MICROBIAL INACTIVATION IN COLD-FILLED ACIDIFIED MAYONNAISE SAUCES

by

PRATIKSHA KOTKAR

(Under the Direction of Faith Critzer)

ABSTRACT

Microbial safety in acidified foods often requires heat treatments, compromising sensory quality. This study investigated cold-fill-hold processing on pathogen inactivation in acidified mayonnaise. Objectives included evaluating survival of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* in five commercial mayonnaise variants at 15.5°C and two lab formulations with 5% acetic acid, with or without 0.1% sodium benzoate, stored at 5°C and 25°C. D- and z-values for each pathogen were calculated based on microbial reduction data to achieve >5 log reductions. *E. coli* O157:H7 was the most resistant pathogen, with the highest D-values. In lab samples, 0.1% sodium benzoate did not enhance pathogen inactivation compared to acetic acid alone, although a pH increase occurred in sodium benzoate samples. Temperature significantly influenced microbial reduction, with lower D-values at 25°C. Cold-fill-hold processing effectively reduced microbial loads, indicating its potential as a non-thermal method for improving food safety in acidified products.

INDEX WORDS: Cold-fill-hold process, Microbial safety, Acidified sauces, 5-Log reduction

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CHAPTER 1

INTRODUCTION

Purpose Of the Study

A. Global consumption of acidified foods

Food products such as fermented and/or acidified vegetables, carbonated beverages, salad dressings, sauces, sports drinks, fruit juices, and salsas constitute the market of the acid and acidified foods and beverages in the United States. Moreover, as studied by Dufort et al. (2017), more than 1,000 novel acidified tomato-based products were introduced in the United States market from January 2008 to October 2012, comprising salsas, pasta sauces, and other table sauces.

B. Microbiological/ Food Safety concerns in acidified foods

Validation for acidified canned foods must be supported by research that ensures a 5-log reduction of *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes*. According to 21 CFR Part 114 set forth by the U.S. Food and Drug Administration (FDA) which covers acidified foods, an acidified food must have an equilibrium pH of 4.6 or less, except for tomato products, which must have an equilibrium pH of 4.7 or less, to prevent the outgrowth of *C. botulinum* (Balestrini et al., 2022). Existence of certain barriers to achieving pH of 4.6 or less, such as inadequacy of acid in cover brines for overcoming buffering capacity of product, alkaline compounds from processing stages of peeling, waxing, oil content, and the dimension of the cut slices in the preparation of acidified foods, may prevent penetration of acids into the food, hence,

raising concerns regarding pathogen growth and toxin production in the final product due to inability to achieve the final equilibrium pH of 4.6 (H. & M., 2013).

C. Challenges faced by producers of acidified foods

The U.S. Food and Drug Administration requires all processors to submit a comprehensive list of scheduled processes before commercializing each acidified food product. The globalization of the food industry has raised growing concerns about food security, particularly due to the lack of innovation and access to capital at local, small-scale levels. The limited understanding and inherent inconsistencies in quality control of largely manual, small-scale processes present significant challenges for manufacturers and entrepreneurs, especially since equipment-intensive thermal processing is typically the preferred method for ensuring acidified foods' shelf-life and microbial stability (De Vries et al., 2018). Alternatively, a cold-filling process under ambient conditions can also achieve microbial reduction due to the inherent properties, such as pH, and the FDA has permitted this process. However, a scientific challenge study based on the scheduled process must be provided, demonstrating the destruction of pathogens and spoilage microorganisms of concern for commercial sterility (FDA). The cost of a challenge study presents an additional financial burden for the small-scale entrepreneur. Consequently, the finite availability of published research on defined critical limits for cold-filled acidified products is an emerging concern of the industry (Breidt et al., 2018).

D. Thermal processing of acidified foods

Thermal processing in acidified foods has often consisted of in-container water or steam pasteurization, or more recently, an inverted hot-fill-hold for destruction of vegetative cells of microorganisms significant to public health, thereby ensuring safety for low pH foods (Dufort et al., 2017). Nonetheless, many undesirable changes in the biochemical and nutritional quality of

fruit juices have been observed due to the high energy transfer through pasteurization in the juices (Vervoort et al., 2011). In addition, recent data indicates significant alterations in the organoleptic characteristics of several acidified foods such as mayonnaises, salad dressings, and a few vegetables pickled products due to the heat treatments (Tola & Ramaswamy, 2018). Studies by Choo et al. (2023) show that using high temperatures in thermal processing techniques, such as pasteurization, might affect the physicochemical properties of juice. A significant reduction in the yield of malic acid, vitamin C (Cheng et al., 2020), and ascorbic acid content has been observed for noni juice after pasteurization. Also, a significant impact on the micro-nutrients such as calcium, zinc, and magnesium, along with reduced total dietary fiber content, was reported for a sensorial and nutritional profiling study of orange pulp by-product. Apart from that, the study discussed lower antioxidant potential after application of thermal treatment on orange pulp, thereby suggesting degradation of major components such as vitamin C, carotenoids, and phenolics. Lastly, a more cooked smell and loss of fresh-like appearance (Cheng et al., 2020) were observed during sensorial analysis (Giavoni et al., 2022).

E. Non-thermal processing of acidified foods

Production of microbiologically safe acidified foods without hampering their fresh organoleptic attributes and without conventional direct thermal treatments has gained increased attention among food manufacturers and consumers. Several novel technologies such as pulsed electric field (PEF) and high pressure (HP) processing, dependent on strong electric fields and high hydrostatic pressures mechanisms, respectively, have received emerging attention for their lethal effect and inactivation of microorganisms, alongside retention of the quality of final product (Vervoort et al., 2011).

Although non-thermal processes portray promising benefits for the acidified food industry, they have drawbacks. Arjeh et al. (2015) reported a significant impact on the color of sour cherry juice post Gamma irradiation treatment over the storage period. A degradation in the red color (anthocyanin content) and increased lightness was attributed to be directly proportional to increased irradiation. Moreover, similar findings in accordance with the study have been reported by Cheng et al. (2020) and Choo et al. (2023) on mandarin and noni juice, respectively. Moreover, curbing microbial growth through acid blanching for vegetables, a prolonged treatment of more than 24 hours under refrigeration, has limitations such as leaching (Tola & Ramaswamy, 2018). Although a better taste profile can be observed after High-Pressure Processing treatment, a hard texture was noted for the mustard pickle compared to the untreated sample. This was attributed to interference from ingredients and an adverse effect of the pressure treatment on the plant cell morphology of the pickle (Chien et al., 2023). Research in many non-thermal processing technologies is still in its infancy, and more research is required to determine their impacts on food safety and quality. Non-thermal processing for the methods reviewed above also requires specialized and expensive equipment, making these technologies out of reach for most small processors.

Cold-Fill Hold Process for Acidified Foods

Cold-filling involves filling food packaging at ambient or chilled conditions and holding the filled packages for a specified amount of time required to achieve a 5-log reduction in microorganisms of public health concern. Because conventional thermal processing is not used, the food may maintain higher quality than its thermally processed counterparts. Several studies conducted to determine the freshness and shelf-life of acidified vegetable products that have been cold-filled indicate temperature as the critical influential parameter. Studies by Lobo et al. (2019) suggested

cold-filling for acidified foods to be an extremely engaging alternative to farmers and small-scale processors, offering reduced capital costs alongside being an alternative to thermal processing with a reduction of any adverse effects on the quality and shelf-life of the food.

According to literature on cold-filling of pickle brine containing acetic and benzoic acids at pH 3.5 and 3.8, respectively, held at 10°C, the time taken for achieving a 5-log reduction was significantly lowered due to the presence of preservative benzoic acid. Apart from that, the time required for a 5-log reduction in bacterial cells of pathogenic bacteria revealed significant dependence on type and the concentration of acid used [Lobo et al., (2019); Breidt et al., (2013)]. Moreover, studies that used a cold-fill-hold at ambient or refrigerated temperatures have observed a linear trend between the brine acid concentration and acidification rate. The method of determining the target pH in an acidified pickled product is reported to be dependent on the points of slowest acidification rate within the product, the product and process parameters such as the brine fill temperature. Additionally, the method should comply with the regulatory requirements (Acosta et al., 2014).

The 5-log reduction times for pathogenic *E. coli* O157:H7 strains have been observed to be almost twice or thrice the time taken for *S. enterica* and *L. monocytogenes* strains at pH less than 3.3, as well as at a pH of 3.5 and 3.8 at a temperature of 10°C [Breidt et al., (2013); Breidt et al., (2007)]. Additionally, the 5-log reduction times for *E. coli* O157:H7 for cucumber pickle was 137.9 hours at 10°C as compared to 8 hours at 25°C for hot pepper sauce, thereby suggesting a product and temperature specific effect on the pathogenic reduction [Lobo et al., (2019); Breidt et al., (2007)]. The study by Dogan et al., (2022) focused on developing mathematical models to predict the survival and inactivation of *Listeria monocytogenes* in soy-based acidified products. This research addressed a knowledge gap regarding how intrinsic product properties, such as pH, NaCl

concentration, and soy sauce content affect pathogen survival, particularly in products that do not undergo thermal treatment. A higher microbial inactivation rate in the survival curve was observed with lower pH, increased soy sauce content, higher soluble solids, or added NaCl. At the same time, other independent variables were kept constant. Hwang et al. (2019) observed prolonged survival of pathogenic *E. coli* O157:H7 and *Listeria monocytogenes* in mayonnaise formulations stored at 23ºC with low concentrations of acetic acid. However, the study suggests that refrigeration at 4ºC reduces the lethality of acidulants against *E. coli* O157:H7 and *Listeria monocytogenes* in acidified mayonnaise and salad dressings.

Effect of Organic Acids and pH on Microbial Inactivation

A. Antimicrobial effects

The food processing industry has been facing severe outbreaks due to the extreme resistance posed by bacterial spores to various physical and chemical treatments (Sokołowska et al., 2013). According to Breidt et al. (2013), acetic acid addition as the primary acidifier and a final pH of 3.3 or lower would allow an acidified food to be considered safe without heat treatment after a suitable ambient hold time. Paudyal et al. (2018) published that organic acids are frequently used for preservation, sterilization, and decontamination in food industries due to their low toxicity and antimicrobial effects. Moreover, strong antimicrobial formulations of oxidizing agents and chlorine-based disinfectants have been studied due to their significant microbial resistance potential (Zarrella et al., 2021).

