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MODELING THE SURVIVAL OF *SALMONELLA* IN SOY SAUCE-BASED
PRODUCTS STORED AT TWO DIFFERENT TEMPERATURES

by

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A THESIS

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MODELING THE SURVIVAL OF *SALMONELLA* IN SOY SAUCE-BASED PRODUCTS STORED AT TWO DIFFERENT TEMPERATURES

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University of Nebraska, 2017

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Acidified foods are defined in the regulations as “low acid foods to which acid(s) has been added to bring finished pH to 4.6 or below”. As the market for these products expands, an increasing number of them are being processed with reduced heat treatment, relying on acid alone to ensure the destruction of pathogens. When considering the survival rate of microbial pathogens in these products, pH, water activity, temperature, salt content and holding time are integral and must be considered together.

Despite improvements in production, handling, and distribution of food products in recent years, protecting consumers from foodborne illness still remains a challenge. Although most food products undergo a kill step at the point of production, there is often a lack of scientific proof. This has created an urgent need in the industry for developing a scientific validation that can better ensure product safety. Predictive models can be used to obtain data on the survival rates of bacteria which can be applied to all stages of the manufacturing process; for instance, new product development, changes to product recipes, etc.

For this study, *Salmonella* has been selected to inoculate soy sauce based products because this pathogen has adaptive responses such as acid tolerance, which is responsible for bacterial survival under extreme acid conditions. Several pH levels (3.0, 4.0 and 5.0), soy sauce (0%, 50% and 100%) and salt (2%, 7% and 14%) were considered. A total of 18

combinations were stored at two different temperatures 18.3°C (65°F) and 23.8°C (75°F). A model was developed to predict the maximum death rate as a response of the interaction of several factors such as pH, storage temperature, salt and soy sauce percentages. The model predicted successfully the response of the pathogen in 83% of the tested cases. The results of this study indicated that the developed model predicts satisfactorily the death rate of *Salmonella* in soy sauce based products and can provide useful quantitative data for the development of safer food products and processes.

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Chapter 1. LITERATURE REVIEW

1. History and background of *Salmonella*

Salmonella are Gram-negative rod-shaped bacteria in the family Enterobacteriaceae that are facultative anaerobic in nature and flagellated bacilli characterized by the H, O and Vi antigens (Wong et al., 2011). These bacteria were discovered and isolated in 1885 by Dr. Salmon, a renowned veterinary surgeon in the U.S., after he was able to isolate the bacterial strain called *choleraesuis* or *enterica* from the intestines of an infected pig (Rabsch, 2002). As can be noted, the bacterial genus was named after its discoverer. According to Wong et al. (2011), the bacteria has also been named according to the places where it was isolated. For instance, there is a strain called *Salmonella Indiana* and another one called *Salmonella London* since they were isolated in Indiana and London, respectively.

The bacteria grow in hosts such as animals (pigs, birds, among others), and on plants (food such as vegetables and fruits), and are eventually ingested by humans when they consume contaminated food or water. Once inside the body of a human, the bacteria replicate in the liver or spleen and cause disease by affecting the intestine. The common symptoms of an ill person are diarrhea, fever, nausea or vomiting, and abdominal cramps depending on the clinical manifestation (enteric fever from *S. Typhi* species; gastroenteritis from *S. Typhimurium* and *S. Enteritidis*; and other non-typhoidal *salmonellosis*) the disease has on the victim (Rabsch, 2002).

Notably, the infections resulting from *Salmonella* are a major public health problem on a global level. For this reason, the infections are a source of negative economic impacts since the disease requires a great investment in the surveillance, investigation, treatment, and prevention (Wong et al., 2011). According to the CDC, there have been 19,119 outbreaks

in the U.S. between 1998 and 2015, in which 373,531 illnesses have been reported resulting in 337 deaths and 14,681 hospitalization cases (CDC, 2017)

1.1 Characteristics and classification of *Salmonella*

The genus *Salmonella* is divided into two different species: *S. bongori* and *S. enterica*. The latter species is the most dominant and has over 2000 strains (Rabsch, 2002). The classification of the strains is further done based on the range of hosts the specific strain attacks. As already mentioned, the strains are characterized by the antigens Vi, H and O. Among the major species, *S. enterica*, subgroup 1 (*enterica*) is the most pathogenic to humans (Rao et al., 2004).

Five of the most recent outbreaks in the U.S. have been caused by strains of *Salmonella enterica*: serotype Braenderup (NVSL-96-12528) whose outbreak was reported in Texas, Tennessee, Iowa and New Mexico in January 2014 and was linked to nut butter; serotype Typhimurium which is a strain that causes a typhoid-like disease in mice and is does not result in fatality in humans and lasts only up to 4 days (Rabsch, 2002); serotype Enteritidis (NVSL 94-13062) which has been a common strain in the U.S. The disease affects chicken and can be transmitted to humans through consumption of infected chicken meat; serotype Heidelberg/Sheldon (3347-1) whose outbreak was reported between 2013 and 2014 sourced from 3 California establishments that dealt with chicken products; and serotype Hadar that was isolated from 12 commercial turkey farms litter and waste samples (CDC, 2017).

Salmonella strains require an optimal temperature of 35-43°C (95-109.4°F) for optimal growth, which means that their functionality is easily disrupted at extremely high or low temperatures. Therefore, the bacteria survive but do not grow in refrigerated conditions. Moreover, under extreme temperatures reaching between 57°C and 60°C or 134°F and 140°F, the bacteria easily die off, and such temperature can be used to kill the pathogen (Rao et al., 2004).

1.2 Infectious dose and disease characteristics

The bacteria affect the human when he or she consumes contaminated food products or through direct contact with infected persons, animals or their environment. The food passes through to the intestines where the bacteria bind to the walls of the intestines from where they penetrate through to the blood to become deposited in the liver and the spleen for growth and multiplication (Deshpande, 2002). After the growth period, the bacteria can now cause infection within the body, especially in the intestine. Excess (or unabsorbed) bacteria may be passed through defecation to the external environment and survive long enough to be transmitted to the next host (animal or person) in poorly sanitized environments (Mastroeni & Maskell, 2006). There are approximately 16 million occurrences of typhoid fever, 3 million deaths as a result of *Salmonella*, and about 1.3 billion occurrences of gastroenteritis (CDC, 2016).

The infectious dose varies with the serotype. For non-typhoidal salmonellosis, the infectious dose is approximately 10^3 bacilli. For enteric fever, the infectious dose is about 10^5 bacilli by ingestion (Mastroeni & Maskell, 2006). Patients with depressed cell-mediated immunity, or who are elderly, may become infected with a lower infectious dose.

The infectious dose may also be dependent on the level of acidity in the patient's stomach (Deshpande, 2002).

1.3 *Salmonella* in acidified foods and mode of resistance

Considering the low pH within the host's stomach due to the presence of HCl, gastric juice (an antibacterial fluid in the body) and other chemicals, *Salmonella* has special adaptations to withstand the acidic conditions. Alvarez-Ordenez et al. (2011) link the survival of *Salmonella* to its nucleophilic nature and its ability to induce an acid tolerance response (ATR) which entails the capacity to undergo adaptive responses that help moderate the pH. Specifically, the response of *Salmonella* to a low internal pH is to activate amino acid decarboxylase systems; synthesizing acid shock proteins; and modification of the membrane fatty acids composition by reducing the membrane fluidity by regulating cadaverine and agmatine in the cell. Notably, through the same mechanism that enables the bacteria to withstand acid conditions in the stomach of the host, the bacteria employ the same process of lowering the internal pH to survive in acidified foods (Alvarez-Ordenez et al., 2011). As a result, the bacteria are able to self-induce an acid-resistance within acidified food products.

2. Contamination in production plants

Salmonella have been linked to contamination of a wide range of animals such as poultry, dairy products, eggs, vegetables and fresh fruits or even water. For instance, it has been found in fruits and vegetables such as lettuce, melon, unpasteurized orange juice, alfalfa sprouts, apple, tomato, parsley, cilantro, celery and mango (Wong et al., 2011).

Consumption of such contaminated foods anywhere within the food chain has led to major outbreaks of the disease on a global scale.

Notably, vector animals such as rats, birds, and flies carry the bacteria and spread it through their feces (Wong et al., 2011). The bacteria can survive in the environment for a long time, and thus they are directly transmitted to animals such as swine, chickens, and cows. Eventually, the vectors and the bacteria find their way to food products within the facility and the food production equipment and products. If uncontrolled before the food gets to the consumers, the contaminated food and food products are consumed and thus cause a direct risk to the consumer which creates high chances of propagating an outbreak.

3. Reduction times of common foodborne pathogens

In research by Breidt et al. (2007), the reduction times for foodborne pathogens was determined in an acidified vegetable product. Specifically, the study was based on testing 5-Log Reduction times for food pathogens in acidified cucumbers during storage at 10 and 25°C. Notably, this research was based on the concerns raised by the increased outbreaks of acid-resistant foodborne pathogens such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* in acid foods such as orange juice and apple cider that usually have pH values lower than 4.0. Based on such concerns, the researchers studied whether acidified vegetable foods with pH values ranging between 3.3 and 4.6 posed a risk to food safety. For that reason, they established and demonstrated how thermal treatments are needed to achieve a 5-Log reduction in acidified vegetable products. However, heat treatment of such acidified food products with a pH value of less than 3.3 would result in poor product quality.

Acetic acid was used with pH of 3.3 or less as the primary acidulant whereby they maintained the vegetable product samples at a minimum equilibrated temperature of 10°C (Breidt et al., 2007). The holding times for the reduction of the *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* strains within the vegetable samples were then determined at these conditions. Eventually, it was established that the *Escherichia coli* O157:H7 was the most acid resistant microorganism even within the acidified conditions and the preset temperature. In this case, *Escherichia coli* O157:H7 took up to 5.7 days to achieve a 5-Log reduction in cell numbers at 10°C. Alternatively, *Listeria monocytogenes* took 0.5 days for a 5-Log Reduction while *Salmonella enterica* took 2.1 days to achieve the same reduction at 10°C. At 25°C, *Escherichia coli* O157:H7 had a 5-Log reduction time of up to 1.4 days (Breidt et al., 2007).

Breidt & Arritt (2013) conducted similar research in 2013. The study also showed that higher storage temperatures can be used to destroy acid-resistant vegetative pathogenic and spoilage bacteria. Dressings and mayonnaises whose pH values were 3.5 or higher were acidified with 1.5- 2.5% or higher acetic acid which was sufficient to cause lethality (Breidt & Arritt, 2013). The study was conducted by determining the 5-Log reduction times of *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* at 10°C using pickling brine medium with a range of benzoic and acetic acid combinations maintained at pH values ranging between 3.5 and 3.8. Fifteen acid-pH combinations were used, and *Escherichia coli* O157:H7 demonstrated significantly higher acid resistance than *Listeria monocytogenes* and *Salmonella enterica* strains (Breidt & Arritt, 2013). Eventually, the results indicated that the addition of benzoic acid added to the efficacy of achieving faster 5-Log reduction times in the target pathogens after 4 days of holding time at pH 3.8 with a

ratio of acetic:benzoic acids being 2.5:0.1% and at pH 3.5 with 2.5% acetic acid and 0.1% benzoic acid. In conclusion, the study considers benzoic acid as an essential constituent for determination of critical controls and supporting filing process during the manufacture of acidified foods (Breidt & Arritt, 2013).

In 2014, Breidt et al (2014) conducted a research study based on the fact that any shelf-stable acidified food with a pH of 4.6 or below requires processing to achieve 5-Log reduction for bacterial pathogens in food products. Therefore, a set of data and models that showed how to destroy acid-resistant vegetative pathogens within non-inhibitory vegetable-based medium (cucumber juice) were developed. The study tested the destruction of 5 different cocktails of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* strains within acid mediums of pH between 4.1 and 4.6. Unlike the previous research studies, *Listeria monocytogenes* was more acid and heat-resistant at temperatures below 74°C. (Breidt et al., 2014)

4. United States regulations for acidified products

Under the Code of Federal Regulations (CFR) 21 Part 114.3, acid foods are defined as those which have a natural pH value of 4.6 or below. CFR refers to acidified foods as “low-acid foods to which acids(s) or acidic food(s) are added... They have a water activity (a_w) greater than 0.85 and have a finished equilibrium pH of 4.6 or below. These foods may be called or may purport to be, “pickles” or “pickled ____.” Carbonated beverages, jams, jellies, preserves, acid foods (including such foods as standardized and non-standardized food dressing and condiment sauces which contain small amounts of low-acid food(s) and

have a resultant finished equilibrium pH that does not significantly differ from that of the predominant acid or acid food, and foods that are stored, distributed and retailed under refrigeration are restricted from the coverage of this part” (U.S. Food and Drug Administration, 2016).

This definition highlights the intrinsic food qualities necessary to prevent the growth of foodborne pathogens and define the processing characteristics necessary to eliminate their presence if not met. A water activity of 0.85 is the upper limit for preventing the growth of *Staphylococcus aureus* and pH 4.6 is the upper limit preventing the growth of *Clostridium botulinum* (Jay, 2000).

It is important to know the amount of acid that is present in the food (pH) and the amount of moisture available to microorganisms (a_w) (Bacon, 2012). These parameters will determine how the food product should be classified, and the classification of the food is what determines how it will be regulated (Figure 1).

It is essential that sufficient acid is added to ensure that pH 4.6 or lower is achieved after all of the components in the finished product have reached equilibrium (Hui et al., 2004).

There are some methods to obtain properly acidified foods: (1) Direct acidification of individual containers; (2) Direct batch acidification; (3) Blanching in an acidified solution; (4) Immersion of blanched products in an acid solution; (5) Addition of an acid food to a low acid food; (Black & Barach, 2015). Regardless of the acidification method used, it is important to establish if there are any critical control points for proper acidification, and if so, it is required to document their control during production (U.S. Food and Drug Administration, 2016).

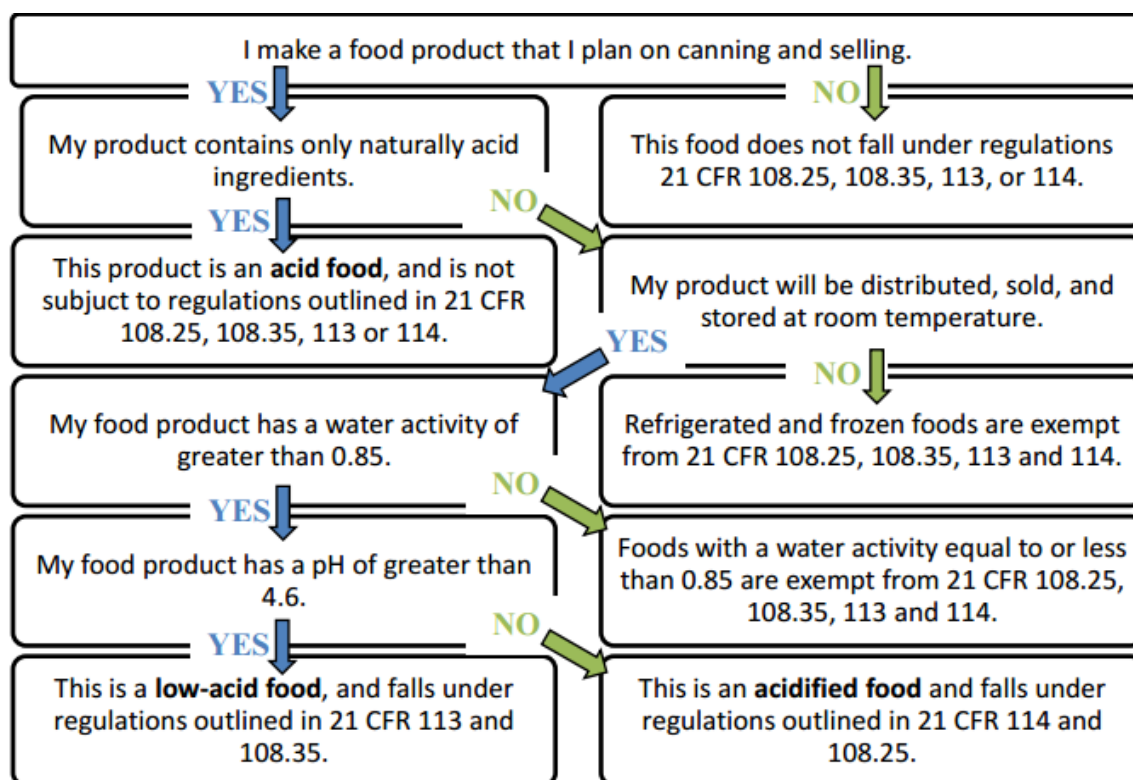


Figure 1. Decision tree for food classification and relevant canned food regulations (Bacon, 2012)

A thermal process requirement for acidified foods is defined in 21 CFR Part 114 to ensure the destruction of vegetative microbial cells. Heat processing also provides additional benefits, such as faster equilibrium of solutes throughout the product, destruction of enzymes that may impact product quality, and the exhaustion of oxygen and a resulting headspace vacuum (Deshpande, 2002). The lower the pH, the less thermal input is needed to ensure commercial sterility (Black & Barach, 2015).

