

INFLUENCE OF LIPIDS ON THE BEHAVIOR OF *SALMONELLA* SPP. IN  
LOW-MOISTURE COCOA-BASED MODEL FOODS

by

MARIA ANUM SOHAIL

(Under the Direction of Joseph Frank)

ABSTRACT

Chocolate and chocolate products have been implicated in numerous outbreaks of salmonellosis and are susceptible to contamination at multiple stages of processing. The behavior of dehydrated *Salmonella* spp. in cocoa-based model foods formulated with monostearin, monolaurin, dilaurin, and trilaurin was evaluated at 22°C over 168 days. Monolaurin caused a reduction in *Salmonella* populations; however, the remaining compounds did not affect cell survival. The effect of 0, 25, and 50% added cocoa fat in model foods with  $a_w$  levels of 0.33 or 0.43 was also determined at 22, 35, and 70°C over 24 h to 168 days. A protective effect of cocoa fat on *Salmonella* spp. was observed at 70°C,  $a_w$  0.33 and 0.43, but protection was not influenced by fat concentration. *Salmonella* survival was not affected by added fat at lower temperatures.

INDEX WORDS: *Salmonella* spp., chocolate, cocoa, cacao, fat, water activity, monolaurin, monostearin, dilaurin, trilaurin, lauric acid

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## DEDICATION

This thesis is dedicated to my parents. Thank you for your love, support, and encouragement.

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## CHAPTER 1

### INTRODUCTION

*Salmonella* spp. are Gram negative, facultatively anaerobic bacteria belonging to the family *Enterobacteriaceae*. They are widespread in the environment, colonize the intestinal tracts of humans and animals, and are spread through the fecal-oral route and water (8). Salmonellae can contaminate a wide variety of foods and ingestion of viable cells causes salmonellosis in humans (8). Children, the elderly, and individuals with compromised immune systems are generally more severely affected by the illness than healthy individuals (9). Of the estimated 9.4 million cases of illness and 1,351 deaths per year associated with the consumption of contaminated food in the United States, approximately 1.0 million illnesses and 378 deaths are caused by non-typhoidal salmonellae (12).

The resilience of *Salmonella* spp. and their ability to grow and survive in unfavorable conditions raises concerns for the food industry. For instance, while optimal growth occurs at temperatures between 35°C and 37°C, pH levels of approximately 6.5 to 7.5, and water activity ( $a_w$ ) levels higher than or equal to 0.93 (11), growth at temperatures between 2°C (5) and 54°C (6) and at pH levels as low as 3.0 (7) has been documented. Of particular concern to the industry is the fact that *Salmonella* spp. can contaminate dry foods and survive for long periods of time (11). Growth of the organism in the food is not necessary in order to cause illness.

*Salmonella* outbreaks associated with low-moisture, high-fat foods have been tied to low infectious doses. Ingestion of as little as one viable cell may cause illness (4). Cells may become entrapped in hydrophobic lipid micelles, protecting them from the acidic conditions of the

stomach and allowing them to eventually pass into the lower intestine to cause illness (15). *Salmonella* spp. in these foods are also more resistant to heat treatment compared to high moisture products (1).

Chocolate, a widely consumed confectionery commodity, has been implicated in numerous outbreaks of salmonellosis for several decades. Chocolate and chocolate products are susceptible to contamination by *Salmonella* spp. throughout the manufacturing process, from harvesting the seeds of the cocoa tree, *Theobroma cacao*, to storing the finished products at home (2). Cells can survive for long periods of time (13, 14). Heat treatments such as roasting and conching may not destroy salmonellae that contaminate products at early stages of processing (10, 3). The survival of *Salmonella* spp. in these products can vary with product composition and moisture content, as well as with the use of different strains and treatment temperatures.

The general effects of food composition on the behavior of microorganisms are known, but more knowledge is needed regarding the role of fat content in *Salmonella* persistence in dry foods. The aim of this study is to evaluate the effect of various lipids on the behavior of *Salmonella* spp. in low-moisture cocoa-based products. To assess the influence of cocoa fat level, product  $a_w$ , and storage temperature, populations of *Salmonella* spp. subjected to various combinations of these factors will also be monitored. The information gained from these studies will be useful for conducting risk assessments and improving mitigation strategies designed to reduce the persistence of *Salmonella* spp. in low-moisture, high-fat foods.

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## CHAPTER 2

### LITERATURE REVIEW

#### ***Salmonella* spp.**

*Salmonella* spp. are Gram negative rod-shaped bacteria belonging to the family *Enterobacteriaceae*. Named after American veterinary scientist Daniel E. Salmon (29), the *Salmonella* genus is organized by the Kauffmann-White classification system into two species: *S. enterica* and *S. bongori*. *S. enterica* contains six subspecies that are further divided into serovars based on surface and flagellar antigenic properties (37). *S. enterica* subsp. *enterica* (I) is the largest subspecies group with over 1,500 known serovars, while *S. enterica* subsp. *salamae* (II) and *S. enterica* subsp. *diarizonae* (IIIb) contain over 500 and 300 serovars, respectively. Today there are over 2,500 recognized serotypes of *Salmonella* (20).

Members of the *Salmonella* genus are facultatively anaerobic and do not form spores. Most are nonmotile, but exceptions such as *S. Pullorum* and *S. Gallinarum* have been identified. As chemoorganotrophs, salmonellae can metabolize organic compounds such as glucose for energy and have both fermentative and respiratory metabolic pathways (29). They can process citrate as a sole carbon source (28) and are catalase positive and oxidase negative (29).

*Salmonella* spp. are widely distributed in the environment and common reservoirs include the intestinal tracts of humans and both domestic and wild animals. Animal products such as poultry, eggs, and beef are therefore likely sources. Cells can be spread through contaminated water (37), in which they can survive for weeks (62) and through the fecal-oral route (37). In ideal conditions, they can survive for years in soil (62). Optimum growth of the bacteria occurs

at temperatures between 35°C and 37°C, pH levels of approximately 6.5 to 7.5, and water activity ( $a_w$ ) levels higher than or equal to 0.93 (62). However, salmonellae are resilient and have the ability to grow and survive in unfavorable conditions. For example, growth at 2°C has been observed for *S. Enteritidis* and *S. Typhimurium* in chicken (23), giving *Salmonella* spp. some psychrotrophic qualities. They can also survive for long periods of time at freezer temperatures (22). Growth at 54°C has been documented for thermally stressed mutants of *S. Typhimurium* (26). *Salmonella* cells that are exposed to pH levels at or below 4.5 after first being exposed to more mildly acidic conditions can experience an acid tolerance response (ATR), allowing them to survive at pH levels as low as 3.0 (32). Growth is generally inhibited by the presence of 3 to 4% NaCl, but increasing growth temperatures between 10°C and 30°C may mitigate this effect (20). Mattick et al. (58) determined that *S. Enteritidis* and *S. Typhimurium* can form filaments at  $a_w$  levels between 0.93 and 0.98. This is a survival mechanism that would allow them to divide rapidly upon the introduction of additional moisture. Various survival strategies utilized by *Salmonella* spp. to overcome environmental stresses such as desiccation and starvation and host defenses such as exposure to high levels of damaging oxygen species and gastric acid (32) illustrate their hardiness and heighten public health concern.