Organic acids, which are naturally present in fruits, show antimicrobial properties through the primary mechanism of reduction in pH, thus inhibiting enzymes, transport of nutrients, and the metabolic activity of the bacterial cell (Sokołowska et al., 2013). The effect of different antimicrobial compounds on microbial growth inhibition, under specific conditions, is expressed

in terms of their Minimum Inhibitory Concentrations (MIC). These attributes of organic acids are primarily characterized by their hydrophobic nature of diffusion across the cell membrane, followed by the degree of dissociation and the pH (Amrutha et al., 2017). In terms of antimicrobial effect, sorbic and benzoic acids at pH 3.2 were observed to have the highest effectiveness on 5-log reduction of *E. coli* O157:H7 strains on acidified foods as compared to the conventional organic acids like acetic, citric, malic, and lactic acid (Breidt et al., 2013).

The effect of SALTEC 514TM, an antimicrobial agent composed of a mixture of formic acid and propionic acid, and surfactants on targeted microbial load reduction of *Salmonella enterica* in fish meal was studied by Pelyuntha et al. (2022). The research demonstrated a successful antimicrobial property, attributable to preventing and controlling the re-contamination by *Salmonella*, thereby stating the potential inhibitory effect of the organic acid formulation on microbial load reduction in fish meal and/or feed ingredients. Additionally, a pH-dependent mechanism of organic acids on the energy metabolism of microorganisms has been reported. A redox reduction in NADPH formation by organic acids due to its penetration through the cell membrane, thereby resulting in the release of hydrogen ions (H+), causes a reduction in the intramembrane pH of the cell membrane.

Ghazanfar et al. (2022) studied the potential substitution of antibiotics for inactivation of *Campylobacter jejuni* in broilers using the bactericidal effect imposed by several organic acids. The results indicate successful reduction of *Campylobacter jejuni* in the guts of broilers by organic acids, used individually and in combinations (Propionic acid, Formic acid, Acetic acid, and Lactic acid) when used at pH 4 and when supplied daily in drinking water.

B. Influence of pKa values

According to studies by Leguerinel et al. (2001), a 10-fold reduction in the D-value (time required for a 1-log reduction of microorganisms) of heat-resistant *Bacillus cereus* spores by the influence of pH has been associated with the acid's dissociated or undissociated form. Moreover, a linear decreasing trend was observed between D-values and pH. The results show higher dissociation of an organic acid at lower pH, characterized by a lower pKa than an organic acid with a higher pKa. Hence, lower pKa values showed a decreased influence of pH of organic acid on D-value.

Additionally, studies indicate *E. coli* has higher malic acid tolerance, followed by lactic and acetic acid in juices equilibrated and acidified to a similar endpoint pH, irrespective of the tested acidulant. The antimicrobial effect of reducing the cytoplasmic pH and intracellular accommodation of acid anions by organic acids is characterized by its pKa value and the external medium's pH. Moreover, the inactivation of the strain required longer reaction times for higher pH values, under constant temperature conditions (Usaga et al., 2014).

C. Effect of organic acids on microbial reduction in acidified foods

According to research (Lobo et al., 2019), an amplified antimicrobial reaction is observed for acetic acid in coalescence with other acidulants, such as citric acid, as a function of its pKa value. Furthermore, a greater than 5-log reduction for *E. coli* O157:H7, *S. enterica*, and *L. monocytogenes* was achieved in less than 24 h after inoculation, for hot pepper sauce (pH of 3.2, acidified with acetic acid). Also, as per the literature by Breidt et al. (2007), a 5-log reduction of acid-resistant pathogens in cucumbers was achieved with acetic acid as the acidulant and pH equilibrated to 3.3, in the absence of thermal treatment. Moreover, products such as banana or jalapeno peppers with a pH of 3.3 or below and acetic acid greater than or equal to 400 mM, are being manufactured in the United States with little or no heat treatment. Nonetheless, studies conducted over a pH range of 3.9 to 5.4, at 37°C, on apple-carrot juice blends indicate that acetic acid, as the acidulant, has

the highest antimicrobial effect on *E. coli* O157:H7 (strain E0139), followed by lactic and malic acid (Usaga et al., 2014).

The antimicrobial mechanism of organic acids, namely citric acid, acetic acid, and lactic acid, on cucumber slices has been studied by Guo et al. (2022). According to the findings, a significant reduction in the bacterial load of three strains of *S. enterica* (*S.* Typhimurium, *S.* Enteritidis, and *S.* Newport) was observed, and 2.37–19.0% destruction of cell membranes was noted using Flow cytometry (FCM). Among the three organic acids, lactic acid showed the highest damage to the integrity of cell membranes, whereas *S.* Newport showed the highest resistance to the bactericidal effect.

The potentiality of citric acid, acetic acid, and lactic acid as anti-quorum agents and inhibitors of biofilm formation on cucumber surfaces was studied for pathogenic *E. coli* and *Salmonella sp*. Lactic acid indicated the highest resistance to biofilm formation and maximum anti-quorum sensing effect, followed by acetic acid and citric acid (Amrutha et al., 2017).

The study by Paudyal et al. (2018) investigated the effect of succinic, lactic, maleic, and acetic acid on the acid resistance mechanism of *L. monocytogenes*. The research focused on studying the combined impact of acidic conditions at pH 3.0 and specific inhibition of acid resistance systems, such as glutamate decarboxylase (GAD) in three different strains of *L. monocytogenes,* namely *L. monocytogenes* 10403S [most acid-resistant strains as stated by Feehily et al. (2014)], LO28 (moderate acid resistance), and EGD-e (one of the most acid-sensitive strains). The results indicated greater antimicrobial potential and partial biofilm removal for the strain at very low levels (1-2 mM) of maleic acid under acidic conditions compared to other organic acids. Moreover, a more substantial effect of maleic acid was observed for inhibition, and the compound's ability to enter the cells of *L. monocytogenes* was observed.

As per studies by Breidt et al. (2013), citric acid primarily functions as a buffer. Hence, it was observed to be the least efficient in pH reduction. Additionally, little or no acid-specific pathogenic inactivation by a completely protonated citric acid solution at lower concentrations for *E. coli* O157:H7 strains, as well as for spoilage microorganisms such as *Pichia manshurica* in hot pepper sauce acidified with citric acid, was observed at a pH of 3.2 [Bjornsdottir et al., (2006); Breidt et al., (2018); Lobo et al., (2019)]. A 5-log reduction of pathogenic *E. coli* O157:H7 in an acidic environment with a pH of 3.5 was achieved in 14.5 days when utilized as buffer along with benzoic acid, whereas at higher concentrations of 50 ± 10 mM protonated acid at pH of 3.2 [Bjornsdottir et al., (2006); Breidt et al., (2013)]. Moreover, the highest MIC of 2% was studied for citric acid as compared to acetic acid at 1.5%, followed by lactic acid at 0.2% against *E. coli* and *S. enterica* for a study of organic acids as anti-quorum agents (Amrutha et al., 2017).

Finally, a cold-fill process, characterized by low pH and high organic acid concentration, would possess significant potential as a suitable packing alternative for a particular formulation without compromising its quality and safety during shelf life. Moreover, enhanced research work would comprehend bridging the gap between the limited availability of research on the potential of organic acids on microbial inactivation to assist processors and regulators in validating cold-fill processes.

Central Hypothesis and Objectives for the Study

The long-term objective is to develop a sustainable and economically beneficial process for farmers and small food businesses that produce value-added products. This is important for sustaining rural farmers and providing them with a steady income stream during famine or the off-season. The proposed work aims to determine the time required for a 5-log reduction of pathogenic *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* in laboratory-scale

prepared acidified mayonnaise. The central hypothesis of the study is that cold-filling in acidic and acidified food through the actions of time, pH, temperature, and titratable acidity of organic acids can inactivate *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes*, thereby causing a 5-log reduction.

The hypothesis has been formulated in large part by existing work showing that a) A cold-fill process would indeed represent a suitable packing alternative for a particular formulation without compromising its microbial stability and safety during shelf life, b) The low pH and high organic acid concentration in most acidified products can inactivate almost all vegetative pathogens while preventing the growth of spore-forming bacteria. The antimicrobial effect of reducing the cytoplasmic pH and intracellular accommodation of acid anions by organic acids is characterized by its pKa value and the external medium's pH, which supports our proposed work (Usaga et al., 2014). We will test this hypothesis using the proposed objectives:

1. **Determine the inactivation at constant storage temperature of 15.5°C for a complete 5-log reduction of pathogenic *E. coli* O157:H7, *S. enterica*, and *L. monocytogenes* in five different variants of commercially prepared mayonnaise-based sauces,**
2. **Determine the effectiveness of different formulations using acetic acid, with and without the addition of 0.1% sodium benzoate, on microbial inactivation of *E. coli* O157:H7, *S. enterica*, and *L. monocytogenes* in lab-scale acidified mayonnaise sauces stored at 25°C and 5°C, and**
3. **Quantify the acid resistance for *E. coli* O157:H7, *S. enterica*, and *L. monocytogenes* in acidified mayonnaise by comparing D-values and z-values.**

CHAPTER 2

MATERIAL AND METHODS

Objective 1

Establishing optimal cold-fill-hold times required to ensure microbial safety and quality of commercially prepared mayonnaise sauce formulations.

A. Preparation and description of cultures

Five cultures, consisting of five strains of *Salmonella enterica*, *E. coli* O157:H7, and *Listeria monocytogenes* isolated from fresh produce or other relevant outbreaks, were used in this study (Table 1). Each strain was prepared independently and later pooled to form a single 5-strain cocktail for separate inoculations of each organism. Cultures were activated by inoculating 9 mL tryptic soy broth (TSB- BD, Bacto, Sparks, MD) with a loopful of culture retrieved from frozen stock stored at -80°C. The resulting mixture was incubated at 37 ± 2°C for 24 ± 3 h. A revived culture loop was then streaked onto a tryptic soy agar (TSA- BD, Difco, Sparks, MD) plate. Plates were incubated at 37 ± 2°C for 24 ± 3 h, then, using a sterile loop, a 10µL loop of cells were scraped into 9 ml of sterile tryptic soy broth, followed by incubation at 37 ± 2°C for 24 ± 3 h. The second overnight culture (0.1 mL) was transferred onto TSA plates and incubated at 37 ± 2°C for 24 ± 3 h. The resulting bacterial lawn was collected by adding 3 ml of 0.1% peptone water (BD, Difco Buffered Peptone Water, Sparks, MD) to each plate and loosening the lawn with a sterile spreader to create a liquid culture. From the resulting liquid culture, 1 ml was transferred to a clean container. This process was repeated for each of the five strains, resulting in a 5 ml volume of inoculum.

Table 1. Pathogenic strains used for the experiments.