Two common methods of heat treatment utilized for acidified foods are:

- (1) Hot-fill-hot procedure, where the hot liquid is injected into a container and then inverted, allowing the heat to sterilize the container and/or the cap (Black &

Barach, 2015). The liquid must be between 194°F and 203°F to ensure sterilization and requires the use of containers, such as those made of glass and certain types of plastic, that do not change form at high temperatures. (Ranken, et al., 1997).

(2) Pasteurization procedure, where the product is filled into a container either cold or hot. The product is heated up to a high temperature for a certain length of time, and then rapidly cooled which is enough to eliminate target microorganisms (Black & Barach, 2015).

If acidified foods are improperly processed and distributed, the health of the consumer may be adversely affected (Hui et al., 2004). There is no adequate way to determine the harmful nature of the foods after they have been released into marketing channels. Therefore, the U.S. Food and Drug Administration (FDA) requires evidence that acidified foods destined for interstate commerce be manufactured and handled in such a manner as to assure safety to the consumer. A critical part of assuring acidified foods are safe is to manufacture products according to a scheduled process (Zhao, 2012). A scheduled process is a process selected by a processor as adequate for use under the conditions of manufacture for a food in achieving and maintaining a food product that will not permit the growth of microorganisms having public health significance (U.S Food and Drug Administration, 2016).

According to the Code of Federal Regulations (CFR) 21 Part 114.33, a scheduled process must be established by a qualified person or a competent process authority, with expert

knowledge acquired through appropriate training and experience in the acidification and processing of acidified foods. Scheduled processes must be followed during manufacture of the food, and critical factors must be monitored under the operating supervision of an individual who has attended and successfully completed a course and has become certified to supervise those operations (U.S Food and Drug Administration, 2016).

5. International compliance with U.S. law

The Food and Drug Administration (FDA or U.S. FDA) is the federal agency that is responsible for overseeing most of the U.S. food supply. FDA is responsible for protecting the public health by ensuring the safety and security of the food supply (U.S. Food and Drug Administration, 2017b).

The United States Department of Agriculture (USDA), also known as the Agriculture Department, is the U.S. federal department responsible for developing and executing government policies that will help farming, agriculture, forestry, and food communities thrive (United States Department of Agriculture, 2017). The Food Safety Inspection Service (FSIS) is the public health agency in the USDA responsible for ensuring that the nation's commercial supply of meat (excluding meat from exotic animals), poultry, and egg products are safe, correctly labeled and packaged (United States Department of Agriculture, 2017).

The FDA continues to provide several guidelines on acidified foods, which offer standardized and non-standardized guidance for food dressings, such as mayonnaise, and

condiment sauces, for example, ketchup (U.S. Food and Drug Administration, 2016). Maintenance of the pH of acidified food below 4.6 prevents germination and outgrowth of *Clostridium botulinum* as contained in the Code of Federal Regulations. Pathogenic bacteria such as *Salmonella*, *Listeria* and Shiga toxin-producing *Escherichia coli* have to be killed during the production and processing of acidified food products. Sterilization of the various food products is through the application of terminal heat sterilization on products (Breidt et al., 2007). However, the same effect is achievable through the acidification of the food product using acetic acid as an acidulant. The acetic acid reduces the concentration of the pathogens by 5-Logs when left for two days at temperatures of 10°C or 25°C. Some acidified foods, for example, dressing, contain compounds with antimicrobial properties. Such compounds include mustard and horseradish (Breidt et al., 2007).

Acidification of the food product is an alternative to reduce the level of microbial contamination in the food products (Zhao, 2012). Exposure of acidified vegetable products to heating produces undesirable properties such as softening and loss of flavor. The use of plastic containers and pouches also renders the products non-sterilizable by heat method (Ranken et al., 1997). Therefore, this may result in survival of pathogens such as *E. coli*, *Salmonella* and *Listeria*, which could lead to outbreaks due to contamination of the food products.

The regulations between the U.S. FDA and other countries are fairly similar. They tend to adhere to the Codex Alimentarius legislations on International Standards. Differences are mainly exhibited on the labelling of the food package. For instance, in the European Union,

salt content is measured in grams whereas in the U.S. it is measured in milligrams (World Health Organization et al., 2017). The food additives permitted by both regulators are different, therefore exporters from the EU to United States can have some of their products refused entry. Different numbers, referred as E numbers, are assigned to the additives used. For example, Sodium Caseinate is labeled in the EU as E469. Such labelling results in rejection of these products by the U.S. FDA. Other countries outside the EU tend to use U.S. FDA guidelines to streamline their products and enable them gain easier entry into the United States food market (U.S. Food and Drug Administration, 2017a).

6. Factors affecting the growth of microorganisms in food

When microorganisms grow in food they cause varying degrees of change in the characteristics of the food as a result of metabolic activity. Some of these changes, like those taking place during fermentation, are desirable, while others, like those resulting in food spoilage and food poisoning, are undesirable changes. The most important factors that affect microbial growth in foods can be summarized in the following categories:

- (1) The intrinsic factors, which include nutrient content, water activity, pH value, redox potential and the presence of antimicrobial substances and mechanical barriers to microbial invasion (Doyle et al., 2013). The food sources can vary, but the microorganisms primarily extract carbon and nitrogen from substances such as proteins, fats and carbohydrates (Jay, 2000). Organisms also prefer certain pH level in the substance or environment in which they grow but if conditions are too acidic, the organism's enzymes break down (Table 1).

- (2) Factors related to the environment in which the food is stored, the extrinsic factors, including temperature of storage and the composition of gases and relative humidity in the atmosphere surrounding the food (Table 2). In general, the higher the temperature, the more easily microorganisms can grow up to a certain point. Very high and very low temperatures both obstruct the enzyme processes microorganisms depend on to survive (Batt et al., 2014).
- (3) Factors related to the microorganisms themselves, the implicit factors, including the interactions between the microorganisms contaminating the food and between these microorganisms and the food (Jay, 2000). For example, their abilities to utilize different nutrient sources, tolerate stresses and produce inhibitors of growth of other microorganisms.
- (4) Processing factors, which include treatments such as heating, cooling, and drying that affect the composition of the food and also affect the types and numbers of microorganisms that remain in the food after treatment. The complex interactions between the factors described in (1) through (4) can also affect the growth of microorganisms in foods.

Table 1: pH values of some foodborne pathogenic bacteria

Microorganism	Minimum pH	Maximum pH	Water Activity (a_w)
<i>Bacillus cereus</i>	4.9	9	0.95
<i>Clostridium perfringens</i>	5	9	0.93
<i>Clostridium botulinum</i>	4.6	8.5	0.97
<i>Escherichia coli</i>	4	10	0.95
<i>Salmonella</i> spp.	3.7	9.5	0.93
<i>Staphylococcus aureus</i>	4	10	0.86

Adapted (Jay, 2000)

Table 2. Growth temperatures of some foodborne pathogenic bacteria

Microorganism	Minimum Temperature (°C)	Optimum Temperature (°C)	Maximum Temperature (°C)
<i>Bacillus cereus</i>	10-15	35-40	47-55
<i>Clostridium perfringens</i>	12-20	30-47	45-51
<i>Clostridium botulinum</i>	12	35	50
<i>Escherichia coli</i>	7	35-40	46
<i>Salmonella</i> spp.	5-10	35-37	45-49
<i>Staphylococcus aureus</i>	5-10	35-40	44-48

Adapted (Jay, 2000)

6.1 Antimicrobial effect of sodium chloride

Sodium chloride (NaCl) is a colorless or white crystalline compound, used in the manufacture of chemicals and as a food preservative and seasoning. Salt reduces water activity of foods, thus controlling microbial growth (Miliotis & Bier, 2003). While a very

few foods, such as raw meats and fish products, are preserved directly by high concentrations of NaCl, salt is used primarily in addition to other processing methods such as canning and pasteurization (Finn et al., 2013).

Salt is commonly used as a flavor enhancer for food and has been used for basic tastes. Salt is used extensively in the preservation of vegetables, such as cucumbers, cabbage and onions (Artes & Allende, 2015). Most preservation methods use salt to reduce the water content by between 10 and 50%, which reduces microbial growth while maintaining palatability of foods (ICMSF, 2005).

In general, foodborne pathogenic bacteria are inhibited by water activity of 0.92 or less (equivalent to 13% NaCl concentration). The exception is *S. aureus*, which has a minimum a_w for growth of 0.83-0.86. Another relatively salt tolerant foodborne pathogen is *L. monocytogenes*, which can survive in saturated salt solutions at low temperatures (Miliotis & Bier, 2003). Additionally, research has demonstrated that *Salmonella* can survive in low-moisture foods, $a_w < 0.7$, for long periods of time. *Salmonella* cross-contamination in low-moisture foods has been traced to factors such as poor sanitation practices, poor equipment design, and poor ingredient control. It is well recognized that *Salmonella* can survive for long periods in low-moisture food products (Finn et al., 2013). The growth of *Salmonella* is generally inhibited in the presence of 3-4% NaCl. No growth was detectable at 5% NaCl. The quantity of salt required to inhibit *Salmonella* decreased with decreasing temperature (Doyle, 1989).

Listeria can survive at 37°C for 15 days in 10.5% NaCl, 10 days in 13% NaCl, and 5 days in concentrations of 20-30% NaCl. When the temperature is lowered to 22°C, survival

times more than double. At 4°C, *Listeria* can survive more than 100 days in 10.5 – 30.5% NaCl. Some reports indicate that it is capable of growing up to 10% NaCl and surviving for 1 year at 16% NaCl when the pH is 6.0 (Finn et al., 2013). *E. coli* grows in 6% NaCl at pH values between 6.8 and 5.6 at temperatures between 35 and 15°C. No growth occurred in media containing 8 or 10% sodium chloride (Doyle, 1989).

7. Microbial challenge studies

According to Ray & Bhunia (2014), microorganisms sense and adapt to changes in their environment. When nutrients are exhausted, some bacteria may become motile to seek out nutrients, or they may produce enzymes to exploit alternative resources. One example is the formation of endospores as a mechanism for survival, not reproduction (Ray & Bhunia, 2014). Therefore, understanding the competition for nutritional resources within a given food or environment is essential to understand the survival of microorganisms (Zhao, 2012).

One of the important concepts to understand in food processing when establishing processing times is lethality, which can be defined as the integrated influence of time and temperature on a microbial population (Vieira, 2013).

Bacterial growth can be suppressed while not necessarily killing the bacteria. Methods used for growth suppression can be divided in two categories, physical and chemical (Weeks & Alcamo, 2008). Physical methods either exclude microbes or reduce their numbers and include filtration, radiation, etc. Chemical methods involve the application of specific chemical agents which inhibit growth, such as preservatives (Weeks & Alcamo, 2008).

Microbial challenge studies are being conducted more frequently by the food industry. The reasons for conducting a challenge study can be summed up in two words: safety and quality, especially in new products or reformulations (Vestergaard, 2001). Microbiological challenge studies must be conducted to validate predictive models, and these studies involve inoculation of foods with the bacteria of interest and simulating the conditions of any stage from preparation to consumer use (National Advisory Committee on Microbiological Criteria for Foods, 2010). Challenge tests determine the ability of the acidified food to inhibit the growth of acid-resistant bacteria, the growth of which can otherwise result in spoilage and infections among consumers due to the consumption of contaminated products (Anderson de Souza, 2017). Selection of the organism and preparation of the inoculum prevent the confounding factors introduced by other bacterial contaminants in the cultures. Inoculation of the acidified foods with carefully prepared bacterial inoculum is conducted by introducing it either directly into the packaging or in a more controlled environment (National Advisory Committee on Microbiological Criteria for Foods, 2010). Intended conditions of storage and use must be considered in designing challenge studies. The storage temperature used in microbial challenge studies should include the typical temperature range at which the product is to be held and distributed (IFT, 2001). The test determines whether there is any growth in the acidified food. If a challenge study is to be conducted on an existing product, it is best to acquire multiple lots of the product produced at different times and account for any known or suspected process variation (Man & Jones, 2000).

Regulations have been put in place in the United States governing the safe manufacture and production of acidic food. The purpose of these regulations was to avert colonization of the acidified products by *Clostridium botulinum*, which causes botulism by releasing high quantities of botulinum toxin into the food (U.S Food and Drug Administration, 2015).

7.1 Selection of appropriate challenge organisms

The selection of types of microorganisms, number of strains and culture conditions for the product validation will depend on the objectives and product parameters, such as whether the formulation is hostile to these microorganisms, requires to controls growth of pathogens, growth limit, etc. (McMeekin, 2003).

Table 3 describes some pathogens that may be considered for use in challenge studies for various products (Vestergaard, 2001). These would typically be useful when the presence of the microorganisms in the food, beverage or ingredient is not acceptable from a scientific, public health or regulatory standpoint, for example, a shelf stable product that has the potential for post processing contamination by *L. monocytogenes*. In this type of product, it may be useful to know how long the pathogen would survive after a potential contamination event during packaging (IFT, 2003).

Table 3. Pathogens that may be considered for use in challenge studies for various products

Food Type	Type of Organism
Salad dressings	<i>Salmonella</i> , <i>Staphylococcus aureus</i>
Modified atmosphere packaged products	<i>Clostridium botulinum</i> , <i>Listeria monocytogenes</i> and enterohemorrhagic <i>Escherichia coli</i>
Bakery items	<i>Salmonella</i> , <i>S. aureus</i>
Sauces and salsas stored at ambient temperature	<i>Salmonella</i> , <i>S. aureus</i>
Dairy products	<i>Salmonella</i> , <i>S. aureus</i> , <i>C. botulinum</i> , enterohemorrhagic <i>Escherichia coli</i> , <i>L. monocytogenes</i>
Confectionery products	<i>Salmonella</i>
Formula with new preservatives	<i>Salmonella</i> , <i>S. aureus</i> , <i>C. botulinum</i> , enterohemorrhagic <i>Escherichia coli</i> , <i>L. monocytogenes</i>

Adapted (Vestergaard, 2001)

The ideal organisms for challenge testing are those that have been previously isolated from similar formulations. Additionally, pathogens from known foodborne outbreaks should be included to ensure the formulation will inhibit those organisms as well (Man & Jones, 2000). For certain applications, surrogate microorganisms may be used in challenge studies in place of specific pathogens. For example, introducing pathogens into a processing facility is not feasible; therefore, it is desirable to use surrogate microorganisms. An ideal surrogate is a strain of the target pathogen that retains all other characteristics except its virulence. (McMeekin, 2003). Cultures can be obtained by either purchase or through culture sharing with other researchers.

7.1.1 Effect of acidity on the survival of microorganisms

Acidity plays a primary role in the preservation of fermented foods and, combined with other factors such as heat, water activity, and chemical preservatives, acts to prevent food deterioration and spoilage. The intensity of acidity of a food is expressed by its pH value. The pH of a food is one of several important factors that determine the survival and growth of microorganisms during processing, storage and distribution. Consequently, food processors are interested in determining the pH of foods and in maintaining it at certain levels to control microbial growth and prevent product deterioration and spoilage.

