin (Baron 1996). Infections caused by *S. Typhi* and *S. Paratyphi* may involve serious complications and require treatment with antibiotics such as cefixime, chloramphenicol, amoxicillin, trimethoprim/sulfamethoxazole (TMP-SMX), azithromycin, aztreonam, cefotaxime or ceftriaxone to prevent death (Kumar 2017) (Table 2).

Table 2. Options for antibiotic treatment of enteric fever caused by *Salmonella enterica Typhi or Paratyphi,* (Kumar 2017)

Susceptibility profile of organism	Drug	Duration of treatment, d
Uncomplicated enteric fever		
Fully susceptible	Ciprofloxacin	5–10
	Amoxicillin	14
	Trimethoprim/ sulfamethoxazole	14
Multidrug resistant*	Ciprofloxacin	5–10
	Cefixime	7–14
Quinolone resistant	Azithromycin	5–7
	Cefixime	7–14
Severe or complicated enteric fever (parenteral antibiotics)†		
Fully susceptible	Ciprofloxacin	10–14
	Ampicillin	14
	Trimethoprim/ sulfamethoxazole	14
Multidrug resistant*	Ciprofloxacin	10–14
	Ceftriaxone	10–14
Quinolone resistant	Ceftriaxone	10–14
	Cefotaxime	10–14
	Ciprofloxacin‡	14

1.6.2 Antibiotic resistance of *Salmonella* isolated from fish related products

Consideration to the inclination of *Salmonella* cultured isolates from catfish may demonstrate antibiotic resistance. There are no significant studies that have been conducted to

investigate the prevalence of antibiotic-resistant *Salmonella* isolates derived from domestic farm raised and wild caught catfish. Similar studies of farmed finfish in the Guangdong Province of China *Salmonella* isolates were resistant to erythromycin and penicillin (Broughton 2009). *Salmonella* isolated from catfish imported to the United States from Thailand were resistant to a variety of antibiotics, including nalidixic acid, streptomycin, tetracycline, and kanamycin (Zhao 2003). The available data indicate that catfish are exposed to antibiotics that might affect bacterial resistance patterns. There is a need for more studies to determine the prevalence of antibiotic-resistant *Salmonella* isolates in domestic farm raised and wild caught catfish. In addition, studies are needed to set a baseline prevalence of *Salmonella* in domestic farm raised and wild caught catfish. Outbreak data in conjunction with results of other reported studies suggest that *Salmonella* may be prevalent in catfish.

1.7 Sanitation indicator microorganisms

In addition to the detection and identification of *Salmonella* spp. in domestic farm raised and wild caught catfish, the estimation of indicator microorganisms in domestic catfish is vital to discovering the quality of sanitation in production, processing, and storage of catfish. Quantification of catfish carcass products for indicator microorganisms can provide predictable, interpretable, and quick information about where the process failed, point of contamination after processing, environmental contamination, and the degree of hygiene maintained while catfish is processed and stored. Detection and estimation of sanitary-indicative microorganisms cannot substitute testing for specific pathogens such as *Salmonella* (Heinitz 2000). However, indictor microorganisms provide qualitative information about the product faster than the time needed to isolate and identify specific pathogens. In its natural habitat or under husbandry conditions, catfish are cultivated in an unrestricted environment which does not limit it to a single

microorganism but rather a multitude of microorganisms that render loss of quality. Therefore, it is practical to determine the microbial load in catfish products as counts of groups of microorganisms using conventional methods to estimate spoilage and degradation.

Microbial activities create undesirable changes like off-flavors, texture and appearance (Johnstone, 1994). Consequences of catfish spoilage are not limited to the loss of quality; it also creates an economic loss due to foodborne illness as a result of consumption. The microbial association with fish compromises safety and the quality for human consumption; particularly critical is when the microorganisms are opportunistic and/or pathogenic in nature (WHO 1996).

Previous studies have demonstrated the presence of indicator microorganisms of fecal pollution, opportunistic and pathogenic bacteria to humans in fish (Cahill 1990, Da Silva 2002, Tsai yung-Hsiang 2002, Ferreira 2006, Tzikas 2007). Common indicator tests include aerobic and anaerobic plate counts, counts of psychrotrophic bacteria, and coliform counts. Testing for indicator bacteria is used by the FSIS-regulated meat and poultry industry to monitor process control as outlined in the 1996 pathogen reduction and hazard analysis critical control point system for raw meat and poultry (USDA FSIS, 1996).

1.7.1 Aerobic Plate Counts

As a result, it is beneficial to estimate group counts such as aerobic plate counts (APC). APC is generally used to determine total numbers of microorganisms in a food product. APC can be used to gauge sanitary quality, organoleptic acceptability, adherence to good manufacturing practices, and to a lesser extent, as an indicator of safety (Leung 1992). APC may also provide information regarding shelf life or impending organoleptic change in a food (Cotton 1998). Environment alterations of incubation or medium selection can be used to screen for groups of

microorganisms such as anaerobic, thermoduric, mesophilic, psychrophilic, thermophilic, proteolytic, and lipolytic.

The focus of concern is the occurrences of psychrophilic/psychrotrophic bacteria above acceptable limits which can result in significant spoilage of seafood products, such as catfish. The specification for bacterial counts of fish which is $<10^5$ CFU/g is considered acceptable (Andrew 1992). High enumerated APC group counts can be contributed to poor hygiene and unsanitary handling of food (Reij, 2004) or cross contamination of viscera and flesh during processing. Catfish has a high viscera bacterial count and is reflected in the microbial counts in catfish flesh (Leung, 1992).

1.7.2 Coliform and *E. coli*

The genera *Escherichia, Klebsiella, Enterobacter, Serratia,* and *Citrobacter* are collectively called coliform bacilli and *Proteus*. Coliforms are rod-shaped gram-negative bacteria that are commonly used as an indicator of sanitary quality. Coliforms are ubiquitous in the environment but are prevalent in the intestine and fecal material of warm-blooded animals. Many species are members of the normal intestinal flora.

Coliforms, including *Escherichia coli (E. coli)*, are destroyed by heat and are reduced during freezing of foods. Food processing plants may have Coliforms in the environment and recontamination can occur to processed foods. Coliform bacteria are used as a component of microbiological standards to monitor the wholesomeness of shellfish and the quality of shellfish-growing waters (Hackney 1994). Justifiably to reduce the risk of harvesting shellfish from waters polluted with fecal materials. The purpose can be forwarded to the assessment of other seafood commodities such as wild caught and farm raised domestic catfish.

E. coli is present in all mammalian feces at high concentrations; it does not multiply appreciably but can survive in water for 4–12 weeks, and so it is useful as an indicator of fecal pollution of water systems (Leclerc 2001). The case for *E. coli* as an indicator in foods and the processing environment is valid because the organism can survive and grow, in certain foods. It can become established in the food processing environment and contaminate foods in the facility (Kornacki 2001). In addition to food industry sanitation and process integrity indicators, *E. coli* is utilized for Hazard Analysis Critical Control Point (HACCP) verification. Quantitative determinations or density of organisms per analytical unit, are specified for the dairy, meats and poultry, bottled water, and shellfish regulations. These practiced standards can be assessable and extended to emerging catfish regulations.

1.7.3 *Staphylococcus aureus*

An essential component of a microbial load count is the enumeration of *Staphylococci*. *Staphylococci* describe a group of spherical, gram-positive bacteria. Enterotoxigenic *staphylococci*, such as *Staphylococcus aureus (S. aureus)* can be present on food products, and time and temperature abuse can allow the development of enterotoxin, which can cause foodborne illness (Le Loir, 2003). *Staphylococcal* food poisoning (SFP) is among the most prevalent causes of gastroenteritis worldwide (Fernandes 1997). The main reservoir is humans, who carry the bacterium in nasal passages, skin, or wounds. *S. aureus* contamination in food, including seafood, is usually due to contamination by a food worker during food preparation (Bryan 1980). Majority of reported cases of illness caused by *S. aureus* enterotoxin were the result of food service and consumer temperature and time abuse, not the direct result of contamination at the farm or processing facility (Le Loir, 2003).

Even though several emergent food pathogens have been identified worldwide in recent years, *S. aureus* food poisoning remains an expressive social problem, causing frequent outbreaks and financial losses. According to the Centre of Surveillance of Health of the State of RS (CEVS/RS), in the US during 1997, it was estimated that $1.5 billion were spent due to *S. aureus* food poisoning (CEVS, 2006). A few decades ago this agent was responsible for 25% of all foodborne outbreaks occurred in the US (Su 1997). The main foods incriminated in *staphylococcal* food poisoning are meat products, poultry and eggs, tuna fish, chicken, pasta, pastries, and dairy products (FDA, 2007).

1.8 Chemical Antimicrobial Treatments

Food processors often modify the intrinsic or extrinsic parameters of a food product such as pH, water activity, temperature of storage, and inhibitory chemicals to prevent growth of undesirable microorganisms. Inhibitory chemicals or antimicrobial treatments are often used during food processing; raw meat may be decontaminated with a variety of acidic rinses. Common food preservatives used for catfish include antibacterial and antifungal agents such as lactic acid (Fernandes 1998), sodium benzoate (Efiuvwevwere 1996), sodium lactate (William, 1995), and sorbic acid (Sofos 2000), and antioxidants such as ascorbic acid and rosemary (Doe 1998). Several researchers have investigated the effects of sanitizing meat and fish surfaces with organic acids that cause sub lethal injury or death to undesirable microorganisms (Marshall, 1995).

1.8.1 Lactic acid

Lactic acid has been considered as a potential alternative to processors than chemical antimicrobial solutions. The FDA classified lactic acid as "generally recognized as safe"

(GRAS). Lactic acid is a natural substance found in various fruits and fermented products and exhibits antimicrobial activity against foodborne pathogens (Beuchat 1989). Additionally, numerous applications for decontamination of meat, fruits and vegetables by lactic acid have been previously described (Dickson 1992).

Lactic acid is lethal to microorganisms via undissociated molecules that flow through the cell membranes and ionize inside. The acidic pH inside the cell causes deformation and damage to enzymatic activities, proteins and DNA structure, thereby damaging the extracellular membrane (Mani-Lopez 2011). In another mechanism, changes in the permeability of the cell membrane hinder substrate transport, while changes in the pH inside the cell suppress NADH oxidation; this affects the electron transport system and leads to the death of the microorganism (Kong 2001). Lactic acids lethal mode of action against pathogens may demonstrate efficacy against *Salmonella* contamination in catfish.

1.8.2 Sodium benzoate

Sodium benzoate is best known as a preservative used in processed foods and beverages to extend shelf life, though it has several other uses. It's an odorless, crystalline powder made by combining benzoic acid and sodium hydroxide. Benzoic acid is an effective preservative alone, however, combining it with sodium hydroxide aids in its dissolving effects. Sodium benzoate does not occur naturally, but benzoic acid is found in many plants, including cinnamon, cloves, tomatoes, berries, plums, apples, and cranberries (WHO 2005). Sodium benzoate is the first preservative the FDA allowed in foods and still widely used as a food additive (Efiuvwevwere 1996). It's classified as GRAS, meaning that experts consider it safe when used as intended. The ingredient is used in food at levels not to exceed good manufacturing practice (GMP). Current

usage results in a maximum level of 0.1 percent in food (FDA). Benzoic acid is effective against bacteria in acid media at a level of 0.1% and in neutral media at 0.2% but inactive in alkaline media (Brul 1999). As the oldest and most utilized preservative approved by the FDA, sodium benzoate can be justified effective against *Salmonella spp.* in catfish carcasses.

1.8.3 Sodium hypochlorite

Chlorine compounds are widely used in the food industry to kill bacteria and disinfect. Examples include treating pasteurizer cooling water, washing fruit and vegetables and disinfecting food contact surfaces. Chlorine is usually combined with inorganic compounds, such as sodium to produce sodium hypochlorite (NaOCl), which are effective disinfectants. Sodium hypochlorite (SH) has been used as antimicrobials at regulated concentrations on poultry in some jurisdictions such as in Asia, Australia, New Zealand and U.S. (AGCL 2013). SH has been labeled GRAS status from the (FDA) at levels permitted for use in foods (del Río 2007).

With recent emphasis by USDA-FSIS on further reducing *Salmonella*, poultry plants have increased their reliance on the water chlorination program in the processing plant including pre-scald bird brushes, equipment rinses, inside/outside bird washers, carcass washes, and as a disinfectant during chilling. SH is commonly used as a poultry carcass wash in processing plants in many countries, its effectiveness maybe demonstrated on seafood carcasses, such as catfish muscle.

1.8.4 Acidified sodium chlorite

Acidified sodium chlorite (ASC) possesses antimicrobial properties and is intended for use primarily as a spray or a dipping solution for poultry, meats, vegetables, fruits and sea foods.

It is also used in poultry chilling water. ASC is produced by the addition of a food-grade acid (e.g., citric acid, phosphoric acid, hydrochloric acid, malic acid, or sodium hydrogen sulfate) to an aqueous solution of sodium chlorite (NaClO2). Combining acid with sodium chlorite solution results in the conversion of chlorite into metastable chlorous acid (HClO2), which can subsequently form a mixture with chlorite (ClO2), chlorine dioxide (ClO2) and chloride (Cl^{-}) that aids in its effectiveness. The reaction, therefore, generates an oxidative solution with oxy-chlorine species with antimicrobial properties. These compounds act by disrupting microbial membranes and oxidizing cellular components. ASC is a GRAS chemical having food processing applications and can be used as a preservative (Ricke 2003). ASC has the ability to maintain antimicrobial efficacy in the presence of organic matter (Cherrington 1992).