Strain Name/ Serovar	ID	Origin
Salmonella Gaminara	F2712	1995 Orange Juice outbreak
Salmonella Stanley	H1256	Alfalfa sprout outbreak
Salmonella Poona	01A 3923	Cantaloupe outbreak
Salmonella Newport	2020K-0778	Onion outbreak
Salmonella St. paul	E2008001236	Pepper/ Tomato outbreak
E. coli O157:H7	35150	Feces, human
E. coli O157:H7	43894	Feces, human
E. coli O157:H7	C7927	Human isolate
E. coli O157:H7	KSU 31	Apple juice outbreak
E. coli O157:H7	CDC 658	Cantaloupe outbreak
Listeria monocytogenes	LCDC 81-861	Coleslaw
Listeria monocytogenes	F8255	Peach isolate
Listeria monocytogenes	548-072	Caramel apple outbreak, Human blood isolate
Listeria monocytogenes	MDD631	Whole cantaloupe
Listeria monocytogenes	ENV 20110 10804-1 (390-1)	2011 Cantaloupe outbreak

B. Mayonnaise sauce formulation and preparation

Five industrially prepared formulations of mayonnaise sauces (Table 2), containing base ingredients such as soybean oil, water, sugar, and egg yolk, were evaluated. These samples were stored in the manufacturer's PET bottles under ambient conditions (22–25°C) until analysis. For each formulation, three independent replications were performed. Approximately 95 g of each sample and 5 ml of inoculum were transferred into a sterile glass jar, mixed thoroughly, and closed with a lid and band. After inoculation, the jars were incubated at 60°F (15.5°C) and stored for up to three days (72 hours).

Table 2. pH of five formulations of the mayonnaise sauces

Sample	Sample code	pH
Original	O	3.68
Original Reduced Calorie	ORC	3.70
Original Spicy	OS	3.68
Ranch Sauce	R	3.70
Japanese Ginger Salad Dressing	G	2.95

C. Enumeration and colony counting

This process was carried out immediately after inoculation to determine the initial concentration of the pathogen in each sample. Containers were removed from the incubator every 24 hours for up to 72 hours, until no colonies were present when plated at the lowest dilution. Each sample (1 mL) was subjected to serial dilutions in sterile 0.1% peptone water and plated in duplicate. Agar plates (TSA) were incubated for 24 hours at 37 ± 2°C for *Salmonella enterica* and *E. coli* O157:H7 strains and for 48 hours for *Listeria monocytogenes* strains.

D. Data Analysis

After incubation, colonies on plates were counted, with values between 25 and 250 CFU converted to log CFU/g. Counts below 25 CFU were adjusted to the limit of quantification (2.4 log CFU/g). Pathogen reduction was assessed by comparing log reductions across different formulations, considering formulation type and pathogen presence variables. To estimate the time required for a 5-log reduction in days, the D-value (negative reciprocal of the inactivation curve slope) was multiplied by 5, and one day was added for a safety margin.

Statistical analysis was performed using R software (R version 4 4 2) using a linear mixed-effects ANOVA (Type III) model from the “car” package model with Satterthwaite's method. The model fitting used the lmer () function from the “lme4” package. The dependent variable, log-transformed

microbial count (CFU/g), measured over time, was treated as the response variable, with pathogen type, formulation, temperature, and time treated as explanatory variables. The significance level (α level) was 0.05. Pairwise comparisons were tested using the Tukey test with a least significant difference to identify important factors influencing pathogen populations across different experimental conditions.

Objective 2

To establish optimal cold-fill-hold times for lab-scale mayonnaise sauce formulations.

A. Preparation of Mayonnaise samples

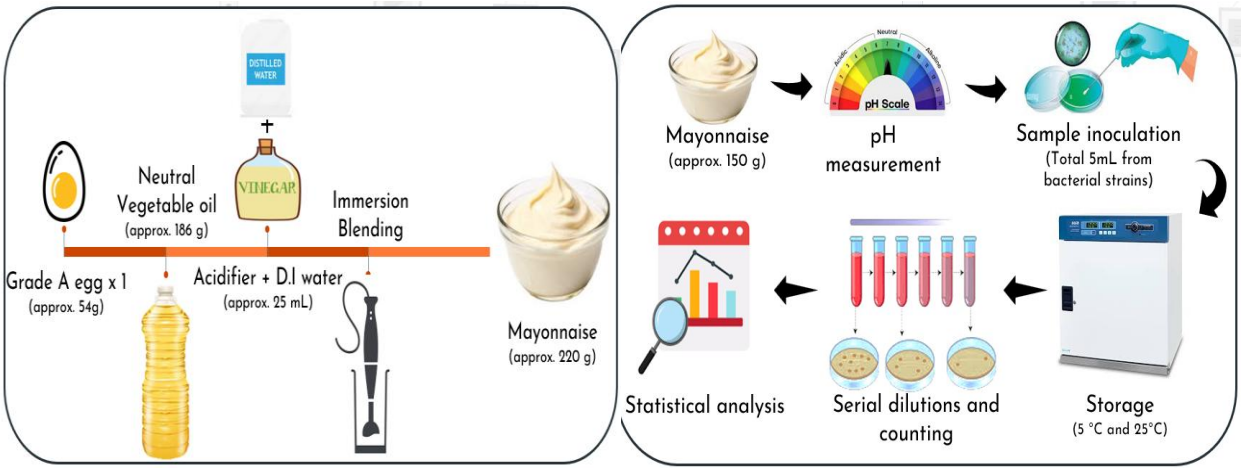

Fig 2.1. Sample preparation and analysis methodology

Grade A table eggs (approximately 54 g each) and neutral vegetable oil (Kroger, Pure Canola Oil) were sourced from a major supermarket. Acidification was achieved using acetic acid (Kroger, Distilled White Vinegar) with 5% acid concentrations. Two formulations were tested for the study, one with 5% acetic acid and the second with the addition of 0.1% Sodium Benzoate as the preservative. To prepare the acidifying solution, specific volumes of deionized (D.I.) water and acid were mixed to reach a total volume of 25 mL (25 g), ensuring an acid concentration of 5.0%. A target acid concentration of 5% was selected to simulate conditions that a small processor would likely use, as this is a typical concentration found in major retail outlets. The acid mixture was

transferred to a glass jar for blending. The egg white and yolk were combined with the acidifying solution and agitated continuously using an immersion blender (KitchenSmith by Bella Immersion Blender) until thoroughly mixed. Vegetable oil (220 mL, approximately 186 g) was gradually incorporated in a thin stream while the immersion blender operated at full speed. This process ensured proper emulsification and yielded a smooth, homogenous consistency. The mixture was blended until it thickened to approximate the texture of commercially prepared mayonnaise. After preparation, the mayonnaise was allowed to stand for 30–60 minutes at room temperature to stabilize the pH.

B. pH and Titratable acidity

The pH of the prepared mayonnaise formulation was measured using a calibrated pH meter (Fisherbrand, Accumet AB315 pH/mV), ensuring the repeatability of our experimental conditions. Titratable acidity (TA) was quantified and expressed as acetic acid based on the acidifier used in the formulation. For analysis, 10 g of the sample was accurately weighed into a 150 mL beaker, and titration was performed using 0.1 N sodium hydroxide (NaOH), with the endpoint determined at pH 8.2. The volume of NaOH consumed was recorded and converted to the percentage of acetic acid equivalent. An expected titration range of 5–15 mL of 0.1N NaOH was anticipated. If the NaOH consumption deviated from this range, the sample amount was adjusted accordingly to ensure accurate titration results. The following equation was used to calculate the percentage of acetic acid in the samples.

$$\%\ acetic\ acid\ = \frac{0.1\ \left(\frac{mEq}{mL\ NaOH}\right)\ \times mL\ NaOH\ used\ \times 60.06\ \left(\frac{mg}{mEq}\right)}{20\ mL\ \times 10\ \left(\frac{mg}{mL}\right)}$$

C. Preparation of cultures and induction of rifampicin resistance

This study used five cultures of *Salmonella enterica*, *E. coli* O157:H7, and *Listeria monocytogenes* isolated from fresh produce outbreaks (Table 1). Rifampicin resistance was induced because preliminary results showed substantial background microflora interfering with microbial analysis. Each culture was prepared independently and later pooled to form a single 5-strain cocktail for separate inoculations of each organism. The frozen stock culture of rifampicin-resistant strains was revived by streaking a loopful of each culture onto rifampicin (80 µg/mL) -supplemented TSA plates (TSA-R). The plates were incubated at 37 ± 2°C for 24 ± 3 h. After incubation, the strains were transferred using a 10 µL sterile loop into 9 mL of sterile tryptic soy broth supplemented with rifampicin (80 µg/mL) (TSB-R), followed by incubation at 37 ± 2°C for 24 ± 3 h. The second overnight culture (0.1 mL) was then transferred onto TSA-R plates and incubated at 37 ± 2°C for 24 ± 3 h. The resulting bacterial lawn was collected by adding 3 ml of 0.1 % peptone water to each plate and loosening the lawn with a sterile spreader to create a liquid culture. Each strain was removed from the plate (1 ml) and placed into a sterile container, and all strains for one organism were pooled, resulting in a 5 ml inoculum volume.

D. Inoculation

Mayonnaise samples (approximately 150 g) were inoculated with a cocktail of five strains of harvested bacterial inoculum (5 mL total), followed by thorough mixing for a uniform suspension. The inoculated sample was transferred to a sterile glass jar and sealed with 2-piece metal lids. No heat treatment was applied, and jars were stored at 5°C and 25°C during sampling. Controls consisted of mayonnaise to which sterile peptone water was added instead of inoculum, and an inoculated control was prepared, where instead of adding acid, only sterile DI water was used to prepare the sample. Except for formulations subjected to microbiological analyses on day 0 (within

30 min after inoculation), control mayonnaise formulations were immediately placed in the refrigerator.

E. Enumeration and colony counting

Samples were enumerated immediately after inoculation to determine the initial concentration of the pathogen. Containers were removed from the incubator and subjected to enumeration every 8 hours for up to 96 hours for samples stored at 25°C and every 24 hours until 360 hours (15 days) when stored at 5°C. For *Listeria monocytogenes,* enumeration was performed every 8 hours at 5 °C for up to 80 hours. The samples stored at 5°C and 25°C were tested until no colonies were present when plated at the lowest dilution, except for *Salmonella enterica* and *E. coli* O157:H7. After sampling, containers were returned to the incubator until the next sampling time. Each sample (1 mL) was subjected to serial dilutions in sterile 0.1% peptone water, and 0.1 mL was plated in duplicate on TSA-R. Agar plates were inverted and incubated at 37 ± 2°C for 24 hours for *Salmonella enterica* and *E. coli* O157:H7 strains, and for 48 hours for *Listeria monocytogenes* strains. After incubation, colonies on plates were counted, with values between 25 and 250 CFU converted to log CFU/g. Counts below 25 CFU were adjusted to the limit of quantification (2.4 log CFU/g).