The definition of pH is a measure of the concentration of hydrogen ions in a solution. The more hydrogen ions that a substance releases in a solution, the lower the pH of the solution, and the more acidic the solution. The values for pH range from 0-14.0, with 0 being the most acid and 14.0 being the most alkaline. The midpoint of the scale, 7.0, is referred to as neutral pH. Most microorganisms grow best in a pH range between 6.0 and 8.0. Too much acid or base disrupts cellular activities.

When an organism adapts to an acidic environment, this may lead to an acid tolerant response (ATR) which may protect against organic acids (Baik et al., 2016). ATR is a complex defense system generated when acid resistance is induced after adaptation to mildly acidic pH conditions to minimize the lethal effects of extremely low pH and also to protect an organism against organic acids. In turn, organic acids enhance survivability of acid sensitive pathogens exposed to low pH by induction of an acid tolerant response (Ricke, 2003). This tolerance is also often linked to increased virulence and concern has risen about implications regarding the use of organic acids (Baik et al., 2016).

7.1.2 Organic acids in tolerance response

Short chain organic acids, including acetic acid, its salts (acetates and diacetates), lactic acid and lactates, propionic acid and propionates, and citric acids and citrates, are commonly utilized in a variety of foods as antimicrobial preservatives or acidulants (Doores, 1993). They may be added directly to foods or in the case of acetic acids, as sprays or dips for surface decontamination of fresh meat and poultry. In the undissociated or protonated form, organic acids, which are weak acids, diffuse across the cell membrane lipid bilayer. Once inside the cell, the acid dissociates because the cell interior (pH_i) has a higher pH than the exterior (pH_o). Microorganisms maintain pH_i near neutrality to prevent conformational changes to the cell structural proteins, enzymes, nucleic acids and phospholipids (Bearson et al., 1997). Protons generated from intracellular dissociation of the organic acid acidify the cytoplasm and must be extruded to the exterior using energy in the form of ATP. The lower the pH_o , the greater the influx of organic acids. This constant influx of protons will eventually deplete cellular energy. Resistance by microorganisms to organic acids and/or low pH must be a response to this mechanism (Bearson et al., 1997). Some foodborne pathogens, when exposed to low pH via short chain organic acids may undergo changes that provide them with varying degrees of resistance to subsequent exposure to normally lethal acidic conditions (Smittle, 2000).

S. typhimurium is well known for its ability to survive extreme pH, but this is dependent on the acid used to acidify the growth medium. The order of development of ATR in *Salmonella* cells by the following acids have been found to increase from ascorbic to citric

in the order: ascorbic < HCl < malic < lactic < acetic < citric (Alvarez-Ordóñez et al., 2011).

7.1.3 Acid tolerant organisms

Acid tolerance has been studied for many years, but mainly in *E.coli* and *Salmonella*, with a few studies in *L. monocytogenes* and other Gram-positive bacteria. Similar to observations in other bacteria, it has been found in *Listeria* that at the same external pH_o, weak organic acids are more damaging than strong inorganic acids such as HCl (Phan-Thanh & Gormon, 1997). Acid adaptation mechanisms, such as those induced by pre-exposing the cells to a moderately acidic environment, have been observed in a number of foodborne pathogens, including *L. monocytogenes*, enterohemorrhagic *E. coli*, *S. typhimurium* and *Shigella flexneri* (Edelson-Mammel et al., 2006). *E. coli* O157:H7, *Salmonella* and other enteric bacteria are capable of adapting to the severe acid stress frequently encountered in animal and human bodies (Lin et al., 1995).

E. coli generally does not readily grow at low pH values (<5.5) but if acid resistance genes are induced, the organism is able to survive pH values as low as 2.0 (Lin et al., 1995). Since the first recognized outbreak due to consumption of fresh apple juice in 1982, *E. coli* O157:H7 has emerged as a serious, potentially life-threatening, human foodborne pathogen (Jordan et al., 1999). Relative resistance to acidic environments is a characteristic of enterohemorrhagic *E. coli* and provides the organism the temporary ability to endure acidic

environments. This ability is enhanced by the presence of several growth phase related and inducible acid resistance systems (Lin et al., 1995).

A significant ATR has been found in *S. typhimurium*, following culture in acidic environments (pH 3.0). The composition of membrane fatty acids is responsible for membrane fluidity, and a number of studies have suggested a relationship between membrane fluidity and stress tolerance for *Salmonella* (Alvarez-Ordóñez et al., 2011). Experiments performed by Álvarez-Ordóñez, et al. (2008) showed that when *S. typhimurium* cells are exposed to acid pH, a membrane adaptation occurs, characterized by a decrease in the unsaturated fatty acid to saturated fatty acid ratio and in membrane fluidity.

Listeria monocytogenes is another major concern to the food industry, due to its inherent resistance to several preservation practices and the high mortality rate of Listeriosis in susceptible populations; the particular concern is its ability to grow at refrigeration temperatures, on dry surfaces, and to tolerate acidic conditions (Barker & Park., 2001).

Shigella is another organism known to survive for extended periods in unfavorable conditions such as high acid or high temperature (Lin et al., 1995). Although *Campylobacter jejuni* cannot survive in extremely acidic conditions (below pH 3.0), it is not affected at pH higher than 3.6 despite the fact that its normal growth pH range is 5.5-5.7 (Chaveerach et al., 2002).

Lactic acid bacteria (LAB) are more resistant to acidic conditions and can tolerate a lower intracellular pH than many other bacteria (Adams & Nicolaidis, 1997)

7.1.4 Development of acid tolerance

Acid adaptation is a complicated process in which many physiological changes occur, such as production of protective acid stress proteins and alterations in cell membranes (Álvarez-Ordóñez et al., 2008). Bacteria can survive in acidic environments depending on their ability to regulate their cytoplasmic pH (pH_i) (Hill et al., 1995). Several factors will determine the ability of a microorganism to survive exposure to an acid environment. These include pH, type of acidulant, concentration of acidulant, temperature, water activity and the presence of other inhibitory substances (Edelson-Mammel et al., 2006). Acid adaptation in *S. typhimurium* can be described as a two stage process, beginning with an initial pre acid shock exposure to a mild pH in the range of 5.0-6.0 and then followed by a post acid shock stage exposure to a pH of 4.5 or below (Tosun & Aktug-Gonul, 2003). The pre shock stage induces an ATR-specific pH homeostasis system which helps maintain the internal pH as the external pH decreases below 4. The post shock phase involves the dramatic reduction of 43 acid shock proteins (ASPs) with predicted importance in the prevention or repair of acid damaged macromolecules (Leyer & Johnson, 1993).

Direct acidification of a food or food ingredient may shock the microflora so they become more acid resistant; for example, lactic acid is added to improve flavor and quality in cheese curd (Shelef & Addala, 1994). Many other organic acids are utilized for improved sensory properties and as antimicrobials in a variety of foods (Doores, 1993). Several research studies have demonstrated that acid adaptation or tolerance may produce pathogens with enhanced survival in fermented foods or foods to which organic acids have been added (Shelef & Addala, 1994).

Salmonella serovars Typhimurium, Enteritidis, Heidelberg and Javiana that were preexposed to pH 5.8 (HCl) demonstrated increased resistance to the food antimicrobials lactic, propionic and acetic acid (Leyer & Johnson, 1993) (Table 4). Acquired resistance is important for microbiological food safety of food products if the resistance is induced by conditions present in the current food processing system and if that resistance enhances survival of those pathogens in foods in which they are normally inactivated (Storz & Hengge-Aronis, 2000).

Table 4. Conditions of organic acid or mineral acid adaptation, tolerance or shock applied to *Salmonella* and their subsequent organic acid, food antimicrobial or acid food tolerance responses

Microorganism	Acid adaptation conditions	Exposure conditions and responses
<i>Salmonella</i> Typhimurium <i>Salmonella</i> Enteritidis <i>Salmonella</i> Heidelberg <i>Salmonella</i> Javiana	HCl, pH 5.8	Increased resistance to lactic, propionic, acetic acids Increased survival in milk fermentation and cheddar, Swiss and mozzarella cheeses
<i>Salmonella</i> Typhimurium	HCl, pH 5.8	Increased resistance to activated lactoperoxidase and NaCl
<i>Salmonella</i> Typhimurium <i>Salmonella</i> Dublin <i>Salmonella</i> Heidelberg	Growth in TSB acidified with lactic acid at pH 5.0 for 24 hours	Inoculated into beef and treated with 1.5 or 3.0% lactic acid No difference in susceptibility to lactic acid by acid tolerant or unexposed cells Acid adapted cells more heat sensitive than unadapted cells

Adapted (Yousef & Juneja, 2003)

7.2 Inoculation level

The inoculum level used in a microbiological challenge study depends on whether the objective of the study is to determine product stability and shelf life or to validate a step in the process designed to reduce microbial numbers (National Advisory Committee on Microbiological Criteria for Foods, 2010). Typically, an inoculum level between 10^2 and 10^3 CFU/g of product is used for microbiological stability of a formulation. If the inoculum level is too low, microorganisms could not grow in the food product because of the increased lag phase. Formulation testing may underestimate the risk or overestimate food safety by using an inappropriately low inoculum level in testing (IFT, 2003). In contrast, at high inoculum levels, microbial growth could be overestimated. For studying lethal processes, higher levels of microorganisms are needed, generally more than 10^6 CFU/gr (U.S Food and Drug Administration, 2015).

7.3 Method of inoculation

The method of inoculation is another extremely important consideration when conducting a microbiological challenge study. Every effort must be made not to change the critical parameters of the product formulation undergoing challenge (Costa, 2009). A variety of inoculation methods can be used depending upon the type of product being challenged. In liquid matrices such as sauces and gravies with high a_w (>0.96), the challenge inoculum may be directly inoculated into the product with mixing, using a minimal amount of sterile water or buffer (McMeekin, 2003). Use of a diluent adjusted to the approximate a_w of the

product using the humectant present in the food minimizes the potential for erroneous results in intermediate a_w foods. Products or components with $a_w < 0.92$ may be inoculated using the atomizer method with a minimal volume of carrier water or buffer (National Advisory Committee on Microbiological Criteria for Foods, 2010). A short post inoculation drying period may be needed for some products before final packaging. A minimum volume of sample should be inoculated so that a minimum of two-three replicates per sampling time is available through the challenge study. In some cases, such as in certain revalidation studies and for uninoculated control samples, fewer replicates may be used (Taormina, 2013).

8. Predictive microbiology

Modeling the survival of a pathogen combines traditional microbiology with statistics, information systems, and specific software to describe microbial growth to prevent food spoilage as well as foodborne illnesses. The behavior of foodborne pathogens in foods is determined by the properties of food such as water activity, pH and storage conditions. The effect of these properties can be predicted by mathematical models derived from quantitative studies on microbial populations. (Presser et al., 1998).

Predictive models are a quick, efficient and cost effective way of assessing the potential for growth of microorganisms. The models developed in predictive microbiology aim at the quantification of the effects of intrinsic, extrinsic and processing factors on the resulting microbial proliferation in food products or food model systems (Fakruddin et al., 2012).

Once a model has been developed it will be the fastest way to estimate the shelf life according to microbial spoilage and to estimate the microbial safety of a product. And immediately, this information can be provided for some different storage conditions or for some slight or more profound recipe changes. (Perez-Rodriguez & Valero, 2013).

8.1 History and classification of predictive modeling

The origin of predictive microbiology is often linked to the work by Bigelow (1921), Bigelow and Esty (1920), and Esty and Meyer (1922) in which a log-linear model was proposed to describe bacterial death kinetics by heat. Their model found a wide application in the food industry, especially in the canning industry (Costa, 2009).

Currently, the results are still applied by the food industry to reduce *Clostridium botulinum* in low-acid canned foods. Other areas, such as fermentation microbiology, have also contributed to the development of predictive microbiology (Batt & Tortorello, 2014).

Several modeling approaches have been published in recent years in the literature (Breidt et al., 2014). These models address different issues of interpreting various microbiological phenomena. Model classifications fall into three main groups based on a number of criteria (Costa, 2009).

8.1.1 Structural characteristics

Based on these characteristics, the models are described as white box or mechanistic (physical) models which are constructed based on theory or underlying mechanisms and are amenable to refinement as knowledge of the system increases (Costa, 2009). Black box or data driven models are purely based on experimental data. Finally, gray box or hybrid models lie at the interface of white box and black box models, combining information from both theory and data and having partly interpretable parameters (Costa, 2009).

8.1.2 Kinetic responses

According to Costa (2009) three models are defined: primary, secondary and tertiary. Primary models describe the response of the microbial load as a function of time (growth, inactivation and survival). Secondary models describe the response of primary model parameters to changes in one or more intrinsic, extrinsic and processing conditions. Tertiary models are a result of the combination of primary and secondary models together with experimental data into a computer software (Costa, 2009).

Traditionally, the environmental variables studied are temperature, pH and a_w but also other important conditions such as the type and concentration of an acid, the relative humidity, the concentration of preservatives or antimicrobials and the redox potential can affect the microbial growth and thus those factors can be included in a model (Batt & Tortorello, 2014).

Kinetic models are useful as they can predict changes in microbial cell density as a function of time, even if a controlling variable, which can affect growth, is changing. However, the main drawback is that kinetic models are difficult to develop as they require a lot of data to be collected to model the interaction effects between the different environmental factors (Doyle et al., 2013).

8.1.3 Categorical responses

Categorical model approaches are developed to characterize the growth/no growth boundary, or to quantify the chance of microbial survival, recovery or spoilage after certain processing treatments. Generally, it is assumed that this boundary is a transition zone where the growth probability increases from 0% to 100% when going from detrimental to more favorable environmental conditions. These types of model have been developed for different types of assessments including: growth/no growth of pathogenic and spoilage microorganisms; survival/death of pathogenic and spoilage microorganisms; recovery/no recovery of pathogenic and spoilage microorganisms and finally, spoilage/no spoilage (Doyle et al., 2013).

8.2 Model validation

The last step of a modeling cycle is the model validation. The application of a mathematical model in more complex systems includes an increase in the error of the predictions. Three

levels of error can be considered as a result of the different environmental conditions or of the microbial diversity (Batt & Tortorello, 2014).

A primary error is due to the difference between the predicted microbial responses and the microbial responses given under similar laboratory conditions. An intermediate error is due to the difference between predicted microbial responses and microbial responses generated in artificially contaminated foods. This error can also be due to competition of microorganisms with a naturally occurring microflora in artificially contaminated foods (Doyle et al., 2013).

An overall error is due to the difference between predicted microbial responses and microbial responses by natural contaminated food products. This is why the generation of an appropriate number of inactivation experiments for determining the model parameters is essential and has to be followed by testing the accuracy of the model with new data using combinations of the examined environmental factors. The validation step works as an auxiliary methodology to evaluate the goodness of fit, to decide on identifying model structure or parameter for improving model accuracy and consider the necessity of generating additional data (Batt & Tortorello, 2014).

9. Asian sauces in the global market

Soy sauce is a derivative product of soybeans and is commonly used as a seasoning product in China, Japan, Indonesia, Malaysia and Korea (McCormick & Company Inc., 2016). The Asian sauce is manufactured by mixing soybeans and wheat and fermenting it using various

microorganisms including *Aspergillus*, yeasts and lactic acid bacteria. The resulting product has a very complex flavor and aroma through biochemical and chemical reactions which occur during the processing and manufacturing steps. Notably, the sauce comes in many flavors with variants resulting from the different fermentation processes, raw materials used, and starter cultures (He et al. , 2013).

The market for the global sauces, dressings, and condiments was worth USD 18.93 billion and has been projected to reach USD 24.91 billion in the year 2021. Sauces and condiments are an integral part of the modern day cooking methods. They have been used to enhance food flavors, and dressing additionally improves the appearance of the food product.

The Asian-Pacific market is experiencing a rapid growth, owing to the rapidly changing consumer patterns within the region (McCormick & Company Inc., 2016). There is an increased preference for fast food and rapid urbanization also drives the consumption of condiments in the region. China and Japan are considered mature markets while Australia, New Zealand, India, South Korea, and Hong Kong have untapped market potential for these commodities. The Asian market is mainly driven by sales of condiment sauces. The majority of these sauces are used in cooked foods or table dips (McCormick & Company Inc., 2016).

