# THE EFFECT OF LIME JUICE ON *VIBRIO PARAHAEMOLYTICUS* AND *SALMONELLA ENTERICA* INACTIVATION DURING THE PREPARATION OF

## RAW FISH DISH CEVICHE

by

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#### ABSTRACT OF THE THESIS

## The effect of lime juice on *Vibrio parahaemolyticus* and *Salmonella enterica* inactivation during the preparation of raw fish dish ceviche

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Ceviche is a Peruvian raw fish dish. It is extremely popular in the South American countries and has recently gained prominence in USA. It can be made with many types of seafood but is most commonly in USA with tilapia. The most characteristic feature of ceviche is the use of lime juice for marinating the raw fish. No ingredient of the dish is cooked in the conventional sense of applying heat. There have been confirmed cases of cholera in Peru, New Jersey and Florida, associated with ceviche. Since lime juice is sole means of controlling risk in ceviche recipes across the world, it is important to study its anti-bacterial effects. Even though the literature is very rich in the use of organic acids as anti-bacterial agents, little data exists for their effect in seafood systems. The objective of the study was to study the anti-bacterial effects of lime juice marination in ceviche as it would be prepared in restaurants or homes. Target pathogens were *Vibrio parahaemolyticus* and *Salmonella enetrica*. Samples were incubated at room temperature (25°C) and refrigeration temperature (4°C) for time intervals up to 150 minutes.

In experiments with *Salmonella* at room temperature, a mean log reduction of 0.8 was observed while at refrigeration temperature a mean log reduction of 2.1 was observed.

Reduction in *Vibrio parahaemolyticus* could only be estimated based on starting levels and detection limits as the plate counts were always below the detection limit for all the times studied (10-150 minutes), both at room and refrigeration temperatures. In experiments at room temperature, log reductions varied from >4.5 to >5.2 while at refrigeration temperature, log reductions varied from >3.5 to >4.3. In experiments testing the inhibitory effect of lime juice, without the fish matrix, more than 5 log reductions in counts was observed on both bacteria.

We conclude that preparing ceviche reduces *Vibrio parahaemolyticus* risk significantly but is less effective for control of *Salmonella enetrica*.

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#### **1. INTRODUCTION**

#### 1.1 Ceviche

Ceviche is a raw marinated fish dish popular across South American countries and similar to products consumed on many Pacific Islands (Fuchs and Sirvas, 1991). It is sometimes spelled as "seviche" or "cebiche". It is widely regarded Peruvian in origin and is a part of country's national heritage (Newspaper article in El Pais, 2008).

There are several varieties of ceviche. However, one underlining factor in all the varieties is the use of lime juice to marinate the raw fish. Other sources of citrus juices like lemons and oranges are also used, but lime juice remains the single common factor across all the recipes and cultures. Traditionally, no ingredient of the dish is cooked by applying heat and citrus treatment is the only "cooking" step involved. The application of citrus juice renders ceviche its distinct white color. In addition, it also causes protein denaturation resulting in loss of muscular integrity and opaqueness. The scientific community has a difference of opinion about equating this protein denaturation to the one caused by conventional heat treatment and whether the dish could be called cooked (NACMCF, 2008). The public perception, however, is to consider ceviche a raw dish.

#### **1.1.1.** Variation in recipes

The classic Peruvian ceviche includes fish pieces cut into small pieces and marinated with lime juice and bitter orange, and it is served after garnishing with onions or scallions, chili, pepper etc. Ceviche recipes vary across countries and cultures. The variations occur mostly in the choice of fish. For instance, Peruvian ceviche is generally prepared from tilapia or sea bass while Ecuadorian ceviche is made from shrimp.



Figure-1, courtesy- <u>http://eauthenticmexicanrecipes.com</u>, is depicting Mexican ceviche prepared from shrimps.

For consistency throughout the remainder of this thesis, the seafood ingredient will be

referred to as fish.



Figure-2, is depicting the traditional Peruvian ceviche. The white color of the fish piece is distinct due to the action of citrus juices. Courtesy-<u>http://cevicherecipe.org</u>

Another major variant is the temperature at which fish is marinated. Some recipes use room temperature marination, while others use refrigerated marination. Time of marination can also be a variant. The traditional recipes have a longer marinating time, from a few hours to overnight, while the more modern recipes are much shorter. The modern recipes are accredited to Peruvian chef Dario Matsufuji in which the fish is marinated for only about 20 minutes to an hour. American ceviche recipes are closer to the Peruvian recipes. Due to the immense popularity of tilapia in the US, most US published recipes use this fish. The amount of lime juice used remains a personal choice. Most recipes describe the amount of lime juice to be used in terms of number of limes and not the volume of the juice.

For our studies, we used a recipe provided to us by a Peruvian restaurant in Phillipsburg, New Jersey that uses tilapia. The marinating time in this recipe was 20 minutes and they did it at room temperature. We, however, have included some variations in these aspects, explained later in this document.

#### **1.1.2.** Potential and reported health risks associated with ceviche consumption

As a raw fish dish, ceviche poses the inherent health threats associated with raw seafood. The Academy of Nutrition and Dietetics (formerly called the American Dietetic Association (ADA)) has issued an advisory against consumption of raw seafood, including ceviche, for pregnant women, elderly people, children and immune compromised individuals (ADA, 2012).

The spread of endemics like cholera, due to uncooked fish, remains a persistent threat in cultures where ceviche is consumed and sanitation is poor (Torres-Vitella et al.,1997) Handling and distribution systems with poor temperature control and lax sanitation can also contribute in the spread of the disease.

Consuming ceviche has been suspected as one of the causes in many cholera outbreaks in Latin American countries. In the US, cholera cases were reported in New Jersey and Florida in 1991 (CDC, 1991).

The patients had travelled to South America, before the onset of the disease, and had consumed raw seafood including ceviche. No samples were available for testing.

Cholera epidemic occurred simultaneously in many South American countries, including Peru, in 1991 (Weber et al., 1993).

Toxigenic *Vibrio* cholera O1, biotype El Tor, serotype Inaba, appeared in Peru (CDC, 1991 and Weber et al., 1993). An estimated 170,000 cases of cholera including 1250 deaths were reported in Peru alone. Raw fish and shellfish, primarily in form of ceviche and concha, were presumed to be the reason for the endemic that spread further due to unhygienic and insanitary conditions. 36% of isolates showed multiple antimicrobial resistances.

Escartin and Torres-Vitella (1996) reported the incidences of *Salmonella* in 89 fixed and mobile vending establishments in Guadalajara, Mexico.

Parasitic diseases have also been associated with consumption of ceviche. If a fish is cooked to an internal temperature of 60°C (140°F), then parasitic infections are unlikely. Therefore the raw or slightly marinated fish dishes like ceviche, sushi and sashimi are potential threats unless made from fish frozen to inactivate parasites. The FDA guidelines for raw or slightly cooked seafood suggest that it must be blast frozen to -31°F (-35°C) or below for 15 hours or regularly frozen to -10°F (-23°C) for 168 hours (7 days). This would be an effective treatment for parasitic infections (FDA, 2001).

Round worms or nematodes are the most common parasites associated with marine fishes. It belongs to the family *Anisakidae* and the disease caused is called Anisakiasis (Sakanari et al., 1989). The symptoms resemble most GI tract associated infections like vomiting and diarrhea that makes the diagnosis hard. It is often misdiagnosed as Crohn's disease. There have been over 50 confirmed cases of *Anisakiasis* in the US. Another common parasite in the US is the tapeworm or *Diphyllobothrium* which is the causative organism of the disease diphyllobothriasis (Sakanari et al., 1989 and Deardoff, 1991).

#### **1.1.3.** Role of acidified substrate in safety of ceviche

As stated before, marinating the fish is the only processing step involved in ceviche preparation. Therefore, it is presumed that the low pH of lime juice is responsible for bacterial load reduction.

In the Spanish language journal "Revista de Biologia Tropical", Mata et al. (1994), studied the effect of lime juice on *V. cholera*e O1 El Tor on fish substrates. The effect was observed both on commercially prepared ceviche (from mahi-mahi fish) and ceviche prepared from the fish contaminated in the lab. The effect of lime juice on *Vibrio* was also tested by adding the bacteria directly to lime juice.