### **Salmonellosis**

There are an estimated 9.4 million cases of illness and 1,351 deaths associated with the consumption of contaminated foods in the United States annually (65). Of these, non-typhoidal *Salmonella* spp. are responsible for approximately 1.0 million cases of illness and 378 deaths (65). Ingestion of viable cells causes salmonellosis in humans, which is an illness characterized by diarrhea, fever, vomiting, and headaches. Although salmonellosis is usually self-limiting, serious complications such as septicemia may occur (37). Some strains may cause typhoid and

typhoid-like fever (37). Children, the elderly, and individuals with compromised immune systems are generally more severely affected (47).

### **Food-related outbreaks**

*Salmonella* spp. have been associated with outbreaks in a large variety of products, including meat and poultry, eggs, dairy (28), and produce (15). Intermediate moisture foods such as cheese (28), candy fondant (59) and salami (17) have also been implicated. Although low  $a_w$  levels prevent the growth of *Salmonella* spp., products such as baked goods, cereal, chocolate, infant foods, peanut butter, potato chips, powdered milk, nuts, and spices have all been associated with outbreaks of foodborne salmonellosis over the last few decades (62). More recently, outbreaks across the United States have been documented in pine nuts (14), pistachios (13), dry cereals (11), and peanut butter (12). Although the number of outbreaks in low-moisture foods is relatively small compared to those in higher moisture products (15), there is often a large health and economic impact associated with them.

### **Infectious dose**

Growth of salmonellae in the food is not required in order to cause illness in humans and, in low-moisture foods, the presence of relatively low numbers of cells has resulted in illness. This has been documented numerous times. Chocolate-related salmonellosis outbreaks were among the first to suggest this with product contamination levels as low as  $\leq 1$  CFU/g (46) and 2.5 CFU/g (18, 24). Levels of 0.04 to 0.45 CFU/g were recovered from paprika and paprika-powdered potato chips in a German salmonellosis outbreak (54). In Canada, an investigation of an outbreak in cheddar cheese suggested that a single *Salmonella* cell can be infective (21).

Infectious doses depend on multiple factors such as variation among serovars, variation among the hosts, and the characteristics of the transmission vehicle, i.e. the food (9). A notable

common factor in the outbreaks tied to low oral infective doses of *Salmonella* spp. is the high level of fat in each implicated food. It is believed that bacterial cells may become entrapped in hydrophobic lipid micelles, protecting them from the acidic conditions of the stomach and allowing them to eventually pass into the lower intestine to cause disease (73). Numerous outbreaks associated with chocolate, dairy, and meat products, which respectively contain cocoa butter, milk fat, and animal fat, contaminated with low levels of salmonellae support this concept (20).

### **Survival of *Salmonella* spp. in dry foods**

Due to their widespread nature, *Salmonella* spp. can contaminate low-moisture foods and raw materials. Studies have shown that the cells can survive for lengthy periods of time in or on these products. For instance, in a dry model system based on anhydrous silica gel ( $a_w = 0.2$ ), *Salmonella* serovars did not experience a 1-log reduction until after approximately 248 to 1,351 days (62), which illustrates their resistance to desiccation. Hiramatsu et al. (39) studied a dry model system based on paper disks ( $a_w = 0.5$  to  $0.6$ ) and reported low declines in *Salmonella* spp. after 22 to 24 months of storage at  $4^\circ\text{C}$ . In dehydrated powdered infant formula, desiccated *S. Enteritidis* survived up to 15 months (5). Burnett et al. (10) reported the survival of five *Salmonella* serovars in peanut butter and peanut butter spreads after 24 weeks of storage at  $21^\circ\text{C}$ . A rapid initial decline in numbers was observed in the study. Beuchat and Heaton (8) reported the survival of *Salmonella* spp. in pecan halves for several weeks at  $21^\circ\text{C}$  and that survival was increased to over 32 weeks at  $5^\circ\text{C}$ . In halva, a low-moisture confection made from sesame seeds and sugar, *S. Enteritidis* survived for eight months under refrigeration (49).

The long term survival of *Salmonella* spp. in chocolate and chocolate products has also been documented. Tamminga et al. (71) recovered *S. Eastbourne* and *S. Typhimurium* in dark

and milk chocolate after nine months of storage at 20°C. The  $a_w$  values of the chocolate bars used in the experiment were reported to be between 0.30 and 0.51. Survival was greater in the milk chocolate used in this study, which contained more fat components and less cocoa solids than the dark chocolate. In another study by Tamminga et al. (70), the contamination of dry raw materials in chocolate production was simulated and artificially contaminated milk powder was used to prepare milk chocolate. *S. Typhimurium* was no longer detectable after 15 months of storage at 20°C, but *S. Eastbourne* was still detectable after 19 months. The characteristics and processing of chocolate will be discussed in a later section.

Studies have been conducted attempting to elucidate the role of fat type and level on the survival of *Salmonella* spp. in foods undergoing various processing treatments. Intermediate to high moisture foods have been studied more than dry foods in this respect. Escriu and Mor-Mur (30) observed differences in sub-lethally injured *S. Typhimurium* cells between minced chicken samples prepared with different fats (tallow, virgin olive oil, and linseed) at 10 or 20%. Samples were subjected to a high-pressure treatment and stored for 60 days at 2 and 8°C. The protective effect of low  $a_w$  was not a factor as the  $a_w$  of the products were reported as 0.990 to 0.998. In another study (42), higher fat levels in ground beef resulted in enhanced thermal resistance of *S. Typhimurium* DT104. Fat levels of 7, 12, 18, or 24% were used and products were subjected to temperatures ranging from 58 to 65°C. It was suggested by the authors that the addition of fat lowered the  $a_w$  of the beef, which may have protected the cells by lowering the heat penetration through the product. The  $a_w$  levels were not reported. Holliday and Beuchat (40) observed a longer survival time for *Salmonella* spp. in yellow fat spreads with higher fat contents than in those with lower fat contents.

Solutes such as salt and sugar in low-moisture foods may also afford protection to salmonellae and enhance their survival over time. In the 2005 study by Hiramatsu et al. (39), survival rates of *Salmonella* spp. were up to 79 times higher in paper disks containing 36% sucrose than in those containing no sucrose. Burnett et al. (10) suggested that the presence of corn syrup solids, soy protein, and vitamin and mineral supplements in peanut spreads may have played a role in the higher survival of *Salmonella* spp. in those products when compared to natural peanut butter.

Certain plant extracts and essential oils used as ingredients in dry foods such as chocolate may also affect the survival of *Salmonella* spp. Kotzekidou et al. (49) reported that *S. Typhimurium* and *S. Enteritidis* were inhibited by various plant extracts and essential oils, including lemon flavor, pineapple, plum, strawberry, and banana. The disc diffusion method was used to determine zones of inhibition. The researchers also evaluated the ability of these substances to inhibit various pathogens in chocolate. Although the efficacy of the compounds was reduced in chocolate, lemon flavor was reported to be the most effective extract for inactivating *E. coli* 0157:H7. The real food system portion of the study did not include salmonellae, but *E. coli* 0157:H7 cells are also Gram negative bacteria from the *Enterobacteriaceae* family. The mechanism of inhibition by plant extracts and essential oils, which involves penetrating the hydrophilic outer membrane and disturbing the cytoplasmic membrane (49), may be similar for these microorganisms.