The growing popularity of soy sauce in the U.S. marketplace has also led to the increasingly widespread availability of wheat-free and reduced sodium versions of this food. For example, it has become relatively common to find "wheat-free tamari" available in U.S. supermarkets, even though tamari is a type of soy sauce traditionally prepared with a small

amount of wheat. It is very common to find information such as "wheat-free" and "reduced sodium" prominently displayed on the product label (He & Chen, 2013).

10. Soy sauce: description

Over the years, China introduced soy sauce to different parts of the world. Later, the Kikkoman Company of Japan promoted soy sauce in various countries. For example, in the United States, Kikkoman Company was the first major foreign company to build a large-scale processing plant to manufacture soy sauce. Now, its products are in almost every supermarket and grocery store in this country. Depending on the raw materials used and manufacturing method applied, there are many different kinds of soy sauce which differ in flavor (Kikkoman Corporation, 2017).

Soy sauce is a liquid made from soybeans, wheat, water and salt made by natural fermentation or chemically produced. The color of soy sauce ranges from light amber to black. Naturally brewed soy sauce is fermented for months or longer which helps to develop new volatile and nonvolatile substances that contribute to the characteristic color, flavor and taste of soy sauce. Chemical or non-brewed soy sauce is produced quickly within days, using a mixture of hydrolyzed soy protein and flavorings such as corn syrup and caramel (Hui et al., 2004).

Among brewed soy sauces, there is another differentiation, the geographic style. Soy sauce styles vary among cuisines including Korean, Indonesian, Thai, Filipino, etc. The general composition of some soy sauces from different parts of the world is shown in Table 5. The

types that consumers are most likely to find at US grocery stores are either Japanese or Chinese in origin or style (Neidleman & Laskin, 1997).

Table 5. General composition of some soy sauces from various parts of the world

Product	NaCl^a	TN^a	RS (IS)^a	Alcohol ^a	Color
<i>Koikuchi shoyu</i> , Japan	17.0	1.70	5.07	2.50	++
<i>Unsuikuchi shoyu</i> , Japan	18.0	1.18	4.00	2.00	+
Soy sauce, Taiwan	15.6	2.05	5.95	0.86	++
Soy sauce, Korea	17.3	1.50	2.10	0.39	++
Soy sauce, Hong Kong	26.2	1.54	4.22	0.00	+++
Soy sauce, Philippines	24.7	0.76	1.06	0.01	++
Soy sauce, Singapore	24.1	1.97	4.81	0.00	+++
Soy sauce, Malaysia	18.3	1.17	8.50	0.03	+++
Soy sauce, US	16.5	1.65	3.70	2.07	++

^aNaCl = Sodium Chloride; TN = Total Nitrogen (g/100ml); RS (IS) = Reducing Sugar (Invert Sugar) (g/100ml); Alcohol = Ethanol (ml/100ml)

Adapted (Neidleman & Laskin, 1997).

Under each ethnic group, soy sauce is further divided based on differences in raw ingredients, methods of preparation or duration of aging. For instance, Chinese soy sauce is divided in traditional soy sauce, modern soy sauce, regular soy sauce, which is made of soybeans and wheat; and mushroom soy sauce, which is made of soybeans, wheat and mushrooms (Hui et al., 2004). Japanese soy sauce, known as shoyu, is brewed with roasted wheat. Chinese soy sauce, which traditionally omits wheat, is nowadays brewed with wheat

flour. This difference in ingredients as well as brewing time gives Japanese soy sauce a slightly sweeter, rounder flavor and Chinese soy sauce a denser, saltier finish. Generally, Chinese sauce also tends to be much thicker and darker than the Japanese style (Airy Rhyme Books, 2014).

Japan has an organized system of classification to define types of soy sauce (Table 6). The difference between these types of soy sauce are quite significant and they are classified based on the proportions of raw materials, soy beans and wheat used to make the sauce (Neidleman & Laskin, 1997).

Koikuchi is made from equal amounts of wheat and soybeans and serves as an all-purpose seasoning. It represents about 85% of total soy sauce production in Japan. *Usukuchi*, is the second most popular type of soy sauce in Japan and is commonly used as a seasoning for foods in which the original flavor and color need to be preserved. The remaining three types of soy sauce are produced and consumed only in isolated localities for special uses in Japan. Among them is *tamari*, which is very similar to the traditional Chinese type of soy sauce. It is made using soy:wheat ratio of 10:1. In contrast to *tamari*, *shiro* is made by using a very high ratio of wheat to soybeans and the fermentation process is set up under conditions that prevent color development. *Saishikomi* is produced by using equal amounts of wheat and soybeans (Neidleman & Laskin, 1997).

Table 6. Percentage compositions of five varieties of soy sauce

Sauce (<i>shoyu</i>)	NaCl^a w/v	TN^a w/v	RS^a w/v	Alcohol ^a v/v	Color
<i>Koikuchi</i>	16.9	1.57	3.0	2.3	Deep brown
<i>Unsuuchi</i>	18.9	1.19	4.2	2.1	Light brown
<i>Tamari</i>	19.0	2.55	5.3	0.1	Dark brown
<i>Saishikomi</i>	18.6	2.39	7.5	trace	Dark brown
<i>Shiro</i>	19.0	0.5	20.2	trace	Yellow-tan

Adapted (Neidleman & Laskin, 1997).

10.1 Fermented soy sauce production

Although there are some variations in making different types of soy sauce, their basic steps are the same, including treatment of raw materials, *koji* making, and brine fermentation, pressing and refining (Figure 2).

The fermentation method takes up to six months to complete and results in a transparent, delicately colored broth with balanced flavor and aroma (Aihara & Aihara, 2013). The taste is mainly from soybean proteins, and the aroma is mainly from wheat starch, created as the microorganisms work on each ingredient (Hui et al., 2004). The characteristic color comes from the combination of amino acids obtained from proteins, and glucose obtained from starch. The flavor enhancing properties, or *umami*, of the soy extract are recognized to help blend and balance taste (Aihara & Aihara, 2013).

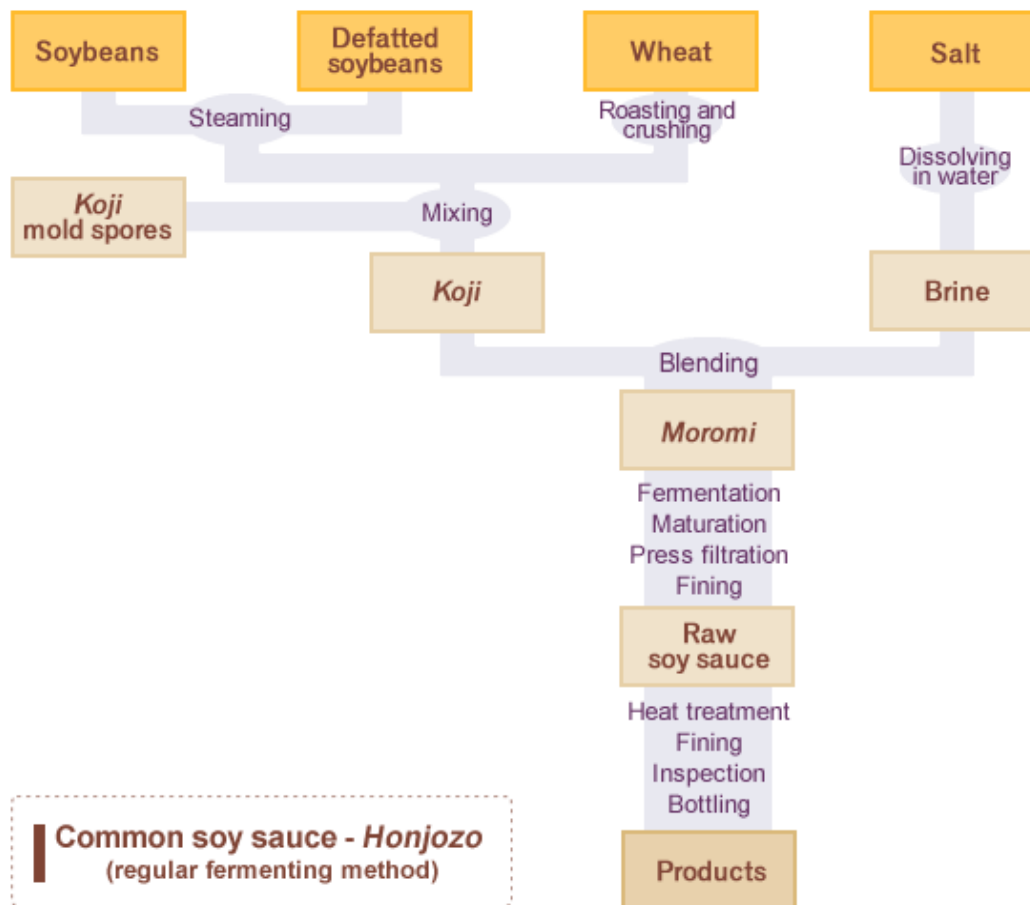


Figure 2. Flowchart of soy sauce manufacturing (Soy Sauce Information center, 2017).

10.1.1 Raw materials

Whole soybeans and wheat are soaked overnight at an ambient temperature. The soaked soybeans are cooked under steam pressure. In the soy sauce manufacturing process, the soybean oil is not utilized. There are two kinds of defatted soybeans. One is the compressed defatted soybean, in which pressure is applied on steamed soy bean to extract soybean oil. The other is solvent defatted soybean, in which the soybean oil is extracted from soybean by means of solvents. The chemical composition for defatted soybeans is described in

Table 7. If defatted soy products are used, they are first moistened by spraying with water in an amount equal to 30% of their own weight. This is followed by pressure and steaming for 45 minutes. The heated soybeans are allowed to cool to less than 40°C within a short period of time (Airy Rhyme Books, 2014).

Solvent defatted soybean contains a large quantity of protein. Among the amino acids, glutamic acid content is very abundant, about 20% of the total soy protein. Solvent defatted soybean does not contain starch; the main components in the carbohydrate fraction are sugars and polysaccharides such as sucrose, raffinose, stachyose, neutral arabinogalactan and arabinan (Neidleman & Laskin, 1997).

Table 7. Chemical composition of solvent defatted soybean and wheat (%)

	Moisture	Crude Protein	Carbohydrates	Crude Fat	Crude Fiber	Ash
Defatted Soybeans	11-13	47-51	19-22	0.1-1.5	3-5	4-6
Wheat	10-14	8-15	68-74	1.8-2.0	2.5-3.5	1.4-2.0

(Hui et al., 2004)

Concurrent with the treatment of soybeans, whole kernel wheat is roasted and cracked in rollers into four or five pieces. When wheat flour and wheat bran are used, they are steamed after being moisturized. Wheat contains only a small amount of protein. However, the glutamic acid content is high among the amino acids in wheat protein, about 32% of the total protein. About 10% of the carbohydrate fraction is pentosane, mainly xylan. This

helps the formation of melanin in the coloration of soy sauce (Hui et al., 2004) . The chemical composition for wheat is described in Table 7.

10.1.2 Koji making

The treated soybeans and wheat are mixed in a certain proportion, depending on the type of the end product to be made; for example, for *koikuchi*, the ratio of soybean:wheat is about 1:1, whereas for *tamari*, the ratio is 10:1. The mixture is inoculated with seed *koji* or a culture containing *Aspergillus oryzae* and *Aspergillus sojae* at a concentration of 0.1-0.2% (Sanchez, 2008).

In traditional *koji* making, the inoculated mixture is put into small wooden trays and kept for 3-4 days in a *koji*-making room. During the mold growth, the temperature and moisture are controlled by manual stirring. In modern *koji* making, however, the culture mix is put into the shallow, perforated vat and kept in a *koji* room where forced air is circulated and temperature and humidity may thus be controllable. After about 3-4 days, when the mixture turns green-yellow as a result of sporulation of the inoculated mold, it becomes mature *koji* (Airy Rhyme Books, 2014).

In the early stage of *koji* making, temperatures as high as 30-35°C are preferable for mycelium growth and the prevention of *Bacillus* as a contaminant. In the later stage, just before spore formation or after the second cooling, a lower temperature (20-25°C) is necessary to allow maximum production of enzymes. Alternatively, *koji* may be prepared at a constant low temperature of 23-25°C for a relatively longer time (66 hours). In any

case, when the temperature rises above 35°C because of active mold growth, it is advisable to cool *koji* material twice, either by hand mixing or by a mechanical device (Sanchez, 2008).

10.1.3 Brine fermentation

Mature *koji* is mixed with an equal amount or more (up to 120% by volume) of a salt solution to form the liquid mash known as *moromi*. The final concentration of NaCl in the mash should be 17-19%. A lower salt concentration promotes the growth of undesirable putrefactive bacteria during subsequent fermentation and aging (Hui et al., 2004). However, a higher salt concentration (in excess of 23%) may retard the growth of desirable halophilic bacteria and osmophilic yeasts. The mash is kept in large wooden containers or concrete vats with aeration device. The temperature of the surroundings can be mechanically controlled. Thus, the fermentation time can be shortened (Hui et al., 2004).

Mixing of the mash introduces fresh air to the mixture, allowing the growth of yeast or other beneficial microorganisms and inhibition of the growth of undesirable anaerobic bacteria. Temperature is also an important factor during brine fermentation. In general, the higher the temperature, the shorter the fermentation time (Aihara & Aihara, 2013). However, lower temperature fermentation results in better products because the rate of enzyme inactivation is slow. A good quality soy sauce can be made by a 6 month fermentation when the temperature of mash is controlled as follows: starting at 15°C for 1

month, followed by 28°C for 4 months and finishing at 15°C for 1 month (Watanabe & Kishi, 1984).

10.1.4 Microorganisms during fermentation

After mixing, there are different kinds of microorganisms living in the mash which are responsible for its fermentation and maturation.

Molds: The molds in the soya *koji* preparation stage will excrete protease, amylase and other enzymes. They are responsible for the hydrolysis of the raw materials. These molds cannot survive the high salt concentration and anaerobic conditions. They will die in three months after mixing of the mash, leaving the enzymes to complete the hydrolysis.

Yeasts: The yeasts that grow in the mash come from the soya *koji* and the environment and they are salt tolerant. They are composed of two groups: those responsible for the main fermentation and those responsible for the maturation. The former is mainly *Zygosaccharomyces rouxii*. It is a round or oval, bottom-fermentation type of yeast. It is capable of alcoholic fermentation and hydrolysis of various amino acids into their respective alcohols, giving the soy sauce its characteristic flavor and odor. At the same time, glutamic acid is converted to succinic acid. During alcoholic fermentation, other organic acids such as acetic, lactic and succinic acids are also produced, giving the soy sauce a rich and bright color. The maturation yeast is mainly *Candida versatilis*. It grows

at the maturation stage of the soy sauce, with the production of odor compounds. It is not salt tolerant and it has a weaker alcoholic production capability.

Bacteria: There are many useful bacteria in the mash. The main ones are lactic acid bacteria. The most important during maturation is *Pediococcus halophilus*, which consists of a chain of four cocci and is an anaerobic or slightly aerobic lactic acid bacterium. It is salt tolerant and grows in 20% salt solution, and has strong fermentation capability, producing lactic acid. Besides the lactic bacteria, there are *Bacillus subtilis* and *Bacillus mesentericus*, with strong fermentation capability, but essentially no presence of acetic acid bacteria (Holt et al., 2000).

10.1.5 Pressing

After months of fermentation and aging (approximately six months), the mash becomes matured. In the case of home processing, raw sauce may be removed from the mash by filtering through cloth under a simple mechanical press. Recently, automatic loading of the mash into a filter cloth or continuous pressing by a diaphragm-type machine has emerged for effective filtration. The filtrate obtained is stored in a tank to separate the sediments at the bottom and the floating oil on the top (Airy Rhyme Books, 2014).