It is allowed to be used for both indirect food contact surface sanitizing and secondary direct antimicrobial food treatment. Indirect food additives and secondary direct food additives are regulated differently by the FDA. Secondary direct additives are applied directly to food, but they only have a technical effect during food processing and handling and do not persist and continue to have a technical effect in the finished food product. The FDA specifically addresses the allowed food contact uses of ASC (§173.325 of the CFR). It may be used in meat, fish, and poultry processing, as well as on both raw and processed agricultural commodities, fruits and vegetables (FDA). Some uses have specific restrictions regarding rinses and maximum rates of use. ASC solutions are commonly used to kill bacteria, viruses, fungi and algae.

Significant research has been conducted that found organic acids and other chemical treatments are effective on food products such as raw meat and fish. A more current investigation is needed to determine if other food antimicrobial treatments such as lactic acid, sodium

benzoate, sodium hypochlorite, and acidified sodium chlorite are effective on domestic farm raised and wild caught catfish carcasses.

1.9 Conclusion

Biological foodborne hazard literature associated with catfish is dated and limited. *Salmonella* appears to be a common etiologic agent for catfish foodborne illness outbreaks (CDC 1991, 2007). This review consists of investigations of specific pathogens in aquaculture, including catfish, on the continuum of production, procession, and retail products. In many of these studies *Salmonella* was identified as a potential foodborne hazard associated with catfish consumption. In addition, the findings in these investigations suggest that *Salmonella* strains found in catfish exhibit antibiotic resistance characteristics (Broughton 2009, Lee, 2010, Zhao 2003). *Salmonella* appears to be the most important microbial hazard associated with catfish consumption. The prevalence of other microbial pathogens such as *E. coli, S. aureus,* and Coliform are included in this review. According to past investigations organic acids and specific chemicals are important components in creating catfish carcass antimicrobial treatments suitable for safe product processing and consumption in the attempt to prevent spoilage, economic loss, and pathogenic illnesses.

Globally the aquaculture industry has rapidly grown over the last decade. Aquaculture is the most rapidly increasing food production system, and the industry will most likely continue to grow through 2025 (Diana 2009). Previous studies support the idea that the continuation of microbial and chemical testing of domestic farm raised and wild caught catfish is needed to ensure the safety of consumers, producers, and processors. The aim of this investigation is to isolate and identify the prevalence of *Salmonella spp.* in retail catfish, determine susceptibility

of clinical treatments to possible isolates, examine the level of sanitation between sample source and retail markets, and measure the effectiveness of antimicrobial chemical treatments to combat contamination in wild caught and farm raised retail catfish.

CHAPTER 2. PREVALENCE: ISOLATION, IDENTIFICATION, AND ANTIBIOTIC RESISTANCE OF *SALMONELLA SPP.* FROM CHANNEL CATFISH CARCASSES

2.1 Introduction

Channel catfish, *Ictalurus punctatus,* is an integral agricultural commodity that ranked as the sixth most frequently consumed aquatic food in the United States (NFI 2009). Channel catfish, order Siluriformes, is a vast differential group of ray finned fish (Ferraris 2007). Ictaluridae catfish are the leading aquaculture-produced seafood, generating approximately half the freshwater aquaculture value in 2014 (NOAA 2016b). Channel catfish can be found primarily within the southern region of the United States (NASS 2010). However, other sources are imported from several countries, including Cambodia, Canada, China, Indonesia, Mexico, Panama, Peru, Thailand, and Vietnam (NASS 2010).

Congressional amendments to the Food Meat Inspection Act (FMIA) classified catfish as a rectifiable species subject to regulation by USDA FSIS (Anonymous 2008). Consequently, imposing risk assessments on domestic catfish products that necessitate the analysis of foodborne pathogenic hazards associated with its consumption. Specific hazards include the prevalence of *Salmonella spp.* in catfish for consumption.

In relation, *Salmonella* is among the leading agents of foodborne disease in the U.S. (CDC 2009). *Salmonella spp.* are gram-negative, rod-shaped bacteria that cause salmonellosis (Adams 2004) that includes but not limited to enteric fever and acute gastroenteritis (Hohmann 2001). *Salmonella* can be introduced into aquaculture ponds from a variety of sources, including fecal contamination from birds and other wildlife (Berg 1972, Koonse 2005, Mikaelian 1997) and contaminated feed (Lunestad 2007). After exposure to *Salmonella*, catfish retained the pathogen

in the intestinal tract for up to 30 days with no clinical signs of infection (Lewis 1975). This act of harboring the pathogen can be a threat to the consumption of catfish by way of processing procedures that may cause cross contamination from the intestine throughout the carcass.

Alternative processing procedure that has resulted in public health concerns is the overexposure to antibiotic treatments. The excessive usage of antibiotics as a therapeutic drug during catfish rearing exacerbates the selective pressure that enriches microbial populations for antibiotic-resistant bacteria. Generally, antibiotic resistance refers to the temporary or permanent capacity of an organism and its progeny to remain viable and multiply under lethal or inhibitory conditions (Cloete 2003). Although antibiotic resistance can be a result of sudden mutation without the presence of antibiotic use (Song 2005), experts and government agencies agree that the increase in antibiotic resistance is associated with an increase in antibiotic use in clinical and agricultural settings. Clinically in 1989, according to the Institute of Medicine, the use of antibiotics estimated annually 50 million pounds. In comparison to the Animal Health Institute estimated that about 20 million pounds of antibiotic were administered to animals in 2003.

Due to the over usage of antibiotics as a clinical or therapeutic drug and the variety of serotypes that make up the genus *Salmonella* it is difficult to generalize about antibiotic resistance in *Salmonella* (CDC 2005). An increase in the resistance of *Salmonella* to commonly used antibiotics is represented in public health and veterinary sectors (Gebre-Yohannes 1985, Molla 2000, Ashenafi 1985). As a resistance factor, serovar prevalence changes should be included when investigating trends in antibiotic resistance, clinically. *Salmonella* serovar Typhimurium DT104 is resistant to at least five antibiotics to date that has been associated with dairy and beef products which resulted from integrons (Cody 1999, Villar 1999). Over recent years, studies found *Salmonella* serovar Newport multidrug resistance MDR-AmpC is resistant

to at least four additional antibiotics and has received attention due to an epidemic spread that sourced from a clonal population. *Salmonella* isolates are usually resistant to ampicillin, aztreonam, cefazolin, cefepime, cefpodoxime, ceftazidime, ceftiofur, cefuroxime, cephalothin, chloramphenicol, streptomycin, sulfamethoxazole, trimethoprim, and ceftriaxone (Muleta 2001).

A growing incidence of extended spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*, and consequently a rapidly increasing antimicrobial resistance (AMR) is a public health concern (WHO 2014, ECD 2016). It is caused by excessive antimicrobial use in humans and animals in addition to inadequate infection prevention and control practices (Holmes 2016). Humans as well as domestic and wild animals harbor ESBL producing bactecteria in the intestine, and those bacteria are more often found in the environment as prevalence increases (Jorgensen 2017). Resistance is encrypted on plasmids and chromosomal island that include two extended spectrum ß-lactamase (ESBL) genes: CTXM-15 and SHV-12 (Hendriksen 2009). *Salmonella* antibiotic resistance is predominantly based on serovar type and rate of mutation.

In addition to determining the incidence of *Salmonella* in retail wild caught and farm raised catfish, it is vital to determine the possible level of antibiotic resistance to potential isolates if clinical and medicinal attention is necessary.

A few outdated studies in which catfish has the potential to harbor *Salmonella* has been explored, some have included comparison of the aquatic environment to catfish, and relative fish alike, microbial loads while others have focused on the processing equipment or retail catfish products. This study covers the presence of *Salmonella spp.* specifically in domestic retail wild caught and farm raised channel catfish, with the caveat that although older reports are available for review, it is unclear that the data accessible reflects the current state of sanitation,

aquaculture, and food technology. Further, the investigation of possible *Salmonella* isolates from the specified matrices may possess antibiotic resistance to specific clinical treatments.

2.2 Materials and Methods

2.2.1 Sample collection, preparation, and enrichment

Fresh channel catfish samples 5 wild caught (WC), 5 farm raised (FR) were randomly collected monthly for 24 months (n = 240) from 22 local, retail stores and seafood markets. The samples were transported in its original package on ice. Once at the laboratory the samples were stored at 4°C for 24 hours maximum. Intact packages were disinfected at the incision sites with 70% isopropanol. Representative catfish tissue samples 25g ±2.5g were aseptically removed and homogenized with 225ml ±4.5ml Buffered Peptone Water (BPW) (Neogen) in a sterile polypropylene bag (Whirl-Pak®) (ca. 24" x 30-36"). The pre-enrichment medium and catfish tissue samples were stomached for 2 minutes and incubated at 35°C±2°C for 22-26 hours.

This study followed a modified combination of USDA FSIS Microbiology Laboratory Guide "Isolation and Identification of *Salmonella* from Meat, Poultry, Pasteurized Egg, and Siluriformes (Fish) Products and Carcass and Environmental Sponges (MLG 4.08) and "FSIS Procedure for the Use of a Polymerase Chain Reaction (PCR) Assay for Screening *Salmonella* in Meat, Poultry, Egg, and Siluriformes (Fish) Products and Carcass and Environmental Sponges" (MLG 4C.07) for screening.

2.2.2 *Salmonella* test screening

Salmonella positive controls groups ATCC 14028 (typical H_2S+) and ATCC 29934 (atypical H_2S-) and a negative control of media were used parallel to the primary experiment to improve validity. Extraction of 5µl of the incubated catfish sample enrichment was added to prefilled

lysis buffer (200µl ± 20 µl) tubes. The sample lysis tubes were heated for 20 minutes at 37°C±2°C using Digital 2 Block Heater (VMR) heating blocks. The sample lysis tubes were then transferred to a 95°C ±3°C Digital 2 Block Heater heating block for 10 minutes. The sample lysis tubes were cooled in a previously refrigerated Molecular Detection Chill Block (3M™) at 4°C ±0.5°C for at least 5 minutes. Propriety primers and probes PCR tablets were hydrated by 50 µl of lysate. The tablets were loaded to the BAX® Q7 PCR instrument for detecting the unique DNA fragment found in *Salmonella spp.* presented in Table 3. Cultural analysis continued for sample enrichment that resulted BAX®-positive, BAX®-indeterminate, or BAX® signal-error result. These samples were labeled presumptive *Salmonella* positives.

Table 3. *Salmonella spp.* gene selected and amplified by BAX Q7 PCR tableted primers and probes.

Salmonella spp. (invA) gene	
Forward	CAACGTTTCCTGCGGTACTGT
Reverse	CCCGAACGTGGCGATAATT
Probe	-CTCTTTCGTCTGGCATTATCGATCAGTACCA-

DuPont Qualicon BAX System User Guide

2.2.3 Cultural analysis of presumptive *Salmonella* positives

2.2.3.1 Selective enrichment and plating media

BAX®Q7-positive catfish enrichment samples and all controls were examined by traditional cultural analysis. Extraction of 0.5ml ±0.005ml of enriched sample and controls were transferred individually into 10ml of Tetrathionate Broth (TT) (Hajna) (Neogen) and 0.1ml

±0.02ml into 10ml Modified Rappaport Vassiliadis Broth (mRV) (Neogen). TT and mRV broths were incubated at 42°C ±1°C for 22-24 hours. The incubated TT and mRV broths were vortexed and streaked for isolation to Hektoen Enteric (HE)(Neogen), Xylose Lysine Desoxycholate (XLD)(Neogen), and Bismuth Sulfite (BS)(Neogen) agar plates using a 10µl loopful of inoculum for each plate. The entire agar plate was streaked with single sample enrichment and incubated at 35°C ±2°C for 18-24 hours.

2.2.3.2 Examination of colonies from selective media

The selective-differential incubated agar plates of the enriched samples and controls were examined for suspect *Salmonella* colonies. Isolated suspected colonies were picked (HE—blue green to blue colonies with (H_2S+) or without (H_2S-) black centers, XLD—pink colonies with (H_2S+) or without (H_2S-) black centers, BS—grey colonies with clear/opaque halo). Triple Sugar Iron agar (TSI)(Hardy) and Lysine Iron agar (LIA)(Hardy) slants were inoculated in tandem with a single pick from a selected colony by stabbing the butts and streaking the slants in one motion. TSI and LIA were incubated at 35°C ±2°C for 24 ±2hours. TSI and LIA slants were then examined after incubation. Observation of the butt and slant colors, blackening of the media, and the presence of gas was documented. Using the positive and negative controls as a reference: typical control (ATCC 14028) on LIA produced a purple butt with H_2S+ or H_2S- blackening of the media, typical control on TSI produced a yellow butt and red slant with H_2S+ or H_2S- blackening of the media. TSI and LIA of samples and controls displaying *Salmonella* biochemical reactions were further tested for molecular serotyping (Table 4). Isolates giving typical *Salmonella spp.* reactions and isolates that are suggestive but not typical of *Salmonella spp.* were confirmed by a combination of biochemical and serological procedures.

Table 4. Potential *Salmonella* reaction criteria requiring biochemical analysis.