F. Data Analysis

Three independent experiments were conducted using three separate batches of each mayonnaise formulation. Pathogen reduction was assessed by comparing log reductions across different formulations, considering variables such as preservative presence, organism, and storage temperature (5°C or 25°C). Microbial inactivation was evaluated by analysing survival curves of log (CFU/g) over time. The time required for a 5-log reduction was estimated by multiplying the D-value (negative reciprocal of the inactivation curve slope) by 5. Additionally, Z-values, which

Table 3. The composition of the lab-scale mayonnaise formulations and sample codes

Sample	*Storage Temperature*	*Sample Code*	*Pathogen*	*Egg (g)*	*Vegetable oil (g)*	*Acidifier (mL)*	*D.I water (mL)*	*pH*	*Total weight (g)*
Formulation A **Acetic Acid**	5°C	AA5	*E.coli O157:H7*	53.27 ± 0.19	186.35 ± 0.26	25	-	4.19 ± 0.03	150.65 ± 0.31
			Listeria monocytogenes	53.18 ± 1.39	186.62 ± 0.35	25	-	4.20 ± 0.04	150.44 ± 0.46
			Salmonella enterica	53.35 ± 0.05	186.40 ± 0.60	25	-	4.18 ± 0.02	150.35 ± 0.24
	25°C	AA25	*E.coli O157:H7*	52.90 ± 0.43	186.58 ± 0.40	25	-	4.19 ± 0.01	151.47 ± 1.06
			Listeria monocytogenes	52.95 ± 0.62	186.67 ± 0.58	25	-	4.18 ± 0.02	151.63 ± 1.33
			Salmonella enterica	52.82 ± 0.75	186.83 ± 0.62	25	-	4.19 ± 0.03	151.87 ± 0.80
Formulation B **Acetic Acid + 0.1% Sodium Benzoate**	5°C	SB5	*E.coli O157:H7*	52.75 ± 0.65	186.85 ± 0.35	25	-	4.38 ± 0.01	152.25 ± 0.05
			Listeria monocytogenes	53.07 ± 0.55	186.70 ± 0.98	25	-	4.36 ± 0.01	152.17 ± 0.98
			Salmonella enterica	54.45 ± 2.05	187.85 ± 0.15	25	-	4.35 ± 0.04	151.90 ± 0.50
	25°C	SB25	*E.coli O157:H7*	53.10 ± 0.54	186.97 ± 0.25	25	-	4.39 ± 0.03	153.23 ± 1.97
			Listeria monocytogenes	53.15 ± 0.28	186.68 ± 0.46	25	-	4.38 ± 0.02	151.05 ± 0.70
			Salmonella enterica	53.10 ± 0.78	186.65 ± 0.71	25	-	4.37 ± 0.03	152.37 ± 1.09
No Acid	5°C	NAA5	*E.coli O157:H7*	52.80	187.10	-	25	7.57	150.40
			Listeria monocytogenes	53.20	186.70	-	25	7.76	150.30
			Salmonella enterica	52.80	186.90	-	25	7.62	151.20
	25°C	NAA25	*E.coli O157:H7*	54.20	188.00	-	25	7.63	150.60
			Listeria monocytogenes	54.60	187.40	-	25	7.96	151.80
			Salmonella enterica	54.60	188.40		25	7.95	151.50
No Inoculum	5°C	NIAA5	-	54.50	186.50	25	-	4.21	153.50
	25°C	NIAA25	-	54.30	186.60	25	-	4.20	152.20
	5°C	NISB5	-	54.30	186.10	25	-	4.34	152.10
	25°C	NISB25	-	54.50	186.10	25	-	4.36	152.80

[a] Values reported as mean ± standard deviation, $n = 3$.

[b] Acetic Acid concentration is 5%.

represent the temperature change needed to achieve a tenfold change in the D-value, were calculated by taking the negative reciprocal of the slope of the line calculated from the logarithm of the D-values as a function of temperature. The Z-value was determined using the formula:

$$z - value = \frac{T_2 - T_1}{\log_{10}(D1) - \log_{10}(D2)}$$

Where T_1 and T_2 are temperatures with corresponding D-values of D_1 and D_2.

Statistical analysis was performed using R software (R version 4 4 2) using a linear mixed-effects ANOVA (Type III) model from the "car" package model with Satterthwaite's method. The model fitting used the lmer () function from the "lme4" package. The dependent variable, log-transformed microbial count (log(CFU/g)), was treated as the response variable measured over time. Pathogen type, formulation, temperature, and time served as explanatory variables. Moreover, pathogen type, formulation (preservative absence or presence), and temperature were categorized to estimate separate means for each level and to examine differences between groups, while time was regarded as a continuous variable. The significance level (α level) was 0.05. Pairwise comparisons were tested using the Tukey test with a least significant difference to identify important factors influencing pathogen populations across different experimental conditions.

CHAPTER 3

RESULTS

Survival of the pathogens in commercially prepared mayonnaise sauce formulations

1. Influence of formulation type on survival of pathogenic microorganisms and D-value

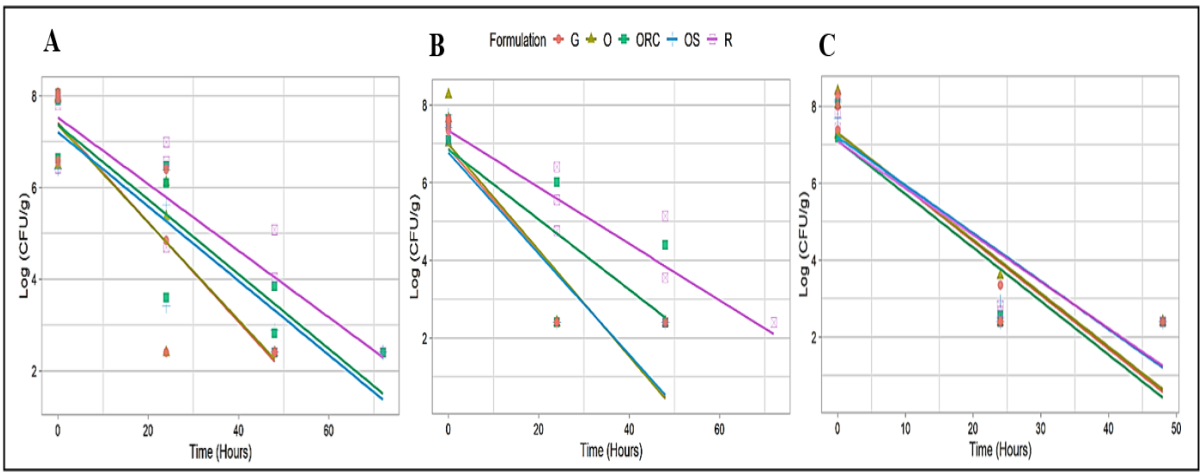

Fig 3.1. Estimated 5-log reduction times of *E. coli* O157:H7 (A), *Listeria monocytogenes* (B), and *Salmonella enterica* (C) for all formulations. The limit of quantification was 2.4 log (CFU/g).

a. Survival *of E. coli* O157:H7

The survival of a cocktail of five strains of *E. coli* O157:H7 in each of the five variants of commercially prepared mayonnaise formulations was studied. The resulting D-values for the formulations were calculated, ranging from 13.77 hours (Ranch Sauce, pH 3.70) to the lowest of 9.22 hours (Japanese Ginger Salad Dressing, pH 2.95). The order of survival of *E. coli* O157:H7, based on the D-values and estimated time required for a 5-log reduction for the different formulations, was R > OS > ORC > O > G (Fig. 3.1A). No significant difference was observed in

the 5-log reduction rate across the various formulations, where the limit of detection was at 2.4 log CFU/g (Table 4).

b. Survival of *Listeria monocytogenes*

The survival of the cocktail of *Listeria monocytogenes* strains showed no significant difference among the five different formulations. The D-values ranged from 13.74 hours (Ranch Sauce, pH 3.70) to 7.33 hours (Original, pH 3.68). The order of survival of *Listeria monocytogenes*, based on the D-values and estimated time required for a 5-log reduction across the different formulations, was R > ORC > OS > G > O. A complete 5-log reduction from the initial pathogen population of an average 7.47 log (CFU/g) to below the limit of detection of 2.4 log CFU/g was observed for all the formulations (Fig. 3.1B).

c. Survival of *Salmonella enterica*

Salmonella enterica's survival was observed to follow the order of R > OS > O > ORC > G. The pathogen was observed to show a complete 5-log reduction from an initial population of 7.88 log CFU/g to a limit of detection of 2.4 log CFU/g, with corresponding D-values ranging from a maximum of 8.22 hours (Ranch Sauce, pH 3.70) to a minimum of 7.15 hours (Japanese Ginger Salad Dressing, pH 2.95). No significant difference was noted for the pathogen's survival among the five variants of mayonnaise sauces (Fig. 3.1C).

Survival of the pathogens in lab-scale mayonnaise sauce formulations

1. Influence of formulation type on pH

The results showed that the pH levels of different mayonnaise formulations varied considerably, with the addition of 0.1% sodium benzoate as a preservative resulting in higher pH levels. The variation in pH levels of the various mayonnaise formulations is shown in Table 5. The pH ranged

Table 4. Initial population (Log CFU/g), D-value (time taken for 1-log reduction), and estimated 5-log reduction times for *E. coli* O157:H7*, Listeria monocytogenes,* and *Salmonella enterica* in commercial mayonnaise samples.

Sample	***Storage Temperature***	***Sample Code***	***Pathogen***	***Initial pH***	***Initial Pathogen count (LogCFU/g)***	***Slope***	***D-Value (Hours)***	***Estimated time for 5-log reduction (Hours)***	***Estimated time for 5-log reduction (Days)***
Original	15.5°C	O	*E.coli O157:H7*	3.68	7.46	-0.1063	9.40	47.02	1.96
			Listeria monocytogenes		7.64	-0.1310	7.63	38.17	1.59
			Salmonella enterica		7.89	-0.1389	7.20	36.00	1.50
Original Spicy		OS	*E.coli O157:H7*	3.68	7.44	-0.0810	12.34	61.70	2.57
			Listeria monocytogenes		7.39	-0.1248	8.02	40.08	1.67
			Salmonella enterica		7.99	-0.1247	8.02	40.08	1.67
Original Reduced Calorie		ORC	*E.coli O157:H7*	3.70	7.53	-0.0814	12.28	61.39	2.56
			Listeria monocytogenes		7.39	-0.0902	11.09	55.43	2.31
			Salmonella enterica		7.77	-0.1393	7.18	35.90	1.50
Ranch Sauce		R	*E.coli O157:H7*	3.70	7.39	-0.0726	13.77	68.83	2.87
			Listeria monocytogenes		7.39	-0.0728	13.74	68.71	2.86
			Salmonella enterica		7.85	-0.1216	8.22	41.12	1.71
Japanese Ginger Salad Dressing		G	*E.coli O157:H7*	2.95	7.54	-0.1084	9.22	46.11	1.92
			Listeria monocytogenes		7.52	-0.1279	7.82	39.09	1.63
			Salmonella enterica		7.90	-0.1398	7.15	35.76	1.49

from 4.36 to 4.38 for formulation type B, compared to a pH of 4.19 for formulation type A, showing that adding sodium benzoate increased the pH. The absence of an acidulant in the no-acid samples resulted in a substantially higher average pH of 7.74. Similarly, the control samples (No inoculum) had average pH values of 4.20 and 4.35 for formulations A and B, respectively. The 5% acetic acid concentration was consistent across all the formulations, except for the no-acid samples, which used D.I. water.