For commercial ceviche, no bacteria were recovered after 15-30 minutes of exposure while 99.99% bacteria were killed (a 4 log reduction) in ceviche prepared in lab after 30 minutes. For addition of *Vibrio* to the lime juice, 99.9% bacteria were eliminated (a 3 log reduction) within 5 minutes and none were recovered after 2 hours.

Another study, in Guinea –Bissau, West Africa, published in the journal Tropical Medicine & International Health, showed that *V. cholerae* O1 was inhibited by lime juice in the sauce eaten with rice (Rodrigues, 2000). Tomato sauce had a similar effect while peanut sauce failed to inhibit and *Vibrio* thrived in its presence. In a meal prepared with lime juice, *V. cholerae* decreased 2.5 fold for 2 limes and 5 fold for five limes instantaneously. It could not survive in curdled milk further underlining the inhibitory effect of low pH for *Vibrio* (Rodrigues, 2000).

The addition of lime juice or citrus products, however, cannot guarantee a safe food. One of the determining factors would be the amount of lime juice used per gram of the food sample. This can vary in various recipes and thus can affect the safety levels. In the study by Mata et al. the fish was immersed in lime juice to get those levels of inhibition which might not be the case in every ceviche recipe. No correlation between the amount of lime juice and reduction could be found in the existing literature.

Another important factor is the quality of fruit used to obtain the juice. Since most ceviche recipes use freshly squeezed citrus juices, the juice is not typically thermally processed. Therefore, sanitary and hygienic factors in the lime juicing process come into picture. Fresh citrus juices have been shown to harbor foodborne pathogens: *Salmonella* and *Shigella* have been isolated from freshly squeezed orange juice, fresh oranges and wiping clothes from public markets and street booths in Guadalajara, Mexico (Castillo et al., 2006). In this study, 14% of orange juice samples were positive for *Salmonella enterica* serotypes Agona, Typhimurium and Anatum while 6% of samples were positive for *Shigella sonnei* and *Shigella dystenteriae*. Contaminants from such juices could be transferred to the fish even if that fish was originally pathogen free.

An 8 log reduction was observed if the fish was boiled in water for 3 minutes (Torres-Vitela et al., 1999). This is not done normally in ceviche recipes with fish but shrimp in ceviche is boiled in a few recipes. It could be encouraging for such recipes.

#### **1.2 Tilapia Aquaculture**

#### **1.2.1.** Market trends

American food writers referred tilapia as the "Fish of the 90s" (Fitzsimmons, 1999). Earlier, the demand was limited to the Oriental restaurants and grocery stores which could be fulfilled by local farmers but in the late 80s and early 90s, the demand grew and large scale imports and trade of tilapia to the US began.

There has been a steady increase in tilapia imports since the 1990s by about 2000-3000 metric tonne (mt) per year and imports have doubled since 2003. According to the recent data by USDA-Economic Research Service, 425,271 mt of total tilapia was imported in USA in 2011 (USDA-ERS, 2012). Mainland China is the biggest supplier of tilapia with around 75% of total imports followed by Taiwan with 7%. The trend has reversed since the early 90s, when Taiwan was the single largest exporter of tilapia to the US (Fitzsimmons, 1999).

The tilapia market is fragmented between live fish, whole frozen fish, frozen fillets and fresh fillets (Fitzsimmons, 1999). The rise in consumption of tilapia by non-Oriental groups also led to decrease in the imports of the raw whole fish. The tilapia imports are dominated by the frozen fillets that constitute 69% of the total imports (USDA-ERS, 2012). The increasing popularity of tilapia among the Americans is also evident by the fact that it is the fifth most consumed seafood in the US now. The per capita consumption of tilapia has increased from 0.3 pounds per person per year in 2000, to 1 pound by 2006 (Fitzsimmons, 2008)



Figure-3, The distribution of imports of tilapia in USA, 2011 (USDA-ERS, 2012)



Figure-4, The per capita consumption of tilapia in USA over the years, (Fitzsimmons, 2008)

#### **1.3 Tilapia Production**

Tilapia is the generic name of *Cichlidae* family. The 3 major species of this family that are grown and farmed commercially are *Tilapia*, *Oreochromis* and *Sarotherodon*.

Tilapia is one of the most widely grown fish in the world. Farmed tilapia constitutes more than 75% of world's tilapia production (Mjourn et al., 2010). The major reason tilapias are preferred by fish farmers is the ease of culturing. Tilapias are highly adaptable and can grow over a wide range of environmental conditions. Their diet can consist of anything from phytoplankton and zooplanktons to detritus. The reproduction rate in tilapia is also very high increasing profitability.

The following table enlists the limits and optima water quality parameters for tilapia (Mjoun et al., 2010)

Parameter	Range	Optimum for growth
Salinity, parts per thousand	Up to 36	Up to 19
Dissolved oxygen, mg/L	Down to 0.1	>3
Temperature, °C	8-42	22-29
рН	3.7-11	7-9
Ammonia mg/L	Up to 7.1	<0.05

Table 1- Growth parameters of tilapia (Kamal Mjoun et al., USDA, 2010)

#### **1.4 Methods of culturing**

All *tilapia* species are nest builders. The two most farmed species, *Oreochromis* and *Sarotherodon* are mouth brooders (Popma, 1999). After fertilization takes place in the nest, parents keep the eggs in the mouth and may even keep their young ones in their mouths even after hatching.

The selection of species is dependent on the culturing method employed. In the US, growth rate and cold tolerance would be significant in deciding the technique of culturing (Rakocy and McGinty, 1989)

Three major techniques are used for farming tilapia: pond culturing, cage culturing and tank culturing

#### **1.4.1. Pond culturing**

Pond culturing is the most popular method of tilapia farming (Rakocy, 1989). It is the closest to the natural environment of the fish and doesn't require supplements to increase growth rate. The ponds are easy to manage and most farmers opt for shallow ponds that are easy to drain when it is the time to catch the young fish.

Uncontrolled reproduction is the major problem with this type of culturing method because it is hard to monitor it. In such a scenario, the frys and fingerlings compete with the each other and parents for the same available nutrients, thereby reducing the overall growth. This also results in stunted growth of the offspring which is detrimental to the farmer. One of the ways to control the rate of reproduction is to limit number of females in the pond. Typically 3-4 females would be stocked per male in the pond. Male monosex culture is also preferred as their growth rate is faster than the females.

Manure is used to supplement the tilapia ponds in many countries. In the US, however, it is not typically used due to adverse public perception. If manure is added, dissolved oxygen (DO) levels have to be monitored closely.

The rate of growth of various species of tilapia in ponds is-*T*.*nilotica* >*T*. *aurea* > *T*. *rendalli* > *T*. *mosambica* > *T*. *hornorum* (Rakocy, 1989).



Figure-5, courtesy- American Tilapia Association, is depicting a tilapia pond culture in CA, USA. (<u>http://ag.arizona.edu/azaqua/engler.jpeg</u>)

#### **1.4.2.** Cage culturing

Cage culturing is a technique used in existing water bodies. Cages are used to farm fishes in water bodies which cannot be drained or seined or cannot be used for farming otherwise (McGinty and Rakocy, 1989). The biggest advantage of such a method is the disruption of breeding cycles of tilapia that can lead to the raising of mixed-sex population in the same cage with no problem of stunted growth. Cage cultures are easy to manage as they retain fish which facilitates feeding and harvesting.

Cage culturing has some disadvantages too. From a regulatory standpoint, some states may restrict the use of public waters for private farming. In the US, the regulatory agencies have been trying to develop laws that can benefit the farmer without compromising the environment (Masser, 2008). Pollution of the water bodies is another major threat (USDA-NRS 2004). The sediments under the cage are exposed to the wastes from the cages which is detrimental to the environment. They can also be harmful to the fishes being raised in the cages.

Though cage cultures have low initial cost, the nutritional requirements of the fishes must be met completely by supplementation as there is no natural food available. The water inside the cages has to be kept fresh and it's DO be monitored (Masser, 1988). The spread of diseases by the wild fishes around the cage in the water body is also a potential threat (Masser, 2008).

The order of growth rates of tilapia species in cage culture is- *T. nilotica* (frequently restricted) > Florida red tilapia > Taiwan red tilapia > *T. aurea* (McGinty and Rakocy, 1989).