### **Heat resistance of *Salmonella* spp. in dry foods**

The resistance of salmonellae to thermal treatments is influenced by multiple factors. The strains and history of the cultures are important, as the temperature at which they are grown, the age of the cells and their growth phase, the growth media, and exposure to stress such as acid can

affect heat resistance (25). For instance, Kirby and Davies (48) observed an increased thermal resistance of *S. Typhimurium* LT2 cells that were dehydrated to a  $a_w$  of under 0.57 prior to heating. Properties of the heating medium such as pH,  $a_w$ , and solute content are also contributing factors to heat resistance (35). Wet heat treatments destroy microorganisms by denaturing nucleic acids, structural proteins, and enzymes. Cytoplasmic membranes and ribosomal RNA also appear to be targets of injury associated with heating (31). With dry heat treatments, higher temperatures and longer treatment times are needed to obtain the same inactivation levels as wet heat treatments (37).

Thermal resistance data for salmonellae reported in wet systems cannot be extrapolated to low-moisture products. Survival and heat resistance generally increases as  $a_w$  decreases. The  $a_w$  of food can be manipulated by physical removal of water through dehydration or by the addition of solutes. Different solutes can have an effect on the heat resistance of *Salmonella* spp. at the same  $a_w$  level (35). For instance, sucrose protected *S. Typhimurium* DT104 from various heat treatments more than glucose-fructose and sodium chloride (58). Differences in heat resistance between strains have also been noted (35). Numerous products such as peanut butter (38), flour (3), dried squid chips (39), almonds (27), crushed cocoa beans and hazelnut shells (41), and egg powders (43) have been investigated and authors observed a protective effect of reduced  $a_w$  levels against the destruction of *Salmonella* spp. by heat.

*Salmonella* spp. in chocolate have also been shown to exhibit thermal resistance. Krapf and Gantenbein-Demarchi (51) studied the thermal inactivation of *Salmonella* spp. during conching in different types of chocolate and reported D-values of  $D_{50^\circ\text{C}} = 999$  min to  $D_{90^\circ\text{C}} = 26$  min for cocoa liquor and  $D_{50^\circ\text{C}} = 1574$  min for dark chocolate. The dark chocolate contained cocoa liquor and sugar. An additional protective effect by the sugar component may have

resulted in the higher D-value when compared to cocoa liquor. Another study reported the  $D_{71^{\circ}\text{C}}$  for *S. Eastbourne*, *S. Senftenberg 775W*, and *S. Typhimurium* as 270, 276, and 396 min, respectively (53). *S. Anatum* was found to be less heat resistant in milk chocolate when water was added (4).

### **Water activity**

Water activity describes the tendency of water molecules to migrate and participate in reactions (64). It has been extensively used by the food industry as a means to predict the microbial stability of intermediate to high moisture products. When the  $a_w$  of a product is below 0.88 to 0.91, most bacteria will not grow (31). The growth of yeasts and molds is generally inhibited below these levels, with 0.60 to 0.62 being the lower limits for osmophilic yeasts and xerophilic molds (31). The response of microorganisms to different  $a_w$  levels is complex. Intrinsic factors such as pH and the use of antimicrobial compounds and extrinsic factors such as temperature and irradiation are influential (52).

Water activity can be described by the following equation:

$$(1) \quad a_w = (p / p^{\circ}) T$$

where  $p$  represents the vapor pressure of water in the material and  $p^{\circ}$  represents the vapor pressure of water at equilibrium (63). The temperature,  $T$ , is held constant. This relationship is derived from thermodynamic principles and applies only when the system is at equilibrium. At low moisture levels, however, the condition of thermodynamic equilibrium is not likely to be met. Instead, a stationary state may be achieved at certain conditions (33). The term relative vapor pressure (RVP) does not assume equilibrium and is therefore sometimes used instead of  $a_w$  (63). It should also be noted that  $a_w$  does not account for the different reaction rates and stabilities associated with the use of different solutes to lower the  $a_w$  of a product (64).

## **Lipids in foods**

Lipids are chemically diverse compounds that are generally insoluble in water and soluble in organic solvents such as hexane and methanol. They can be found in living organisms. Some substances that are considered lipids do not fit into this definition, such as short chain fatty acids, which are soluble in water and insoluble in organic solvents (60). Lipids that are solid at room temperature are called fats, while lipids that are liquid at room temperature are called oils. Fats and oils are found at different levels in a variety of foods and are a source of essential fatty acids and energy.

Some lipids have long been recognized for their antimicrobial activity (44). They have the ability to inhibit bacteria, viruses, molds, and other microorganisms. In addition to serving a number of functions, lipids can therefore be used as protective agents in foods, especially as part of a hurdle approach that utilizes different processing methods to achieve a stable and safe product.

## **Fatty acids and their esters**

Fatty acids are lipids that contain an aliphatic chain and a carboxyl group. Most of the fatty acids that occur in nature have straight chains with an even number of carbon atoms. Shorthand nomenclature lists the number of carbons in the fatty acid, a colon, the number of double bonds on the aliphatic chain, and, if applicable, the Greek character  $\omega$  followed by the position of the double bond near the methyl end (60). Free fatty acids are generally undesirable in foods and are usually present in the form of mono-, di-, or triacylglycerols. These are glycerol molecules that have been esterified with one, two, or three fatty acids. Among the three forms, triacylglycerols are the most common in foods. They may contain the same fatty acids or any combination of different fatty acids.

The antimicrobial properties of fatty acids and their esters have been investigated. In general, fatty acids and monoacylglycerols can inhibit microorganisms, while diacylglycerols and triacylglycerols cannot (69). It should be noted that dietary triacylglycerols are hydrolyzed by gastric and pancreatic lipases to fatty acids and monoacylglycerols, which can inhibit microorganisms in the small intestine. Sprong et al. (68) reported that the ingestion of triacylglycerols containing capric acid (C10:0) or lauric acid (C12:0) might increase resistance against pathogens in the intestines.

The antimicrobial activity of acylglycerols is generally less than that of free fatty acids (7). However, monoacylglycerols of medium-chain saturated fatty acids are more bioactive than the free fatty acids, especially monolaurin. Due to the protection afforded by the lipopolysaccharide layer, Gram negative bacteria are not greatly affected by lipids unless their chain lengths contain six carbons or less (66). Some lipid-sensitive Gram negative bacteria have been observed. *Helicobacter pylori*, for instance, was reported to be susceptible to medium-chain monoacylglycerols and free fatty acids (C12:0) (61).

The relationship between structure and antimicrobial properties of fatty acids and their derivatives have been studied. The degree of unsaturation, hydrocarbon chain length, and presence of additional functional groups can affect the activity of these compounds (45). In a study by Petschow et al. (61), *H. pylori* was more susceptible to monolaurin than a C12:1 monoacylglycerol, suggesting that saturation plays a role in the antimicrobial activity of these compounds. Furthermore, only the *cis* form of unsaturated fatty acids has been found to be bacteriostatic, not the *trans* form (45).