Triple Sugar Iron (TSI)			Lysine Iron (LIA)		O Group and H Antigen		Testing/Disposal
Butt	Slant	H_2S	Butt	H_2S	O	H	
Y	R	+	P	+	+	+	BcT
Y	R	+	P	+	+	-	BcT
Y	R	-	P	-			BcT
Y	R	-	Y	-	+	+	BcT
Y	R	-	Y	-	-	-	BcT
Y	R	+	Y	+/-			BcT
Y	Y	-	Y or P	-			Discard
Y	Y	+	P	+			BcT
NC	NC						Discard

[a] Triple Sugar Iron (TSI) and Lysine Iron (LIA): Y= yellow (acid (A) reaction); R= red (alkaline (K) reaction); P=purple (alkaline (K) reaction)
[b] Testing/Disposal: Bct= biochemical testing (VITEK®2 Compact System); NC= no change in color from uninoculated medium.

2.2.4 Serological Tests—somatic (O) and flagellar (H) antigen agglutination

A loop of presumptive *Salmonella* culture growth from TSI or LIA slants was tested with 1 drop of polyvalent O antiserum on slides. Negative control, dispensed 1 drop of saline, control for autoagglutination, and 1 drop of polyvalent O antiserum, mixed thoroughly. The slides were rotated for 1 minute and determined for agglutination. Positive isolates were identified by individual O group results. If the isolate test positive for multiple individual O groups, the isolates were identified by the poly group. The saline control was noted as an auto-agglutinater if it reacted with antiserum. Isolates that are recovered as serologically poly H+ and is non-reactive with O group antisera was reported as "*Salmonella* non-A-I + Vi". The isolates were then tested for flagellar (H) antigen agglutination using Oxoid *Salmonella* Latex Test kit.

2.2.5 Biochemical identification-VITEK®2 Compact

Culture growth from TSI slants were individually suspended into 3ml sterile saline (aqueous 0.45-0.5% NaCl, pH 4.5-7.0) in clear 12 x 75 mm polystyrene test tubes. The homogenous organism suspension tubes were read by DensiChek Plus instrument to measure the optical density of the suspension using the McFarland units. Tubes were diluted (decreased) or inoculated (increased) to a density of 0.5±0.06 McF. Positive controls *Salmonella* ATCC 14028 and ATCC 29934 were identified parallel to the primary test. Suspected *Salmonella* isolates and positive controls were loaded into the VITEK®2 Compact with individual gram-negative (GN) biochemical test cards for sample identification.

2.2.6 Antibiotic susceptibility testing

Antibiotic susceptibility testing (ampicillin, amoxicillin/clavulanic acid, ampicillin/sulbactam, piperacillin/tazobactam, cefazolin, ceftazidime, ceftriaxone, cefepime, ertapenem, imipenem, gentamicin, tobramycin, ciprofloxacin, levofloxacin, nitrofurantoin, and trimethoprim/sulfamethoxazole) was conducted using pure culture inoculums of confirmed isolates from the *Salmonella* screening test of wild caught and farm raised retail catfish. Pure isolates were selected from Tryptic Soy agar (TSA) with 5% sheep blood plates (Thermo Scientific). 3.0 ml of sterile saline (aqueous 0.50% NaCl, pH 7.0) was aseptically transferred into clear polystyrene test tubes (12 mm x 75 mm). A swab transfer of sufficient similar morphological colonies was inoculated into an organism suspension with a density equivalent of 0.5±0.06 McF using the DensiCheck™ Plus. A second polystyrene tube with 3.0 ml sterile saline is inoculated with 145 µl of the original organism suspension. The secondary tubes and antibiotic susceptibility test gram-negative (AST-GN69) cards were loaded into the VITEK ®2 Compact.

The criteria used to select the antibiotic drugs to be tested were based on local clinical need and use for treating salmonellosis. *E. coli* ATCC 25922, ESBL negative, was used as a reference strain that is susceptible to all antibiotic drugs tested and was examined parallel to the confirmed *Salmonella* isolates derived from screening. Two independent analyses were conducted for AST.

2.2.7 Statistical analysis

All data were analyzed using Pearson's Chi Squared analysis (SAS Institute Inc., Cary, NC, USA). Statistical significance occurred at $P<0.05$. Experiments were a random categorical survey, each with primary determination.

2.3 Results and Discussion

Due to the concerns of pathogens such as *Salmonella,* the FSIS placed greater emphasis on risk assessments of catfish processors. Such assessments require the evaluation of the occurrence of *Salmonella* in retail market catfish samples.

2.3.1 Prevalence of Salmonella in domestic retail channel catfish

Generally, screening of retail channel catfish samples (n = 240) resulted in low incidences of *Salmonella* contamination throughout the 24-month survey (Table 5). Presumed *Salmonella* occurrence appeared in 10 out of 240 samples. Survey by BAX Q7 PCR resulted in 4 false positives that were confirmed serologically. However, biochemical identification analysis determined 4 BAX Q7 PCR presumptive positives as *Hafnia alvei, Vibrio cholera non01 non0139, Pseudomonas aeruginosa, and Proteus mirabilis,* respectively.

Confirmatory and biochemical identification analysis concluded 6 of 240 samples proved *Salmonella spp.* positive. The prevalence of *Salmonella* contamination in domestic retail catfish

in this study is 2.5%. Previous studies have detected *Salmonella* in catfish ponds, processing plants, and retail products. Studies at these assorted sites along the farm-to-fork continuum have rendered vast differences in results for *Salmonella* prevalence. In one study, *Salmonella* was detected in densely stocked ponds at 40% occurrence, two out of five skin and viscera samples, and 11 of 15 dressed (fully skinned and eviscerated), 73%, catfish samples (Wyatt 1979). A more recent study, *Salmonella* was not isolated from catfish pond waters, also stocking density did not affect *Salmonella* levels (MacMillan 1990). Changes of a period of time, sampling, testing methods, storage, and production may be responsible for the variations among results with the same objective of determining the occurrence of *Salmonella* in domestic channel catfish.

Table 5. Occurrence of presumptive and confirmed *Salmonella spp.* in wild caught (WC) and farm raised (FR) retail catfish.

Sample	Sample ID	Type	BAX*	Vitek 2**	Result
10	10-MTN-FR110215	FR	Positive	*Hafnia alvei*	Negative
60	60-ALB-FR032216	FR	Positive	*Vibrio cholera non01, non0139*	Negative
89	89-TNY-WC071816	WC	Positive	*Salmonella*	Positive
90	90-TNY-WC071816	WC	Positive	*Salmonella*	Positive
103	103-BCS-WC091316	WC	Positive	*Salmonella*	Positive
55	55-CCS-WC032817	WC	Positive	*Salmonella*	Positive
89	89-WLM-FR062017	FR	Positive	*Pseudomonas aeruginosa*	Negative
90	90-ALX-FR062017	FR	Positive	*Proteus mirabilis*	Negative
105	105-BCS-WC082317	WC	Positive	*Salmonella*	Positive
106	106-BCS-WC082317	WC	Positive	*Salmonella*	Positive

* BAX Q7 screen results are presumed *Salmonella* positive in leu of serological confirmation.
**Vitek2 identification results are final confirmation post serological confirmation.

2.3.2 Significance of catfish source—wild caught and farm raised

Salmonella prevalence of WC (*n = 120*) and FR (*n = 120*) were significantly affected by sample sources. WC sample type resulted in 60% *Salmonella* presumptive occurrences among all sample type isolates (*n = 10*), yet, 5% within the total surveying type (*n = 120*) (Table 6). WC presumptive positive isolates were confimed six out of six times via serological analysis and biochemical identification. FR, however, resulted in four presumptive *Salmonella* isolates that were identified biochemically as other gram-negative relative bacteria. FR sample type did not have any *Salmonella* positive occurrences confirmed or identified (Figure 2). The sample sources are significantly different, p-value = 0.002. Hence, the null hypothesis is rejected. The conclusion that there is a compelling relationship between channel catfish sample types and both types are dependent. However, the probability value is bias and unbalanced due to zero confirmed positve isolates sourced as FR. Continued screening is recommended for a more balanced statistical analysis using Fisher chi squared analysis.

Contrary to the findings, previous studies concluded a distintly different result. In a study, catfish samples from both retail and processing facilities to compare fresh farm raised and commercially wild caught catfish and catfish imported from Mexico and Brazil, 21% (11 of 52) farm raised fish were positive for *Salmonella* in comparison to 5% (2 of 40) wild caught fish were positive for *Salmonella* (Wyatt 1979). The imported fish samples (*n = 61*) were negative for *Salmonella.* In order to improve consistancy in determining the prevalence of *Salmonella* in wild caught and farm raised catfish more investigative surviellance is required.

Table 6. Prevalence of *Salmonella* isolates from WC and FR catfish sample source.

Type	Presumptive Positive	Confirmed Positive
Farm raised	4	0
Wild caught	6	6
Total	10	6

2.3.3 Seasonal influence on the presence of *Salmonella* within confirmed isolates

Examination of WC and FR channel catfish samples (*n = 240*) displayed a small correlation between confirmed *Salmonella* isolates and annual season change. According to Farmer's Almanac, seasons are defined as spring (March 20th – June 20th), summer (June 21st – September 22nd), fall (September 23rd – December 20th), and winter (December 21st – March 19th). Categorically, confirmed *Salmonella* isolate incidences displayed an increased occurrence in the summer months (Figure 3). During the defined summer months seven channel catfish samples were presumed positive for *Salmonella* via PCR of ten presumptive positives in totality (Table 7). Confirmatory methods concluded during the summer months five of seven presumptive *Salmonella* isolates were identified as the targeted pathogen. During the cooler months, fall and winter, incidence of presumptive *Salmonella* contamination was isolated in one sample. However, the isolate was identified to be a relative bacterial contamination but not *Salmonella.* Therefore, there were no incidences of *Salmonella* contamination during the fall and winter months. The occurrence of one confirmed isolate was detected during fall as the warmer season approached.

Studies of the variations of *Salmonella* growth throughout the year yielded conflicting results. In one study, an increased incidence of *Salmonella*–positive farm raised catfish product

collected from July through September (summer) was found compared with products collected from January to March (Andrews 1977). In contrast, *Salmonella* surveyed off of a production line at various processing plants showed no seasonal effects on growth (McCaskey 1998). *The* results, in this study, of the interaction between seasonal months and the prevalence of *Salmonella* in channel catfish reveal there may be a trend if more isolates were detected throughout screening. Although there is an initial distinction between warmer and cooler months and their impact on *Salmonella* occurrence, further investigation is required to conclude such a theory.

Table 7. Presumptive and confirmed *Salmonella* occurrences within four seasons.

Season	Presumptive Positive	Confirmed Positive
Fall	1	0
Spring	1	1
Summer	2	5
Winter	0	0
Total	4	6

2.3.4 Antibiotic susceptibility testing

The prevalence of *Salmonella* in wild caught and farm raised channel catfish samples from 21 retail markets were 2.5%. For these *Salmonella* isolates, resistance occurred in cefazolin, gentamicin, and tobramycin from all isolates (Table 8). This demonstrates multidrug resistance (MDR) (resistant to three or more antimicrobial agents) and showing resistant trends in *Salmonella*. The resistance to cefazolin was ≤4 μg/ml, where the MIC range is 4-64 μg/ml. Gentamicin and tobramycin was ≤1 μg/ml with a MIC range of 1-16 μg/ml.

Table 8. Minimum Inhibitory Concentration (MIC) and resistance interpretation of confirmed *Salmonella* isolates treated with selected antibiotics in vitro diagnostics.

Antibiotics [a]	*Salmonella* isolates [c]					
	55-1	89-2	90-5	103-5	105-1	106-2
	MIC (µg/ml) / Interpretation [b]					
AM	≤ 2 / S	≤ 2 / S	≤ 2 / S	≤ 2 / S	≤ 2 / S	≤ 2 / S
AMC	≤ 2 / S	≤ 2 / S	≤ 2 / S	≤ 2 / S	≤ 2 / S	≤ 2 / S
SAM	≤ 2 / S	≤ 2 / S	≤ 2 / S	≤ 2 / S	≤ 2 / S	≤ 2 / S
TZP	≤ 4 / S	≤ 4 / S	≤ 4 / S	≤ 4 / S	≤ 4 / S	≤ 4 / S
CZ	≤ 4 / R	≤ 4 / R	≤ 4 / R	≤ 4 / R	≤ 4 / R	≤ 4 / R
CAZ	≤ 1 / S	≤ 1 / S	≤ 1 / S	≤ 1 / S	≤ 1 / S	≤ 1 / S
CRO	≤ 1 / S	≤ 1 / S	≤ 1 / S	≤ 1 / S	≤ 1 / S	≤ 1 / S
FEP	≤ 1 / S	≤ 1 / S	≤ 1 / S	≤ 1 / S	≤ 1 / S	≤ 1 / S
ETP	≤ 0.50 / S	≤ 0.50 / S	≤ 0.50 / S	≤ 0.50 / S	≤ 0.50 / S	≤ 0.50 / S
IPM	≤ 0.25 / S	≤ 0.25 / S	≤ 0.25 / S	TRM	≤ 0.25 / S	≤ 0.25 / S
GM	≤ 1 / R	≤ 1 / R	≤ 1 / R	≤ 1 / R	≤ 1 / R	≤ 1 / R
TM	≤ 1 / R	≤ 1 / R	≤ 1 / R	≤ 1 / R	≤ 1 / R	≤ 1 / R
CIP	≤ 0.25 / S	≤ 0.50 / S	≤ 0.25 / S	≤ 0.25 / S	≤ 0.25 / S	≤ 0.50 / S
LEV	≤ 0.12 / S	≤ 0.12 / S	≤ 0.12 / S	≤ 0.12 / S	≤ 0.12 / S	≤ 0.12 / S
FT	≤ 16 / S	≤ 32 / S	≤ 32 / S	32 / S	≤ 16 / S	≤ 16 / S
SXT	≤ 20 / S	≤ 20 / S	≤ 20 / S	TRM	≤ 20 / S	≤ 20 / S

[a]Antibiotic treatments ampicillin (AM), amoxicillin/clavulanic acid (AMC), Ampicillin/Sulbactam (SAM), Piperacillin/Tazobactam (TZP), Cefazolin (CZ), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefepime (FEP), Ertapenem (ETP), Imipenem (IPM), Gentamicin (GM), Tobramycin (TM), Ciprofloxacin (CIP), Levofloxacin (LEV), Nitrofurantoin (FT), and Trimethoprim/Sulfamethoxazole (SXT) applied to confirmed *Salmonella* isolates to examine resistance or susceptibility response.
[b]Minimum Inhibitory Concentration (µg/ml) (MIC), Interpretations: S (susceptible), R (resistant), TRM (drug terminated, insufficient growth in positive control well).
[c] *Salmonella* isolates identification, agar position from source: 55-1 (55-CCS-WC032817), 89-2 (89-TNY-WC071816), 90-5 (90-TNY-WC071816), 103-5 (103-BCS-WC091316), 105-1 (105-BCS-WC082317), 106-2 (106-BCS-WC082317).