Figure-6, courtesy- American Tilapia Association, is depicting a cage culture system in Egypt. (<u>http://ag.arizona.edu/azaqua/egypt.jpeg</u>)

#### 1.4.3. Tank culturing

When lack of space, water or funds for investment are a concern, tank cultures offer a viable alternative to pond cultures. Tilapias are a highly adaptable species and can tolerate the high densities in a tank culture system (Rakocy, 1989). Their slimy coat prevents bacterial infections and abrasion in a tank (DeLong et al., 2009). Disruption of breeding cycles due to crowding is a significant advantage over pond cultures. Like cage cultures, mixed-sex populations can be grown in the same tank. As with cage culture, due to lack of natural food, all the dietary needs must be supplied by the farmer, adding to the cost of operation. The pumping and filtration of water, along with aeration equipment can also add significantly to the cost. The dissolved oxygen (DO) levels have to be monitored too.

The growth rate of tilapia species in a tank culture is same as in cage cultures. Legal status and cold tolerance are the major deciding factors for the growth rate.

The order of growth rates of tilapia species in tank culture is-*T. nilotica* (frequently restricted) > Florida red tilapia > Taiwan red tilapia > *T. aurea* (Rakocy, 1989).



Figure-7, courtesy- American Tilapia Association, is depicting tank culture in a farm in Safford, AZ, USA (<u>http://ag.arizona.edu/azaqua/safford.jpeg</u>).

#### **1.4.4. Integrated farming practices**

Integrated farming practices are common and have been practiced a long time in Asia. Integrated farming consists of farming fishes or shrimps in conjunction with chicken, pigs, ducks etc (Vincke, 1991). With most of the seafood in the US being imported from the Asian countries, primarily China, it becomes imperative to study the impacts of this practice. Integrated agricultural practices have the basic principle of utilizing the wastes of one farm activity as a useful component of the other (Siaw-Yang, 1991). The wastes (feces and urine) from the animal farms are used as the organic manure for the fish ponds or tanks. Some integrated systems incorporate plants too and are more complex utilizing both animal wastes as manure and water from fish ponds for irrigation.

In principle, these practices are eco-friendly as they result in complete conservation and utilization of the farm resources. They also present financial impetus for the farmer to have multiple farming systems in the same facility. They emerged in Asia, primarily, because fish farming was a part-time activity for the peasant farmers (Vincke, 1991). For developed countries, financial constraints may not be a primary concern. However, integrated farming still saves the additional costs of collection, transport, storage and distribution of manure in ponds. However, more importantly, it presents an effective way of reducing water pollution by utilizing the farm wastes that would otherwise be released in rivers. If these wastes are used on land as fertilizers, excessive usage still poses a threat of eutrophication.

The nutritional advantages of using animal wastes as manure in integrated farming systems can be summed up as: the nutrition derived from the breakdown of organic matter of the manure by bacteria that results in growth of phytoplankton and zooplanktons which, in turn, are consumed by the fish and direct consumption of manure by the fish (Siaw-Yang, 1991)



Figure-8, is the pictorial representation of a typical integrated farming system. The pigs/poultry/livestock is at a higher level while the fish ponds are beneath the farms receiving the fecal droppings from the animals above. Courtesy- New Zealand Digital Library (<u>http://www.nzdl.org/gsdl/collect/hdl/index/assoc/HASHb35d.dir/p038.png</u>)

#### Microbiological impact of integrated farming methods

The addition of manure to the fish cultures or the integrated farming methods have always been a cause of concern in the developed countries especially the US. The public perception in this scenario has also been negative. However, in strict microbiological sense, studies suggest contrasting views on whether to supplement the ponds is advisable or not. The points of consideration in integrated systems are the potential sources of contamination.

The fish being raised can be exposed to pathogens like *Salmonella* or *E. coli 0157:H7* in two ways: First, all the livestock fecal wastes are expected to be the hosts for pathogens (Little and Edwards, 2003) and second, the animal feed used to raise the poultry/livestock used in integrated farming systems can infect the animal being raised and can later appear in its feces.

Both livestock and fish have been implicated with a score of human diseases (Little and Edwards, 2003). It is sufficiently established that a number of diseases have entered the human populations through animals (Morse, 1990 cited by Little and Edwards, 2003). The stakes on integrating livestock and fish are higher than before because of both have been implicated with the irregular pandemics of influenza. Though literature suggests that common pathogens of warm-blooded animals do not cause diseases in fish (Guelin, 1962 cited by Buras, 1985), their role in transfer of these pathogens during farming processes is not clear and needs further attention.

The recent emergence of a variant of Creutzfeldt-Jakob disease in humans has brought back the attention on animal feed as a potential contaminant. It is a prion disease associated with cattle fed with meat and bone meal derived from sheep infected with spongiform encephalopathies (Crump et al., 2002).

Historically, poultry feed has been associated commonly with *S. enterica* globally (Crump et al., 2002). In 1993, FDA detected *S. enetrica* in 56% of the 101 animal-protein based samples of poultry feed tested. Earlier, in the 1970s, *S. enterica* serotype Agona emerged as a major public health threat associated with poultry meal derived from Peruvian fish meal. By the year 2000, nearly 29000 cases of *S. enterica* serotype Agona were reported (Crump et al., 2002). Feed ingredients and dust are considered to be the major sources of contamination in feed mills (Jones et al., 2002).

It has been sufficiently established that chicken and other poultry animals shed *Salmonella* in their droppings. Studies have shown that *Salmonella typhimurium* and *E.coli 0157: H7* can survive in a poultry manure for 1-2 days at 37°C and 6-22 weeks at 4°C (Himathongkham et al., 2000). If the poultry integrated with the fish farms (directly or as a source of manure) is infected by pathogens due to the meal feeds, it can contaminate the water in which the fish is raised and the fish can subsequently act as a vector of transport of the pathogen.

A contrasting view emerges when other possible sources of contamination of the farming water are taken into account. Buras et al. (1985) established a concept of threshold concentration over which micro organisms could be recovered from the muscles of the fish when it is raised in treated and untreated waste water.

It defined the total number of bacteria in the fish, sampled through blood, kidney, liver, spleen, bile gastro-intestinal tract and muscles. The research outlined the fact that fish can tolerate a certain threshold level of bacteria in water. Above that, its immune system fails to cope up and, bacteria appear in its organs and finally muscles. The concentration in water that is required to reach these threshold levels varies between 1 and 5 x  $10^4$ / ml (Buras, 1985). Trials in Asian Institute of Technology suggest that the threshold concentrations are not generally crossed in routine manure loading rates. More microorganisms are recovered from the GI of the fish than those present in the water suggesting that it is more likely for a fish to get infected by handling practices than the contaminated water. Birds are also a potential source of contamination by *Salmonella* in fish ponds (Little and Edwards, 2003).

The importance of organic supplements is highlighted by the difference in weights of *T. aurea* farmed without any organic fertilizers, 46 grams, and when supplied with poultry wastes, 304 grams (Burns et al., 1980). In the same study, the samples collected from the ponds with poultry wastes, were found free from any *Salmonella*. This also points at another significant factor. It is not the presence of pathogens in the water, but their ability to cause diseases in humans which should be the critical factor. The large variation in daily temperatures, pH and dissolved oxygen in tropical fish ponds cause rapid decrease in pathogen numbers (Somnasang et al., 1990 cited by Little and Edwards, 2003).

Despite of varied views in the scientific community and the cited literature, integrated farming remains one of the most important farming methods. Chicken and duck are the two most common poultry animals used for integrated farming.

This became the basis of choosing our *Salmonella* strains for the study. The strains were 4 clinical strains of *S. enterica* isolated from chicken because of its common usage in these farming systems.



Figure-9, courtesy- Department of Fisheries and Allied Aquaculture, Auburn University, Auburn, AL, USA, is depicting an integrated farming system involving chicken and fish (http://www.ag.auburn.edu/fish/image\_gallery/data/media/94/fishchicken.jpg).

## **1.5 Pathogens of interest**

Estimates suggest that 26.5% of all foodborne illnesses in the US is a result of contaminated seafood (Drake et al., 2007). The propensity towards raw or slightly marinated seafood can adds to this risk.