Members of the *Enterobacteriaceae* family are generally resistant to the bactericidal activities of fatty acids and monoacylglycerols at neutral pH (7). The antimicrobial activity of

various fatty acids and their monoesters specifically against Gram negative microorganisms, including *Salmonella* spp. and *E. coli* O157:H7, was evaluated by Altieri et al. (2). One observation from that study was that chain length influenced antimicrobial activity against Gram negative bacteria, with lauric acid and monolaurin being the most effective. The efficacy of these compounds also appeared to be species or strain dependent. Lauric acid and monolaurin effectively controlled *E. coli* O157:H7 and *Yersinia enterocolitica*, but only moderately controlled *Salmonella* spp. In addition, myristic acid (C14:0) and palmitic acid (C16:0) effectively inhibited *E. coli* O157:H7 in broth, but were not effective at inhibiting the growth of *Salmonella* spp. or *Y. enterocolitica*. Another significant observation was that at low concentrations, fatty acids and their monoglycerides inhibited Gram negative microorganisms in broth, but this effect declined after 24 h (2).

### **Lauric acid and monolaurin**

Lauric acid (C12:0) is a naturally occurring fatty acid found in coconut oil and other coconut products. It is also a major antimicrobial component of human breast milk (55) and has been extensively studied for its antimicrobial properties. Monolaurin, a monoester of lauric acid, has been recognized as a significant antimicrobial lipid since the 1960s (55) and is said to have a high potential for use in foods (44). Although both forms are antimicrobial, monolaurin is more active in inhibiting bacteria and viruses than lauric acid (55). Compared to Gram negative bacteria, monolaurin is normally more active against Gram positive bacteria. The use of monolaurin in a microemulsion system has been shown to inhibit the growth of *E. coli* (34). Raising the fat or carbohydrate content of a food system appears to decrease the effective inhibition concentration, which may be why high monolaurin concentrations ranging from 3,000 to 5,000 ppm have been used in meat or starch products (34).

## **Mode of action**

Lipids have the ability to disturb cellular membranes. The antibacterial effects of fatty acids are due to their undissociated forms and not their anions (44). As pH levels decrease, the concentration of the undissociated forms increase. The addition of acids such as citric acid in the food may remove a barrier in the cell wall outer membrane, allowing the lipids to penetrate the membrane and access the inner membrane. The interruption of cellular processes such as the electron transport chain or the inhibition of nutrient uptake and enzyme activity results in growth inhibition. When fatty acids are present in high concentrations, they have a detergent effect that can cause cell lysis (7).

## **Chocolate production**

Chocolate is a widely consumed confectionery commodity produced from the seeds of the cocoa tree, *Theobroma cacao*. The tree produces pods that contain pulp and beans. The beans are removed from the pod and fermented, dried, and roasted. Cracking and deshelling of the seeds yields cocoa nibs, which are considered pure chocolate in its rough state after grinding into a cocoa mass (6). Cocoa mass may be further processed into its two major constituents, cocoa solids and cocoa butter. These are the key components of formulated chocolate (1).

Sugar, milk, emulsifiers, flavor extracts and other ingredients are often added to cocoa solids and cocoa butter to produce items such as sweetened dark chocolate, milk chocolate, etc. Typical plain chocolate can consist of approximately 40% cocoa mass, 48% sugar, 12% additional cocoa butter, and minimal amounts of additional ingredients such as lecithin (36). In the United States, requirements for cocoa products are outlined in Title 21, Part 163 of the Code of Federal Regulations established by the Food and Drug Administration (FDA). The European Union allows chocolate processors to substitute 5% of the cocoa butter in chocolate with

alternative fats. The fats approved for this use are illipe, shea, sal, palm oil, mango kernel, and kokum gurgi.

### **Cocoa butter**

Cocoa butter is the pale yellow solid fat derived from the cocoa bean. It melts between 32 and 35°C and is hard and brittle at room temperature (6). Cocoa butter is composed of mostly SOS type triacylglycerols. The S refers to saturated fatty acids in the 1- and 3- positions on the glycerol moiety and the O refers to an oleoyl chain in the 2- position. These types of triacylglycerols make up approximately 70 to 90% of cocoa butter. The three main triacylglycerols in cocoa butter are 1, 3-dipalmitoyl-2-oleoyl-*sn*-glycerol (POP), 1-palmitoyl-2-oleoyl-3-stearoyl-*sn*-glycerol (POSt), and 1, 3-distearoyl-2-oleoyl-*sn*-glycerol (StOSt) (56). In terms of fatty acid composition, cocoa butter typically consists of 37% oleic acid, 33.6% stearic acid, 24.4% palmitic acid, 3.4% linoleic acid and 1.6% others (56). It should be noted that cocoa butter produced in different countries can have slight differences in composition.

Cocoa butter can crystallize in six polymorphic forms (I-VI), which are a function of the triacylglycerol composition. The principal forms are  $\alpha$ ,  $\beta$ , and  $\beta'$ , with the  $\beta$  form being the most desirable in a properly tempered chocolate (6). Cocoa butter contributes to the desirable snap in chocolate, melts completely in the mouth, and has a smooth texture. Its distinctive properties are due to its particular triacylglycerol composition (36). The presence of different types of fats used in the chocolate system could potentially change the physical properties of the product. For example, chocolate products may soften, bloom, lose gloss and color, snap, or contract as a result.

### ***Salmonella* spp. in chocolate processing**

Many steps throughout the chocolate-making process can present microbiological hazards. The fermentation and drying step for cocoa beans, for example, results in high microbial loads (6). Fermented, dried cocoa beans contain mostly members of the *Bacillus* genus (19). The possibility of the presence of *Salmonella* spp. exists at this step. Studies investigating the internal microflora of cocoa beans found yeasts, molds, and bacteria, with bacterial loads of approximately  $10^3$  to  $10^8$  CFU/g (19). Additional raw materials for chocolate manufacture such as nuts and dyes may also be contaminated. Heat treatments may not destroy *Salmonella* spp. that contaminate the product at the early stages of processing. Krapf and Gantenbein-Demarchi (51) determined that even a conching process using relatively high temperatures was inadequate at completely removing *Salmonella* cells. In another study, roasting was shown to be an ineffective means of destroying *Salmonella* cells (19). This emphasizes the importance of raw material inspection, which is the first line of defense for chocolate manufacturers (16). *Salmonella* spp. can survive for long periods of time in chocolate and are more resistant to heat in the product. Prolonged thermal processing may have deleterious effects on the organoleptic properties of chocolate, so the chocolate industry faces a challenge in ensuring both the safety and quality of its products.

In addition to insufficient control or handling of ingredients, contamination of the product may occur due to inadequate sanitation methods, poor facility and equipment design, poor maintenance, and lack of good manufacturing practices (GMPs) (62). The risk of *Salmonella* spp. in chocolate should be addressed with stringent food safety plans that incorporate GMPs and a Hazard Analysis and Critical Control Points (HAACP) approach (16).

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**CHAPTER 3**  
**INFLUENCE OF LIPIDS ON THE BEHAVIOR OF *SALMONELLA* SPP. IN**  
**LOW-MOISTURE COCOA-BASED MODEL FOODS**

**Introduction**

In the United States, an estimated 1.0 million illnesses and 378 deaths are associated with the consumption of foods contaminated with non-typhoidal *Salmonella* spp. each year (11). Salmonellae are widespread in nature and can contaminate a variety of foods. Of particular concern to the food industry is their ability to contaminate low-moisture, high-fat foods and survive for long periods of time. Even at low levels, the presence of *Salmonella* spp. can cause illness.