Table 5. Initial pH across different formulations

Sample type	*Sample Code*	*Average pH*
Formulation A[a]	AA5	4.19
	AA25	4.19
Formulation B[b]	SB5	4.36
	SB25	4.38
No acid	NA5	7.65
	NA25	7.84
No inoculum A[a]	NIAA5	4.21
	NIAA25	4.20
No inoculum B[b]	NISB5	4.34
	NISB25	4.36

[a] Acetic Acid samples are Formulation A.

[b] Acetic Acid + 0.1% Sodium Benzoate samples are Formulation B.

2. Influence of formulation type on titratable acidity

Table 6. Titratable acidity and pH of different formulations over time

Formulation	*Temperature*	*Sample Code*	*Day 0*		*Day 5*		*Day 15*	
			TA	*pH*	*TA*	*pH*	*TA*	*pH*
Formulation A Acetic Acid	5°C	NIAA5	0.51	4.21	0.50	4.19	0.43	4.24
	25°C	NIAA25	0.51	4.20	0.48	4.20	0.60	4.17
Formulation B Acetic Acid + 0.1% Sodium Benzoate	5°C	NISB5	0.33	4.34	0.52	4.31	0.43	4.34
	25°C	NISB25	0.33	4.36	0.41	4.30	0.63	4.30

[a] TA indicates titratable acidity (%)

The percentage of titratable acidity in the two formulations was calculated using the formula at three time points: 0 Day, 5 Days, and 15 Days. The initial percentage TA for formulation A was higher (0.51%) than for formulation B (0.33%). Additionally, the final percentage of TA at 25°C was observed to be 0.63% and 0.60% for samples with and without the addition of preservative, respectively, and it followed an increasing trend regardless of the initial percentage of TA and pH. In contrast, a final percentage TA of 0.43% was noted when stored at 5°C for both formulations, with the value gradually decreasing when tested for acetic acid-only samples.

3. ANOVA for Main Effects and Interactions

A significant ($p < 0.001$) interaction among the main effects of pathogen type, formulation type, temperature, and time on microbial reduction was observed using a mixed effects ANOVA model (Table 7). The duration of exposure (Time) reflected the most substantial impact, indicating that time is a highly influential variable for pathogenic reduction in the mayonnaise formulations. All interactions that included time showed significance. Additionally, the pathogen showed statistical significance ($P = 0.000150$), indicating differences between strains that should be accounted for in designing these studies. The three-way and four-way interactions among pathogen type, formulation type, temperature, and time indicate significant interactions, suggesting complex dependencies of the main effects.

Table 7. Type III ANOVA Table

Main Effects	***Sum Sq***	***Mean Sq***	***DF***	***F value***	***Pr(>F)***	***Signif. Codes*** [a]
Pathogen	3.14	1.57	2	9.11	0.000150	***
Formulation	1.22	0.61	2	3.53	0.0307	*
Temp	0.340	0.34	1	1.96	0.162	
Time	293	293	1	1699	0.000	***
Pathogen:Formulation	1.28	0.320	4	1.85	0.119	

Pathogen:Temp	1.30	0.650	2	3.77	0.0244	*
Formulation:Temp	0.370	0.180	2	1.06	0.347	
Pathogen:Time	76.3	38.2	2	221	0.000	***
Formulation:Time	208	103.8	2	602	0.000	***
Temp:Time	139.7740	140	1	810	0.000	***
Pathogen:Formulation:Temp	0.810	0.200	4	1.18	0.321	
Pathogen:Formulation:Time	44.2	11.1	4	64.1	0.000	***
Pathogen:Temp:Time	29.6	14.8	2	85.7	0.000	***
Formulation:Temp:Time	103	51.5	2	298	0.000	***
Pathogen:Formulation:Temp:Time	21.9	5.48	4	31.8	0.000	***

[a] Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

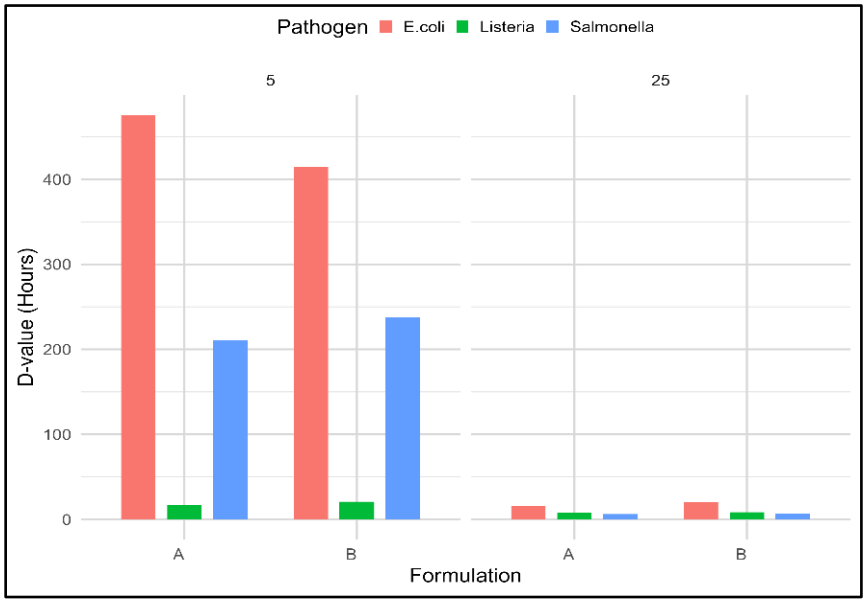

Fig 3.2. Calculated D-values for *E. coli* O157:H7, *Listeria monocytogenes, and Salmonella enterica* for formulations A and B at 5°C and 25°C. The limit of detection was 2.4 log (CFU/g).

4. Influence of storage temperature and formulation on D-values

The D-values (Table 8) for the pathogens at different temperatures and formulation combinations ranged from 475 hours for *E. coli* O157:H7 (Formulation A, at 5°C) to 6.28 hours for *Salmonella enterica* (Formulation A, at 25°C). Additionally, for the formulation-temperature combinations of each pathogen, the D-values for *Listeria monocytogenes* varied from 20.7 hours (Formulation B,

at 5°C) to a low of 7.49 hours (Formulation A, at 25°C). A significantly high acid resistance was observed for *E. coli* O157:H7, with D-values ranging from 475 hours for *E. coli* O157:H7 (Formulation A, at 5°C) to a minimum of 15.82 hours (Formulation A, at 25°C). Nonetheless, *Salmonella enterica* exhibited the highest acid resistance, with a D-value of 237 hours (Formulation B, at 5°C), and the least resistance was recorded at a D-value of 6.28 hours (Formulation A, at 25°C) (Fig. 3.2).

5. Influence of formulation type on survival of pathogenic microorganisms

The effect of formulation type on pathogen survival was evaluated by inoculating mayonnaise formulations A (5% acetic acid) and B (5% acetic acid + 0.1% sodium benzoate) with *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* and storing them at 5°C and 25°C. No significant differences were observed in the survival rates of *E. coli* O157:H7 between formulations A and B at either temperature. At 5°C, the 5-log reduction time was estimated to be 86.4 days for formulation B, whereas for formulation A, the inactivation time was slightly longer at 99 days. Similarly, at 25°C, the 5-log reduction was achieved in 3.30 days for formulation A and 4.23 days for formulation B, indicating minimal effect of sodium benzoate on pathogen inactivation compared to acetic acid alone. A similar trend was observed for *Listeria monocytogenes* across the formulations. At 5°C, the complete 5-log reduction was achieved in 3.59 days for formulation A and 4.31 days for formulation B. At 25°C, the inactivation times were 1.56 days (A) and 1.76 days (B), respectively. Adding sodium benzoate did not significantly reduce the inactivation time compared to acetic acid alone. For *Salmonella enterica*, the time required for a 5-log reduction was approximately 43.8 and 49.5 days at 5°C for formulations A

and B, respectively. At 25°C, the inactivation times were reduced to approximately 1.60 days, with no significant differences between the formulations (Fig. 3.3).

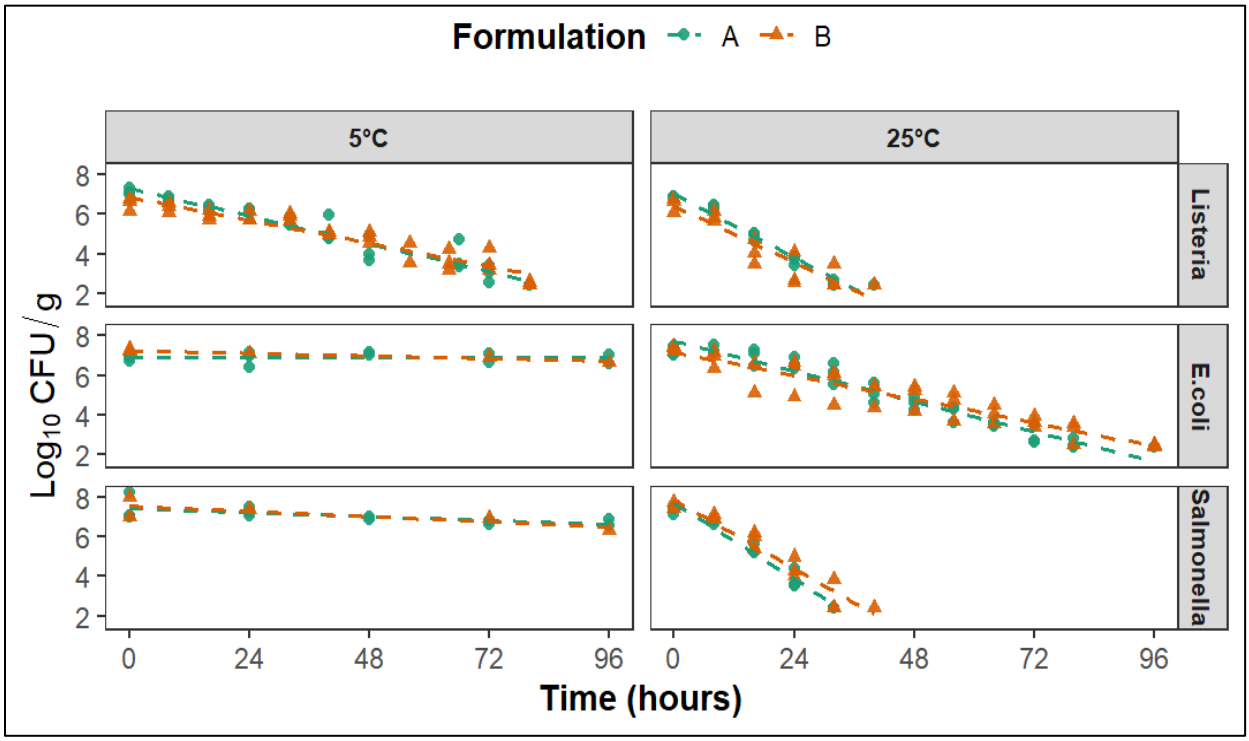

Fig 3.3. Survival curves for *E. coli* O157:H7, *Listeria monocytogenes, and Salmonella enterica* for formulations A and B at 5°C and 25°C. The limit of detection was 2.4 log (CFU/g).