On the basis of our knowledge of the natural flora of the fish and farming methods, we have two pathogens of interest for this study: *Vibrio parahaemolyticus* and *Salmonella enterica*.

#### **1.5.1.** Vibrio parahaemolyticus

*Vibrio parahaemolyticus* is gram-negative halophilic bacteria which inhabits brackish saltwater. Both pathogenic and non-pathogenic strains have been isolated from fish and shellfish from these habitats. It causes gastrointestinal illness in humans called *V*. *parahaemolyticus*-associated gastroenteritis. It is spread by raw or undercooked seafood. It can also cause a disease if an open wound is exposed to seawater (FDA, 2009).

*V. parahaemolyticus* causes watery diarrhea along with abdominal cramps, nausea, vomiting, fever and chills (CDC, 2009). The symptoms occur within a day of ingestion and last for about 3-4 days. It could be severe for immune-compromised individuals otherwise the disease is self-limiting.

Between 1988 and 1997, CDC reported 345 cases of *V. parahaemolyticus* infections, and 59% of these infections were gastroenteritis, 88% of which were caused by raw oysters' consumption in the week before the infection. Another 34% were wound infections while 2% were from other routes of exposures (Daniels et al., 2000).

#### Classification and pathogenicity of V. parahaemolyticus strains

*V. parahaemolyticus* is classified as pathogenic or non-pathogenic based on the genes coding for hemolysin (Drake et al., 2007).

The strains are considered pathogenic if the thermostable direct hemolysin (*tdh*) gene is present. The protein TDH coded by the gene *tdh* lyses the red blood cells on Wagatsuma agar. This is referred to as Kanagawa phenomenon (KP). This is the most distinct and well known test for the pathogenicity of the *V.parahaemolyticus* strains. Almost all clinical strains are KP-positive while only 1-2% environmental strains are KP-positive. Strains that are not KP-positive are referred to as KP-negative strains TDH has been characterized as a protein of molecular 42000 Daltons with 2 equal subunits (Joseph et al., 1982). It contains no carbohydrates, lipids or organic phosphorus. It can be inactivated by pepsin and trypsin enzymes and is activated by  $Ca^{2+}$  ions.

Due to cardio-toxicity exhibited by TDH protein, volunteer studies with *V*. *parahaemolyticus* were only performed prior to 1975. A dosage of  $10^6$  cells have shown to cause illness. Volunteers became sick within 6 to 8 hours when challenged with a KP+ strain while a KP- strain caused illness in 18 hours (Kothary and Babu, 2001). A dose of  $10^5$  cells caused abdominal discomfort in 25% volunteers. The risk assessment of a pathogen is affected the most by the food matrix used (FDA, 2005). For instance, buffered foods like cooked rice or antacids can reduce the number of microorganisms needed to cause disease by increasing the gastric pH.

Pathogenicity has also been reported by a TDH-related hemolysin (TRH) producing gene designated as *trh*. The genes *tdh* and *trh* have around 69% similarity in nucleotide sequence (Honda et al., 1987 cited by Drake et al., 2007). However, recent findings indicate that *tdh* and *trh* genes may not be necessary for virulence (Drake et al., 2007). CDC reported some strains associated with more severe cases of gastroenteritis lacking both the genes.

It is, therefore, postulated that some other factors are also responsible for virulence like adhesiveness. All *Vibrio* strains causing disease have the adhesive property to attach to the human fetal intestinal (HFI) cells.

Being a halophile, *Vibrio* spp. requires saline environment to survive. NaCl has a protective effect for *Vibrio parahaemolyticus* even at temperatures as high as 48°C, with 3% to 12% salt. Cells were recovered even after 30 days of storage in presence of salt in the growth media (tryptic soy broth) (Covert and Woodburn, 1972). Surprisingly, similar results were observed when cells were suspended with fish homogenate. Fish homogenate was found to be more protective than TSB alone. This shows that if a fish is infected with this bacterium, the fish system itself can act as a protective matrix for the bacteria.

*Vibrio* spp. is susceptible to inactivation high (cooking) temperatures. FDA suggests seafood to be cooked for prevention against illness from these bacteria.

#### 1.5.2. Salmonella enterica

The *Salmonella* family includes over 2400 serotypes of bacteria. They are rod shaped, facultatively anaerobic, motile gram negative bacteria which are non-spore formers. *Salmonella* can survive and grow in a wide range of environmental conditions with temperatures ranging from 2-54°C. They do not require salt to grow but can survive in a concentrations up to 4%. These properties allow them to be a potent foodborne pathogen which can infect a variety of foods. *Salmonella* have been found in raw meats, seafood, eggs, peanut butter, sauces, pepper and chocolate. *Salmonella* Enteritidis is the most common serotype in the world and in the US.

Infection can be caused by water or food contaminated with animal waste. There are 1.3 billion cases of gastroenteritis and 3 million deaths worldwide due to *Salmonella* (Pui et al.,2011). CDC estimates that *Salmonella* accounts for 28% of total foodborne deaths in USA (Scallan et al., 2011). The symptoms include fever, abdominal cramps and diarrhea 12-72 hours after ingestion. The disease last for 4-7 days and is treatable without antibiotics unless the patient is immune-compromised (CDC, 2010)

#### **Classification of** Salmonella

Epidemiological classification of *Salmonella* serotypes is based on the host preferences (Pui et al., 2011). Those restricted to only one host, like *S*. Typhi that infects only humans, constitute the first group. The second group includes the host-adapted serotypes that are associated with one species but can cause disease in other host serotypes like *S*. Pullorum. The remaining serotypes are classified in the third group. More than 99% of disease causing *Salmonella* in humans belongs to *Salmonella enterica* subtype *enterica*.

#### Salmonella in seafood

Huss et al. (2000), classified seafood into risk categories. Molluscs and raw fish constituted the highest risk category followed by crustaceans and fish that is to be eaten after cooking. The low risk group consisted of lightly preserved, semi preserved, mildly heat processed and heat processed fish and fish products. *Salmonella* has been found to cause illness related to seafood worldwide. FDA collected and analyzed imported and domestic seafood samples for a 9 year period between 1990 and 1998 (Heinitz et al., 2000). It included raw seafood, ready-to-eat seafood and raw shellfish.

The incidence of *Salmonella* for imported seafood was 7.2% while for domestic it was 1.3%. About 10% of imported raw seafood and 2.8% of domestic raw seafood was found to be positive for *Salmonella*. The incidences of positive ready-to-eat imported seafood were 2.6% while this was number was placed lowly at 0.47% for domestic food. The biggest incidence of *Salmonella* was in imported raw fish at 12.2%.

*Salmonella* is likely to infect seafood due to farming methods. Integrated farming practices in Asia can lead to such infections. Even though *Salmonella* does decline in *Tilapia aurea*, it can survive for 16 days (Baker et al., 1983). The dose–response model of *Salmonella*, which incorporated the data from all outbreaks, had an infection ID50 of 7 CFUs and illness ID50 of 36 CFUs (Teunis et al., 2010). This indicates much higher pathogenicity than the volunteer studies with lab adapted strains. Preventive measures for *Salmonella* infections would depend on the food; however, cooking would be a sufficient step in most of the cases.

#### **1.6 Role of Organic Acids**

#### 1.6.1. Anti-bacterial effects of organic acids

The basic premise of this study was to test the effect of lime juice on pathogen reduction in ceviche preparation. Citric acid is the major constituent of lime juice. It is naturally present in lime juice at a concentration of about 1.4g/oz, while the concentrates contain 1.1g citric acid/oz of lime juice (Penniston et al., 2008). In commercially available lime juice, it ranges from 0.03 to 0.22 g/oz. In ceviche recipes, freshly squeezed lime juice is commonly used and is the most distinct ingredient in ceviche preparation. Organic acids have historically been used as food preservatives and additives for perishable food items but all of them have anti-microbial activity. They have also been used as sprays on pre and post harvest processing and production. Organic acids have GRAS (Generally Recognized as Safe) status and hence are used extensively in food systems. As a group, these compounds primarily are saturated straight chained monocarboxylic acids and their derivatives. They are referred to as fatty acids, volatile fatty acids or weak or carboxylic acid (Ricke, 2003).