10.1.6 Refining

Raw soy sauce may be adjusted to the standard salt and nitrogen concentrations. It is then pasteurized at 70-80°C to inactivate enzymes and microorganisms, enhance the unique product aroma, darken color and induce formation of flocs, which facilitates clarification (Aihara & Aihara, 2013). Overall, the pasteurization process serves two purposes: it helps prolong the shelf life of the finished product, and it forms additional aromatic and flavor compounds (Watanabe & Kishi, 1984).

Table 8. Comparison of soy sauce of different grades

Composition	Grade A	Grade B	Grade C
Nature of product	Color, odor and flavor characteristic, fermented soy sauce, absence of undesirable odor or taste	Color, odor and taste of typical soy sauce, absence of undesirable odor and taste	Good color, taste, odor and absence of undesirable odor and taste
Total nitrogen (g/100ml)	1.4 or above	1.1 or above	0.8 or above
Amino nitrogen (g/100ml)	0.56 or above	0.44 or above	0.32 or above
Total solids less salt (%)	13 or above	10 or above	7 or above
Foreign matter	None	None	None
Contents	Same as identified	Same as identified	Same as identified

(Hui et al., 2004)

After heating, the soy sauce is clarified by either sedimentation or filtration. The clear supernatant is packed immediately into cans or bottles. In some cases, preservatives such as sodium benzoate and paraoxy-benzoate may be used (Hui et al., 2004).

Good soy sauce products should have a clear red color, possess characteristic odor and flavor, be absent of burnt flavor and offensive odor, sour, astringent bitter taste and have a proper composition. Table 8 shows a comparison of the composition of three grades of soy sauce in Taiwan.

10.2 Chemical soy sauce production

Traditionally, soy sauce is made by fermentation as described. However, soy sauce can also be made by acid hydrolysis. The resulting product is known as chemical soy sauce or protein chemical hydrolysate. Briefly, defatted soy products or other proteinaceous materials are first hydrolyzed by heating with 18% hydrochloric acid for 8-12 hours. After hydrolysis, the hydrolysate is neutralized with sodium carbonate and filtered to remove the insoluble materials (Sanchez, 2008). The resulting product is a clear dark-brown liquid. However, chemical soy sauce does not have the flavor and odor of fermented *shoyu*. Therefore, to improve its quality, chemical soy sauce is often blended with fermented *shoyu* to become a semichemical product before being sold (Hui et al., 2004).

10.3 Soy sauce composition

According to Feng et al. (2013), the chemical composition of soy sauce is rather complex and varies with types and even batches. In a typical Japanese fermented soy sauce, the soluble solids are divided almost equally between inorganic (46%) and organic components

(47%). Sodium and chlorine are the principal inorganic constituents (Sanchez, 2008). Amino acids are the principal organic components, comprising almost 25% of the total soluble solids. They are followed by carbohydrates 13%, polyalcohols 5% and organic acids nearly 3%. Of the total nitrogen, about 40-50% is in the form of amino acids, 40-50% peptides and peptones, 10-15% ammonia and less than 1% protein (Feng et al., 2013).

There are 18 amino acids present, and glutamic acid and its salts are the principle flavoring agents. Sugars present are glucose, arabinose, xylose, maltose and galactose; whereas sugar alcohols are glycerol and mannitol. Organic acids found are lactic, acetic, succinic, citric, formic and pyroglutamic (Feng et al., 2013).

Summary

Manufacturers of acidified products must ensure the safety of their products by thermal processing or by including a holding time that is sufficient to ensure that the product does not develop results outside the acceptance criteria, that it is safe for consumption and there is no presence of organisms of public health significance. Holding times must be determined through challenge studies that are conducted at the temperature they are likely to experience after production. However, with the number of unique products being produced, challenge studies are an inefficient means of determine safe holding time. Soy sauce-based Asian condiments are one type of products growing in popularity in the U.S. Currently, as predictive models are evolving from the basic research laboratory to use by industry and regulatory agencies, modeling should be considered as an initial estimator of

microbial behavior and guides for evaluating potential problems. The choice of maximum holding period should be supported by relevant and documented research; therefore, the main goals of this research study are:

Objectives:

- Determine the 5-Log reduction time for *Salmonella* spp. in soy sauce-based products stored at 18.3°C (65°F) and 23.8°C (75°F).
- Create a model using R software that can be used to define safe parameters for new products based on pH, salt and soy sauce concentration.
- To validate the model by conducting a challenge study on test products.

Chapter 2. MATERIALS AND METHODS

2.1 Bacterial strains

Five strains of *Salmonella enterica* [Serotype Braenderup (NVSL-96-12528, isolated from 10% salted yolk), serotype Typhimurium (ATCC 14028, isolated from chicken), serotype Enteritidis (NVSL 94-13062, isolated from liquid chicken eggs), serotype Heidelberg/Sheldon (3347-1, isolated from chicken) and serotype Hadar (S 24, isolated from turkey)] were used in this study. All of these serotypes have been linked to foodborne illnesses in the US. The stock cultures were stored in at -80°C (Ultra Low Temperature Freezer model U535 Innova, New Brunswick Scientific Co., Inc., Edison, NJ) in Tryptic Soy Broth (Neogen Corp., Lansing, MI) supplemented with 20% glycerol (Fisher Scientific, Pittsburg, PA).

2.2 Preparation of *Salmonella* cocktail

Each strain of *Salmonella enterica* was transferred to test tubes containing 20 ml of Tryptic Soy Broth + 1% glucose (Sigma Aldrich Co., St. Louis, MO) to lower the pH in the broth (induce acid resistance) by static growth in the presence of a fermentable sugar. Inoculated broth was incubated in a 42°C incubator (Mechanical Convection Incubator 30M, Thermo Scientific Co., Waltham, MA) overnight. After growth, the cells were harvested by centrifugation (Sorvall ST 16R Centrifuge, Thermo Scientific Co., Waltham, MA) for 10 minutes at 5000 rpm and resuspended in Butterfield's Phosphate-Buffered dilution water. Fifteen milliliter of each strain of *Salmonella* was combined into a sterile tube in order to obtain a "*Salmonella* Inoculation Cocktail" at the target level (i.e., above 7.0 log₁₀ CFU/g).

2.3 Preparation of treatments

The soy sauce (Kikkoman Co., Hoffman Estates, IL) for this experiment came from new bottles to make sure that the product was in good condition. Low sodium soy sauce (50% regular soy sauce) with the following characteristics: Brix degree of 18.3%, pH 4.77 and 6.9% salt was used to prepare 6 treatments with pH adjusted to 3.0, 4.0 and 5.0 and two different salt concentrations of 7 and 14%. Regular soy sauce with a Brix degree of 35.2%, pH 4.71 and salt percentage of 13.7 was used to prepare three treatments with pH of 3.0, 4.0 and 5.0. Butterfield's Phosphate-Buffered dilution water was used to prepare 9 treatments with adapted salt percentages of 2, 7, and 14% and three levels of pH 3.0, 4.0 and 5.0. Table 9 shows the list of 18 treatments including the description of each formulation. Butterfield's Phosphate-Buffered dilution water was used as a control.

Sodium chloride (Fisher Scientific) was used to adjust the salt concentration, acetic acid (Fisher Scientific) to lower the pH, and a solution of 1 N NaOH (Fisher Scientific) to increase the pH. Duplicated samples for each treatment were aseptically weighed into 500 milliliters sterile bottles then distributed into sterile whirl-pak bags. Twenty five grams of each treatment and the control were weighed. Each bag was labeled with the treatment identification and testing day (1, 2, 4, 7, 10 and 15 days), except for treatments with pH 3.0 which were tested hourly (0.5, 1, 2, 3, 4, 6 and 8 hours).

For each treatment, the pH was measured by using a Eutech PC 700 pH meter (Fisher Scientific). The pH meter was calibrated with the specific standards pH 4.0 and 7.0. Ten milliliters of each sample were used to confirm the desired pH 3.0, 4.0 and 5.0 ± 0.05 .

Table 9. Formulation (pH, salt concentration and soy sauce) for each treatment

Treatment	Salt %	pH	Soy Sauce %
A	2	3.0	0
B	2	4.0	0
C	2	5.0	0
D	7	3.0	0
E	7	4.0	0
F	7	5.0	0
G	7	3.0	50
H	7	4.0	50
I	7	5.0	50
J	14	3.0	0
K	14	4.0	0
L	14	5.0	0
M	14	3.0	50
N	14	4.0	50
O	14	5.0	50
P	14	3.0	100
Q	14	4.0	100
R	14	5.0	100

2.4 Inoculation and storage

Each sample was inoculated with 250 μ l of *Salmonella* cocktail (1% of total volume). Into each whirl-pak bag, the inoculum was added drop-wise to several different locations. Bags were agitated to homogenize the inoculum with the product. The inoculum level was targeted above 7.0 log₁₀ CFU/g to achieve a final concentration in the product of 6.0 log₁₀ CFU/g or above. Samples representing the first time point from time 0 were plated immediately after inoculation. The remaining bags were stored at 18.3°C and 23.8°C respectively, for each pull day (1, 2, 4, 7, 10 and 15 days) and pull hour (0.5, 1, 2, 3, 4, 6,

and 8 hours). Control samples were inoculated and plated immediately, the counts (CFU/g) were used as an initial point for survival curves

2.5 Procedure for enumeration of microorganisms

Samples were plated for *Salmonella* immediately after the bags were pulled at a specific time points (1, 2, 4, 7, 10 and 15 days) and (0.5, 1, 2, 3, 4, 6, and 8 hour). To each sample, 225 ml of Butterfield's Phosphate-Buffered dilution water was added followed by stomaching for 2 minutes to assure homogeneity. To quantify the viable bacterial population, each sample was serially diluted with Butterfield's Phosphate-Buffered dilution water and spread plated onto Tryptic Soy Agar (TSA) added with ferric ammonium citrate (Sigma Aldrich Co., St. Louis, MO) and sodium thiosulfate (Acros Organics, Fisher Scientific). TSA is a non-selective agar that recovers sub-lethally injured cells and allows differentiation by addition of ferric ammonium citrate and sodium thiosulfate based on the reaction with hydrogen sulfide gas (H₂S) produced by *Salmonella* spp.

The black colonies formed on the plates were counted after 24 hour incubation period at 42°C. Each treatment was plated in duplicate.

2.6 Development of the model

All experiments were carried out with two independent replicates and each treatment was sampled in duplicate. The data from plate counts were transformed to log₁₀ values. For each combination of pH, salt and soy sauce, viable counts (log₁₀ CFU/g) at two different temperatures (18.3°C and 23.8 °C) were plotted versus the storage period in hours for treatments at pH 3.0 and versus days for treatments at pH 4.0 and 5.0. DMFit Version 3.5

(Institute of Food Research, Norwich, UK) was used as a resource for primary modeling, fitting data to the Baranyi and Robert model (Baranyi et al., 1994) (Equations (1) and (2)).

The growth of *Salmonella* was expressed as a function of time.

$$\ln(N(t)) = \ln(N_0) + \mu \max A(t) - \ln \left[1 + \frac{e^{\mu_{\max} A(t)} - 1}{e^{(N_{\max} - N_0)}} \right] \quad (1)$$

$$A(t) = t + \frac{1}{\mu_{\max}} \ln \left(\frac{e^{\mu_{\max} t} + q_0}{1 + q_0} \right) \quad (2)$$

Where: $\ln(N(t))$ = log of cell concentration at time t (h) (CFU/g); $\ln(N_0)$ = log of initial cell concentration (CFU/g); μ_{\max} = exponential growth/death rate (\log_{10} CFU/g/h); $\ln(N_{\max})$ = log of maximum cell concentration; q_0 = parameter expressing the physiological state of cells when $t = t_0$ (McKellar et al., 2004).

The Baranyi and Robert model has 4 main parameters: rate, lag, y_0 and y_{end} . Rate is the potential maximum rate of the model which can be negative when it is considered as a death rate (\log_{10} CFU per gram per day or hour). Lag time is usually defined as the intersection between the tangent to the exponential growth phase and the initial value and its unit is the same as the unit used for the time data. y_0 represents the upper asymptote, which corresponds to the initial bacterial counts (\log_{10} CFU per gram); while y_{end} , represents the lower asymptote, which corresponds to final bacterial counts (\log_{10} CFU per gram) (Baranyi, 2017). One of the advantages of the Baranyi and Robert model is that is readily available as a series of differential equations that allow modeling in a dynamic environment.

For the secondary model, a multiple linear regression was performed to fit the death rate (\log_{10} CFU per gram per hour) of *Salmonella* as a function of temperature, pH, salt and soy

sauce percentages using R software version 3.4.0 for Windows (R Core Team, Vienna, Austria) following a quadratic equation:

Death rate (\log_{10} CFU/g/hour) = $\alpha - \beta_1(\text{temp}) - \beta_2(\text{salt}) - \beta_3(\text{pH}) - \beta_4(\text{soy sauce}) + \beta_5(\text{salt})^2 + \beta_6(\text{pH})^2 + \beta_7(\text{soy sauce})^2 - \beta_8(\text{temp}*\text{salt}) - \beta_9(\text{temp}*\text{pH}) - \beta_{10}(\text{temp}*\text{soy sauce}) + \beta_{11}(\text{salt}*\text{pH}) + \beta_{12}(\text{salt}*\text{soy sauce}) + \beta_{13}(\text{pH}*\text{soy sauce})$, where α is the intercept and β_1 - β_{13} are estimated coefficients.

The summary function in R software provides valuable information regarding regression analysis, including t-test, F-test, R-squared, residual, and significance values. All of this data were collected for the data set under evaluation and can be used to answer important research questions related to the linear model. For each regression analysis, the 95% confidence limit interval (95% CLI) of the predictions for each observed value used in model development was also obtained. The 95% CLI provides the upper and lower boundaries of the predictions, which accommodate the variability in the predicted values and the variability in the observed values.

A response surface methodology (RSM) was performed to have a visual understanding of *Salmonella* survivability as a function of the environmental and intrinsic factors such as temperature, pH, soy sauce and salt concentrations. RSM explores the relationship between explanatory and response variables to obtain an optimal response.

2.7 Model validation

Different formulations including different soy sauce content, salt percentage and pH, were prepared in the laboratory. Procedures for preparation of treatments, *Salmonella* cocktail and inoculation were followed as described before. It is important to validate models with

data independent of those used for developing the model; therefore, the concentrations for each parameter were defined based on the formulation of different soy sauce based products such as teriyaki, ponzu and sweet and sour sauces (Table 10).

To validate the model, the observed populations of *Salmonella* obtained after the inoculation in the formulations described in Table 10, were compared to the predicted populations, which were estimated by using the Baranyi and Robert model (Baranyi et al., 1994) with kinetic parameters calculated at some combinations of temperature, pH, soy sauce and salt percentages. The model validation was performed to ensure that the information entered into the model equation agrees with the observed data obtained from laboratory experiments.

Table 10. Formulation of products used for model validation

Product	Soy Sauce %	Salt %	pH
Teriyaki sauce	60	9	4.2
Ponzu dressing	30	6	3.8
Sweet and sour sauce	0	3	3.4

Chapter 3. RESULTS

3.1 Effect of pH and storage temperature

A total of 36 survival curves of *Salmonella* corresponding to different combinations of pH (3.0, 4.0 and 5.0), temperature (18.3°C and 23.8°C), salt (2, 7 and 14%) and soy sauce (0, 50 and 100%) were generated as a result of the average of four replicates. The inoculum level applied to the samples at pH 3.0 was 7.1 log₁₀ CFU/g. The reduction in viable cell counts in log₁₀ CFU/g for each treatment at pH 3 stored at 18.3°C (65°F) are shown in Figure 3. According to the results obtained, *Salmonella* survived more than 4 hours in treatments with 0% soy sauce (A, D and J). However, treatments that contained 50 and 100% soy sauce (G, M and P) affected the survival rate of *Salmonella* after 30 minutes of inoculation. By 6 hours *Salmonella* was in the detection limit of 10 CFU/g for all the treatments.