Moreover, the statistical significance ($p < 0.05$) based on letter separation for Formulations A and B at 25°C and 5°C for all three target pathogens falls into the same letter group, indicating no statistically significant difference between Formulations A and B. On the contrary, formulation with no acid component consistently appeared in higher letter groups (c/ d), indicating poor inactivation compared to formulation containing 5% acetic acid. Overall, the inclusion of 0.1% sodium benzoate in formulation B did not significantly impact the survival times of any of the three pathogens compared to formulation A, which contained only 5% acetic acid (Fig. 3.4).

6. Influence of storage temperature on the survival of pathogenic microorganisms

The acid-based inactivation of pathogens was found to be most effective at higher storage temperatures (25°C). For each pathogen, the statistical significance ($p < 0.05$) based on letter separation (Fig. 3.4), when stored at refrigeration conditions of 5°C, is significantly higher (groups b to d) compared to 25°C (groups a to b). Moreover, the No Acid (NA) formulations consistently appear in higher groups, with *E. coli* O157:H7 and *Salmonella enterica* falling into the "d" group, exhibiting the least reduction in log (CFU/g) of the pathogens at 5°C.

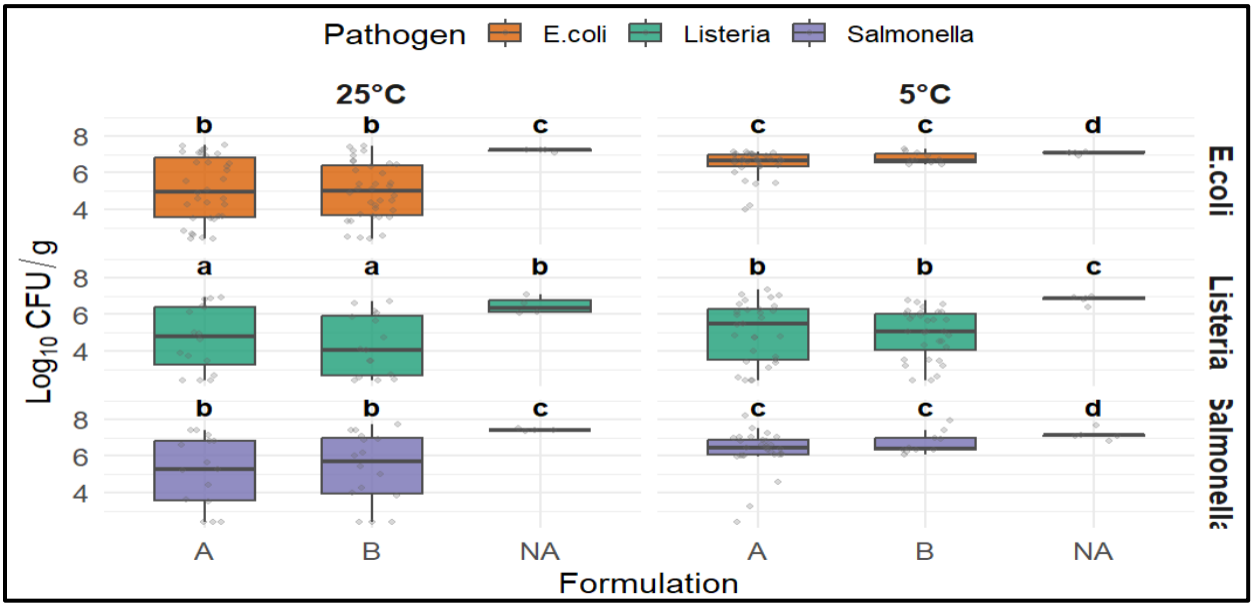

Fig 3.4. Comparison of Log (CFU/g) for *E. coli* O157:H7, *Listeria monocytogenes, and Salmonella enterica* across formulations A, B, and NA at 5°C and 25°C. Boxplots display the distribution of microbial counts for each condition. Compact letters displayed above boxes indicate statistical significance based on Tukey-adjusted comparisons ($p < 0.05$).

a. Survival *of E. coli* O157:H7

E. coli O157:H7 exhibited the longest survival time among the tested pathogens, particularly under refrigeration conditions. The effect of storage temperature was highly significant ($p < 0.0001$) for both formulations. At 5°C, the 5-log reduction time for formulation A was 99 days, while formulation B's inactivation time was reduced to 86.4 days. Conversely, at 25°C, the 5-log

reduction was achieved in 3.30 days for formulation A and 4.23 days for formulation B, indicating a considerable reduction in survival time under ambient conditions (Fig. 3.4, 3.5).

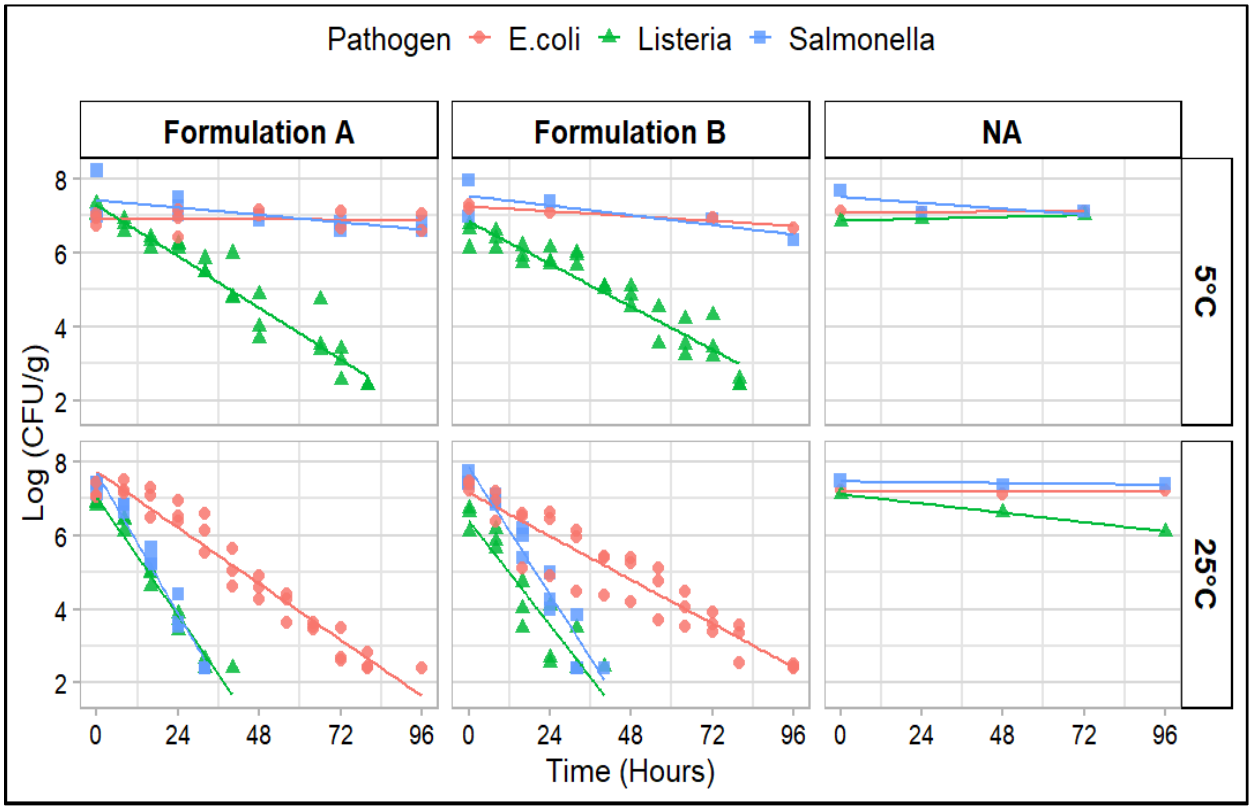

Fig 3.5. Survival curves for *E. coli* O157:H7*, Listeria monocytogenes, and Salmonella enterica* in formulations A, B, and NA at 5°C and 25°C. The data for *E. coli* O157:H7 *(circles), L. monocytogenes (triangles), and S. enterica (squares*) depict the reduction of initial log (CFU/g) values from an average of 7.11 log (CFU/g) to the limit of detection of 2.4 log (CFU/g). The solid lines represent the linear regression model applied to the data.

Table 8. Initial population (Log CFU/g), D-value (time taken for 1-log reduction), and estimated 5-log reduction times for *E. coli* O157:H7*, Listeria monocytogenes,* and *Salmonella enterica* in lab-scale mayonnaise samples.