Reference strain *E. coli* 25922 was extended spectrum ß-lactamase (ESBL) negative and susceptible to all applied antibiotics as anticipated (Table 9). *S. typhimurium* 14028 and *S. entrica subsp. diarizonae 29934* expressed MSR to cefazolin, gentamicin, and tobramycin as the screened isolates from the catfish samples. *V. cholera non01, non 0139* were resistant to

ampicillin ≥32 μg/ml, MIC range 2-32 μg/ml and nitrofurantoin ≤16 μg/ml with a MIC range of 16-512 μg/ml. In a chicken egg study of antibiotic resistand of *Salmonella enterica subsp. enterica* serovars *Typhimurium, Enteritidis,* and *Typhi* isolates were resistant to cefazolin at 36.5 % of screened isolates (Al 2016).

In relation to MDR pattern to cefazoilin, gentamicin, and tobramycin, it is noteworthy that the range of drugs to which resistance was acquired is wide and of concern, showing the seriousness of the emergence of this pathogen's antibiotic resistance. In an additional study, cefazolin was only effective against *Salmonella* isolates 15.4% from a pig slaughter processing plant in Romania (Morar 2015). MDR was shown in cefazolin, gentamicin, and tobramycin as a trend in an antibiotic resistance study from chicken carcass *Salmonella* isolates in retail markets in Myanmar. In this study the MDR pattern of the subjected antibiotics were cefazolin 7.2%, gentamicin 8%, and tobramycin 8.7% (Moe 2017). Previous studies exhibit different incidences of antibiotic resistance in *Salmonella* isolates of various matrices at different percentages. However, in this investigation *Salmonella* had 100% MDR patterns to cefazolin, gentamicin, and tobramycin, it is apparent to continue surveying retail wild caught and farm raised channel catfish for the prevalence of *Salmonella* contamination and its resistance to clinical antibiotics as treatment.

Table 9. Minimum Inhibitory Concentration (MIC) and resistance interpretation of control isolates *Salmonella* ATCC 14028, *Salmonella* ATCC 29934, *E. coli* ATCC 25922, and *V. cholera* treated with selected antibiotics in vitro diagnostics.

Antibiotics [a]	Reference strains [c]			
	Sal 14028	*Sal* 29934	*Ec* 25922*	*V. chloera*
	MIC (µg/ml) / Interpretation [b]			
AM	≤ 2 / S	≤ 2 / S	8 / S	≥ 32 / R
AMC	≤ 2 / S	≤ 2 / S	4 / S	≤ 2 / S
SAM	≤ 2 / S	≤ 2 / S	4 / S	4 / S
TZP	TRM	TRM	≤ 4 / S	≤ 4 / S
CZ	≤ 4 / R	≤ 4 / R	≤ 4 / S	≤ 4 / S
CAZ	≤ 1 / S	≤ 1 / S	≤ 1 / S	≤ 1 / S
CRO	≤ 1 / S	≤ 1 / S	≤ 1 / S	≤ 1 / S
FEP	≤ 1 / S	≤ 1 / S	≤ 1 / S	≤ 1 / S
ETP	≤ 0.50 / S	≤ 0.50 / S	≤ 0.50 / S	--------
IPM	≤ 0.25 / S	≤ 0.50 / S	≤ 0.25 / S	0.5 / S
GM	≤ 1 / R	≤ 1 / R	≤ 1 / S	≤ 1 / S
TM	≤ 1 / R	≤ 1 / R	≤ 1 / S	≤ 1 / S
CIP	≤ 0.25 / S	≤ 0.50 / S	≤ 0.25 / S	≤ 0.25 / S
LEV	≤ 0.12 / S	≤ 0.12 / S	≤ 0.12 / S	≤ 0.12 / S
FT	≤ 16 / S	≤ 32 / S	≤ 16 / S	≤ 16 / R
SXT	≤ 20 / S	≤ 20 / S	≤ 20 / S	≤ 20 / S

[a]Antibiotic treatments ampicillin (AM), amoxicillin/clavulanic acid (AMC), Ampicillin/Sulbactam (SAM), Piperacillin/Tazobactam (TZP), Cefazolin (CZ), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefepime (FEP), Ertapenem (ETP), Imipenem (IPM), Gentamicin (GM), Tobramycin (TM), Ciprofloxacin (CIP), Levofloxacin (LEV), Nitrofurantoin (FT), and Trimethoprim/Sulfamethoxazole (SXT) applied to confirmed *Salmonella* isolates to examine resistance or susceptibility response.

[b]Minimum Inhibitory Concentration (µg/ml) (MIC), Interpretations: S (susceptible), R (resistant), TRM (drug terminated, insufficient growth in positive control well).

[c] Reference isolates: *Sal* 14028 (*Salmonella typhimurium* ATCC 14028 H_2S+), *Sal* 29934 (*Salmonella enterica subsp. diarizonae* ATCC 29934 H_2S-), *Ec* 25922 (*E. coli* ATCC 25922), *V. cholera* (*non01, non0139).*

* Extended-Spectrum ß-Lactamases (ESBLs) tested negative.

2.4 Conclusion

This investigation demonstrated that there is a minimal occurrence of *Salmonella* contamination in domestic, retail wild caught and farm raised channel catfish. Although there

have been conflicting studies regarding the correlation between the targeted pathogen and catfish, there was infinitesimal evidence that *Salmonella* is a substantial threat to consumers. There is however, an interaction between wild caught catfish and *Salmonella* contamination within the limited occurrences. Confirmatory and biochemical identification proved essential in determining false positives and actual *Salmonella* contamination. Seasonal changes have an effect on the incidence of *Salmonella* contamination in wild caught and farm raised channel catfish. Although primitive data have fluctuating results on the effects of seasonal changes, warmer months displayed an increased occurrence of *Salmonella* contamination in channel catfish. Antibiotic resistance is abundant in limited and select drug treatments of *Salmonella* surveyed isolates in vitro. More investigations are necessary to identify primary sources of *Salmonella* contamination in channel catfish, evaluations of the potential seasonality of *Salmonella* in channel catfish, any impact of aquaculture practices on the growth of this pathogen and determine antibiotic resistance to specific clinical treatments.

CHAPTER 3. PRESENCE OF SANITARY INDICATOR MICROORGANISMS ON CHANNEL CATFISH CARCASSES

3.1 Introduction

Catfish is a source of high-quality protein (Ferreira 2006). The degradation of fish is accelerated by microorganisms associated with aquatic environments as well as contaminants during post-harvest handling (Jay 1992). Postmortem, microorganisms on fish surfaces including the gut and gills begin to utilize the fish protein (Ames 1992). Microbial activities create undesirable alterations such as off-flavors, texture and appearance (Johnstone 1994). The rate of bacterial spoilage is dependent on the initial microbial load, ambient temperature and improper handling. Therefore, proper storage is critical in maintaining a high standard of quality when processing fish (Jay 1992).

Loss of quality in catfish may not be limited to one microorganism but rather to a variety of microorganisms due to its unrestricted environment. Bacterial groups can be subgroup into two categories: indigenous and post-harvest bacteria (Purvis 2002). The microbiological diversity of fresh fish muscle depends on the fishing grounds and environmental factors around it (Cahill 1990). Preceding studies have also shown incidence of indicator microorganisms of fecal pollutants, opportunistic and pathogenic bacteria to humans in fish (Da Silva 2002, Tsai 2002, Ferreira 2006, Tzikas 2007).

In such cases it is often more practical to determine the counts of groups of microorganisms most likely to cause spoilage and/or illness. The outcome of fish spoilage is important, and it is greater than the loss of protein. Foodborne illnesses have resulted in great

economic losses because of contaminated fish consumption. The association with microbes and fish endangers quality and safety for consumption; it is especially important when microorganisms are pathogenic and opportunistic (WHO 1996). Indicator bacteria tests are necessary to monitor food, water, foodborne pathogen contamination and microbiological quality. The quantification of indicator bacteria equates to the level of fecal contamination, a sign of deviation from set process control standards and good manufacturing practices (GMP), to determine the organoleptic characteristics of food degradation (Doyle 1997). Indicator microorganisms commonly tested are aerobic plate counts (APC), coliform including *E. coli,* and *Staphylococcus spp.*

APC or standard plate count is used to assess the sum number of microorganisms in a food product. The use of APC can screen for groups of organisms that are anaerobic, thermoduric, mesophilic, thermophilic, psychrophilic, proteolytic and lipolytic. Levels of APC of refrigerated perishable food products such as milk, meat, poultry, and fish may be used to indicate equipment condition, time/temperature of storage and distribution (Doyle 1997). However, APC values only measure live cells. Therefore, is not beneficial to determine the quality of heat processed foods. Furthermore, APC is essential when used to index sanitary and organoleptic quality, and safety (Silliker 1963).

Coliforms, including *E. coli* may exist in food processing plants, surrounding environments, and ultimately in processed foods. *E. coli* signifies a risk of enteric pathogens in food because it is a part of the normal bacterial flora found in the intestinal tracts of humans and animals (Doyle 1997). Coliform is used as a component of microbiological standards that asses the quality of seafood and aquaculture waters (Hackney 1994). Coliforms can become

established on equipment and contaminate food that are processed by the equipment. *E. coli* is widely used as an indicator of fecal contamination. However, detection of *E. coli* does not assure the absence of enteric pathogens (Silliker 1976).

Enterotoxigenic *staphylococci* can be present on food product. Time and temperature are key factors in the formation of enterotoxin, which can cause illness (Le Loir 2003). The prevalence of *Staphylococcus* food illnesses is caused by consumer temperature and time abuse, and food service not the direct result of contamination at the farm or processing facility (Le Loir 2003).

The key factors of sanitary indicator microorganism pose the need to investigate such opportunistic and/or pathogenic bacteria. The enumeration of sanitary indicator microorganisms, aerobic bacteria, *Escherichia coli*, coliforms, and *Staphylococcus aureus* of various retail wild and farmed channel catfish carcasses will offer insight into their prevalence.

3.2 Material and Methods

3.2.1 Sample Collection and Preparation

Catfish samples (5 wild, 5 farmed) were randomly collected monthly for 12 months (*n =120*) from 21 local, retail stores and seafood markets. The samples were transported in its original package on ice and stored at 4°C up to 24 hours. Intact packages were disinfected at the incision sites with 70% isopropanol.

3.2.2 Determination of Microbial Counts

3.2.2.1 Aerobic Plate Counts and E. coli / Coliform Counts

Aerobic plate counts (APC) and *Escherichia coli*/Coliform Count (EC) were analyzed using 3M™ Petrifilms for refrigerated raw meats, poultry and seafood. The method was followed as described by the manufacturer. Representative catfish tissue samples 25g ±2.5g were homogenized with 225ml ±4.5ml Phosphate Buffered Saline (PBS) in sterile stomacher bags. Serial dilutions in PBS were made five times consecutively. Aliquots of diluted PBS (1ml) were distributed onto Aerobic Count and *E. coli*/Coliform Count Plates 3M™ Petrifilm™ as duplicates and incubated at 35°C ±2°C for 48 hours (APC, AOAC Official Method 990.12) and 24 hours (EC, AOAC Official Method 998.08), respectively. Interpretation of red colonies on AC 3M™ Petrifilms was counted as APC. Blue colonies associated with gas on EC 3M™ Petrifilms were counted as *E. coli*. Red colonies with gas were counted as non-*E coli* coliforms. Total coliform count was the sum of red and blue colonies with gas. Colonies counted in the range of 10-250 were recorded, calculated, and expressed as log CFU/g.

3.2.2.2 Staphylococcus spp.

Aliquots of 0.1 ml from PBS sample mixture dilution stock was spread over the surface of Baird-Parker Agar (BPA)(Hardy) plates, supplemented with egg yolk tellurite emulsion, in duplicates up to 10^{-2}. BPA plates were incubated at 35°C ±2°C for 24-48 hours. Plates were observed for characteristic colonial morphology and color at 24 hours. If growth was not detected, negative for *staphylococci*, the plates were re-incubated for an additional 24 hours at 35°C ±2°C and read again. Colony forming units of black, circular colonies with off-white margins resulted in *Staphylococcus spp.* isolates. Colonies counted in the range of 5-250 were recorded, calculated, and expressed as log CFU/g.