#### Mechanism of action

The anti-bacterial properties of organic acids are attributed to their low pH. It is sufficiently established that un-dissociated forms (uncharged or protonated forms) of organic acids can penetrate the bacterial membranes while the dissociated (non-protonated or charged forms) forms cannot (Davidson, 2007).

The pH at which the concentrations of charged and uncharged acids are equal is called the pK<sub>a</sub>. One pH unit above the pK<sub>a</sub>, there are 10 fold fewer protonated acid molecules. Organic acids are limited to work in a narrow range of pH, generally pH <5.5. The cytoplasm of the bacterial cell is neutral. Once the uncharged acid enters the cytoplasm, it dissociates into anions and protons. The cell has to spend energy in the form of ATP to keep its neutrality and hence growth is impeded (Cherrington, 1991 and Davidson, 2007).

The role of dissociation in activity of organic acids on bacteria was elucidated in a study with meat spoilage bacteria by Ouattara et al. (1997). The inhibitory effect of common organic acids like acetic, benzoic, citric, lactic, propionic and sorbic acids were studied on six common meat spoilage bacteria.
On the basis of weight, acetic acid was found to be the most inhibitory, followed by, propionic, lactic and citric acid. Inhibition resulted in longer lag periods, lower growth rates or lower bacterial count. The order of effectiveness was reversed when concentrations were expressed in molar terms. This was because, with concentrations in moles, the effectiveness was expressed in terms of un-dissociated acid molecules. When the effectiveness of organic acids was compared to hydrochloric acid by lowering the pH, the former were more inhibitory. This explains that the inhibition is not just by acidification but also due to the ATP usage in case of organic acids. Some acids, like citric and propionic acid, may have a lesser ability to enter the bacterial cell, but they make up for it by more dissociation, once inside the cell.

The precise effectiveness of organic acids in foodborne pathogens can be difficult to study due to the multiplicity of the factors involved (Ricke, 2003). The results may vary due to the species involved, physiological state, growth media etc. Most of the foodborne pathogens can grow in a various ecosystems and thus their response to organic acids may vary.

#### Effect of low pH on Vibrio spp.

One of our target pathogens was *Vibrio parahaemolyticus* due to its presence in the natural flora of seafood. Other species of *Vibrio* like *V. cholerae* and *V. vulnificus* have also been studied for similar reasons. The response of *Vibrio* spp. towards low pH is generally in the same pattern, with some variations with respect to the species, environment and matrix in question.

All *Vibrio* were found to be susceptible to low pH but gradually developed an acid tolerance response pattern if the stress was regulated (Beuchat, 1976, Wong et al., 1998, Koga et al., 1999, Merrel et al., 1999 and 2001, Koo et al., 2001 Koo et al., 2000 and Bang et al., 2005). Adaptation to one stress also seems to trigger other physiological adaptations, therefore, signaling a complex protein synthesis phenomenon rendering adaptability to multiple stresses.

Beuchat (1973) studied the effect of pH, temperature and salt concentration on growth and survival of *Vibrio parahaemolyticus*. Six strains of *V. parahaemolyticus* were grown in TSB containing either 7% or 3% NaCl. The pH values were adjusted to values from 4.7 to 8, by using HCl or NaOH. Temperatures 2,5,9,13,21 and 30°C were observed. No growth was observed at 2°C and growth at 5°C and 9°C was restricted to alkaline pH only. The growth was better at lower pH if the incubation temperatures were higher. The lowest pH tolerance observed in the study was 4.8. The study was performed in liquid broth and some differences may be observed if a food system is used due to potential buffering. The pH trend, however, is supported by a previous study (Vanderzant et al., 1972) in which the organism was found very sensitive below pH 6.0.

*Vibrio parahaemolyticus* has shown sensitivity towards organic acids. Beuchat (1976) studied the effects of organic acids present in lemons and tomatoes on the organism in solution. Inhibition was observed in case of all the acids studied (citric, ascorbic and malic) in form of extended lag phases from 5.2 < pH < 7.2. The degree of inhibition was a factor of pH of the medium and not the acid used to reach that pH. No viable cells were recovered from tomato sauce at pH 4.4 after 24 hours at 10 and 22°C or after 4 hours at  $35^{\circ}$ C.

However, the interesting results were observed with tomato sauce at pH 5.0-6.5 with lack of significant reduction of the organism. In another study (Bradshaw et al., 1974) with cooked seafood and sauce cocktail, similar survival was observed over a period of 48 hours. Both these unexpected results could be explained only by the buffering action of other ingredients present. Therefore, if tomato sauce is added to contaminated seafood, with other ingredients, it may not result in significant reduction. The roles of spices used with sauces become important in such circumstances.

Even though various spices have shown varied levels of inhibition, thyme and oregano have shown high bactericidal activity against *Vibrio parahaemolyticus* in solution (Beuchat, 1976).

#### Effect of low pH on Salmonella

*Salmonella* encounters low pH environments at various phases of its life cycle including the highly acidic gastric juices. During its pathogenesis, it can survive the extreme acid environment of stomach, the volatile fatty acids of intestines, the milder acidic stress of epithelial cells and finally in macrophage phagolysosomes (Foster, 1995). It can grow at pH values ranging from 4.5 to 9.5. Some of the common organic acids have a bactericidal effect on *Salmonella*. Propionic and acetic acids were found to be more effective than citric and lactic acids which are associated more with food (D'Aoust and Maurer, 2007). The antibacterial effect of the organic acids decreases with increase in chain length.

The optimum pH for *Salmonella* is 5.5 but it has shown growth down to pH values as low as 4 (Foster, 1995). Survival depends on the ability to keep the internal pH constant or in a tolerable range (bacterial homoeostasis) (Foster and Hall, 1991).

When the external pH is changed to 4.5, the difference between internal and external pH becomes important. *Salmonella* keeps it's internal pH in the range of 6, but when external pH is dropped further, internal pH starts to change too. When the difference between the two reaches a maximum of 1.8, the culture starts to lose its viability. However, it still survives due to the activation of acid tolerance response ATR.

#### **1.6.2.** Acid tolerance response (ATR)

The uses of organic acids as anti-bacterial agents have been extensively studied and established in pathogens like *E. coli* and *S. typhimurium*. Acid tolerance response, commonly referred as ATR, is a result of synthesis of proteins that confer resistance to stresses to the bacteria. Various mechanisms like inducible pH homeostasis, DNA repair, chaperonins and replacement of acid-sensitive cell constituents with acid-stable homologs can be the potential mechanisms for acid defense (Foster, 1995)

### ATR in Vibrio spp.

In *V. parahaemolyticus*, acid tolerance responses have been observed. In a study by Wong et al. (1998), cells grown at pH 7.5 were adjusted to pH 5.8, 5.5 and 5.0 for 30 minutes and were subsequently challenged at pH 4.4. Expectedly, cells adapted at the gradual pH intervals showed better survival than non-adapted cells. The average death time was not affected significantly by acid-adaptation, but intestine/body weight ratio in suckling mice showed significant increase in enteropathogenicity for acid-adapted cells. The more important result was however the trigger of other stresses' responses. The acid adapted cells showed greater survival at low salinity and after thermal inactivation. Similar responses have been observed as a result of nutrition starvation including survival at lower temperatures (Jiang et al., 1996). Interestingly, heat adapted cells showed no increased acid resistance (Koga et al., 1999).

The acid tolerance response of *Vibrio cholerae* is more well characterized than in *V. parahaemolyticus*. It is attributed to a system of genes, like *cadA* and *toxR*, that code for both organic and inorganic ATR. The inorganic ATR results in its survival at low pH, organic ATR results in its survival in at low pH in presence of organic acids (Merrel et al., 1999 and 2001).

Another foodborne bacteria, Vibrio vulnificus, has been shown to have tolerance to low acid environment. It is not tolerant to low pH environment naturally but adapts if the stress is increased gradually. Bang et al. (2005) showed that in a media acidified by HCl, at pH 5, the number of Vibrio vulnificus didn't decline for considerably for 10 hours but a 5 to 6 log reduction occurred at pH below 4 and below. Koo et al. (2000) showed a 5 log reduction of Vibrio vulnificus within 1.7 minutes at pH 2 and within 18 minutes at pH 3. The natural tolerance of V. vulnificus towards organic acids is similar as in case of HCl. Bang et al. (2005) showed a reduction of 6 log within 15 minutes at pH 3.5 with citric acid. At pH 3.5 the order of inhibition was acetic acid > citric acid > hydrochloric acid. However at pH 4.0, hydrochloric acid had more inhibitory effect than citric acid. In the same study it was shown that the organism adapts to low pH for both hydrochloric acid and organic acids. The adaptation was slow and acid resistance increased for pH 3.5 after 5 and 10 hours adaptation at pH 5 with HCl. The adaptation increased for citric acid irrespective of time for adaptation. V. vulnificus didn't show any increased heat tolerance for acid adapted cells.