Sample	***Storage Temperature***	***Sample Code***	***Pathogen***	***Initial pH***	***Initial Pathogen count (LogCFU/g)***	***Slope***	***D-Value (Hours)***	***Estimated time for 5-log reduction (Hours)***	***Estimated time for 5-log reduction (Days)***
***Formulation A* Acetic Acid**	5°C	AA5	*E.coli O157:H7*	4.19 ± 0.03	6.90	-0.0021	475	2376	99
			Listeria monocytogenes	4.20 ± 0.04	7.13	-0.0581	17.2	86.1	3.59
			Salmonella enterica	4.18 ± 0.02	7.42	-0.0048	210	1051	43.8
	25°C	AA25	*E.coli O157:H7*	4.19 ± 0.01	7.19	-0.0632	15.8	79.1	3.30
			Listeria monocytogenes	4.18 ± 0.02	6.85	-0.1334	7.49	37.5	1.56
			Salmonella enterica	4.19 ± 0.03	7.32	-0.1593	6.28	31.4	1.31
***Formulation B* Acetic Acid + 0.1% Sodium Benzoate**	5°C	SB5	*E.coli O157:H7*	4.38 ± 0.01	7.25	-0.0024	415	2073	86.4
			Listeria monocytogenes	4.36 ± 0.01	6.50	-0.0483	20.7	104	4.31
			Salmonella enterica	4.35 ± 0.04	7.47	-0.0042	237	1187	49.5
	25°C	SB25	*E.coli O157:H7*	4.39 ± 0.03	7.35	-0.0493	20.3	102	4.23
			Listeria monocytogenes	4.38 ± 0.02	6.48	-0.1181	8.47	42.3	1.76
			Salmonella enterica	4.37 ± 0.03	7.52	-0.1438	6.95	34.8	1.45
No Acid	5°C	NAA5	*E.coli O157:H7*	7.57	7.10	-0.0004	2709	13549	565
			Listeria monocytogenes	7.76	6.85	-0.0013	747	3733	156
			Salmonella enterica	7.62	7.68	-0.0015	658	3289	137
	25°C	NAA25	*E.coli O157:H7*	7.63	7.23	0.0002	N/A	N/A	N/A
			Listeria monocytogenes	7.96	7.10	-0.0061	164	822	34.2
			Salmonella enterica	7.95	7.48	-0.0004	2647	13237	552

b. Survival of *Listeria monocytogenes*

The survival of *Listeria monocytogenes* was significantly affected by storage temperature ($p < 0.0001$) for both formulations. At 5°C, the 5-log reduction times were 3.59 days for formulation A and 4.31 days for formulation B. Under ambient storage at 25°C, the reduction times were 1.56 days for formulation A and 1.76 days for formulation B. Additionally, the significantly lower mean log CFU/g (group "a") indicated a substantial decrease in survival time as the storage temperature increased (Fig. 3.4, 3.5).

c. Survival of *Salmonella enterica*

The survival period for *Salmonella enterica* varied significantly ($p < 0.0001$) between the two storage conditions of 5°C and 25°C. The pathogen's microbial population was reduced below the detection limit of 2.4 log CFU/g, reaching 1.34 and 1.31 days for formulations with and without the preservative, respectively, when stored at 25°C. In contrast, when stored under refrigeration at 5°C, the time required for a complete 5-log reduction of *Salmonella enterica* was 46 days, resulting in a significant difference ($p < 0.0001$) (Fig. 3.8). Similar patterns were observed in the NA formulation, highlighting the temperature-dependent nature of pathogen inactivation across all formulations (Fig. 3.4, 3.5).

Influence of storage temperatures on z-value

Table 9 presents the thermal resistance constant, "z-value", an essential factor for consideration in temperature-based inactivation calculations. The z-values were estimated from the D-values for all the formulations (Tables 1 and 4). The z-values for *Listeria monocytogenes* and *Salmonella enterica* exhibited a similar and gradually increasing trend, as the increase in storage temperature affected the D-values, indicating strong resistance to the rise in temperature.

Table 9. Average calculated D-values (time taken for 1-log reduction) and z-value for *E. coli* O157:H7*, Listeria monocytogenes,* and *Salmonella enterica* in all formulations of mayonnaise samples.

Pathogen	***D-Value 5°C (Hours)***	***D-Value 15.5°C (Hours)***	***D-Value 25°C (Hours)***	$Z_{(5°C \rightarrow 25°C)}$
***E.coli* O157:H7**	444.92	11.40	18.06	14.37
Listeria monocytogenes	18.97	9.47	7.98	53.20
Salmonella enterica	223.86	7.55	6.61	13.08

Additionally, for *E. coli* O157:H7 and *Salmonella enterica,* a sudden drop in the D-value from 5°C to 15.5°C has been observed, indicating higher sensitivity to higher temperatures, with *Salmonella enterica* being comparatively more stable than *E. coli* O157:H7. A lower z-value of 6.6°C is estimated for *E. coli* O157:H7 compared to a rise of 34.82°C for *Listeria monocytogenes,* for the thermal death time curve to traverse 1 log cycle between 5°C to 15.5°C. On the contrary, for 1-log reduction between 5°C and 25°C, the lowest z-value was observed for *Salmonella enterica*, while *Listeria monocytogenes* still portrayed stronger resistance to a rise in temperature.

CHAPTER 4

DISCUSSIONS

The time required for 5-log reduction of *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes,* using a cold-fill hold process in acidified mayonnaise by the action of storage temperature, time, pH, titratable acidity, and addition of a preservative, was studied. Previous research on the cold-fill-hold process indicated the influence of acid concentration, acid type (Lobo et al., 2019) and storage temperature (Hwang et al., 2019) on the inactivation of pathogens in acid or acidified foods. Also, previous studies on cold-fill-hold processes established processing conditions for acidified foods at a broad pH range of 3.3 to 4.6 for foods such as mayonnaise, salad dressings, carrot juice, and cucumber pickle. The use of a cocktail of *E. coli* O157:H7*, L. monocytogenes,* and *S. enterica* as the inoculum for the survival study of pathogens was per previous research on acidified foods [(Lobo et al., 2019), (Dufort et al., 2017), (Breidt et al., 2013) and (Breidt et al., 2007)] and relevance to acidified food processing.

The study utilized a constant concentration of 5% acetic acid, as high acid concentrations are studied to produce faster inactivation rates (Acosta et al., 2014), with initial pH, undissociated organic acid concentration (% TA), and storage temperature being the major dependent factors (Manios et al., 2014). The samples that contained preservatives had a higher initial average pH of 4.35 and a lower % TA of 0.33 compared to a lower initial average pH of 4.2 and a higher % TA of 0.51 (Tables 5 and 6). The increase in the % TA of samples stored at 25°C has been observed in similar inactivation studies for Fava beans spread stored at 25°C (Manios et al., 2014). Studies by Khurana et al. (2006) revealed the efficacy of acetic acid in increasing %TA and its bactericidal

effect on samples with a pH range of 4.2 to 4.6. Similarly, the output can be examined in the present study as well, as the samples, regardless of the addition of preservatives, were observed to have increased acidity.

The present study investigated the inactivation rates of *E. coli* O157:H7, *L. monocytogenes*, and *S. enterica* in commercially prepared and laboratory-prepared mayonnaise formulations. The commercially prepared mayonnaises were acidified using lactic acid, with sodium benzoate and potassium sorbate as preservatives, and had pH values ranging from 2.95 to 3.70, stored at 15.5°C. On the other hand, the lab-prepared mayonnaise sauces were tested as combinations of two formulations (with and without 0.1% sodium benzoate) and two temperature conditions (5°C and 25°C), acidified with acetic acid at 5% concentration (Table 1 and Table 4). As per 21 CFR part 582, the inhibition of microorganisms of concern can be achieved using permitted preservatives, when in accordance with the allowed limits. The addition of 0.1% sodium benzoate had no significant effect ($p > 0.0001$) on the reduction of *E. coli* O157:H7*, L. monocytogenes,* and *S. enterica* in the formulations A (no preservative) and B (with preservative) (Fig. 4.1). The results from previous studies by Breidt et al. (2013), contrast with the current study, which states a significant effect of 0.1% benzoates on the 5-log reduction of bacterial pathogens, for acidified pickle brine with pH 3.5 and 3.8 at 10°C. Although no significant effect on pathogen survival was observed between our formulations, the addition of preservative (Formulation B) helped to achieve a similar 5-log reduction rate as that of formulation with acetic acid alone (Formulation A), with higher average pH of 4.37 (acetic acid + 0.1% sodium benzoate) than that of average pH of 4.19 (only acetic acid). Guo et al. (2022) revealed disruption of the cell barrier and damage to the bacterial cell membrane's integrity by the action of organic acids (acetic acid, lactic acid, and citric acid) on cucumber slices.

The analysis of D and z-values (Table 9) in the current study suggests that *E. coli* O157:H7 exhibits higher temperature resistance compared to *L. monocytogenes* and *S. enterica*. The highest D-values for *E. coli* O157:H7 ranged from 445 hours, 11.4 hours, and 18.1 hours at 5°C, 15.5°C, and 25°C, respectively. Additionally, the letter groupings (Fig. 3.4) utilizing Tukey-adjusted pairwise comparisons demonstrated the impact of both formulation type and temperature on microbial reduction. A significant difference ($p < 0.0001$) is observed in the inactivation rate of the three pathogens when the temperature increases from 5°C to 25°C, indicating temperature dependence with weak acid treatment, and the results are consistent with those of Lu et al. (2013). The 5-log reduction times were observed to be about 90.3 hours (3.76 days), 39.9 hours (1.66 days), and 33.1 hours (1.38 days) for *E. coli* O157:H7, *L. monocytogenes*, and *S. enterica*, respectively, when stored at 25°C. The results are in agreement with studies by Hwang et al. (2019) and (Beuchat et al., 2006), which revealed that the population of pathogenic *E. coli* O157:H7 at ambient storage conditions of 23°C (acetic acid, pH 4.31) and 25°C, decreased to below 2.2 log CFU/g in mayonnaise sauce within 72 hours (3 days). Similar inactivation periods of about 96 hours (4 days) for mayonnaise sauce (2% acetic acid, pH 3.91) were studied for a two-strain cocktail of *E. coli* O157:H7 when stored at 22°C. (Raghubeer et al., 1994). D-values for *L. monocytogenes* and *S. enterica* were low, with the order of survival time being *L. monocytogenes* (9.47 hours and 7.98 hours) > *S. enterica* (7.55 hours and 6.61 hours), at higher temperatures of 15.5°C and 25°C, respectively. The results are in accordance with the findings by Dufort et al. (2017), revealing significantly less heat tolerance by *S. enterica* when compared to *E. coli* O157:H7 and *L. monocytogenes* at pH 4.1 for pickled brines. Contrastingly, *L. monocytogenes* showed the fastest inactivation (19.0 hours) as compared to *E. coli* O157:H7 (445 hours) and *S. enterica* (224 hours) when stored at 5°C (Table 4). The results coincide with the studies by Breidt

et al. (2007), as a similar trend of faster inactivation period of 11.2 hours by *L. monocytogenes* was recorded in comparison with 51.3 hours by *S. enterica*, for acidified cucumbers stored at a refrigeration temperature of 10°C.