3.2.3 Statistical Analysis

Data was analyzed using Vector t-test (SAS Institute Inc., Cary, NC, USA). Statistical significance occurred at $P<0.001$ and $P < 0.05$ between different experimental factors. All experiments were repeated independently twice, each with two replications. The analysis of variance (ANOVA) was performed to compare the differences in microbial (log CFU/g) (SAS Institute Inc., Cary, NC, USA). The General Linear Model (GLM) procedure was used and Tukey's comparisons. Differences between the mean values were considered significant ($P<0.05$). Statistical significance occurred at $P < 0.05$.

3.3 Results and Discussion

Testing for levels of indicator bacteria has been done to monitor food and water contamination and microbiological quality. Identifying and computing the presence of sanitary indicator bacteria in wild caught and farm raised channel catfish from various retail markets can add value to domestic aquaculture industry practices by determining critical points of contamination, modifying hygienic practices, and preventing consumer illnesses.

3.3.1 Screening of WC and FR channel catfish for the presence of indicator bacteria

Sanitary indicator microorganisms observed include but are not limited to grouped mesophilic bacteria such as APC, in addition, coliform including *E. coli*, and *Staphylococcus.* WC catfish samples expressed a slightly elevated indicator bacterial load than FR catfish among the surveyed samples (Figure 4). Both sample sources, WC and FR, are relatively similar within APC, *E. coli, Salmonella,* and *Staphylococcus* log CFU/g measurements. In both WC and FR catfish samples, *E. coli, Salmonella,* and *S. aureus was not present*. APC on FR samples was significantly different ($P < 0.05$) than WC (Table 8). APC in FR resulted in lower counts of 5.94 ± 1.59 log CFU/g and 6.44 ± 0.96 log CFU/g for WC catfish sample types. Coliform counts

were considerably different ($P < 0.01$) between WC and FR catfish samples. WC coliform counts of 1.96±1.42 log CFU/g were significantly lower than FR coliform counts of 0.71±1.23 log CFU/g. Similarly, *E. coli* population was also significantly different ($P < 0.05$) between FR, less than the lower detection limit of < 1.00 log CFU/g, and WC, 0.51±0.96 log CFU/g., where WC catfish had an increased load in comparison to FR catfish *E.coli* counts. Among WC and FR catfish samples there were a substantial significantly different ($P < 0.01$) bacterial load of *Staphylococcus spp.* FR *Staphylococcus* counts were less than the lower limit of detection of <1.00 log CFU/g. In contrast to FR catfish samples, WC catfish sample counts of *Staphylococcus* were 0.55±0.78 log CFU/g.

Table 10. Indicator microorganism counts of FR and WC retail channel catfish

Sample Type [x]	Sanitation Indicator Bacteria[y] (log CFU/g)			
	APC	COLI	EC	STAPH
FR	5.95 ± 1.59	0.71 ± 1.23	< DL[z]	< DL[z]
WC	6.44 ± 0.96	1.96 ± 1.42	0.51 ± 0.96	0.55 ± 0.78

[x]Sample type: farm raised (FR), wild caught (WC).
[y]Sanitation indicator bacteria: aerobic plate count (APC), coliform (COLI), *E. coli* (EC), *Staphylococcus* (STAPH).
[z] DL: Detection limit < 1.00 log CFU/g.

In previous studies, aerobic bacteria have been detected on catfish fillets within a consistent range of 3.00 to 6.20 log CFU/g of fish throughout the year (Fernandes 1997). As a final retail product, catfish fillets have generated 7% of fresh and 5.5% of frozen samples with APCs in excess of 7.00 log CFU/g (Andrews 1977). According to this study, the range of APCs was 3.80 to 8.30 log CFU/g in both fresh and frozen catfish (Andrews 1977). Other studies have shown APCs were 4.30 to 6.00 log CFU/g (Martin 1993).

The high APC bacterial counts in WC in contrast to FR support the argument that the water source in use during degutting becomes highly contaminated with psychrophilic/psychrotrophic bacteria (Leung 1992).

A processing plant study showed catfish fillet testing resulted in coliform counts of 0.80 to 3.20 log CFU/g (Fernandes 1997). In additional studies, catfish fillets were purchased at local markets and via the internet, where coliform mean value of 2.30 log mpn/g (Pao 2008). A study focusing on domestic frozen channel catfish and imported Vietnamese basa fish samples showed that 80% of 30 frozen catfish samples and 90% of 30 imported basa fish samples had mean coliform values below 0.48 log CFU/g (Pal 2009). All 60 samples had coliform counts below 6.00 log CFU/g (Pal 2009).

A common indicator organism used to determine fecal contamination is *E.coli* because it is a part of the normal bacterial flora in the intestinal tracts of humans and most animals (Doyle 1997). In a fish study, *E. coli* levels 2.00 to 7.50 log CFU/g were detected during the summer months, whereas levels during other seasons ranged from 0 to 4.0 log CFU/g., annual *E. coli* counts averaged 2.20 log CFU/g (Fernandes 1997).

Staphylococcus-induced illnesses have not been reported to be in association with catfish consumption specifically. However, a large catfish study of 240 fillets collected throughout the year from three processing plants showed an increase in *Staphylococcus aureus* during summer months, suggesting *Staphylococcus* has seasonal prevalence in catfish (Fernandes 1997).

3.3.2 Microorganism population of various channel catfish retail markets

3.3.2.1 Aerobic plate count

APCs of 21 various retail markets resulted in the range of 3.98 to 7.31 log CFU/g (Table 9). Sample markets WM1206, RM135, WM0532, WD1590, WFM304, and HS are of the lowest APC. These values ranged from 3.98±0.33 log CFU/g, 4.41±3.44 log CFU/g, 4.76±0.03 log CFU/g, 5.01±1.11 log CFU/g, 5.22±0.09 log CFU/g, 5.26±0.02 log CFU/g, respectively. Retail market WM5056 had the highest APC load of 7.31±0.02 log CFU/g. The average APC was significantly (P<0.001) different among retail stores supporting the notion that harvesting, processing, and storage contamination is dependent on individual retail outlet practices.

Table 11. Indicator microorganism counts of channel catfish samples of 21 retail markets.

Retail [x]	Sanitation Indicator Bacteria[y] (log CFU/g)			
	APC	COLI	EC	STAPH
AHM	6.86 ± 0.90a	0.72 ± 1.12cd	< DL[z]	< DL[z]
ALB3747	6.73 ± 0.89ab	2.45 ± 0.52abc	1.23 ± 1.42 ab	< DL[z]
ALB709	5.30 ± 1.14bcde	0.67 ± 1.04cd	< DL[z]	< DL[z]
BCS	6.69 ± 0.70ab	2.40 ± 1.04abc	0.65 ± 1.05bc	0.76 ± 0.94ab
CBS	6.34 ± 0.03abc	2.45 ± 0.21abc	< DL[z]	1.27 ± 0.32 a
CCS	5.64 ± 0.77abcd	1.95 ± 1.16abc	0.49 ± 0.98bcd	< DL[z]
CSM	6.41 ± 1.25ab	2.17 ± 1.83abc	0.81 ± 1.14b	0.51 ± 0.72abc
HS	5.26 ± 0.02bcde	< DL[z]	< DL[z]	0.52 ± 0.74abc
LAM	6.32 ± 0.75abc	2.48 ± 2.87ab	1.90 ± 2.20a	0.97 ± 1.18ab
LFM	6.78 ± 1.12a	0.91 ± 1.14cd	< DL[z]	< DL[z]
RM135	4.41 ± 3.44de	< DL[z]	< DL[z]	< DL[z]
TFM74	6.95 ± 0.82a	0.37 ± 0.88d	< DL[z]	0.47 ± 0.71abc
TJ	6.19 ± 0.07abcd	< DL[z]	< DL[z]	< DL[z]
TSMD	6.39 ± 1.09 abc	3.18 ± 0.69a	0.50 ± 1.00bcd	< DL[z]
WD1463	6.41 ± 1.21ab	0.32 ± 0.82d	< DL[z]	< DL[z]
WD1590	5.01 ± 1.11cde	1.12 ± 1.31bcd	< DL[z]	< DL[z]
WD463	NA	NA	NA	< DL[z]
WFM304	5.22 ± 0.09bcde	< DL[z]	< DL[z]	< DL[z]
WM5056	7.31 ± 0.02a	< DL[z]	< DL[z]	< DL[z]

table cont.

Table 12. Indicator microorganism counts of channel catfish samples of 21 retail markets.

Retail [x]	Sanitation Indicator Bacteria[y] (log CFU/g)			
	APC	COLI	EC	STAPH
WM0532	4.76 ± 0.03cde	< DL[z]	< DL[z]	< DL[z]
WM1206	3.98 ± 0.33e	0.50 ± 1.00cd	0.50 ± 1.00d	< DL[z]

[x] Various random south Louisiana retail markets that sell WC and FR channel catfish.
[y] Sanitation indicator bacteria: aerobic plate count (APC), coliform (COLI), *E. coli* (EC), *Staphylococcus* (STAPH).
[z] DL: Detection limit < 1.00 log CFU/g.
Based on two independent experiments and two replications per experiment. Means ± SD within each column followed by the lowercase letter(s), different letters indicate signifcant differences in the same column ($P < 0.05$).

Previously, domestic retail channel catfish and Vietnamese basa fish average APCs were 4.40 log CFU/g for channel catfish and 3.80 log CFU/g for basa (Pal 2009). The occurrence of APC bacteria above acceptable limits is concerning. The specification for bacterial counts of fish which is <105 CFU/g, is considered tolerable (Andrew 1992). The heighten APC bacterial counts in between the two systems of cultivation of channel catfish may suggest poor handling during the gutting and scaling process. In another study, APC ranged between 2.0 x 10 and 2.0 x 10 CFU /g in large fish along the market distribution channel, the differences were not significant ($p>0.05$) (Ganegamaarachchi 2000). This study also found that APC of *Katsuwonus pelamis* were in the range of 104-106 CFU/g along the market distribution channel in Negombo Sri Lanka. A farmed tilapia market study in Egypt, found that the mean APC (6.2 x 10 CFU/g) for samples collected from the whole sale market was lower than that from the retail markets and the latter was lower than that from street vendors (Mahmoud 2018). The pre and post-harvesting contamination and handling of the tilapia samples were identified as potential sources of contamination (Eltholth 2015). Previous studies suggest that current investigations are necessary for the interpretation of grouped bacteria enumeration such as APC, which aids in the interpretation of quality and spoilage in U.S. retail channel catfish.

3.3.2.2 Coliform

In the case of coliform, 10 of 21 stores maintained lower counts (Table 9). 5 of 21 markets ranged in intermediate counts of 0-2 log CFU/g. Markets with high contamination counts >2 log CFU/g were ALB3747, BCS, CSM, CBS, TSMD, and LAM with maximum values of 2.45±0.52log CFU/g, 2.40±1.04 log CFU/g, 2.17±1.83log CFU/g, 2.45±0.21log CFU/g, 3.18±0.69log CFU.g, 2.48±2.87log CFU/g, respectively. Among 21 retail stores, coliform was significantly (P<0.001) different. Markets are susceptible to contamination such as fecal matter as a direct result poor manufacturing practices, unacceptable hygiene routines, and temperature abuse.

A study in Pakistan of the microbial quality of farmed fish in markets determined the total coliform counts (TCC) in samples of *L. rohita* and *H. molitrix* collected from retailers shops (143.88 MPN and 149.38 MPN) were significantly higher than the samples collected from main fish markets (78.00 MPN and 70.76 MPN) (Begum 2010). At a retail market, catfish products in the distributor display case had fecal coliform levels of <0.48 to 3.4 log mpn/g in fresh catfish and <0.48 to 2.4 log mpn/g in frozen catfish (Andrews 1977). The latter investigation supports the need to study the prevalence of coliform contamination in the present state of retail channel catfish.

3.3.2.3 Escherichia coli

Overall, *E. coli* did not have a significant presence in catfish retail markets. Detection limits of < 1.0 log CFU/g were found in 17 of 21 retail stores (Table 9). Counts > 1.0 log CFU/g were in ALB3747 and LAM, with maximum values of 1.23±1.42 log CFU/g and 1.90±2.20 log

CFU/g. *E. coli* was significantly ($P<0.05$) different among the 21 various markets, expressing evidence of influence in comparison of individual retail store *E. coli* count means.

In contrast, according to a study on retail fish markets in India, on average around 14 CFU g^{-1} of *E. coli* was present in retail market, which indicates a considerable number of fecal contaminations were observed in the retail markets (Murugadas 2016). In an additional study, an *E. coli* average 1.70 log mpn/g was determined in retail catfish (Pao 2008). A 2014 fresh farm fish study in Pakistan revealed, *E. coli* were found in all fish samples, showing similar increasing trend from main fish market to retailers' shops ranging from 3.00 MPN g^{-1} to 29.40 MPN g^{-1} and were beyond acceptable limits of FAO for human consumption (< 10 MPN g-1) in samples collected from both marketing points of main fish market (Jan 2014). *E. coli* was reported as a good sanitary indicative of fish quality in 1930s (Griffiths 1936) and is currently applied as strong parameter of microbial fish quality especially related to fecal contaminations (Silva 1993, Jeyasanta 2012).

3.3.2.4 Staphylococcus spp.

Staphylococcus mean counts were not detected for 10 of 21 retail markets (Table 9). *Staphylococcus* counts were detected at LAM, BCS, HS, and CSM. The maximum log measurements found in these markets were 0.97±1.18 log CFU/g, 0.76±0.94 log CFU/g, 0.52±0.74 log CFU/g, 0.51±0.72 log CFU/g, respectively. *Staphylococcus* was not significantly ($P>0.05$) different among the retail stores, mean counts shows minimal indication that there is an influence between retail stores.