Chocolate and chocolate products have been associated with outbreaks of salmonellosis for decades (9). Some serotypes have survived in artificially contaminated chocolates after nine months of storage at 20°C (16) and in milk chocolate for over 19 months (15). Fat is thought to protect bacterial cells from the acidic conditions of the stomach, allowing them to eventually pass to the lower intestine and cause disease (18). The presence of fat may also help *Salmonella* cells survive over time in dry products.

Some fatty acids and their derivatives have been shown to possess antibacterial properties. The degree of unsaturation, hydrocarbon chain length, and presence of additional functional groups can affect the activity of these compounds (7). Lauric acid and monolaurin are antimicrobial lipids with a high potential for use in foods (6). Disruption of the cellular membrane is thought to be the main mechanism. Some lipids such as diacylglycerols and

triacylglycerols are naturally present in or added to foods but have not been shown to inactivate microorganisms (14).

There is a lack of quantitative data relating influence of specific lipids on the behavior of *Salmonella* spp. in low-moisture products. Such information could help food manufacturers perform risk assessments and improve *Salmonella* control measures by formulating safer products. In this study, monostearin, monolaurin, dilaurin, and trilaurin were substituted for portions of the natural cocoa fat in chocolate. The objective is to determine the influence of the different lipids on the behavior of *Salmonella* spp. in cocoa-based foods with a  $a_w$  level of 0.33 stored at 22°C for 24 weeks.

## **Materials and Methods**

The methods involving preparation of media and inoculum, moisture adjustment, sample inoculation, and sample packaging are based on those developed by Santillana Farakos et al. (11).

### **Raw materials**

Organic cocoa powder (Dagoba Organic Chocolate, Ashland, OR), organic cocoa butter (Tisano, Costa Mesa), and cocoa mass (Barry Callebaut, Wieze, Belgium) were screened in triplicate for the presence of *Salmonella* spp. using methods outlined in Chapter 5 of the Food and Drug Administration's Bacteriological Analytical Manual (FDA BAM). Monostearin (62.6%), monolaurin (> 98.0%), alpha, alpha'-dilaurin (> 96.0%), and trilaurin ( $\geq$  98.0%) were obtained from TCI America (Portland, OR).

### **Preparation of media**

Two different types of media were used for *Salmonella* enumeration to distinguish between healthy and injured cells. To recover both healthy and injured cells, tryptic soy agar

(TSA; Becton, Dickinson and Company, Sparks, MD) was supplemented with 3 g yeast extract (Becton, Dickinson and Company, Sparks, MD), 0.8 g ammonium iron (III) citrate (Sigma-Aldrich Co., St. Louis, MO) and 6.8 g sodium thiosulfate, 5-hydrate, crystal (Avantor Performance Materials, Center Valley, PA) per liter. The media was steamed to dissolve the agar component and then autoclaved at 121°C for 15 min. This formulation is hereby referred to as mTSA (modified TSA) or nonselective media. Yeast extract was utilized in order to provide nutrients and aid in cellular repair. Ammonium iron (III) citrate and sodium thiosulfate were used as differentiating ingredients, resulting in the formation of colonies with black centers due to the production of hydrogen sulfide (17).

To determine the level of healthy cells, 2.5 g of sodium desoxycholate were added per liter of mTSA after autoclaving and cooling to 50°C. This medium is hereby referred to as mTSA-D (modified TSA plus desoxycholate) or selective media. Sodium desoxycholate is a selective agent that inhibits Gram-positive bacteria and non-enteric Gram-negative bacteria (17). This ingredient also inhibits the growth of *Salmonella* cells that have incurred damage to their cellular membranes. The cells that grow on this medium can be considered healthy. The level of injured cells was calculated using the colony counts from mTSA and mTSA-D originating from the same sample with the following formulas:

- (1)  $\text{mTSA (CFU/g)} - \text{mTSA-D (CFU/g)} = \text{sublethally injured cells (CFU/g)}$
- (2)  $[\text{sublethally injured cells (CFU/g)} / \text{mTSA (CFU/g)}] * 100 = \text{sublethally injured cells as percentage of entire population (\%)}$

### **Preparation of salt solution**

Magnesium chloride hexahydrate (Fisher Scientific, Fair Lawn, NJ) was placed in vacuum desiccators (Thermo Scientific, Rochester, NY). Distilled water was added in increments until at

least 25% of the mixture was free liquid. At 21°C, the equilibrium relative humidity of this salt solution is approximately 33% (12).

### **Sample formulation and moisture adjustment**

Samples were prepared in 50 g batches according to the formulations listed in Table 3.1. For samples 3, 4, 5, and 6, the test lipids were first melted individually in glass beakers. Cocoa butter was mixed in next, followed by cocoa powder. Sterile wooden rods were used to stir the samples until evenly mixed. Sample 2 was melted individually in a glass beaker. Immediately after heating and mixing, all samples were covered in foil and cooled on ice for 5 min. After cooling, the samples were placed in the previously prepared desiccators, vacuum sealed, and stored in the dark for 24 h at 22°C. Due to its low fat content, sample 1 did not require heating and cooling and was placed directly in a prepared desiccator.

Following the 24 h storage period, samples 2 through 6 were ground with a sterile metal rod (6 mm in diameter). All samples (1 through 6) were then distributed in 5 g portions on weigh boats, moved back to the vacuum desiccators, vacuum sealed, and stored at 22°C away from light. Samples were allowed to equilibrate to a water activity ( $a_w$ ) level of approximately 0.33 for at least 30 d. The adjusted level was measured using an AquaLab 4TE water activity meter (Decagon Devices, Inc., Pullman, WA).

### ***Salmonella* spp.**

Four serovars of *Salmonella enterica* were used in this study. Each serovar was previously implicated in outbreaks in low-moisture foods: *Salmonella* Agona (isolated from dry cereal), *Salmonella* Montevideo (isolated from pistachios), *Salmonella* Tennessee (isolated from peanuts), and *Salmonella* Typhimurium (isolated from peanuts). All cultures were obtained from the Food Science and Technology Department at the University of Georgia.

### **Preparation of inoculum**

Stock cultures were maintained on cryogenic beads at -80°C. To activate, 10 µl of each isolate was inoculated into 9 ml of tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD) and incubated at 37°C for 24 h. A 1 ml portion from each tube was transferred to 9 ml of TSB and incubated at 37°C for 24 h. A 3 ml portion from each tube was then transferred to 225 ml of TSB and incubated at 37°C for 24 h. The resulting cultures were centrifuged (Forma Scientific, Inc., Marietta, OH) at 2784 x g for 30 min at 21°C in sterile 500 ml centrifuge bottles. The supernatant fluid was discarded and each pellet was resuspended in 2 ml of 1% peptone (Becton, Dickinson and Company, Sparks, MD). The suspensions were poured separately into weigh boats, placed in vacuum desiccators containing anhydrous calcium sulfate (W.A. Hammond DRIERITE, Co. LTD, Xenia, OH), and dried for a minimum of 48 h. The dried cells were ground to a powder using a sterile metal rod and combined in equal amounts to prepare a four strain cocktail.