They however showed increased tolerance for freeze/thaw and cold storage, signaling trigger of cross tolerance. The acid adaptation in bacteria is dependent on the gradual nature of exposure and the degree of change. It is exposed to various pH changes in the GI tract and adaptation can happen *in vivo* as well. Koo et al. (2001) showed that *Vibrio vulnificus* showed better growth in presence of antacid as compared to the simulated natural GI tract environment.

### ATR in Salmonella

*Salmonella* spp. has been the most extensively studied bacteria for ATR studies. It has shown a vast range of stress tolerance levels and they have been well characterized to the gene level. The ATR in *Salmonella* is controlled by a series of acid shock inducible proteins (ASPs). There are 51 ASPs for log phase and 15 for stationary phase ATR. A sigma factor is needed to express 8 of these proteins that lead to an extended survival in stationary phase (Baik et al., 1996). Most ASPs are released for heat shock as well. While a heat shock doesn't induce a acid tolerance reverse does occur (Foster, 1995).

The concept of ATR brings forth the idea that cell might be naturally prepared for a more severe stress once it is subjected to a stress. The ATR in *Salmonella* is different from the high acid resistance of other pathogens like *E. coli* and *Shigella*. ATR can operate in minimal media unlike acid resistance that requires components of complex medium for induction (Lin et al., 1995). A study by Baik et al (1996) showed that at pH 4.4, *Salmonella* couldn't survive the addition of organic acids and volatile fatty acids.

However, acid adapted cells (at pH 4.4 for 1 hour) could successfully survive organic acids' addition indicating that ATR induced by acidification protects also from organic acids.

However, the extent and conditions may differ for different organic acids. For example, ATR in *Salmonella* provided protection against propionate and acetate but not against benzoate even at lower concentrations of the latter (Baik et al., 1996). This supports the role of dissociated and un-dissociated acids in anti-bacterial effects of organic acids. However ATR is not merely caused by inhibition of growth and an acid stress is mandatory. Inhibition of growth by other stresses like osmotic, temperature or peroxide shock doesn't provide cross protection against acid stress (Foster and Hall, 1990).

## **1.7 Hypothesis**

Based on the literature review, existing recipes and the knowledge of natural flora of tilapia we make the following hypothesis:

• The low pH of lime juice is effective in inactivation of pathogens of interest on tilapia during ceviche preparation.

# 1.8 Rationale

Ceviche recipes are a part of South American heritage for thousands of years. Barring minor variations, across countries and cultures, they remain primarily the same. The use of lime juice is distinct in all these recipes. Despite of some sporadic cases associated with the dish, no widespread outbreaks have been reported. It is reasonable to assume that something in the traditional recipes may be reducing foodborne illness risk from consumption of this dish. There is limited scientific research in support of this assumption, so we set out to study pathogen survival during the preparation of ceviche under real life settings.

## 2. MATERIALS AND METHODS

### **2.1 Materials**

### 2.1.1. Frozen tilapia fillets

Frozen tilapia fillets were purchased from local supermarkets. The best before date of each fish package was noted and only fresh packages were purchased. After purchase, each package was transported using ice packs and promptly frozen to prevent spoilage. For all the experiments no fish stored for more than 2 days was used.

## 2.1.2.Limes

In accordance with the ceviche recipes, only freshly squeezed lime juice was used in this study. Limes were purchased from local supermarkets. Quality and freshness was based on visual inspection. Limes were stored refrigerated prior to use.

### 2.1.3. Media for culturing Vibrio parahaemolyticus

**Peptone water (0.1%)** was prepared by dissolving 1.5 grams of peptone powder (Difco, Becton, Dickinson and Company, MD, USA) in purified distilled water. *Vibrio* spp. is halophilic and it requires salt for its optimum growth. Hence 3% w/v NaCl was added to the peptone solution. The solution was divided in 9ml aliquots in glass tubes. It was sterilized by autoclaving at 121°C for 15 minutes in an autoclave.

**Tryptic soy broth** was prepared by suspending 30 grams of tryptic soy broth powder (Difco, Becton, Dickinson and Company) in 1 liter of purified distilled water. Because of halophilic nature of *Vibrio* spp. 3% w/v NaCl was added to the solution. It was sterilized by autoclaving at 121°C for 15 minutes.

**Thiosulfate-Bile-Sucrose (TCBS)** agar was prepared by dissolving 89 grams of TCBS powder (Difco, Becton, Dickinson and Company) in 1 liter of purified distilled water. TCBS is a media selective for the isolation of enteropathogenic *Vibrio* spp. Additional agar powder was added to enhance the solidification in plates. It was sterilized by boiling and then cooling to 45-50°C prior to pouring plates.

**Tryptic soy agar** was prepared by dissolving 40 grams of tryptic soy agar powder (Difco, Becton, Dickinson and Company) in 1 liter of purified distilled water. NaCl was added to the agar for *Vibrio* spp. It was sterilized by autoclaving at 121°C for 15 minutes.

**Sodium chloride** (Acros Organics, NJ, USA) used in the media for *Vibrio* was a lab grade extra pure salt.

### 2.1.4. Media for culturing Salmonella

**Peptone water (0.1%)** was prepared by dissolving 1.5 grams of peptone powder (Difco, Becton, Dickinson and Company) in purified distilled water. It was divided in 9ml aliquots in glass tubes. It was sterilized by autoclaving at 121°C for 15 minutes in an autoclave.

**Tryptic soy broth** was prepared by suspending 30 grams of tryptic soy broth powder (Difco, Becton, Dickinson and Company) in 1 liter of purified distilled water. It was sterilized by autoclaving at 121°C for 15 minutes.

For the antibiotic resistant strains of *Salmonella* (*S. typhimurium* -248b and *S. kentucky*-129a), tetracycline was added to the broth after autoclaving at a concentration of 0.01gram/liter.

**Xylose lysine tergitol-4 (XLT-4) agar** was used as a selective media for *Salmonella* spp. It is selective for non-typhi *Salmonella*. It is prepared by dissolving 59 grams of powder (Difco, Becton, Dickinson and Company) in 1 liter of purified distilled water. It is then boiled for sterilization, and cooled to 45-50°C before pouring in petri plates.

**Tryptic soy agar** was prepared by dissolving 40 grams of tryptic soy agar powder (Difco, Becton, Dickinson and Company) in 1 liter of purified distilled water. It was sterilized by autoclaving at 121°C for 15 minutes.

## 2.2 Methods

## 2.2.1. Experiments with lime juice and fish

### A. Testing the efficacy of lime juice in bacterial reduction

### **Preparation of fish**

Tilapia was purchased as packets of the frozen fillets from a local supermarket. It was stored frozen at -18 to -23°C and thawed for about 20 minutes at room temperature before use. To replicate existing ceviche recipes, the thawed fillets were cut into cubical pieces ~ 2.5 cm in length. Each cube weighed approximately 4.5 grams. Sterilized cutting boards and knives were used for preparing the fish for the experiments.



Figure-10 (i), depicts the frozen tilapia fillets purchased from market. Figure-10 (ii), depicts the thawed and cut cubical pieces of fish  $\sim 2.5$  cm in length, weighing 4.5 grams

# **Preparation of lime juice**

Freshly squeezed lime juice was used for the study. The amount of lime juice to be used was determined by referring to ceviche recipes. Based on various recipes, a suitable standardized ratio of lime juice to fish was determined to be 1 milliliter per 1.5 grams of the fish used for all experiments.



Figure-11, showing the limes, sterilized squeezer, cutting board and knife

The pH of the lime juice was an important factor to consider in the study. The pH of the lime juice was measured for every experiment using a bench-top pH meter (Accumet AB 15/15+ bench-top meter, Fisher Scientific, Fair Lawn, NJ, USA). The pH of the lime juice used in this study varied from 2.2 to 2.5.