In fish markets with similar environmental conditions the prevalence of *Staphylococcus spp.* in whole and gutted tilapia samples were 41% and from street vendors in Gaborone, Botswana was 62% (Mhango et al., 2010). Complementary to this study, an investigation of various market fish concluded *Staphylococcus* were not detected in any fish samples collected from all fish markets (Begum 2010) which is also in line with the findings of Jan *et al.* (2014), who did not find *Staphylococcus* in *Labeo rohita* and *Wallogo attu* sampled from Peshawar fish markets, Pakistan. An additional study isolated *Staphylococcus* from tilapia fish samples, where the highest prevalence was in frozen tilapia 86% from the supermarkets and whole tilapia 62% from street vendors (Ogbondeminu 1993). The contradictions in many studies of the prevalence of *Staphylococcus* in nonmarine fish in various countries prove the need to conclude the presence of *Staphylococcus* in channel catfish.

3.4 Conclusion

This study demonstrated that sanitary indicator bacteria have an elevated load in WC retail channel catfish samples than FR catfish samples. Among all indicator bacteria WC samples were higher than FR samples. It can be considered that environmental factors such as an uncontrolled habitat of WC catfish may contribute to higher indicator bacteria counts in comparison to FR samples. The processing and production facility may factor in the results of sanitary indicator bacterial loads. WC catfish are typically processed and sold in small, modest seafood markets without established Hazard Analysis and Critical Control Point (HACCP) that would aid in the avoidance of such bacteria and potential pathogens. FR catfish samples in this study did present some concern of contamination during processing, production, and storage within retail markets. Evidently, the fluctuation of sanitary indicator counts among the screened

markets support the idea that there is a need to further investigate retail stores that sell domestic channel catfish to consumers to create a preventative standard and to avoid illnesses.

CHAPTER 4. EFFICACY OF ANTIMICROBIAL TREATMENTS ON CHANNEL CATFISH (*Ictalurus punctatus)* FOR THE CONTROL OF *Salmonella enterica*

4.1 Introduction

Catfish are a lean protein source and highly nutritious food commodity with wide consumer acceptance. Catfish production is the largest finfish aquaculture industry in the U.S. and a critical part of economies of many southeastern states (Hargreaves 2002; Stankus 2010). The catfish aquaculture industry in the U.S. began primarily with reproduction of channel catfish, *Ictalurus punctatus.* In the U.S., Alabama, Arkansas, Louisiana, and Mississippi account for over 95% of the nation's channel catfish production, exceeding over 270 thousand ton in live weight among food-size farm-raised sales in 2005 (National Agricultural Statistics Service, 2005). However, similar to other fish products, channel catfish can have significant economic losses as a result of its highly perishable nature, especially if they are not preserved in some way (Wood, 1981).

Salmonella spp. are Gram-negative, rod-shape bacteria that cause salmonellosis. In addition, it is a resilient microorganism that adapts to various atomspheric conditions (temperatures 2°C-54°C, pH 4.0-9.5) (Airoldi 1988). Such growth characteristics raise concerns about food safety

through bacteriostasis. These concerns are further heightened by the widespread refrigerated storage and shelf life.

These pathogenic bacteria caused enteric fever and acute gastroenteritis in humans (Hohmann 2001). The symptoms include mild to severe gastroenteritis, with an incubation period of 6–72 h (Hohmann 2001). *Salmonella* carried by fish and other aquaculture products has been indicated as a vehicle for a growing number of enteric disease outbreaks (Amagliani 2012, Olgunoglu 2012). Salmonellosis outbreaks have been reported in several countries as a result of fish consumption. The association of *Salmonella enterica* in a variety of fishes and shellfishes have been established by the FDA (Brands 2005, Duran 2005, Heinitz 2000). Human or animal activities and environmental factors commensed a variety of hazards related with cultured fish (Heinitz 2000). Fish, such as channel catfish can be a pathway for *Salmonella* transmission which can be pathogenic to humans and have the ability to express antimicrobial resistance via plasmids (Hradecka 2008). The ability of antibacterials to cause development of resistance in fish pathogens is of concern worldwide (Schnick, 2001).

In order to prevent the transmission of various infectious pathogens, effective protocols are required on farms and in processing facilities for disinfecting equipment and instruments, and sanitizing storage materials with antimicrobial and preserving agents often used as biocidal agents in aquaculture and laboratory facilities (Birkbeck 2006, Treasurer 2005). Antimicrobial and perservative treatments are divided into groups of oxidizing agents containing peroxides and halogens, and reducing agents containing formaldehydes, acids, alkalis, alcohols, and phenols (Birkbeck 2006). Accepted food preservatives used for catfish include antibacterial and antifungal agents such as lactic acid (Fernandes 1998), sodium benzoate (Efiuvwevwere 1996), sodium hypochlorite (Yang 2001), and acidified sodium chlorite (Harris 2012).

Refrigerated foods treated with acid offer preservative effects during storage. Organic acid dips such as lactic, citric, and propionic are favorable in controlling undesirable microorganisms on refrigerated foods (Ray 1992, Kim1995). Sanitizing meat and fish surfaces with organic acids result in sublethal injury or death to pathogenic microorganisms (Ingham 1989, Anderson 1990, Kim 1995). The degree of injury varies with type and concentration of acid, microbial species, product, and storage condition.

Lactic acid (LA), ($C_3H_6O_3$) is typically used as a flavor enhancing ingredient, it is also utilized as an antimicrobial treatment and pH control agent in food products. The maximum allowable amount of LA as a carcass rinse or chiller wate concentrant is 5% (USDA FSIS 2012). Research have been conducted on the efficacy of LA as a sanitizer on meat and poultry carcasses to reduce or eliminate pathogens, such as *Salmonella.* LA sprays or dips at 0.2-2.5% in most cases are effective in reducing contamination on beef, veal, pork, poultry, and fish and improving shelf life (Barboza de Martinez 2002, Bautista 1997, Dickson 1992, Jangho 2001, Smulders 1986, Snijders 1985). Despite previous studies of the efficacy of LA against microorganisms as an inexpensive, generally reconized as safe (GRAS) ingredient, studies are limited on LA affect on *Salmonella* on channel catfish.

Other adulterants are utilized as antimicrobial agents in the food industry to preserve and defend consumable products from microorganisms. Chemical treatments such as sodium hypochlorite (SH), (NaOCl), also known as chlorine, is an inorganic sodium salt and chlorine compound often used as a disinfectant in which hypochlorite is the counterion. Although variety of sanitizing agents are approved by USDA FSIS for use during immersion-chilling of carcasses in the USA, SH is by far the most commonly used antimicrobial agent (McKee 2011, Nagel 2013, USDA-FSIS 2016). According to USDA-FSIS, maximum of 50 ppm SH concentration is

permissible in process water during carcass chilling (Russell, 2012, USDA-FSIS, 2012). There are factors that effect the antimicrobial activity of SH such as pH of the applied matrix, contact time and the amount of organic matter of the contaminant (Tsai 1992). The organic matter can create a chlorine demand causing significant loss of free-chlorine resulting in reduced antimicrobial activity and increased survival of food-borne pathogen such as *Salmonella* (Mohamed 2015, Oscar 2013, Tsai 1992, Yang 2001). Consequently, researchers have relied on in vitro models to study the effects of such intrinsic factors on anti-microbial activity of SH against bacterial pathogens including *Salmonella*. However, there is a lack of studies available analyzing these factors against *Salmonella* on channel catfish carcasses.

Sodium benzoate (SB), ($C_7H_5NaO_2$) the sodium salt of benzoic acid, is a common food preservatives used primarily as anti-fungal agents but to some extent inhibit bacteria depending on the concentration and degree of dissociation (Anon. 1980, Eklund 1989). SB, up to 0.1% (USDA FSIS 2016) is GRAS and is used as an antimicrobial in beverages, acidic condiments such as pickles, fruit salads, dressings and in the storage of vegetables (Chipley 2005). The use of SB as an antimicrobial agent against *Salmonella* on channel catfish carcasses have not been reported.

Acidified sodium chlorite (ASC), has antimicrobial properties and is intended for use primarily as a spray or a dipping solution for poultry, meats, vegetables, fruits and seafoods (Gill 2004). Food-grade acids such as citric acid is used to lower the pH of aqueous sodium chlorite (NaClO2) solution depending on the desired concentration resulting in ASC formulation (Gill 2004, Lim 2004). Combining citric acid and sodium chlorite solution produce a conversion of chlorite to metastable chlorous acid (HClO2), which form a mixture with chlorite (ClO2), chlorine dioxide (ClO2) and chloride (Cl^{-}) (Lim 2004). The reaction, therefore, generates an

oxidative solution with oxychlorine species having antimicrobial properties. ASC is intended for use to control microbial loads on food stuffs and to treat pre-chilling and chilling water at relatively low levels of 50 to 150 ppm (USDA FSIS 2016).

Previous studies have reported the use of LA, SH, SB, and ASC antimicrobial properties on beef, pork, poultry chill water, and various nonmarine seafood products and/or the effects of these antimicrobial chemical treatments against *E. coli 0157:H7, Listeria monocytogenes,* spoilage bacteria, yeast and mold. However, more data is necessary to study how effective the subjected antimicrobial and preservative chemicals are against selected serovars of *Salmonella enterica subsp. enterica* on retail channel catfish.

As a result, the aim of this study is to evalute the antimicrobial effect of lactic acid, sodium hypochlorite, sodium benzoate, and acidified sodium chlorite at various concentrations on retail channel catfish against inoculated selected serovars of *Salmonella enterica subsp. enterica* during storage at 4°C for 8 days.

4.2 Materials and Methods

4.2.1 Bacterial strains and culture conditions

The antimicrobial activity of lactic acid, sodium hypochlorite, acidified sodium chlorite, and sodium benzoate was determined against a total of four bacterial strains combined. The combinations of gram-negative strains were *Salmonella enterica subsp. enterica sers. typhymiruim* (ATCC 14028), *concord* (FN $C_1$86), *infantis* (CDC H3517), and *senftenberg* (ATCC 43845). Bacterial stock cultures were stored in cryogenic vials at -80°C in 30% (wt/wt) glycerol Tryptic Soy Broth (TSB) and working cultures were maintained on Brain Heart Infusion (BHI) agar slants at 4°C with routine transfers to new slants. Frozen cultures were activated by

successive passages in Brain Heart Infusion (BHIB) broth. The cultures were streaked for isolation on Xylose Lysine Deoxycholate (XLD) agar. XLD agar plates were incubated at 37°C for 24 h. A single colony of each *Salmonella* stain with expected morphology characteristics was transferred to 10 ml of BHI broth and incubated at 37°C for 24 h. 100 µl of each culture was used to inoculate 4 lots of 100 ml BHIB and incubated at 37°C for 18-24 h. Twenty ml of each culture was centrifuged at 4°C for 10 min at 5000 rpm x g, supernatant was discarded and recovered with 20 ml of Phosphate Buffered Saline (PBS), and centrifuged as before. The cell pellet of each strain was suspended in 20 ml PBS and combined to make a 4-strain cocktail. The 4-strain cocktail was diluted with PBS to obtain an initial inoculation of >5.5 log CFU/ml.

4.2.2 Preparation of catfish and inoculation

Fresh refrigerated channel catfish fillets were purchased from a local market a day prior to the experiment and stored at 4°C. Catfish fillets were cut aseptically into 10 g pieces and separated into 75 portions in sterile 500 ml beakers. To obtain approximately 5.5-6.0 log CFU/g inoculum level on the fillet surface, 100 µl of the 4-strain cocktail was applied to the surface of 70 sample portions (excluding 5 pieces for negative control). The sample portions were allowed to dry for 5 min under a biohazard laminar flow hood.

4.2.3 Preparation of antimicrobial solutions

A stock of solution for each chemical treatment was diluted in sterile distilled ice water to obtain different concentrations before application. Twelve freshly prepared solutions at various concentrations were used as treatments. Lactic acid (LA) was prepared at concentrations of 15000 ppm, 25000 ppm, 50000 ppm (wt/v), sodium hypochlorite (SH) 50 ppm, 100ppm, 200ppm (v/v), sodium benzoate (SB) 250 ppm, 500ppm, 1000 ppm (wt/v), acidified sodium

chlorite (ASC) (pH 2.5) 30 ppm, 40 ppm, 50 ppm (wt/v). The highest treatment concentration of each treatment is the maximum allowable limit according to the USDA FSIS Directive "Safe and Suitable Ingredients Used in the Production of Meat, Poultry, Eggs and Siluriformes Products".

4.2.4 Antimicrobial treatment

Inoculated catfish pieces were submerged in 250 ml of 1.5, 2.5, 5.0 % LA, 50, 100, 200 ppm SH, 250, 500, 1000 ppm SB, 30, 40, 50 ASC, and sterile distilled ice bath (control nontreated ice bath) (CNTRL NTIB) individually at 4°C for 12 h. Treated pieces were drained and allowed to dry under a biohazard laminar flow hood for 30 min. Treated pieces representing each treatment and concentration, including control nontreated (inoculated catfish, sterile distilled ice bath)(CNTRL NTIB), control nontreated (inoculated catfish only) (CNTRL NT), and negative control (catfish, sterile distilled ice bath) were placed in individual WhirlPak® bags and stored at 4°C for upto 8 days.