### **Preparation of samples**

For one replicate of the experiment, four uninoculated samples (negative controls) and eleven inoculated samples were prepared for each formulation using the moisture-adjusted samples. For non-inoculated control samples, 0.05 g portions of peptone powder (Becton, Dickinson and Company, Sparks, MD) were placed in the pouches prior to the addition of the moisture-adjusted samples. For inoculated samples, 0.05 g portions of the dry inoculum were placed in metal retort pouches, followed by the addition of the moisture-adjusted samples. Pouches were gently tapped to guide samples to the bottom and mix the contents. To evenly incorporate the inoculum or peptone powder into the sample, pouches were heated for 1 min in a water bath held at 66°C and massaged with metal tongs. The pouches were then placed on ice for 15 s and immediately dried,

placed in quart size FoodSaver Heat Seal Pre-Cut Bags (Sunbeam Products, Inc., Boca Raton, FL) and vacuum sealed using a FoodSaver GameSaver® Silver vacuum sealer (Sunbeam Products, Inc., Boca Raton, FL). Care was taken to avoid letting water enter the pouches during the heating and cooling steps. An impulse sealer (PackworldUSA, Nazareth, PA) was used to thermally seal the metal pouch through the FoodSaver bag (180°C for 2 s). The resulting sample pouches were stored at 22°C in sealed desiccators containing the saturated salt solution for various lengths of time.

### **Recovery of *Salmonella* spp.**

*Salmonella* spp. in inoculated samples were enumerated on days 0 (immediately after inoculation and vacuum sealing), 14, 28, 84, 112, 140, and 168 of the study. Negative controls were enumerated on days 3 and 168. Samples were removed from the pouches using a sterile metal spatula and washed twice with sufficient 1% peptone (Becton, Dickinson and Company, Sparks, MD) prewarmed to 37°C to produce a 1:100 dilution. The diluted samples were stomached for 1 min at 270 rpm using a Stomacher® 400 Circulator (Seward Limited, Worthing, West Sussex, UK). Serial dilutions (1:10) were performed with 1% peptone. Samples were surface plated (0.1 ml in duplicate or 0.25 ml in quadruplicate) on selective and non-selective media. Plates were incubated at 37°C for 48 h before colonies were counted.

### **Measurement of water activity ( $a_w$ )**

The  $a_w$  of each formulation was measured at 25°C prior to mixing the sample with peptone or inoculum on day 0. Negative controls were used to measure  $a_w$  on day 3 to determine equilibrium  $a_w$  and on days 84 and 168 to observe any changes in  $a_w$  during storage.

## **Statistical analysis**

The experiment was replicated three times. Final counts were analyzed after  $\log_{10}$  transformation using the analysis of variance (ANOVA) approach in Minitab 16 (Minitab Inc., State College, PA). Significant differences in means ( $p < 0.05$ ) were separated using Tukey's multiple comparison method.

## **Results**

### **Analysis of raw materials and control samples**

Samples of cocoa powder, cocoa butter, and cocoa liquor did not test positive for the presence of *Salmonella* spp. Plating of all uninoculated control samples on days 3 and 168 of the experiment yielded levels of  $<100$  CFU/g on non-selective media.

### **Measurement of water activity ( $a_w$ )**

Upon receipt, the  $a_w$  of the cocoa powder was  $0.42 \pm 0.01$  and the  $a_w$  of cocoa liquor was  $0.45 \pm 0.02$ . The equilibrated  $a_w$  levels of uninoculated control samples are presented in Table 3.2. The  $a_w$  was similar for all samples at each time point.

### **Enumeration of *Salmonella* spp. in inoculated samples**

Results of the population study in samples inoculated with *Salmonella* spp. are presented in Tables 3.3 and 3.4. When population means at all time points were combined, *Salmonella* in the monolaurin formulation had significantly lower counts than in cocoa powder when using selective and non-selective media ( $p < 0.05$ ). From days 0 to 168, *Salmonella* spp. in cocoa powder declined from 9.38 to 9.20 log units in non-selective media (Table 3.3) and from 9.04 to 8.79 log units in selective media (Table 3.4). In the monolaurin formulation, *Salmonella* spp. declined from 9.04 to 8.88 log units when determined using non-selective media (Table 3.3) and from 8.66 to 8.52 when determined using selective media (Table 3.4).

The initial population of *Salmonella* in the monolaurin formulation was significantly lower than in cocoa powder when determined using non-selective media, but not when using selective media. No significant difference was found between initial population levels in cocoa powder and the monostearin, dilaurin, trilaurin, or cocoa liquor formulations when using either culture medium. After 168 days of storage the populations of *Salmonella* in the monolaurin formulation were significantly lower than in cocoa powder when determined using non-selective media (Table 3.3.), but not when using selective media (Table 3.4). Additional comparisons of population levels in the various formulations after 14, 28, 84, 112, and 140 days of storage are presented in Tables 3.3 and 3.4. Populations of *Salmonella* in the monostearin, dilaurin, trilaurin, and cocoa liquor formulations were not significantly different than those in cocoa powder over time.

Non-selective media provided significantly higher *Salmonella* counts than did selective media ( $p < 0.05$ ). Sublethally injured cell populations are presented as a percentage of total recovered populations in Table 3.5. No significant difference was found between the percentage of sublethally injured cells in cocoa powder and the monostearin, monolaurin, dilaurin, trilaurin, and cocoa liquor formulations.

## **Discussion**

Pure cocoa liquor, made from deshelled cocoa beans, is naturally comprised of approximately 50% fat. In this experiment, model foods were formulated by substituting portions of the cocoa fat with monostearin, monolaurin, dilaurin, or trilaurin and the influence of these lipids on the behavior of *Salmonella* spp. at 22°C over 168 days was evaluated.

*Salmonella* spp. cannot grow at  $a_w$  levels below 0.93 but can survive for long periods of time in dry foods (9). This was observed in the present study, which is in agreement with other

reports of long term survival in chocolate and other low-moisture products (3, 16, 15, 5, 4). The history of the inoculum is one important consideration when making comparisons with the results of other studies. The slow decline over time in *Salmonella* populations in all samples, including low fat cocoa powder, can largely be attributed to the desiccation of cells prior to use. This process of desiccation injures cells and may inactivate less stress tolerant cells in the culture, resulting in a higher level of cells in the inoculum that are more tolerant to stress. This increased tolerance helps account for the resilience of *Salmonella* populations under the experimental conditions. In chocolate processing environments, dehydrated, injured cells may reside on equipment and other surfaces and be present in raw materials. The desiccation of cells prior to the start of the study simulates this scenario.

Monostearin and monolaurin are monoacylglycerols containing a long chain saturated fatty acid (stearic acid, C18:0) and a medium chain saturated fatty acid (lauric acid, C12:0), respectively. Dilaurin and trilaurin are di- and triesters of lauric acid. Lauric acid has been recognized for its antimicrobial properties and is more active against bacteria in its monoester form (8). Monolaurin was therefore expected to cause a greater decline in *Salmonella* spp. compared to the other lipids. The effect of unsaturation, which increases the bioactivity of fatty acids (7), was not evaluated in this study.