Figure 4 shows the reduction of *Salmonella* at pH 3.0 stored at 23.8°C (75°F). Treatments that contained 50 and 100% of soy sauce and a high salt content of 7 and 14% (G, M and P) showed a reduction in viable cell counts after 30 minutes of inoculation. It should be emphasized that for treatments D and J, the reduction rate was faster during higher storage temperature at 23.8°C (75°F) than if it is compared with *Salmonella* population at 18.3°C (65°F). Accordingly, during storage at relatively high temperatures (23.8°C), the bacterial population decreases, while lower temperatures (18.3°C) protects *Salmonella*. It has been suggested that the protective effect of low temperatures on survival of acidification arises from alteration of the kinetics of protein denaturation (Russell & Gould, 2003).

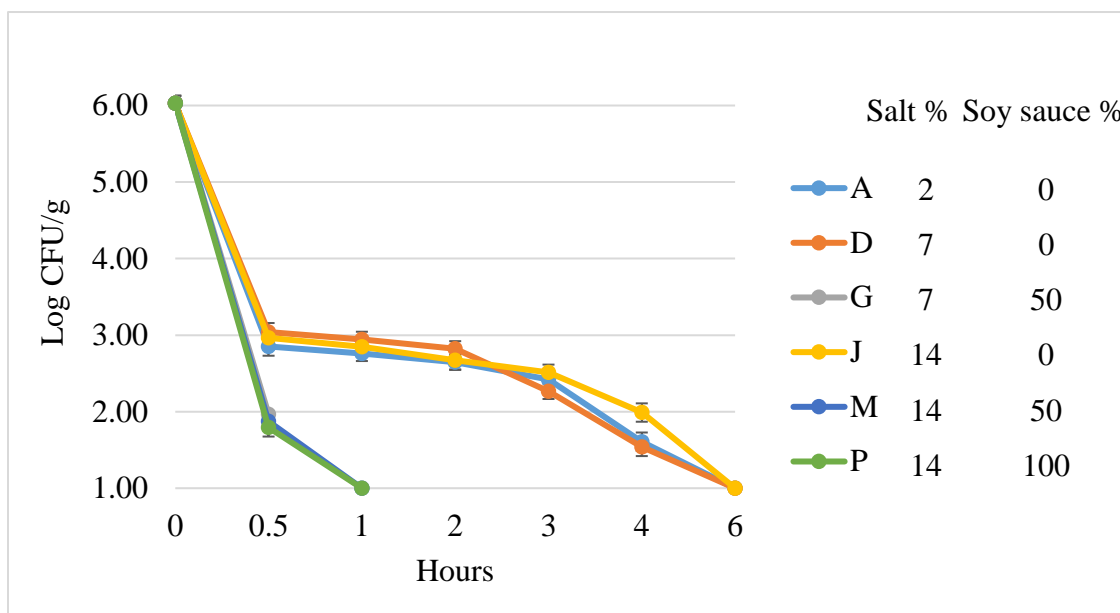


Figure 3. Survival curve of *Salmonella* at pH 3.0 stored at 18.3°C (65°F)

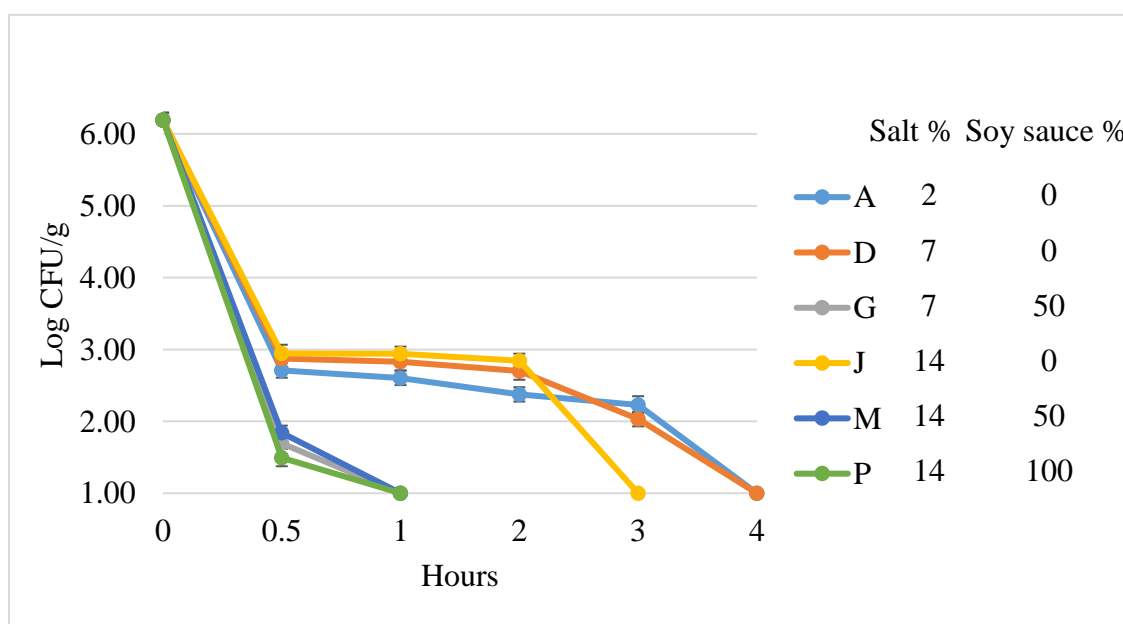


Figure 4. Survival curve of *Salmonella* at pH 3.0 stored at 23.8°C (75°F)

The inoculum level applied to the samples at pH 4.0 and 5.0 was $7.6 \log_{10}$ CFU/g. The reduction in viable cell counts in \log_{10} CFU/g for each treatment at pH 4.0 stored at 18.3°C (65°F) are shown in Figure 5. According to the results obtained, *Salmonella* population decreased immediately after inoculation and were below the detection limit (<10 CFU/g) by Day 1 of storage in treatments with 50 and 100% soy sauce and a high salt content of 7 and 14% (H, N and Q). Treatments with 0% soy sauce and 7 and 14% salt (E and K), showed a reduction of *Salmonella* by Day 2. Finally, a concentration of 2% salt and 0% soy sauce (treatment B) allowed *Salmonella* to survive up to 15 days.

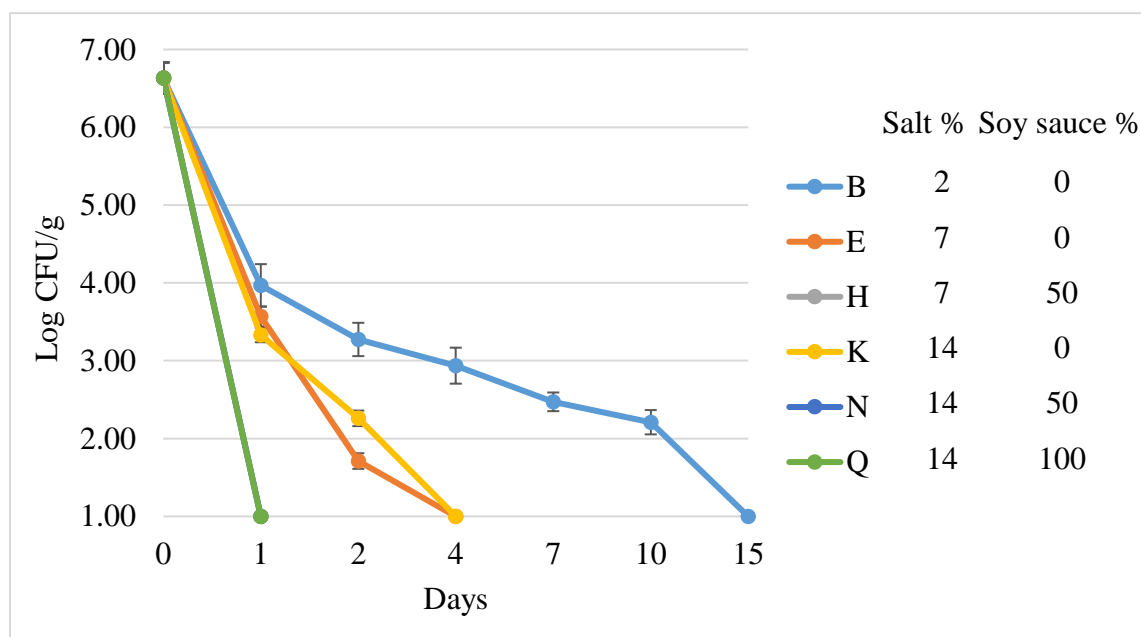


Figure 5. Survival curve of *Salmonella* at pH 4.0 stored at 18.3°C (65°F)

Figure 6 shows the reduction of *Salmonella* at pH 4.0 when formulations were stored at 23.8°C (75°F). *Salmonella* population decreased considerably by Day 1 in treatments with 50 and 100% soy sauce and a high salt content of 7 and 14% (H, N and Q). Treatments

with no soy sauce but with 7 and 14% salt (E and K), showed a reduction of *Salmonella* below the detection limit (<10 CFU/g) by Day 2.

By Day 7 *Salmonella* was below the detection limit of <10 CFU/g for all treatments. The reduction rate was faster during the higher storage temperature at 23.8°C (75°F) when compared to *Salmonella* populations at 18.3°C (65°F), as expected (Russell & Gould, 2003).

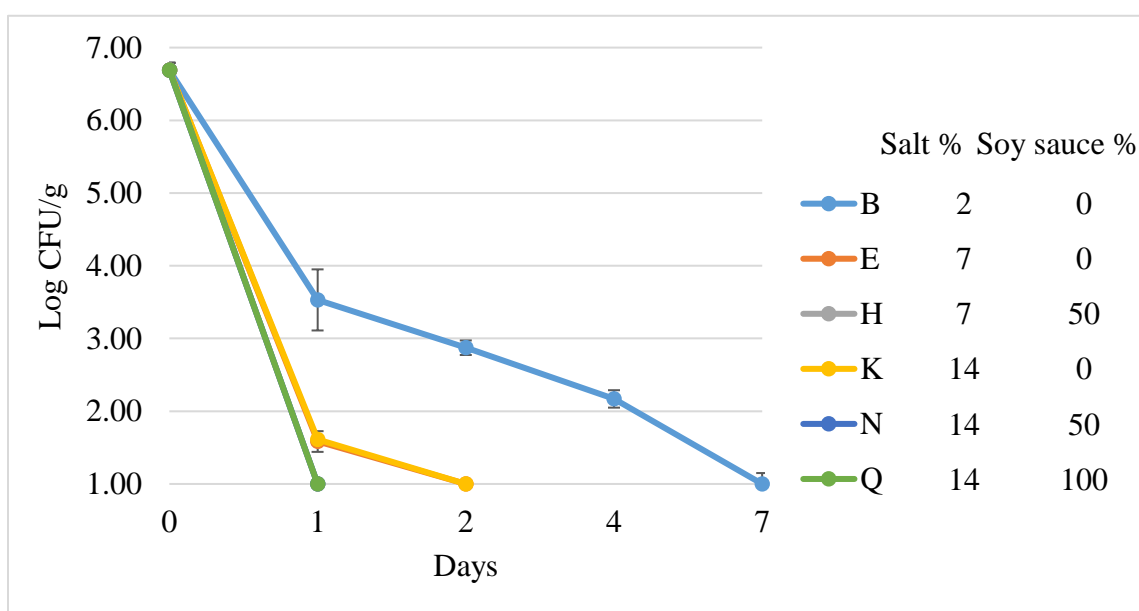


Figure 6. Survival curve of *Salmonella* at pH 4.0 stored at 23.8°C (75°F)

Figure 7 shows 3 logs reduction of *Salmonella* stored at 18.3°C (65°F) by Day 4 for treatments (F, L, O, R) that combined high salt percentage (7 and 14%) and soy sauce at pH 5.0. By Day 10, *Salmonella* was below the detection limit of <10 CFU/g for all treatments, except treatment C that had 2% salt and 0% soy sauce. Since pH 5.0 is closer to neutral, it represents less of a barrier against *Salmonella* survival. Also, this pathogen is generally inhibited in the presence of 3-4% NaCl (Doyle, 1989); therefore, it was not

surprising that *Salmonella* was still detected in 2% salt samples even after 15 days of storage.

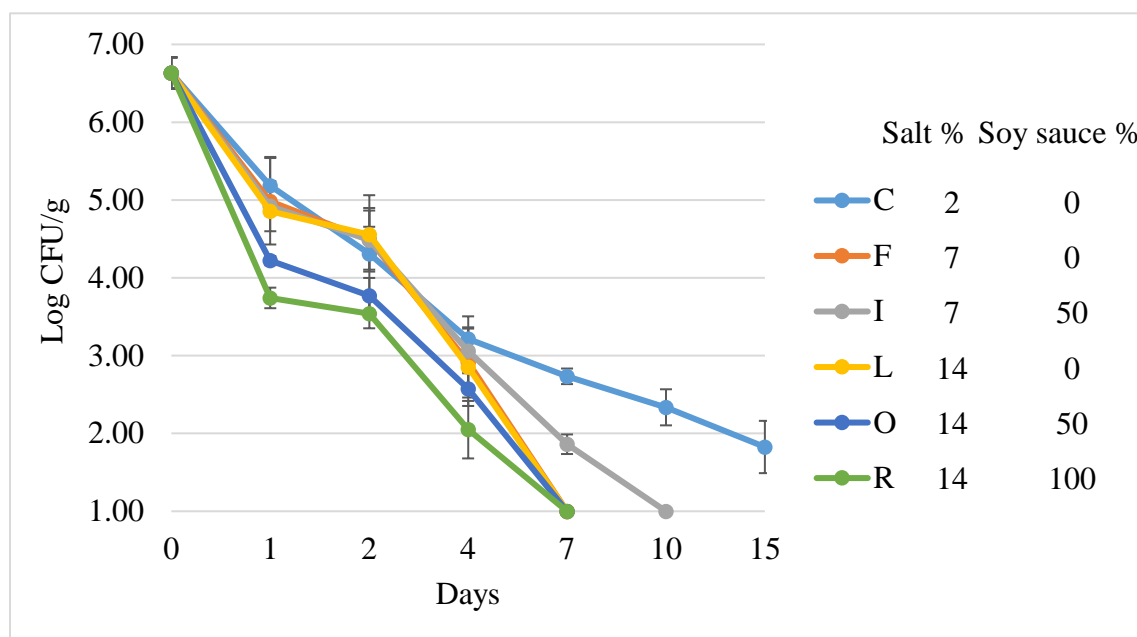


Figure 7. Survival curve of *Salmonella* at pH 5.0 stored at 18.3°C (65°F)

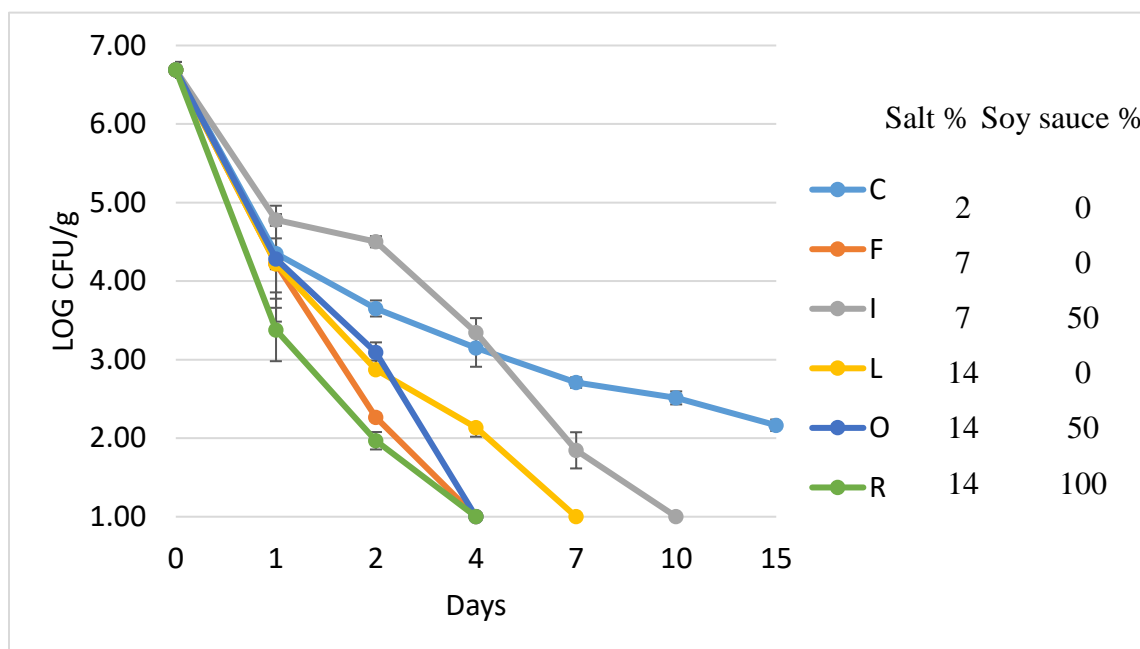


Figure 8. Survival curve of *Salmonella* at pH 5.0 stored at 23.8°C (75°F)

The reduction in viable cell counts in \log_{10} CFU/g for each treatment at pH 5.0 stored at 23.8°C (75°F) are shown in Figure 8. *Salmonella* showed a 4 \log_{10} reduction by Day 2 for treatments (F, L, O, R) that combined high salt percentage (7 and 14%) and soy sauce. By Day 10, *Salmonella* was below the detection limit of <10 CFU/g for all treatments, except treatment C that had 2% salt and 0% soy sauce.