4.2.5 Microbiological analysis

At days 0, 2, 4, 6 and 8 portioned catfish pieces were analyzed for *Salmonella* counts. For the enumeration of *Salmonella* on catfish pieces, previously weighed samples (10 g) were diluted with 90 ml PBS. Samples were then stomached for 1 min, serially diluted and surface plated on duplicate pre-poured plates of XLD and incubated at 37°C for 24 h, and colony counts were expressed as log CFU/g. Two independent trials were conducted to examine the effect of antimicrobial agents on microbiological changes in catfish.

4.2.6 Statistical analysis

The analysis of variance (ANOVA) was performed to compare the differences in microbial (log CFU/g) and physiochemical attributes among treated and control catfish samples using (SAS Institute Inc., Cary, NC, USA). The General Linear Model (GLM) procedure was used and Tukey's comparisons. Differences between the mean values were considered significant ($P<0.05$). Statistical significance occurred at $P < 0.05$. All experiments were repeated independently twice, each with two replications.

4.3 Results and Discussion

Evaluating antimicrobial and preservative chemical food additives as treatments on retail channel catfish carcasses may improve U.S. aquaculture safety provisions by protecting processors and consumers against contamination by selected strains of *S. enterica subsp. enterica.* Individually, each treatment significantly reduced the presence of *Salmonella.* However, the effectiveness of the antimicrobial treatments is dependent upon the concentration of each solution (Table 1). Food preservation and antimicrobial activity is best achieved when antimicrobial type and concentration are taken into account of intrinsic, extrinsic, and process factors. (Gould 1989).

The mean of untreated control (CNTRL NT) samples was used as the baseline for determining population reductions produced by the chemical treatments. CNTRL NT is intended to represent a population baseline that accounted for the referenced amount of *Salmonella* inoculum to compare the effects of each treatment application. Adherence of *S. enterica* to the sample surface was not a factor in killing microorganisms by sterile ice water alone. Pre-

refrigerated storage (Day 0) of control nontreated ice bath (CNTRL NTIB) was 0.88 ± 0.02 log CFU/g less than control nontreated (CNTRL NT) the two means were significantly different (P < 0.05). A negative control of untreated catfish, sterile ice bath and no inoculation was evaluated parallel with the study, targeted microorganisms were undetectable in the negative control.

Table 13**.** Antibacterial activity (log CFU/g) of twelve treatments and three controls against *S. enterica* on the surface of retail channel catfish carcasses stored at 4C for 8 days.

Treatments[x]	Days[z]				
	0	2	4	6	8
LA 15000	3.31 ± 0.02 i	< DL[z]	< DL[z]	< DL[z]	< DL[z]
LA 25000	< DL[z]	< DL[z]	< DL[z]	< DL[z]	< DL[z]
LA 50000	< DL[z]	< DL[z]	< DL[z]	< DL[z]	< DL[z]
SH 50	4.25 ± 0.05 f	3.96 ± 0.03 d	3.13 ± 0.03 g	3.12 ± 0.03 f	3.02 ± 0.03 g
SH 100	4.22± 0.02 fg	3.69 ± 0.06 e	3.10 ± 0.00 g	2.87 ± 0.06 g	2.70 ± 0.09 i
SH 200	4.12 ± 0.04 g	3.58 ± 0.03 f	2.77 ± 0.06 h	2.86 ± 0.08 g	2.41 ± 0.01 j
SB 250	5.06 ± 0.06 b	4.42 ± 0.18 b	4.07 ± 0.03 b	4.00 ± 0.01 c	4.33 ± 0.03 b
SB 500	4.89 ± 0.10 c	4.47 ± 0.09 b	3.89 ± 0.04 c	3.91 ± 0.04 d	4.04 ± 0.03 c
SB 1000	4.85 ± 0.13 c	4.43 ± 0.02 b	3.63 ± 0.05 d	3.95 ± 0.04 cd	3.87 ± 0.03 d
ASC 30	4.59 ± 0.10 e	3.74 ± 0.03 e	3.92 ± 0.05 c	4.07 ± 0.01 b	3.52 ± 0.02 e
ASC 40	3.76 ± 0.11 h	3.77 ± 0.07 e	3.88 ± 0.07 c	3.25 ± 0.02 e	3.15 ± 0.10 f
ASC 50	4.20 ± 0.02 fg	3.49 ± 0.04 f	3.25 ± 0.03 f	2.86 ± 0.05 g	2.89 ± 0.04 h
CNTRL NTIB[y]	4.74 ± 0.04 d	4.25 ± 0.30 c	3.55 ± 0.07 e	4.07 ± 0.02 b	4.32 ± 0.05 b
CNTRL NT[y]	5.62 ± 0.02 a	5.65 ± 0.01 a	5.64 ± 0.02 a	5.05 ± 0.04 a	5.19 ± 0.03 a

[x] Different concentration of antimicrobial treatment solutions formulated in sterile ice water (LA 15000, 25000, 50000 ppm), (SH 50, 100, 200 ppm), (SB 250, 500, 1000 ppm), (ASC 30, 40, 50 ppm) to treat *Salmonella enterica subsp. enterica* ser cocktail inoculated retail channel catfish carcass samples.

[y] CNTRL NTIB (untreated inoculated control of catfish and sterile ice water), CNTRL NT (untreated inoculated control of catfish only.

[z] DL: Detection limit < 1.00 log CFU/g.

Based on two independent experiments and two replications per experiment. Means ± SD within each column followed by the lowercase letter(s), different letters indicate signifcant differences in the same column (P < 0.05).

4.3.1 Effects of lactic acid on channel catfish carcass

S. enterica was unable to grow on the surface of channel catfish at 4°C under LA treatments at all applicable concentrations and concluded non-detectable (Table 1). However, pre-refrigerated storage allowed minimal growth at 15000 ppm (1.5%) of 3.31 log CFU/g. LA at concentration of 25000 ppm (2.5%) and 50000 ppm (5.0%) effectively destroyed the bacteria at pre-refrigerated storage (Day 0). Despite the enumerable growth on day 0 under LA 15000 ppm, there was a large significant difference ($P < 0.001$) in *S. enterica* compared to the non-treated inoculated catfish. LA 15000 ppm reduced S. *enterica* counts by 2.31 log CFU/g lower than the non-treated inoculated catfish on Day 0. *S. enterica* was not detectable after LA treatments of all three concentrations (15000, 25000, 50000 ppm) from day 2 throughout storage. The antibacterial properties of organic acids such as lactic acid are attributed to pH and their undissociated (uncharged or protonated) forms, which can penetrate bacterial membranes (Davidson 2001). The undissociated form of lactic acid can invade the cell membrane lipid bilayer then create a lower pH within the cell interior than exterior. This mechanism of action is effective because *Salmonella* generally maintain an internal pH near neutral to prevent configurational changes to the cell structure (Mitchell 1969).

Ingham showed that catfish fillet pieces dipped in 2.55% lactic acid for 10 min lowered microbial numbers in comparison to controlled samples stored for 6 days on ice. Catfish fillets treated with a 2.5% culture of *Lactococcus lactis spp*. cremoris after dipping in 3.0% lactic acid for 1 minute had lower gram-negative bacterial numbers during storage at 4°C and 10°C for 9 days (Kim, 1995). In a study conducted by Fernandes, 1998, the effect of 0 to 4% acetic acid on catfish microflora during a 20-minute suspension period resulted in a maximum of 2.5 log CFU/ml reduction. Significant research has been conducted to conclude that organic acids are effective on catfish and raw meat products against *Salmonella.*

4.3.2 Effects of sodium hypochlorite on channel catfish carcass

Salmonella treated with SH over the 8-day storage period was reduced significantly ($P < 0.001$) in comparison to non-treated inoculated catfish. SH treated catfish resulted in lower counts before refrigerated storage on Day 0 at all concentrations. SH 50, SH 100, and SH 200 treatments reduced *Salmonella* counts on Day 0 by 1.37 log CFU/g, 1.40 log CFU/g, 1.50 log CFU/g, respectively. Throughout the storage period, samples responded significantly to the treatment of SH on all levels of concentration. Further, the levels of treatment affected the reduction of bacterial counts throughout storage, the more concentrated SH levels the more *Salmonella* counts were reduced. For instance, on Days 2, 4, 6, and 8 of SH 200 expressed a reduction of 2.07 log CFU/g, 2.87 log CFU/g, 2.19 log CFU/g, 2.78 log CFU/g, respectively. In comparison to the lowest level of SH concentration on Days 2, 4, 6, and 8 with reductions of 1.69 log CFU/g, 2.51 log CFU/g, 1.93 log CFU/g, 2.17 log CFU/g, respectively. Among SH treatments, factors that substantially affect reduction activity are concentration levels and days of storage at 4°C.

Similarly, a study by Afari and Hong examining the effectiveness of sodium hypochlorite and electrolyzed water treatments against *Salmonella* in various food matrices found, free chlorine concentration, time and temperature were significant variables ($p < 0.05$) in estimating NaOCl effectiveness. A total reduction for NaOCl was 4.37 log CFU/g on eggshells. In the case of the treatment of *Salmonella* on lettuce, the estimated reduction for NaOCl application was 1.08 log CFU/g.

4.3.3 Effects of sodium benzoate on channel catfish carcass

Among the concentrations of SB 250, 500, 1000 ppm of pair-wise testing, there was no significance ($P > 0.05$) between concentration levels. Increasing the dosage of SB did not notably affect the reduction of *Salmonella.* However, SB concentrations were significantly different ($P < 0.01$) among all the other treatments. Multiple comparisons of means expressed SB is the least effective treatment among the other treatment at all levels of concentrations. Although SB antimicrobial performance was not substantial, their effect to reduce *Salmonella* was significant ($P < 0.05$) in comparison to initial inoculation of non-treated inoculated catfish. Without temperature and time as a factor, SB reduced *Salmonella* counts by 0.56 log CFU/g at 250 ppm, 0.73 log CFU/g at 500 ppm, and 0.77 log CFU/g at 1000 ppm. The most effective time factor of SB was Day 4 of storage, where SB 250, 500, 1000 ppm reduced *Salmonella* counts by 1.57 log CFU/g, 1.75 log CFU/g, 2.0 log CFU/g, respectively.

Previous studies of the antibacterial effectiveness of sodium benzoate on cherry tomatoes showed the minimum inhibitory concentration (MIC, ppm) against *Salmonella enterica* was not significant (Chen 2018). In addition, the MIC at pH 7.0 did not show a significant antibacterial activity against the targeted strains at up to 10,000 ppm (Chen 2018). Similarly, in Chen's study, at a concentration of 1000 ppm there was < 0.5 log CFU/g reduction for not only *Salmonella enterica* but all three tested pathogens including *E. coli* O157:H7 and *Listeria monocytogenes*. However, in his study, SB 1000 ppm expressed significant log reduction of 2.14 log CFU/g with a lowered pH of 2.5. The magnitudes of log reductions of *Salmonella enterica* by SB are similar to a recent study applying ozone to reduce pathogens in apple juice adjusted to pH 3.0 and 5.0 (Song 2015). At pH 3.0, no log reduction of *S. Typhimurium, E. coli O157:H7*, and *L. monocytogenes* was obtained, while the population of pathogens was below the detection limit for the treatment at pH 3.0 plus ozone for 4 min.

4.3.4 Effect of acidified sodium chlorite on channel catfish carcass

Salmonella count reduction among acidified sodium chlorite is dependent upon the level of concentration. ASC of 50 ppm was the most effective concentration. However, ASC 40 ppm was less effective than 50 ppm, but had significant difference ($P < 0.01$) from non-treated inoculated catfish on Day 0. ASC antibacterial activity at all concentrations is slightly unstable in comparison to the other chemical treatments. The most effective ASC concentration and time of storage was ASC 50 on Day 4 of storage with a bacterial reduction of 2.39 log CFU/g, ASC 40 on Day 8 of 2.04 log CFU/g reduction, and ASC 30 on Day 2 of 1.91 CFU/g log reduction. These commutative trends suggest that ASC may require low temperature and time to enhance its effectiveness. The low pH of ASC concentrations is also partially responsible for the antimicrobial actions of ASC.