The higher overall levels of *Salmonella* spp. in non-selective media compared to selective media indicates the presence of sublethally injured cells. The lack of decline in levels of healthy cells for all samples, as shown in Table 3.4, indicates that healthy cells were unaffected by the lipids throughout the storage period. The population differences observed in the non-selective media between monolaurin and cocoa powder (Table 3.3) suggest that monolaurin lethally injured a greater number of cells. These cells likely incurred membrane damage during the

desiccation process, making them more susceptible to the compound. It appears that monolaurin effectively inactivated some *Salmonella* spp. at the start of the experiment but did not exert a strong antimicrobial effect with time. In fact, the population on day 168 may reflect an initial reduction in cells by monolaurin and not an effect over time. These results are similar to those of Altieri et al. (1), who observed a moderate control of *Salmonella* by low concentrations of lauric acid or monolaurin (20 ppm) in broth at 37°C. Inhibition was highest initially within 10-24 h, followed by a more steady inhibition of growth for 96 h. Although the study involved the growth of *Salmonella* spp. in a liquid medium for a relatively short period of time, their results are similar in that the presence of monolaurin caused an initial decline followed by a moderate inhibition. It is possible that the brief heat step during sample preparation enhanced interaction of monolaurin with sublethally injured *Salmonella* cells, which may explain the initial decline in *Salmonella* populations. Subsequent cooling and crystallization of the fat would allow less interaction with cells, resulting in a decrease in antimicrobial activity.

Chain length and molecular weight of lipids did not influence the behavior of *Salmonella* spp. Altieri et al. (1) reported that myristic acid (C14:0) and palmitic acid (C16:0), as well as their monoacylglycerols, did not affect the growth of *Salmonella* spp. in broth at 37°C. This suggests that monostearin, which is similar in structure to palmitin, may not affect the behavior of *Salmonella* spp. Karaba et al. (7) reported that stearic acid, 1,3-dilaurin, and trilaurin were not inhibitory in broth against various Gram positive microorganisms. *Salmonella* cells are surrounded by a lipopolysaccharide layer that affords protection against entry by various compounds (13) and they are therefore generally more resistant to the antimicrobial effects of lipids than Gram positive bacteria. This may explain the observation that no differences were found between *Salmonella* populations in cocoa powder and samples containing monostearin,

dilaurin, or trilaurin. The level of uninjured cells at the start of the study was unaffected by these compounds. The relative hydrophobicities of the compounds should also be noted.

Triacylglycerols are more hydrophobic than mono- and diacylglycerols, which contain hydrophobic and hydrophilic regions and are often used as emulsifiers to disperse fat and water molecules in foods. Water bound by lipids is unavailable to *Salmonella* cells at the molecular level, which may affect survival. The behavior of *Salmonella* in this study could not be related to lipid hydrophobicity; however, it is possible that differences in populations as a result of relative hydrophobicity may be observed with longer storage periods.

The high portion of added lipids used in this study does not simulate a realistic chocolate formulation. Therefore, the survival of *Salmonella* in cocoa liquor, also referred to as unsweetened baking chocolate, was determined. The survival of *Salmonella* spp. in this product did not differ from that observed in cocoa powder, meaning that the presence of fat did not influence the long term survival of the microorganisms at 22°C. A possible synergistic effect of fat level and  $a_w$  should be investigated. This information is useful in gaining a better understanding about the behavior of *Salmonella* spp. in controlled food systems in order to perform risk assessments and formulate safer foods.

Monolaurin, the only lipid to exert an influence on the cells in this experiment, is not an effective antimicrobial in low-moisture products stored at moderate temperatures; however, additional evaluation using higher holding temperatures and longer storage periods may be useful. It should be noted that low levels of *Salmonella* spp. have been recovered from low-moisture products associated with outbreaks of illness, but a high level of inoculum was used in this study. Additionally, the length of storage in the current study could be increased as chocolate products have a long shelf life.

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Table 3.1. Sample formulations used in this study, expressed as percent of total weight

Sample	Cocoa Powder (%)	Cocoa Butter (%)	Test Lipids (%)	Cocoa Liquor (%)
Cocoa powder	100	0	0	0
Cocoa liquor	0	0	0	100
Monostearin	50	25	25 (monostearin)	0
Monolaurin	50	25	25 (monolaurin)	0
Dilaurin	50	25	25 (dilaurin)	0
Trilaurin	50	25	25 (trilaurin)	0

Table 3.2. Water activity of uninoculated control samples stored at 22°C<sup>a</sup>

Sample	Storage time (days)		
	3	28	168
Cocoa powder	0.32 ± 0.05	0.37 ± 0.05	0.35 ± 0.06
Cocoa liquor	0.30 ± 0.05	0.38 ± 0.02	0.41 ± 0.01
Monostearin	0.28 ± 0.06	0.38 ± 0.02	0.40 ± 0.01
Monolaurin	0.29 ± 0.06	0.38 ± 0.02	0.39 ± 0.01
Dilaurin	0.28 ± 0.06	0.38 ± 0.01	0.38 ± 0.02
Trilaurin	0.29 ± 0.06	0.38 ± 0.02	0.39 ± 0.02

<sup>a</sup>Mean ± SD, n = 3. Mean values in the same column are not significantly different (p < 0.05).

Table 3.3. *Salmonella* spp. recovered using non-selective media<sup>a</sup> in products stored at 22°C<sup>b</sup>

Storage time (days)	Sample and population (log <sub>10</sub> CFU/g sample)					
	Cocoa powder	Cocoa liquor	Monostearin	Monolaurin	Dilaurin	Trilaurin
0	9.38 ± 0.01 a	9.36 ± 0.03 a	9.34 ± 0.07 a	9.04 ± 0.03 b	9.34 ± 0.04 a	9.36 ± 0.09 a
14	9.14 ± 0.03 ab	9.24 ± 0.04 a	9.25 ± 0.04 a	9.02 ± 0.09 b	9.16 ± 0.02 ab	9.18 ± 0.07 ab
28	9.15 ± 0.19 a	9.04 ± 0.07 a	9.05 ± 0.05 a	8.72 ± 0.42 a	9.01 ± 0.16 a	9.03 ± 0.14 a
84	9.00 ± 0.05 a	8.95 ± 0.05 a	9.15 ± 0.27 a	8.89 ± 0.06 a	8.92 ± 0.11 a	8.96 ± 0.05 a
112	9.04 ± 0.30 a	9.07 ± 0.24 a	9.02 ± 0.20 a	8.88 ± 0.26 a	9.02 ± 0.22 a	8.95 ± 0.03 a
140	8.92 ± 0.03 a	8.98 ± 0.10 a	8.96 ± 0.07 a	8.78 ± 0.05 a	8.89 ± 0.14 a	8.88 ± 0.22 a
168	9.20 ± 0.06 a	8.97 ± 0.13 ab	9.05 ± 0.07 ab	8.88 ± 0.11 b	9.09 ± 0.05 ab	9.07 ± 0.10 ab

<sup>a</sup>Trypticase soy agar modified with yeast extract, ammonium iron (III) citrate, and sodium thiosulfate

<sup>b</sup>Mean ± SD, n = 3. Mean values in the same row are significantly different if the letters that follow are different (p < 0.05).