### **Preparation of overnight cultures**

### Preparation of Vibrio parahaemolyticus

Five clinical isolates of *Vibrio parahaemolyticus*, as shown in Table-2, were used to make the cocktail of overnight culture, used for this study. These strains were kindly provided by Dr. Haiqiang Chen (University of Delaware, Newark, Delaware, USA).

The overnight cultures used for the study were prepared from the aforementioned strains which were stored in 24% glycerol with 1% NaCl as stock cultures at -80°C. They were thawed and TSB broth with 3% NaCl was inoculated with a loop full of culture. All strains were individually grown in 15 ml falcon tubes overnight at 37°C. Two ml from each overnight culture was added to a fresh tube of TSB with 3% NaCl.

Strain ID Number	Source
ATCC 17803	Shirasu Food poisoning in
	Japan
ATCC 27519	Shrimps involved in food
	poisoning in Louisiana,
	USA
ATCC 33845	Patient with food poisoning
	in Japan
ATCC 43996	Cockles causing fatal food
	poisoning in England
DIE-12	United States Department of
	Agriculture (USDA)

Table-2- V. parahaemolyticus strains used in this study. Courtesy- Dr. Haiqiang Chen, University of Delaware

Three hundred micro liters of the overnight culture from each of the tubes was added to new tubes with fresh TSB media and were incubated for an additional 24 hours at 37°C. Duplicate tubes of this overnight cocktail were centrifuged for 5 minutes at 3500 rpm at 4°C. The pellets obtained were re-suspended in 9 ml 0.1% peptone buffer with 3% w/v NaCl and spun again. The buffer was then discarded and the pellets were re-suspended in fresh 0.1% peptone water with 3% w/v NaCl were then combined and used to inoculate tilapia samples

## Preparation of Salmonella enterica serovar cocktail

Four clinical strains of *Salmonella enterica* were used to prepare the cocktail used in this study. These strains were generously provided by Dr. Jianghong Meng of University of Maryland, Baltimore, Maryland, USA.

Salmonella eneterica serovar	Resistant to
Kentucky 129a	Streptomycin and tetracycline
Typhimurium 248b	Amoxicillin/clavulanic acid, ampicillin,
	cephalothin, ceftiofur, cefoxitin,
	streptomycin and tetracycline
Kentucky 3a	Antibiotic-susceptible
Typhimurium 14a	Antibiotic-susceptible

Table-3- Salmonella enterica strains used in the study. Provided by Dr. Jianghong Meng, University of Maryland

The overnight cultures were prepared from the strains that were stored in 24% glycerol stocks at -80°C. Two of the strains used were antibiotic resistant so for these strains, a TSB broth with 0.01gram/liter tetracycline was used. The antibiotic-susceptible strains were grown in TSB broth without antibiotic. The strains were thawed and the appropriate TSB broth was inoculated with loop full of the cultures. They were then incubated at 37°C for 18-24 hours. The cocktail was prepared the following day by adding 2.5 ml of each overnight culture to another tube.

The cocktail was centrifuged to obtain pellets by the same process as employed with the *Vibrio* cocktail. The pellets from 2 tubes were suspended in 0.1% peptone water buffer. The pellets obtained by spinning down the overnight culture of bacteria and suspended in 0.1% peptone water buffer were then used for all studies.

#### **Inoculation of the fish sample**

Selecting an inoculation method for a particular study, is based upon the expected sources of contamination of the sample to be studied. For our studies there could be three possible sources of contamination: natural flora of the fish, farming methods of the fish and handling of the fish till the point of consumption. Studies suggest that the internal tissue of the animals harvested in unpolluted water remains sterile (Buras et al., 1985, 1987). Even for the fish raised in treated domestic waste water, bacteria are not recovered from blood or muscle.

Considering this, a surface inoculation technique was used. One inch (~2.5 cm) cubes of fish were inoculated with 400 micro liters (0.4 milliliters) of the appropriate cocktail and the inoculum was spread evenly on the fish surface using a sterile plastic loop. Samples were held for less than one minute prior to lime juice treatment. Starting concentrations were generally 6-8 CFU/gram of fish.

## **Application of lime juice**

The juice of 15 limes was used to treat 800 grams of tilapia fillets, or 3 ml per 4.5 gram of fish or a fish to lime ratio of 1.5. This ratio approximates that found in many ceviche recipes.

## Tossing the fish

Ceviche recipes involve tossing the fish in ceramic containers after applying the lime juice. In our study we used plastic weigh boats (Fisher Scientific) (See Figure-12) to toss the individual fish pieces. These weigh boats were sealed by multiple layers of lab tape to avoid spills.

Since the experiments involved pathogens, inoculation, lime treatment and tossing fish steps were all performed in a bio-safety hood (The Baker Company, Sanford, ME, USA).



Figure-12, depicts the sealed weigh boats containing the inoculated fish piece.

## Marinating time and temperature

Traditional ceviche recipes have longer marinating times (~3 hours) while modern recipes have much shorter marinating times (~20 minutes), so to incorporate the possibilities of both kinds of recipes, 10 time intervals ranging from 10 minutes to 150 minutes were studied for all the experiments. The shorter time periods were studied within intervals of 5 minutes each (10, 15, 20, 25 and 30 minutes) while the longer times were studied at intervals of 15 or 30 minutes (45, 60, 90, 120 and 150 minutes).

Ceviche is marinated either at room temperature or at refrigeration. Both the conditions were used in our study. Room temperature was recorded as 25°C and refrigeration as 4°C.

#### Homogenization, dilution and enumeration

After lime juice treatment, the fish samples were placed in a sterile filter bag (Fisher Scientific) and diluted appropriately using 0.1% peptone water, either with or without 3% w/v NaCl depending on the organism. The amount of buffer added to the fish sample is an important step in recovering bacteria after the lime treatment before plating for enumeration. While the bacterial count was expected to be lower after the lime treatment and over dilution could further reduce it, the presence of a fish matrix that is hard to homogenize required a minimum amount of liquid in the filter bag for proper homogenization. Considering these factors, the buffer volume was fixed at 9 ml per fish piece (4.5 grams) for all our experiments. The dilution factor in the filter bag, therefore, was 1:3. The samples were then stomached (Tekmar Company, Cincinnati, Ohio) for fifteen minutes. The samples were spread plated, in duplicate, on the respective selective media and incubated at 37°C overnight and enumerated.

The amount to be plated on the petri-dishes varied for the bacteria in question. In *Salmonella* experiments, 0.1 ml of the sample was plated while for *Vibrio* experiments, the amount of sample plated was 0.3 ml. The increase in the amount was to counter the problem of low counts of recoverable bacteria in *Vibrio* experiments

### **Detection limits and controls**

Detection limit is defined as the minimum amount of analyte that can be detected in a particular experiment. The detection limits were different for *Vibrio parahaemolyticus* and *Salmonella* experiments for our studies. The detection limit for *Vibrio parahaemolyticus* was 1.7 log CFU/gram of fish, while the detection limit for *Salmonella* was 2.17 log CFU/gram of fish.

The difference between T=0 counts (~8 log CFU *Salmonella*/gram of fish and ~6 log CFU *V. parahaemolyticus* of fish) and the number of bacteria recovered after treatment gives the efficacy of lime treatment. If the counts were below the detection limit, the difference starting concentration and the detection limit gave the theoretical log reductions. Theoretical log reductions give the minimum amount of reduction that can be recorded for a given technique and the actual log reductions could be equal to or greater than this value. These reductions indicate that the lower limit of the efficacy of a process Negative controls were used to check the initial total bacterial count on the fish sample purchased from the super market. Positive control experiments were used to determine the amount of bacteria present on a fish piece after surface inoculation

## B. Testing the buffering potential of the fish matrix

The buffering effect of shrimp tissues (Bradshaw et al., 1974) has been postulated as a reason for reducing the anti-bacterial effect of low pH of cocktail sauces, thus tests of the buffering potential of the fish matrix were performed. Fish was cut into pieces of similar size and weight as used in previous experiments (4.5 grams).