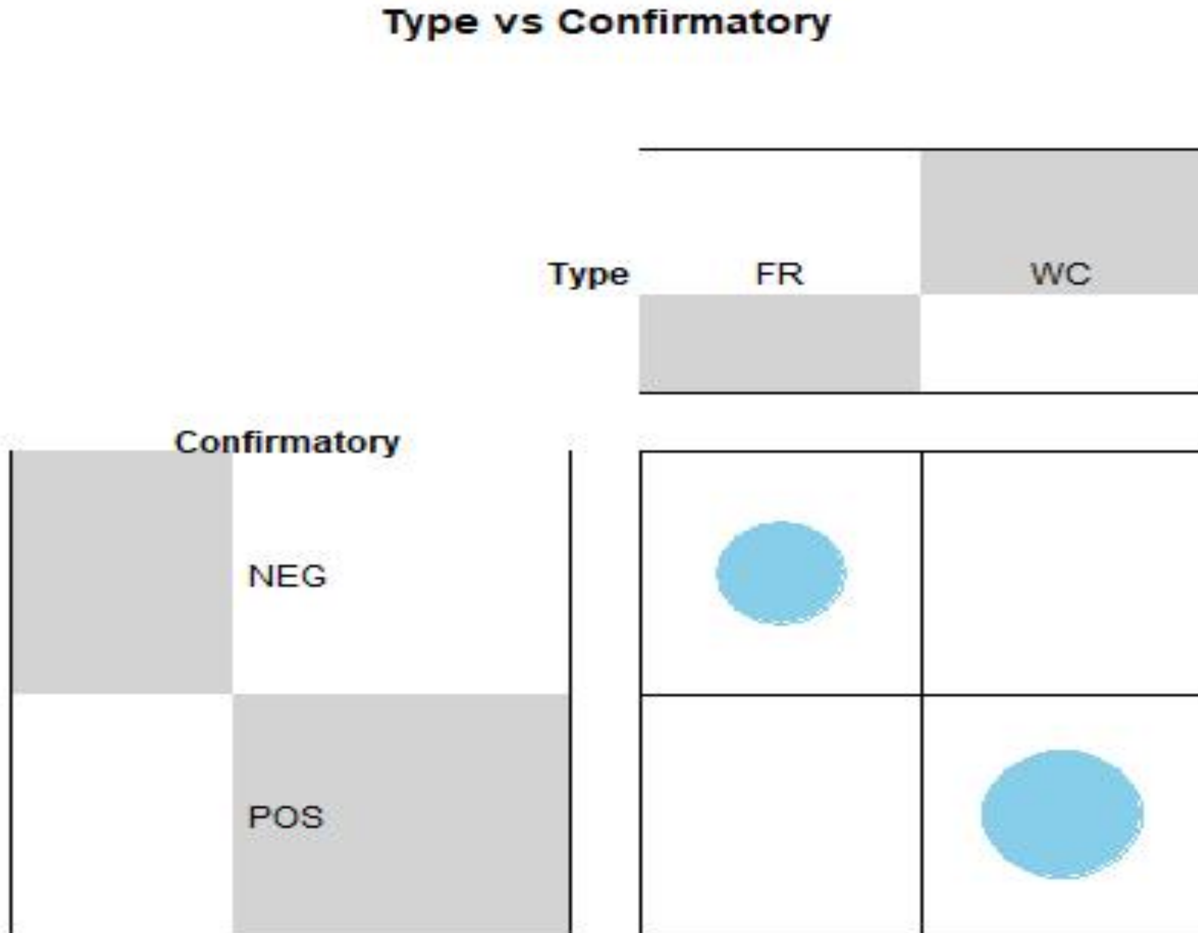
It has been observed that the MIC of ASC is higher than the minimum regulated concentrations (Alonso-Hernando 2009). Adaptations of *Salmonella* and *Listeria* strains to chlorine compounds have been demonstrated (Alonso-Hernando 2009, Davidson 2002), concentrations of ASC sufficient to ensure that *Salmonella* is eradicated and not subjected only to sub-lethal levels should be used (Davison 2002). However, insufficient concentrations frequently occur because of inadequate distribution of ASC, lack of storage time or out-bound temperature, and/or an excessive amount of organic matter, known to inactivate chlorine disinfectants, in the poultry dipping (Alonso-Hernando 2009). The antimicrobial activity of ASC is associated with chlorous acid, which derives from the conversion of chlorite ions into an acid form from the addition of citric acid. Chlorous acid kills microorganisms by direct action on the cell membrane and by the oxidation of cell constituents (Castillo 1999, SCVPH 2003).

4.4 Conclusion

This investigation evaluated the antimicrobial effects of lactic acid (LA), sodium hypochlorite (SH), sodium benzoate (SB), and acidified sodium chlorite (ASC), against *S. enterica* on retail channel catfish carcass during storage at 4°C for 8 days. The data in this study found that all chemical antimicrobial treatments applied decrease the presence of *S. enterica* or reduced it to nondetectable pre and post refrigerated storage for the entirity of the investigation. LA proved to be the most effective treatment where at 1.5%, 2.5%, and 5.0% reduced the presence of *S. enterica* on catfish throughout pre and post storage to nondetectable. Concentration levels were a major factor in the efficiency of SH, SB and ASC. SH was most effective at its highest concentration, due to the organic content of catfish (protein and fat), this effects the availability of free chlorine to be active. SB, at all concentrations, were most effective on Day 4 of storage, suggesting that low temperature and time is required for antimicrobial capacity. The marginally unstable results of ASC indicate that time and temperature may be a factor of effeciency. Yet, its acidic component may also contribute to its ability to reduce *S. entrica* on catfish carcasses. This study conclude that 12 antimicrobial treatments are benefical in reducing *S. enterica* in retail catfish carcass. Additionally, the antimicrobial effectiveness of each treatment varied among concentration levels, refrigerated time, and the presence of organic acids.

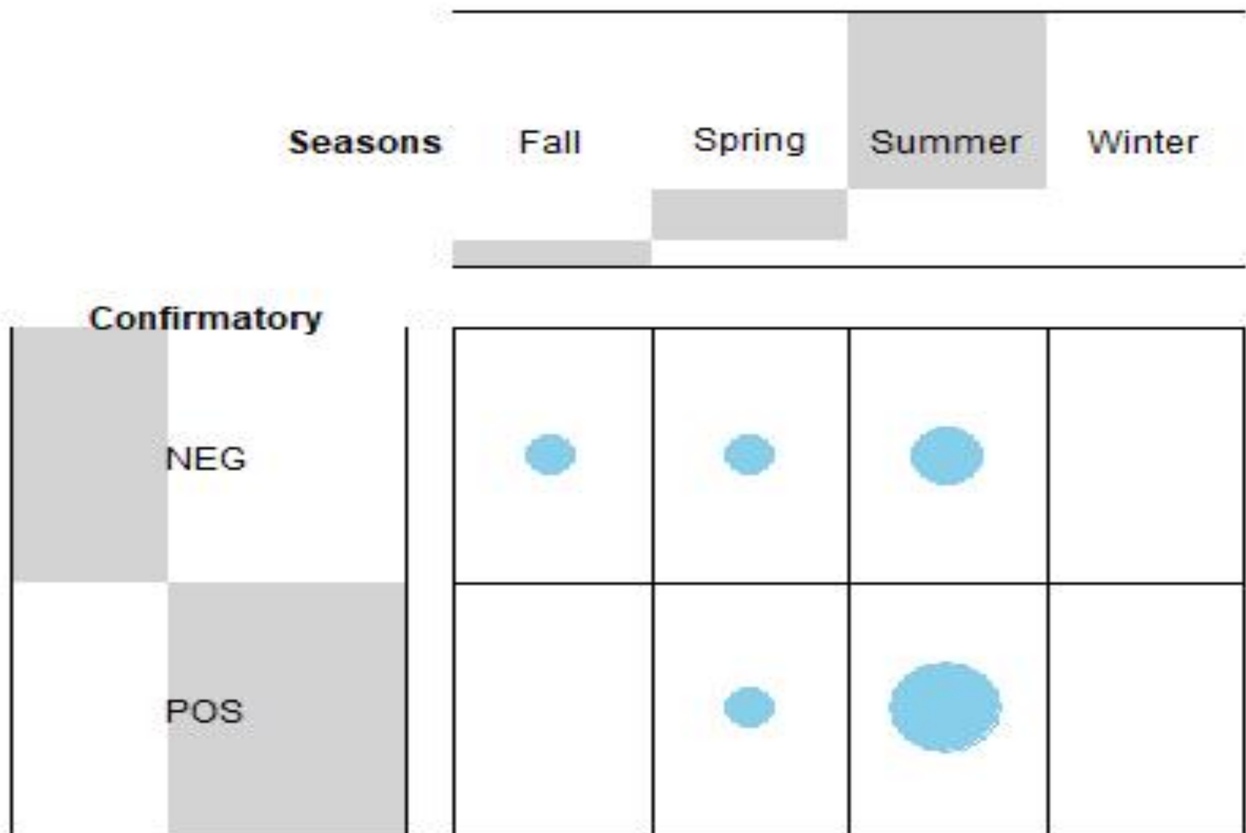
APPENDIX. SUPPLEMENTAL DATA

A.1. Chapter 2 supplemental data



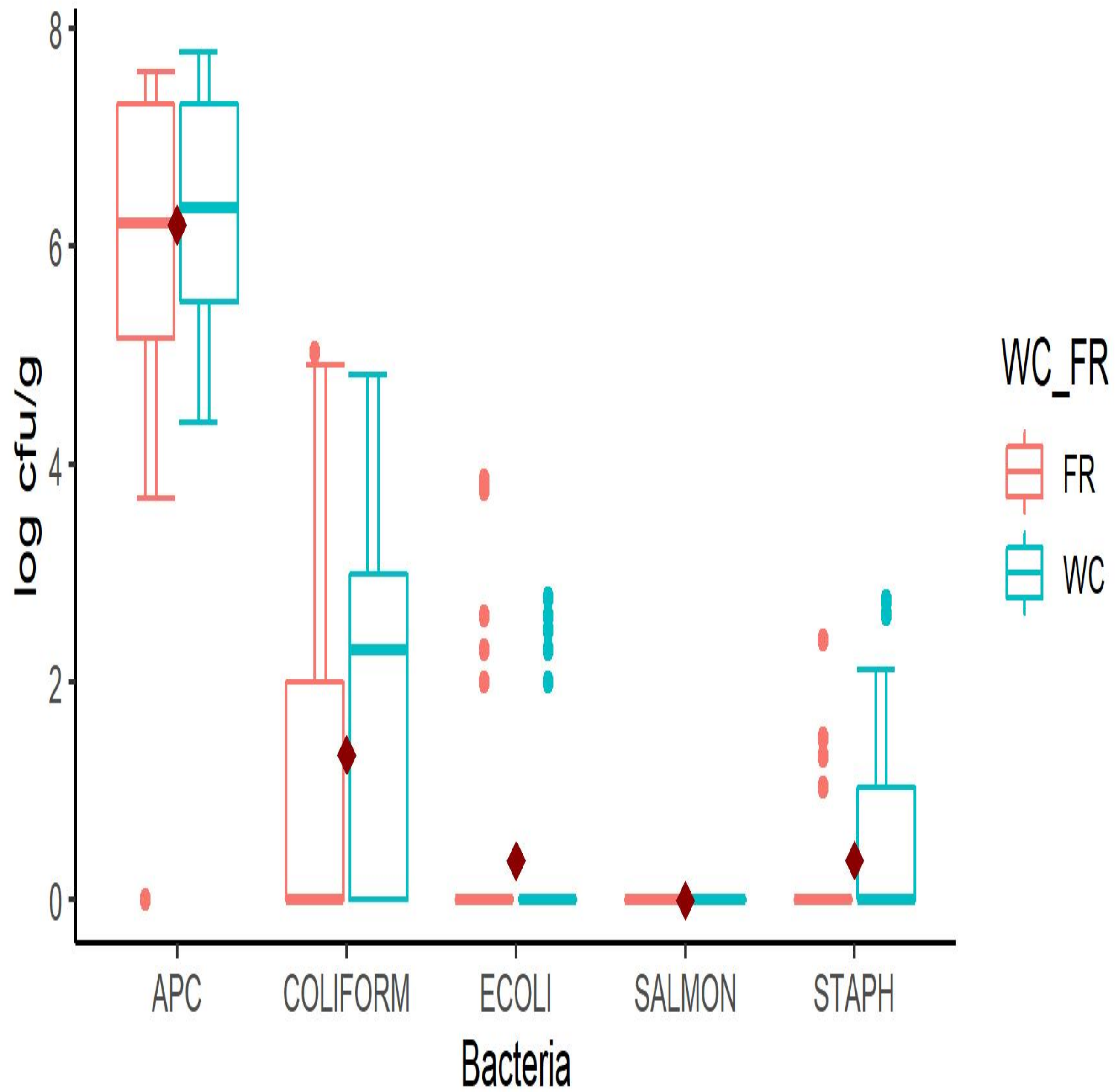
A.1. **Significance of catfish type (WC, FR) regarding the presence of *Salmonella. d.f.* = 1, p = 0.0016.**

A.2. Chapter 2 Supplemental Data



A.2. Season effects on the presence of *Salmonella* confirmed isolates.

A.3. Chapter 3 Supplemental Data



A.3. Enumeration of sanitary indicator microorganisms (log CFU/g) among survey WC and FR retail channel catfish.

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VITA

Bilan Costley Jessie was born in Vacherie, Louisiana in December 1982. She is the daughter of Barbara Costley and Bruce Mitchell. She was raised by her grandparents Benny and Shirley Costley, Jr. She is the mother of two daughters: Kennedy Anacia Jessie and Sydney Taylor Jessie. In May 2005 she received a Bachelor of Science degree in Biology from Dillard University in New Orleans, Louisiana. She began employment as an Agricultural Analyst at Intertek Caleb Brett. In 2010, she began employment at Louisiana Department of Agriculture and Forestry in Baton Rouge, Louisiana where she currently holds a position as a Animal Health Specialist III and Safety Manager at Louisiana State University Agricultural Chemistry Laboratory. She began taking courses to learn about food science as a part-time student in 2012. She enrolled in the graduate program as a PhD student in 2016 in the School of Nutrition and Food Sciences at LSU under the guidance of Dr. Marlene Janes. Her research was the detection and isolation of *Salmonella spp.* in wild caught and farm raised *Ictalurus puntatus,* testing the antibiotic resistance of *Salmonella* isolates from channel catfish, evaluating the effectiveness of common food grade antimicrobial treatments on channel catfish against *Salmonella enterica* and assessing the presence of sanitary indicator microorganisms in retail channel catfish carcasses. While a graduate student at LSU she was accepted into the Honor Society of Agriculture, Gamma Sigma Delta. As a candidate to receive her Doctorate of Philosophy in December 2019, she plans to enhance her professional career as an agricultural scientist.