3.2 Development and validation of the model

A total of 36 survival curves of *Salmonella* corresponding to different combinations of pH (3.0, 4.0 and 5.0), temperature (18.3°C and 23.8°C), salt (2, 7 and 14%) and soy sauce percentages (0, 50 and 100%) were generated with the Baranyi model.

In Table 11, the outputs of the model are shown. The estimated parameter, death rate (DR) (\log_{10} CFU per gram per hour), for each individual survival response were further expressed as function of the controlling factors. Also, the standard error of fitting (SE of fit) and the adjusted R-square statistics of the fitting (R^2) are included.

The temperature of storage, pH, salt and soy sauce percentages influenced the kinetic characteristics of *Salmonella* (Table 12). Indeed, by using response surface methodology, it was possible to generate figures that show the death rate of *Salmonella* in soy sauce based on the parameters studied.

Table 11. Outputs of Baranyi model for the survival of *Salmonella*

Conditions				Output		
Temp (°C)	Salt %	pH	Soy Sauce %	Maximum Death Rate ^a	Std. Error of fit	R ²
18.3	2	3.0	0	-0.60	0.53	0.77
18.3	2	4.0	0	-0.01	0.66	0.79
18.3	2	5.0	0	-0.02	0.33	0.95
18.3	7	3.0	0	-0.63	0.13	0.98
18.3	7	4.0	0	-0.06	9.55E-05	1.00
18.3	7	5.0	0	-0.03	0.07	1.00
18.3	7	3.0	50	-5.03	4.08E-07	1.00
18.3	7	4.0	50	-0.23	1.45E-06	1.00
18.3	7	5.0	50	-0.02	0.15	0.99
18.3	14	3.0	0	-0.59	0.22	0.95
18.3	14	4.0	0	-0.05	0.13	0.99
18.3	14	5.0	0	-0.03	0.11	1.00
18.3	14	3.0	50	-8.31	4.02E-07	1.00
18.3	14	4.0	50	-0.23	6.43E-07	1.00
18.3	14	5.0	50	-0.03	0.09	1.00
18.3	14	3.0	100	-8.47	3.67E-07	1.00
18.3	14	4.0	100	-0.23	1.19E-06	1.00
18.3	14	5.0	100	-0.03	0.19	0.99
23.8	2	3.0	0	-0.89	0.48	0.77
23.8	2	4.0	0	-0.03	0.39	0.94
23.8	2	5.0	0	-0.07	0.30	0.93
23.8	7	3.0	0	-0.93	0.07	0.99
23.8	7	4.0	0	-0.12	9.83E-05	1.00
23.8	7	5.0	0	-0.06	0.00	1.00
23.8	7	3.0	50	-5.19	4.34E-07	1.00
23.8	7	4.0	50	-0.24	1.26E-06	1.00
23.8	7	5.0	50	-0.02	0.10	1.00
23.8	14	3.0	0	-0.95	0.09	0.99
23.8	14	4.0	0	-0.12	5.60E-05	1.00
23.8	14	5.0	0	-0.03	0.40	0.95
23.8	14	3.0	50	-5.19	3.89E-07	1.00
23.8	14	4.0	50	-0.24	5.42E-07	1.00
23.8	14	5.0	50	-0.06	0.10	1.00
23.8	14	3.0	100	-5.19	3.65E-07	1.00
23.8	14	4.0	100	-0.24	8.20E-07	1.00
23.8	14	5.0	100	-0.06	0.01	1.00

Table 12. t values and probability for the single parameters and interactions which affect the survival of *Salmonella* in soy sauce based products

Parameters	t value	Pr(> t) ^a
Temperature (°C)	1.1699	0.0917*
Salt %	-1.283	0.2017*
pH	10.151	<2e-16*
Soy sauce %	-8.730	1.07e-4*
Temp (°C)*salt %	0.995	0.3215
Temp (°C)*pH	-2.435	0.0162*
Temp (°C)*soy sauce%	2.100	0.0377*
Salt %*pH	1.122	0.2640
Salt %*soy sauce %	-1.256	0.1295
pH * soy sauce %	11.515	<2e-16*

*Significance at 0.01 indicating parameter or interaction included in the model

Figure 9 shows a graphical visualization of the fitted surface in terms of the interaction of temperature and pH; therefore, to obtain the higher death rate the temperature should be in the range of 19.5 to 21°C and the pH needs to be between 3.0 and 3.1 as the pH increases from 3.0 to 5.0, the reduction rate is gradually reduced. On the other hand, if soy sauce percentage and temperature are the factors limiting *Salmonella* survival in a product; to maintain the same death rate as the storage temperature increases, the soy sauce percentage should be increased (Figure 10). According to the counterplot in Figure 10, the highest death rates for *Salmonella* which were observed when soy sauce was above 40% and the temperature was between 18.3-22.5 °C.

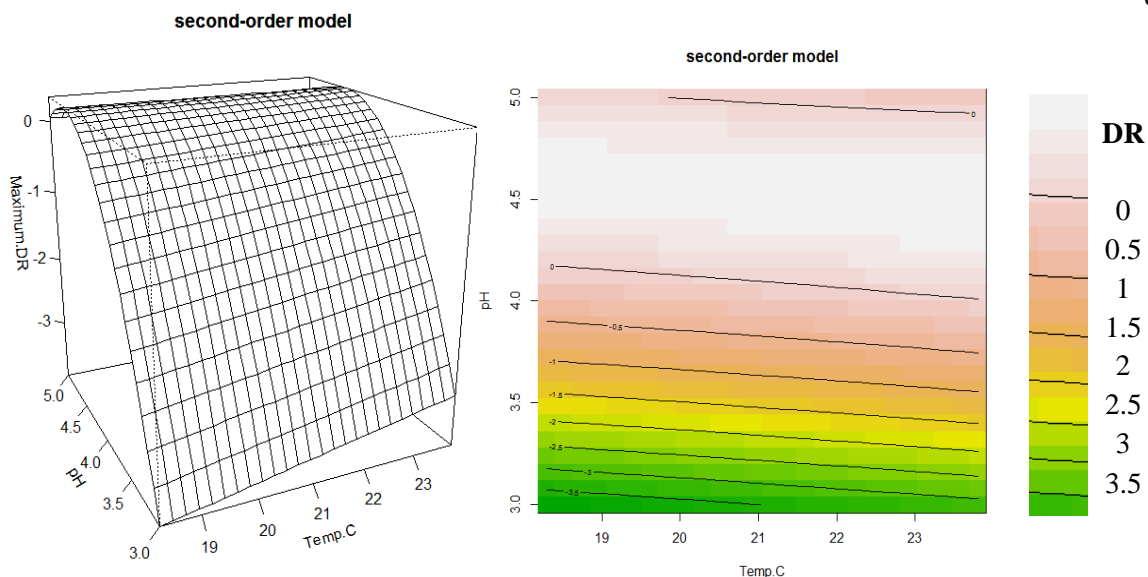


Figure 9. Quadratic response surfaces predicting the death rate (DR) of *Salmonella* in soy sauce based products as a function of temperature and pH

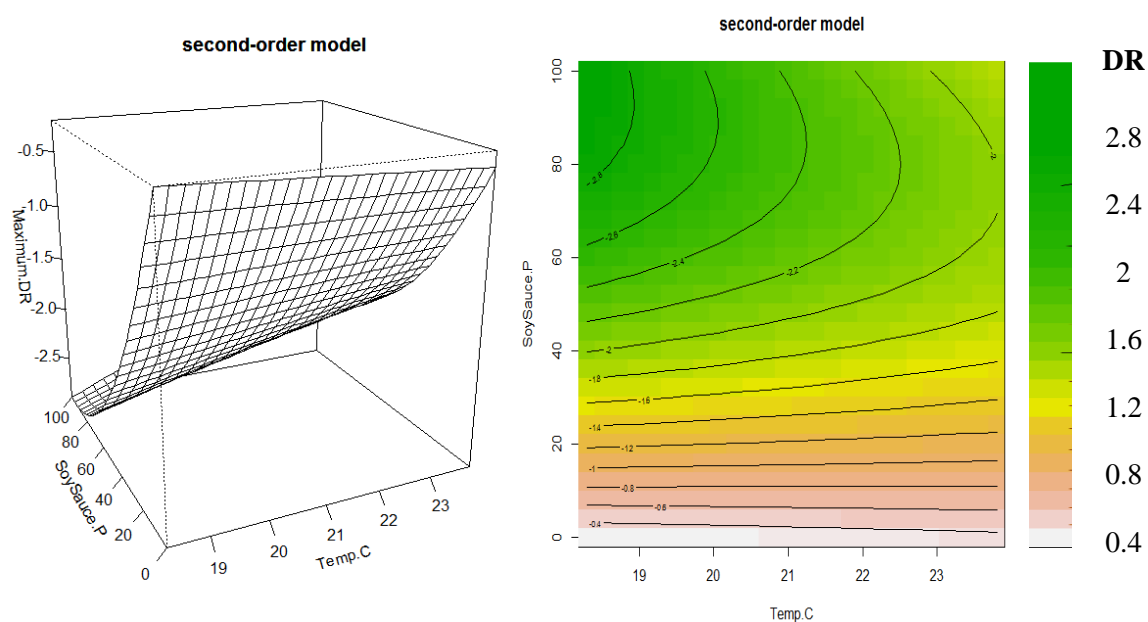


Figure 10. Quadratic response surfaces predicting the death rate (DR) of *Salmonella* in soy sauce based products as a function of temperature and soy sauce

Salmonella survival is more likely to occur when the pH is above 3.8; therefore, an additional barrier against pathogen growth should be considered. For example, the use of soy sauce as an ingredient is one option due to the high salt content that reduces water activity which prevents microbial growth in food products, thus increasing shelf life. Moreover, when pH and soy sauce percentages are considered, to maintain a high death rate, the content of soy sauce should be increased if the pH is increased in the formulation (Figure 11). According to the counterplot in Figure 11, it is worth mentioning that pH values above 3.5, regardless of the soy sauce concentration, has a minimum effect on *Salmonella* inactivation as shown by death rates approaching zero.

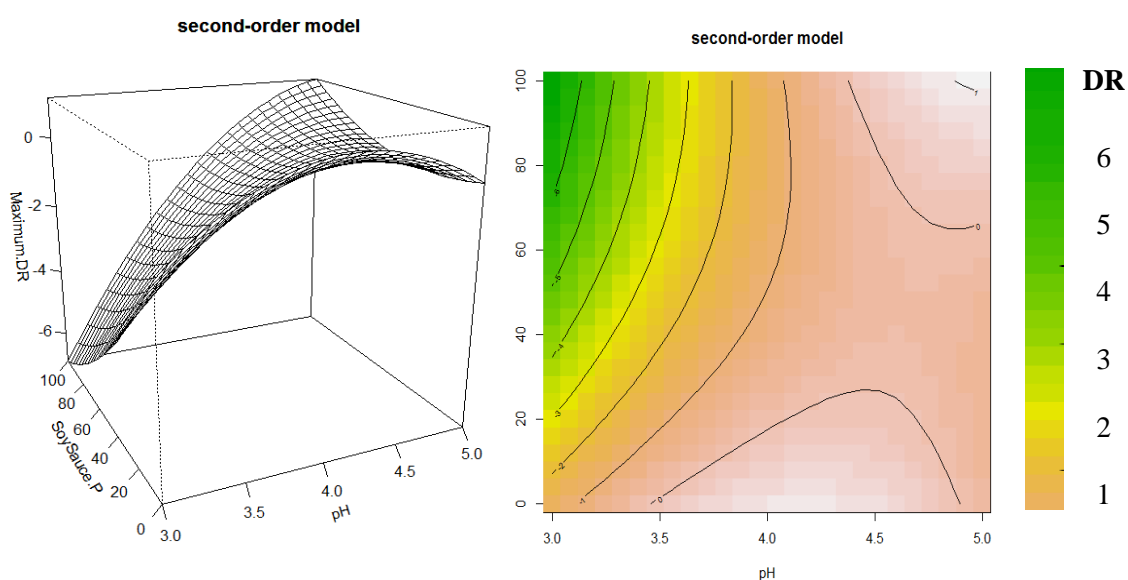


Figure 11. Quadratic response surfaces predicting the death rate (DR) of *Salmonella* in soy sauce based products as a function of pH and soy sauce percentage

Figures 9 to 11 were described as a part of the results because they affected the survival of *Salmonella* in soy sauce based products and the interactions were statistically significant. The equation to predict the death rate (\log_{10} CFU per gram per hour) of *Salmonella* as a