S. enterica had the lowest z-value, while *L. monocytogenes* remained the most resistant to changes in temperature, as indicated by its higher z-value (Table 9) and consistently lower letter grouping derived from Tukey-adjusted pairwise comparisons (Fig. 3.4). Additionally, the pathogen *E. coli* O157:H7 was found to be the most acid-resistant at all tested pH levels (2.95 to 4.39) of various formulations used in this study. These findings are in accordance with the studies on acidified cucumber (acetic acid, pH ≤ 3.3) by Breidt et al, (2007), pickled brines (2.5% acetic acid, pH 3.8) by Breidt et al, (2013), and low-fat mayonnaise-based hibachi sauce (0.5 g lactic acid/100g, pH 4.31) by Hwang et al, (2019).

As per the U.S. Food and Drug Administration and the acidified vegetable industry, the increasing concerns regarding foodborne disease outbreaks caused by vegetative cells of acid-resistant food pathogens are alarming (Breidt et al., 2007). Moreover, the work by Breidt and colleagues, as well as the promulgation of 21 CFR part 120, has studied food products with pH values between 3.5 and 4.0 in response to outbreaks of disease caused by *Escherichia coli* O157:H7 and *Salmonella enterica* strains. According to a survey by Australian manufacturers, a potential source of microbial contamination for cold-filled acid sauces and dressings has been linked to the addition of frozen or dried ingredients, such as spices, herbs, and onions (Chapman et al., 2013). Overall, the present study conducts the evaluation of conditions such as different storage temperatures, efficacy of different organic acid on microbial load reduction and use of preservatives, to provide a useful study for food industries on inactivation of pathogenic *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes* using cold-fill-hold

process, and hence to be selected as a thermal processing alternative. As a recommendation for future work, a formal shelf-life analysis and sensory test are highly recommended before standardizing the base formulation and selection of acidifiers and their impact on final product pH.

REFERENCES

Acosta, O., Gao, X., Sullivan, E. K., & Padilla-Zakour, O. I. (2014). Pickled egg production: Effect of brine acetic acid concentration and packing conditions on acidification rate. *Journal of Food Protection*, *77*(5), 788–795. https://doi.org/10.4315/0362-028X.JFP-13-362

Amrutha, B., Sundar, K., & Shetty, P. H. (2017). Effect of organic acids on biofilm formation and quorum signaling of pathogens from fresh fruits and vegetables. *Microbial Pathogenesis*, *111*, 156–162. https://doi.org/10.1016/j.micpath.2017.08.042

Arjeh, E., Barzegar, M., & Ali Sahari, M. (2015). Effects of gamma irradiation on physicochemical properties, antioxidant and microbial activities of sour cherry juice. *Radiation Physics and Chemistry*, *114*, 18–24. https://doi.org/10.1016/j.radphyschem.2015.05.017

Balestrini, C., Bresnahan, D., Lou, Y., Ocasio, W., Ramaswamy, R., & Weise, K. (n.d.). *An Examination of the Acidified Foods Rule with Regard to the Acid versus Acidified Foods Classification*.

Beuchat, L. R., Ryu, J.-H., Adler, B. B., & Harrison, M. D. (2006). Death of Salmonella, Escherichia coli O157:H7, and Listeria monocytogenes in Shelf-Stable, Dairy-Based, Pourable Salad Dressings. In *Journal of Food Protection* (Vol. 69, Issue 4).

Breidt, F., Hayes, J., & Mcfeeters, R. F. (2007). Determination of 5-Log Reduction Times for Food Pathogens in Acidified Cucumbers during Storage at 10 and 25C † ‡. In *Journal of Food Protection* (Vol. 70, Issue 11).

Breidt, F., Kay, K., Cook, J., Osborne, J., Ingham, B., & Arritt, F. (2013). Determination of 5-log reduction times for escherichia coli o157:h7, salmonella enterica, or listeria monocytogenes in acidified foods with PH 3.5 or 3.83. *Journal of Food Protection*, *76*(7), 1245–1249. https://doi.org/10.4315/0362-028X.JFP-12-528

Chapman, B., Scurrah, K. J., & Ross, A. T. (n.d.). *Contemporary Formulation and Distribution Practices for Cold-Filled Acid Products: Australian Industry Survey and Modeling of Published Pathogen Inactivation Data*.

Cheng, C. xiang, Jia, M., Gui, Y., & Ma, Y. (2020). Comparison of the effects of novel processing technologies and conventional thermal pasteurisation on the nutritional quality and aroma of Mandarin (Citrus unshiu) juice. *Innovative Food Science and Emerging Technologies*, *64*. https://doi.org/10.1016/j.ifset.2020.102425

Chien, H. I., Lee, Y. C., Yen, Y. F., Wei, P. C., Hwang, C. C., Kuo, C. H., Yen, F. L., & Tsai, Y. H. (2023). Replacing the Addition of Sulfite in Mustard Pickle Products by High-Hydrostatic-Pressure Processing to Delay Quality Deterioration during Storage. *Foods*, *12*(2). https://doi.org/10.3390/foods12020317

Choo, Y. X., Teh, L. K., & Tan, C. X. (2023). Effects of Sonication and Thermal Pasteurization on the Nutritional, Antioxidant, and Microbial Properties of Noni Juice. *Molecules*, *28*(1). https://doi.org/10.3390/molecules28010313

De Vries, H., Mikolajczak, M., Salmon, J. M., Abecassis, J., Chaunier, L., Guessasma, S., Lourdin, D., Belhabib, S., Leroy, E., & Trystram, G. (2018). Small-scale food process engineering — Challenges and perspectives. In *Innovative Food Science and Emerging Technologies* (Vol. 46, pp. 122–130). Elsevier Ltd. https://doi.org/10.1016/j.ifset.2017.09.009

Dufort, E. L., Etzel, M. R., & Ingham, B. H. (2017). Thermal Processing Parameters to Ensure a 5-log Reduction of Escherichia coli O157:H7, Salmonella enterica, and Listeria monocytogenes in Acidified Tomato-based Foods. In *Food Protection Trends* (Vol. 37, Issue 6).

Giavoni, M., Villanueva-Suárez, M. J., Peña-Armada, R. D. la, Garcia-Alonso, A., & Mateos-Aparicio, I. (2022). Pasteurization Modifies the Sensorial Attributes and Nutritional Profile of Orange Pulp By-Product. *Foods*, *11*(13). https://doi.org/10.3390/foods11131973

Guo, C., He, Y., Wang, Y., & Yang, H. (2022). NMR-based metabolomic investigation on antimicrobial mechanism of Salmonella on cucumber slices treated with organic acids. *Food Control*, *137*. https://doi.org/10.1016/j.foodcont.2022.108973

H., F., & M., A. (2013). Acidified Foods: Food Safety Considerations for Food Processors. In *Food Industry*. InTech. https://doi.org/10.5772/55161

Hwang, D., White, E., Purohit, A., Mohan, A., & Mishra, A. (2019). Survival of Escherichia coli O157:H7 and Listeria monocytogenes in an acidified low-fat mayonnaise based hibachi sauce. *LWT*, *108*, 297–300. https://doi.org/10.1016/j.lwt.2019.03.060

Khurana, A., Awuah, G. B., Taylor, B., & Enache, E. (2006). Efficacy of Selected Acidulants in Pureed Green Beans Inoculated with Pathogens (Escherichia coli O157:H7 and Listeria monocytogenes). In *Journal of Food Protection* (Vol. 69, Issue 8).

Leguerinel, I., Mafart´´, P., & Mafart´´, M. (2001). Modelling the influence of pH and organic acid types on thermal inactivation of Bacillus cereus spores. In *International Journal of Food Microbiology* (Vol. 63). www.elsevier.nl/locate/ijfoodmicro

Lobo, A., Zúñiga, C., Worobo, R. W., Padilla-Zakour, O. I., & Usaga, J. (2019a). Fate of spoilage and pathogenic microorganisms in acidified cold-filled hot pepper sauces. *Journal of Food Protection*, *82*(10), 1736–1743. https://doi.org/10.4315/0362-028X.JFP-19-071

Lobo, A., Zúñiga, C., Worobo, R. W., Padilla-Zakour, O. I., & Usaga, J. (2019b). Fate of spoilage and pathogenic microorganisms in acidified cold-filled hot pepper sauces. *Journal of Food Protection*, *82*(10), 1736–1743. https://doi.org/10.4315/0362-028X.JFP-19-071

Lu, H. J., Breidt, F., & Pérez-Díaz, I. (2013). Development of an Effective Treatment for A 5-Log Reduction of Escherichia coli in Refrigerated Pickle Products. *Journal of Food Science*, *78*(2). https://doi.org/10.1111/j.1750-3841.2012.02968.x

Manios, S. G., Lambert, R. J. W., & Skandamis, P. N. (2014). A generic model for spoilage of acidic emulsified foods: Combining physicochemical data, diversity and levels of specific spoilage organisms. *International Journal of Food Microbiology*, *170*, 1–11. https://doi.org/10.1016/j.ijfoodmicro.2013.10.021

Paudyal, R., Barnes, R. H., & Karatzas, K. A. G. (2018). A novel approach in acidic disinfection through inhibition of acid resistance mechanisms; Maleic acid-mediated inhibition of glutamate decarboxylase activity enhances acid sensitivity of Listeria monocytogenes. *Food Microbiology*, *69*, 96–104. https://doi.org/10.1016/j.fm.2017.07.013

Raghubeer, E. V, Ke, J. S., Campbell, M. L., & Meyer, R. S. (n.d.). Fate of Escherichia coli 0157:H7 and Other Coliforms in Commercial Mayonnaise and Refrigerated Salad Dressing. In *Journal of Food Protection* (Vol. 58, Issue 1).

Sokołowska, B., Niezgoda, J., & Chotkiewicz, M. (2013). Opportunities to Germinate and Grow of Alicyclobacillus acidoterrestris Spores in the Presence of Organic Acids. In *www.fmfi-journal.org Focusing on Modern Food Industry (FMFI* (Vol. 2). www.fmfi-journal.org

Usaga, J., Worobo, R. W., & Padilla-Zakour, O. I. (2014). Thermal resistance parameters of acid-adapted and unadapted Escherichia coli O157:H7 in apple-carrot juice blends: Effect of organic acids and pH. *Journal of Food Protection*, *77*(4), 567–573. https://doi.org/10.4315/0362-028X.JFP-13-371

Vervoort, L., Van Der Plancken, I., Grauwet, T., Timmermans, R. A. H., Mastwijk, H. C., Matser, A. M., Hendrickx, M. E., & Van Loey, A. (2011). Comparing equivalent thermal, high pressure and pulsed electric field processes for mild pasteurization of orange juice: Part II: Impact on specific chemical and biochemical quality parameters. *Innovative Food Science and Emerging Technologies*, *12*(4), 466–477. https://doi.org/10.1016/j.ifset.2011.06.003