The surface pH of all the fish pieces was recorded by using a bench-top pH meter (Accumet AB 15/15+ bench-top meter, Fisher Scientific). The pH was recorded for all the faces of the cube shaped fish piece and a mean value was calculated.

Three ml of lime juice was then applied to each fish piece, and as in other experiments, held at room and refrigeration temperatures for time intervals ranging from 10 minutes to 150 minutes. The average pH was again calculated based on measurement of each face of the fish cube.

## 2.2.2. Experiments with lime juice alone

The aim of these experiments was to observe the ability of lime juice alone to inactivate *V. parahaemolyticus* and *Salmonella*. Four tenths (0.4) ml of the spun down overnight cocktail of bacteria was added to 9 ml of the buffer, as specified above and 3 ml of lime juice was added. So, the lime juice to bacteria ratio was kept same as with the experiments involving fish for uniformity. The solutions were then incubated for the same time and temperature combinations, as above and serially diluted plated, in duplicate. The bacteria were enumerated by the plate count method, taking plating factors and dilution factors in account.

## 3. RESULTS AND DISCUSSION



### 3.1 Results for Salmonella enetrica serovar as contaminant

Figure-13, Log reductions at different time intervals when fish pieces were inoculated with a cocktail of *Salmonella enetrica* at room temperature

Figure 13 shows the effect of lime juice on *Salmonella* in ceviche marinated at room temperature. The effect of lime juice is seen immediately after application and a reduction  $\sim 0.5$  logs was observed in the first 10 minutes. The reductions remain almost constant till time 60 minutes. An extended incubation for 150 minutes produced a log reduction of 1.3.

The time for which fish was treated with lime juice doesn't seem to bear a significant impact on the log reductions, at least within the first hour.

In figure 13, the values of log reduction remain in the same range irrespective of increase in time of exposure to low pH.



Figure-14, Log reductions at different time intervals when fish pieces were inoculated with a cocktail of *Salmonella enetrica* at refrigeration temperature

Figure 14 shows the effect of marination in lime juice in experiments at refrigeration temperature. A ~2.2 log reduction was observed in the first 10 minutes and remained remarkably consistent despite of increase in time of exposure to lime juice. A mean log reduction of 2.1 was observed when log reductions were averaged over all marinating times. There is a > 1 log difference in the reductions at room temperature and at refrigeration. This is a significant factor considering the relatively low log reductions in general. It could be postulated that when cells are exposed to 2 stresses simultaneously (acid and refrigeration), this results in greater reduction.

It is well established that *Salmonella* can survive in environments with pH below 4 due to activation of acid tolerance response (ATR) mechanism if the cells are adapted to a moderate acid pH before exposing them to acidity below 4 (Foster, 1995). However, in our studies, the exposure to low pH is sudden that remains consistent for the whole incubation period without letting the cells gradually adapt to the acid shock. It is, therefore, less likely that an ATR mechanism would be the reason for the relatively lower log reductions. *Salmonella* has been shown to be protected from organic acids when attached to poultry skin (Tamblyn and Conner, 1996). The relatively lower log reductions observed in our studies might be a result of such an association. The survival of *Salmonella* in our experiments was in agreement with Escartin and Torres-Vitela (1996) where they isolated *Salmonella* from two of the eight samples below pH value of 4.

Results with *Salmonella*, both at room temperature and refrigeration, indicate that time of exposure to low pH doesn't impact the safety of ceviche in a significant way. For a particular fish to lime juice ratio, the decrease in bacterial load wouldn't be affected even if the marinating time is prolonged.

### 3.2 Results for Vibrio parahaemolyticus as contaminant

All treatments with lime juice resulted in *V. parahaemolyticus* inactivation to below the detection limit. Such data also poses a problem of representation as no variable, except the starting concentration and detection limit, is quantifiable. Results were grouped based on time of treatment to represent results of a particular experimental condition. The shorter time periods(less than 30 minutes) formed one group while times above 60 minutes formed another.



Figure-15, Minimum calculated Log reduction in *Vibrio parahaemolyticus* at room temperature

A reduction of  $>=5.2 \log$  CFU was observed in the first data set (T=10, 15, 20, 25, 30 minutes) while a reduction of >=4.5 was observed for times above 60 minutes. This difference is not a true difference and is simply attributed to the difference in starting concentration between the two sets of experiments.

At refrigeration temperature, the minimum calculated log reductions for *V*. *parahaemolyticus* were observed to be  $>= 4.3 \log \text{CFU/gram}$  while for the longer times it was  $>= 3.5 \log \text{CFU/gram}$ . As with the room temperature experiments, this is not a true difference, and is simply attributed to the differences in starting concentration between two sets of experiments.



Figure-16, Minimum calculated Log reduction in *Vibrio parahaemolyticus* at refrigeration temperature

The observed susceptibility of *Vibrio parahaemolyticus* to low pH was in agreement with the studies by Beuchat (1973) in liquid broth and Vanderzant et al. (1972) in shrimp and shrimp homogenate. The organism was found very susceptible to pH values lower than 4.8 and 6, respectively, in these studies. The observations by Beuchat (1976) with the organic acids present in lemons and tomatoes also had similar results in form of extended lag phases from 5.2 < pH < 7.2. In fish substrates, these results are in accordance with the studies involving *V. cholerae* O1 by Mata et al. (1994) where a 4 log reduction of the organism in ceviche was observed in 30 minutes by the addition of lime juice.

## **3.3 Buffering potential of fish matrix**



Figure-17, depicts the change in appearance of the fish sample after lime treatment highlighting the loss in muscular integrity and the white color

The change in surface pH of fish was observed to be very rapid and consistent. General surface pH of fish was observed to be in a range of basic to neutral (6.8 -7.2). After treatment with lime, it drops rapidly in just 10 minutes of exposure time to a highly acidic value and remains consistent thereafter. The final surface pH was observed to be ~3.6. This change was not affected by the temperature of incubation.

The lime juice used in our experiments had an average pH of 2.3. This study suggests that the buffering potential of fish reduces the effect of organic acid by about one pH unit. Such buffering effect can significantly enhance the survival of *Vibrio parahaemolyticus* as observed by Bradshaw et al. (1974) with shrimp and cocktail sauce where few cells were recovered even after 48 hours. Covert and Woodburn (1972) also showed that *Vibrio parahaemolyticus* cells were even more stabilized by fish homogenates than by TSB without salt.

Another effect of lime juice treatment was observed in the change in appearance of the fish piece. It loses its muscular integrity, becomes opaque and whitish in color, i.e. the characteristic ceviche appearance. The lost in muscular integrity suggests protein degradation. The appearance and observed texture change was not significantly affected by the time of exposure.

### 3.4 Results for experiments with lime juice alone

The log reductions obtained by treatment of bacteria with lime juice in solution, without the fish matrix, were of the order of  $\geq=5 \log CFU/gram$  for both *Salmonella enterica* and *Vibrio parahaemolyticus*. The effect of lime juice in bacterial reduction is in agreement with previous studies by Mata et al. (1994) and Rodrigues (2000) in which *V. cholerae* O1 was inhibited by lime juice, respectively, in solution and in a sauce eaten with rice.

The detection limit for *Salmonella* experiments was 2.78 log CFU/ml while for *Vibrio parahaemolyticus* it was 2.30 log CFU/ml.

## 4. CONCLUSIONS

From our studies, for the volume of lime juice used per gram of fish in this study, we can conclude that during ceviche preparation, *Salmonella* risk is reduced but not eliminated, while the *Vibrio parahaemolyticus* risk is significantly reduced. Lime juice is found to be effective in inactivation of both *Vibrio parahaemolyticus* and *Salmonella enterica* when used in solution. Refrigeration while marinating the fish is found to have a significant impact in *Salmonella* reduction while time of treatment with the lime juice doesn't affect the reductions of either of the bacteria studied and the appearance and observed texture changes significantly.

## 5. FUTURE WORK

Due to the variations in recipes, the study could be repeated with different volumes of lime juice per gram of fish to develop a ratio between the two that can be used to develop a Hazard Analysis Critical Control Point plan for the preparation of ceviche. Marinating at different temperatures, especially for *Salmonella*, can provide an insight in the difference of reductions observed at room temperature and refrigeration. Use of other sources of citrus could be used in conjunction with lime juice to study the effect of other common organic acids in bacterial reduction in ceviche.

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