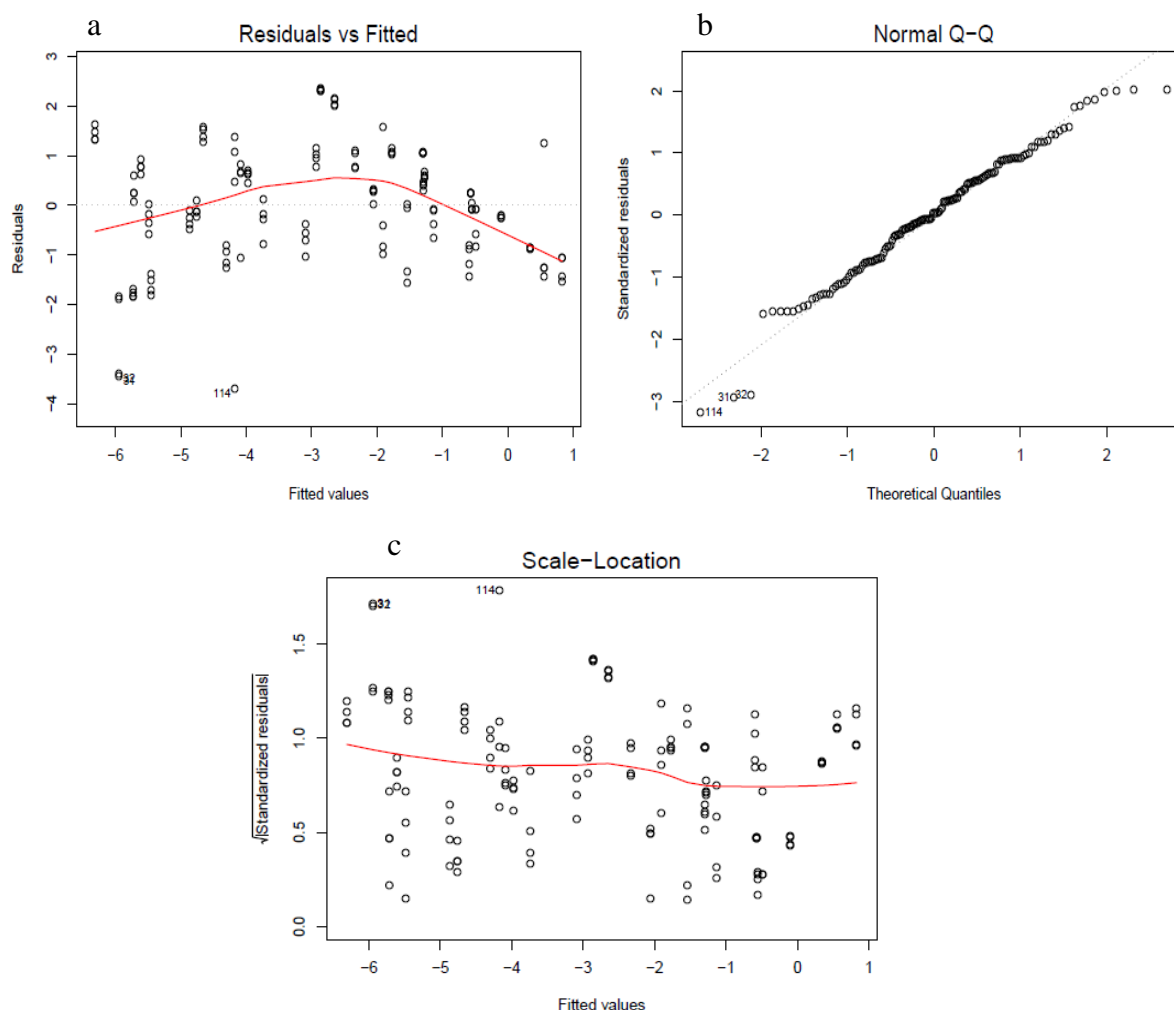
function of several factors such as temperature, pH, salt and soy sauce concentration is as follows:

$$\begin{aligned} \text{Death rate (log}_{10} \text{ CFU/g/hour)} = & 31.89 - 0.25(\text{temp}) - 0.26(\text{salt}) + 15.05(\text{pH}) - 0.21(\text{soy} \\ & \text{sauce}) + 7.0\text{e-}04(\text{salt})^2 - 1.62(\text{pH})^2 + 4.05\text{e-}04(\text{soy sauce})^2 + 6.80\text{e-}03(\text{temp}*\text{salt}) - \\ & 8.20\text{e-}02(\text{temp}*\text{pH}) + 1.79\text{e-}03(\text{temp}*\text{soy sauce}) + 2.58\text{e-}02(\text{salt}*\text{pH}) - 1.62\text{e-}03(\text{salt}*\text{soy} \\ & \text{sauce}) + 3.30\text{e-}02(\text{pH}*\text{soy sauce}). \end{aligned}$$

Multiple R-squared: 0.8638 Adjusted R-squared: 0.8502 P-value: < 2.2e-16

After running the regression analysis, it was confirmed that the model works well for the data based on the information obtained through diagnostic plots. Figure 12a shows a residuals vs fitted graphic where the residuals do not seem to have an obvious trend and they appear to have the same variance everywhere. In regression analysis, the difference between the observed value of the dependent variable (y) and the predicted value (\hat{y}) is called the residual (McPherson, 2001). Therefore, one of the most important assumptions in multiple linear regression model is that the residuals do not follow a specific tendency and are normally distributed. Figure 12b shows the normal Q-Q plot where the residuals are normally distributed because they are lined well on the straight dashed line. Finally the scale-location plot (Figure 12c) shows that residuals are spread equally along the ranges of predictors.

Figure 12. Diagnostic plots for linear regression analysis (a)residuals vs fitted (b)normal Q-Q (c)scale-location



The observed death rates (\log_{10} CFU/gram/hour) were obtained during laboratory experiments. The predicted reduction rates (\log_{10} CFU/gram/hour) were the result from the equation. A model prediction is considered acceptable, and the model therefore applicable, if an observed value is within the 95% CLI of the predicted value. As shown in Table 13, the values of *Salmonella* death rates are within the 95% confidence limit interval, with one exception.

For validation purposes different conditions were selected and the predicted values for death rates were obtained, using the model equation. Those predictions were acceptable in 83% of the cases. However, for one combination of temperature (18.3°C), pH (4.2), salt (9%) and soy sauce (60%), the observed value was not in the 95% confidence limit interval (Table 13).

Table 13. Predicted and observed values of *Salmonella* death rate in soy sauce based products under different conditions (validation)

Conditions				Death rate (log CFU/gram/hour)		95% CLI	
Temp (°C)	Salt %	pH	Soy sauce %	Observed	Predicted	Lower	Upper
18.3	9	4.2	60	-0.18	-0.72	-1.16	-0.28
18.3	6	4.4	30	-0.06	-0.08	-0.32	0.48
18.3	3	3.4	0	-0.10	-0.04	-0.51	0.42
23.8	9	4.2	60	-0.20	-0.30	-0.74	0.14
23.8	6	4.4	30	-0.08	-0.07	-0.39	0.41
23.8	3	3.4	0	-0.22	-0.07	-0.54	0.39

Chapter 4. DISCUSSION

Inactivation of microorganisms has been essential to the production of foods that are safe and of quality. The success of a food preservation technique depends on the mechanism of microbial inactivation (Breidt et al., 2010). Death rate kinetics of microorganisms are usually used to explain the effects of processing factors and to achieve their optimization. The food matrix that surrounds the microorganisms also has an extremely large influence on death rates, especially its pH, water activity and concentration and type of components. Microbial cell death is caused by heat, pressure, acid, chemicals, bacteriocins and other factors (Lee, 2004).

Kinetic models can be used for describing changes in microbial population based on linear reduction of microbial cells. For example, an initial microbial population is reduced to a final number of population (N) after a process time (t) at a constant process temperature. The plot of $\log_{10} (N)$ versus time gives a linear survival curve, which follows a first order decrease in the number of microorganisms and provides data on the rate of destruction in specific conditions. Inactivation models assume that all of the cells in a population have identical resistance to processing conditions (Fakruddin et al., 2012).

The multiple linear regression model developed in this study describes the influence of salt and soy sauce content, storage temperature and pH on the death rate of *Salmonella* in soy sauce-based products. *Salmonella* survival rate increases rapidly at lower temperatures, which can increase the risks associated with food safety.

The U.S. FDA expressed safety concerns about the survival of acid resistant pathogens in acidified products because of outbreaks in some acid foods, such as apple cider and orange juice (CDC, 2017). Therefore, linear models predicting the minimum times and temperature combination needed for safety, in terms of destruction of *E. coli* O157:H7,

Salmonella, and *Listeria* have been developed for application in commercial processes for shelf stable acidified foods (Breidt et al., 2010). The acid present in some products may be sufficient to assure a 5-Log reduction in numbers of acid resistant pathogens. For this reason, products with acetic acid as the primary acidulant and a pH below 3.3 do not require a heat process, but do require a temperature dependent holding time to assure safety (Breidt & Arritt, 2013).

Breidt & Arritt (2013), used acetic acid in their research to determine 5-Log reduction times for *Salmonella* in pickling brines, because these brines are among the ingredients in dressings, pickled peppers and other acidified foods. When the pH was reduced from 3.8 to 3.5, there was a decrease as well in the time to obtain a 5-Log reduction. That research concluded that a 2.8-fold reduction in survival was observed for acid solutions containing 2.5% acetic acid when initial pH was reduced from 3.8 to 3.5 (Breidt & Arritt, 2013).

The organic acid used in this study to reduce the pH was acetic acid because it is an effective microbial inhibitor (Koutsoumanis et al., 2004) and it is an ingredient used in the soy sauce making process. A faster log reduction was observed at pH 3.0 with *Salmonella* levels in product being reduced below the limit of detection of the method within 4 hours. Treatments at pH 4.0 required several days (7-15) to show the same decrease *Salmonella* populations (Figures 3-6). The minimum pH at which *Salmonella* can survive is dependent on the storage temperature, presence of salt and the type of acid present. Therefore, it is not surprising that there is a rapid log₁₀ reduction in *Salmonella* in treatments with high salt content (7-14%) (Figures 3-8). One of the ingredients that contribute to the final salt content of the product is the addition of soy sauce. Soy sauce has been expected to possess the ability to inhibit the deterioration of food (Sumague et al., 2008). The effects of soy

sauce on foodborne and other common pathogens such as *E. coli*, *Shigella flexneri*, *Salmonella typhi*, *Salmonella paratyphi A*, *Salmonella enteritidis* and *Vibrio cholera* have been studied (Sumague et al., 2008). Previous research has shown that initial cell concentrations of 10^3 , 10^5 , and 10^7 of bacteria in 1 ml of soy sauce decreased to undetectable levels within 4–6 hours, 24–48 hours and 5–7 days, respectively. Results showed that the antimicrobial activity of soy sauce is mainly based on the combined effects of NaCl, ethanol, pH, preservatives and temperature (Akiba et al., 1957)

Studies on the modeling of pathogens are built with higher starting initial populations (10^7 CFU/g) of pathogens than that of naturally contaminated foods (10^2 CFU/g). The survivability of several strains of *Salmonella* have been monitored the purpose of developing a model as a function of temperature, pH and water activity to get a better understanding about the importance of the interaction between these factors (Koutsoumanis et al., 2004). The overall results showed that small differences in temperature, pH or water activity could change the probability of growth dramatically. Overall, predictions of the model agreed with the observed response of the pathogen in 90% of the tested cases (Koutsoumanis et al., 2004). In the present research study, after conducting a validation process, the predictions were acceptable in 83% of the cases. Although there have been no known outbreaks of acid resistant pathogens in acidified soy sauce based products, the results reported in the present study may be used to help maintain the long history of safe production in this area. The results of this research showed an interaction of the factors on the survival limits of *Salmonella*.

The objective of this study was to determine log reduction of *Salmonella* as a response to the combination of several parameters (storage temperature, pH, salt and soy sauce

concentrations). As a result of the modeling, it was possible to determine a 5-Log reduction of *Salmonella* in soy sauce-based products and the necessary storage temperature and holding time combination. Table 14 shows the hours and days required to achieve a 5-Log reduction under several conditions used in the validation of the model. By comparing the time for achieving the required 5-Log reduction in two different temperatures, it is possible to observe that more time is required for conditions at 18.3°C (65°F) than for those at 23.8°C (75°F). For example, at 18.3°C (65°F), if the salt percentage is 6, the pH is 4.4 and the soy sauce content is 30%, 6 days are the necessary time to achieve a 5.8 log₁₀ CFU/g reduction. However, if the same formulation is held at 23.8°C (75°F), only 3 days are the necessary time to achieve a 5.8 log₁₀ CFU/g reduction. Depending on the interactions and concentrations of each parameter, the time to achieve the 5-Log reduction rate will change.

Table 14. Time (days or hours) to achieve a 5-Log reduction in *Salmonella* based on parameters used for validation

Conditions				Output		
Temp °C	Salt %	pH	Soy Sauce %	Death rate (log ₁₀ CFU/g/h)	Log ₁₀ CFU/g	Time
18.3	9	4.2	60	-0.30	-5.1	17 hours
18.3	6	4.4	30	-0.04	-5.8	6 days
18.3	3	3.4	0	-0.29	-5.2	18 hours
23.8	9	4.2	60	-0.45	-5.4	12 hours
23.8	6	4.4	30	-0.08	-5.8	3 days
23.8	3	3.4	0	-0.38	-5.3	14 hours

Chapter 5. CONCLUSIONS AND FUTURE RESEARCH

Currently most of the available shelf stable food products utilize more than one microbial hurdle such as thermal treatment, moisture, salt, low temperature storage and preservatives. Food may be spoiled by chemical and biological agents. Biological spoilage can result from the action of inherent enzymes, growth of microorganisms, invasion of insects, contamination with parasites, etc. About one-fourth of the world's food supply is lost through the action of microorganisms alone. Chemical spoilage results from purely chemical reactions, such as browning and oxidation reactions. The chance of food spoilage and association of new types of microorganisms have greatly increased due to new marketing trends, new processing techniques, extending shelf life and changes of temperature between production and consumption of foods. Many food materials are processed to destroy enzymes and microorganisms, thus prolonging their shelf life for hours, days, months or even years.

Predicting the fate of microbial pathogens in foods is a goal of all food safety specialist employed by food companies. Therefore, predictive microbiology has emerged as an important scientific discipline for estimating the consequences of diverse food handling and processing operations on the growth, survival and inactivation of microbial pathogens. However, no published documented research exists in modeling for soy sauce based products. This lack of documentation is challenging to all areas of the industry including small food processors that may not have the resources to support challenge studies conducted by private laboratories.

Expanding the currently available science based information regarding product parameters (storage temperature, pH, salt, water activity) and microbial survival will enhance the

production of safer foods, increase consumer confidence in the safety and quality of products and reduce the investment in challenge studies. A validation of a model that would assist food safety decisions during product development would be a great tool for the food industry. Nowadays, in the U.S. and other nations, predictive models are used to develop HACCP plans, improve product formulations and conduct risk assessments. Thus, substantial savings by reducing human disease and production costs will subsequently be achieved.

This project focuses on ensuring food safety of soy sauce based products. The objectives of this study were to determine a variety of safe combinations of parameters for acidified foods with pH values of 3.0 to 5.0, salt percentages of 2% to 14%, soy sauce concentration of 0% to 100%, which are representative conditions of acidified foods. Because bacterial survival in acidified foods is enhanced as temperature decreases, two storage temperatures were evaluated: 18.3°C (65°F) and 23.8°C (75°F).

The response of the pathogen to the parameters evaluated was monitored in a total of 36 treatments, where storage temperature, pH, salt and soy sauce conditions were varied. Treatments at pH 3.0 were monitored for 8 hours, while treatments at pH 4.0 and 5.0 were evaluated for 15 days. At pH 3.0, *Salmonella* counts were below the detection limit (<10 CFU/g) of the method by 4 hours of inoculation time. At pH 4.0, treatments stored at 23.8°C (75°F) with 7% and 14% salt content and 50% and 100% soy sauce showed a faster death rate (4 days) than treatments stored at 18.3°C (65°F) (2 days). However, pH 5.0 allowed *Salmonella* to survive longer, up to 7 days, and in some treatments longer (10 or 15 days). Since pH is a very important parameter that determines the growth and survival of pathogens,

it is feasible to conclude that pH it is an important factor for increasing the death rate of *Salmonella* in soy sauce-based product. Also, the concentration of salt which can be used as an ingredient in its original form or through the addition of soy sauce, will contribute to inhibit the growth of *Salmonella*.

A total of 36 survival curves of *Salmonella* were generated with the Baranyi model using DMFit program which provided the death rate (DR) (\log_{10} CFU/g/hour) as an output. The DR obtained from the inactivation models were used as an input for statistical analysis in R program and the output being a model equation. The statistical analysis also determined the significance of the interaction between the parameters studied (storage temperature, pH, salt and soy sauce percentages). As a result, based on the P value, it was concluded that the effect of temperature and pH were statistically significant on the DR of *Salmonella*. Also, interactions between temperature and soy sauce, salt and pH, and pH and soy sauce statistically affected the death rate of the organism of interest.

Finally, a validation study was conducted and the model predicted successfully the behavior of the pathogen in 83% of the tested cases. The results of the study indicated that the developed model predicts satisfactorily the death rate of *Salmonella* in soy sauce-based products. Additionally, the model provides an accurate description of the conditions that can be applied to control a process or specify the product formulation for safer food processing. This model provides a faster and more cost effective alternative to laboratory studies to estimate the effects of storage temperature, pH, soy sauce and salt percentages on the safety of soy sauce-based product. Such models can be beneficial to the food industry because they can describe the necessary combination of parameters to specify a

formulation in order to minimize the risk of pathogen growth or to enhance survival microbial reduction.

While the modeling for soy sauce-based products demonstrated a high precision in predicting the reduction rate of *Salmonella*, future research is necessary to generate models that describe the probability of pathogen survival based on product characteristics. A number of new approaches to address this issue would include:

- Development of new models for soy sauce-based products with pathogens also considered as a high risk in food products, such as *E. coli* (considered as an acid resistant microorganism)
- Develop new models for soy sauce-based products to predict *Listeria* death rate because of the ability to grow in cold temperatures
- Further model validation by comparison conducting challenge studies on test products

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