Table 3.4. Uninjured *Salmonella* spp. recovered using selective media<sup>a</sup> in products stored at 22°C<sup>b</sup>

Storage time (days)	Sample and population (log <sub>10</sub> CFU/g sample)					
	Cocoa powder	Cocoa liquor	Monostearin	Monolaurin	Dilaurin	Trilaurin
0	9.04 ± 0.06	9.04 ± 0.07	8.89 ± 0.22	8.66 ± 0.20	8.99 ± 0.15	8.95 ± 0.17
14	8.73 ± 0.12	8.90 ± 0.05	8.80 ± 0.11	8.69 ± 0.10	8.74 ± 0.06	8.74 ± 0.08
28	8.60 ± 0.17	8.78 ± 0.08	8.81 ± 0.03	8.35 ± 0.62	8.62 ± 0.15	8.70 ± 0.21
84	8.72 ± 0.08	8.68 ± 0.15	8.87 ± 0.32	8.52 ± 0.11	8.53 ± 0.24	8.51 ± 0.08
112	8.72 ± 0.32	8.74 ± 0.25	8.60 ± 0.18	8.44 ± 0.31	8.57 ± 0.24	8.53 ± 0.10
140	8.59 ± 0.17	8.65 ± 0.09	8.60 ± 0.12	8.51 ± 0.11	8.50 ± 0.10	8.51 ± 0.20
168	8.79 ± 0.08	8.74 ± 0.06	8.69 ± 0.17	8.52 ± 0.12	8.66 ± 0.12	8.65 ± 0.23

<sup>a</sup>Trypticase soy agar modified with yeast extract, ammonium iron (III) citrate, sodium thiosulfate, and sodium desoxycholate

<sup>b</sup>Mean ± SD, n = 3. Mean values in the same row are not significantly different (p < 0.05).

Table 3.5. Sublethally injured populations of *Salmonella* spp. expressed as percentage of entire population<sup>a</sup>

Storage time (days)	Sample and sublethally injured cells (%)					
	Cocoa powder	Cocoa liquor	Monostearin	Monolaurin	Dilaurin	Trilaurin
0	53.2 ± 6.8	51.9 ± 3.6	61.3 ± 14.9	55.2 ± 17.2	53.0 ± 16.0	59.8 ± 11.4
14	59.9 ± 7.6	55.0 ± 3.0	63.8 ± 6.9	53.4 ± 2.5	60.9 ± 6.7	62.9 ± 2.7
28	70.2 ± 8.3	44.7 ± 4.1	43.4 ± 3.5	53.0 ± 18.2	58.1 ± 10.1	51.8 ± 12.6
84	46.7 ± 7.3	43.9 ± 18.8	47.6 ± 6.7	54.4 ± 17.1	58.0 ± 11.3	64.2 ± 6.3
112	52.0 ± 4.9	53.1 ± 5.1	61.3 ± 2.5	63.5 ± 5.6	64.0 ± 2.4	61.7 ± 6.2
140	48.7 ± 21.9	53.1 ± 7.2	55.4 ± 7.6	45.8 ± 7.7	58.6 ± 6.1	57.0 ± 2.9
168	58.8 ± 14.7	40.6 ± 10.3	55.5 ± 10.6	56.3 ± 7.9	61.7 ± 6.4	60.8 ± 10.2

<sup>a</sup>Mean ± SD, n = 3. Mean values in the same row are not significantly different (p < 0.05).

## CHAPTER 4

# INFLUENCE OF FAT LEVEL ON THE BEHAVIOR OF *SALMONELLA* SPP. IN COCOA-BASED MODEL FOODS AT THREE TEMPERATURES AND TWO WATER ACTIVITIES

### Introduction

Consumption of chocolate, a low-moisture, high-fat food, has been associated with salmonellosis outbreaks for decades. *Salmonella* spp., the bacteria responsible for the illness, grow optimally at water activity ( $a_w$ ) levels higher than or equal to 0.93 and at temperatures between 35 and 37°C (12). However, salmonellae may contaminate chocolate and chocolate products during various stages of the manufacturing process and survive for long periods of time. The low moisture level and high fat content is thought to impart a protective effect on the cells.

While the inherently low  $a_w$  of chocolate does not support the growth of *Salmonella* spp., growth in the product is not needed in order to cause illness in humans. Salmonellae in chocolate have been shown to exhibit thermal resistance. They may withstand typical heat treatments such as roasting and conching (10, 3). With added moisture, however, cells generally become more susceptible to heat treatments (2).

The survival and thermal inactivation of *Salmonella* spp. in chocolate and other low-moisture foods have been studied, but the effect of fat level on their behavior is yet to be elucidated. Moreover, because heat resistance changes with food formulation, relating *Salmonella* survival to products with controlled  $a_w$  and fat levels is of interest. This information will be helpful in improving *Salmonella* control measures used by the food industry. The

objective of this study is to determine the influence of 0%, 25%, and 50% added cocoa fat on the behavior of *Salmonella* spp. in cocoa-based foods with  $a_w$  levels of 0.33 and 0.43 stored at 21°C, 35°C, and 70°C. Total storage times vary from 24 h to 24 weeks.

## **Materials and Methods**

The methods involving preparation of media and inoculum, moisture adjustment, sample inoculation, and sample packaging are based on those developed by Santillana Farakos et al. (13).

### **Raw materials**

Organic cocoa powder (Dagoba Organic Chocolate, Ashland, OR), organic cocoa butter (Tisano, Costa Mesa), and cocoa liquor (Barry Callebaut, Wieze, Belgium) were obtained. Prior to use, each of these products was screened in triplicate for the presence of *Salmonella* spp. using methods outlined by Chapter 5 of the Food and Drug Administration's Bacteriological Analytical Manual (FDA BAM).

### **Preparation of media**

Selective and non-selective media were prepared as described in Chapter 3.

### **Preparation of salt solutions**

Saturated magnesium chloride hexahydrate solutions were prepared in vacuum desiccators as described in Chapter 3. Potassium carbonate (anhydrous, granular; J.T. Baker, Phillipsburg, NJ) solutions were also prepared. Distilled water was added in increments to the salt in a vacuum desiccator until at least 25% of the mixture was free liquid. At 21°C, the equilibrium relative humidity of this salt solution is approximately 43% (14).

## **Sample formulation and moisture adjustment**

Samples were prepared in 50 g batches according to the formulations listed in Table 4.1. For samples 2 and 3, cocoa butter was first melted individually in glass beakers. Cocoa powder was added and combined by stirring with a sterile wooden rod. Immediately after heating and mixing, the samples were covered in foil and cooled on ice for 5 min. After cooling, the samples were placed in the previously prepared desiccators containing saturated solutions of magnesium chloride hexahydrate or potassium chloride, vacuum sealed, and stored in the dark for 24 h at 21°C. Due to its low fat content, sample 1 did not require heating and cooling and was placed directly in a desiccator. Following the 24 h storage period, samples 2 and 3 were ground with sterile metal rods (6 mm in diameter). All samples were then distributed in 5 g portions to weigh boats, moved back to the appropriate desiccator, vacuum sealed, and stored at 21°C away from light. Samples were allowed to equilibrate to a  $a_w$  level of approximately 0.33 or 0.43 for at least 30 d. The water activity was measured using an AquaLab 4TE water activity meter (Decagon Devices, Inc., Pullman, WA).

## ***Salmonella* spp.**

The isolates used in this study were the same as described in Chapter 3: *Salmonella* Agona, *Salmonella* Montevideo, *Salmonella* Tennessee, and *Salmonella* Typhimurium.

## **Preparation of inoculum**

The inoculum was prepared as described in Chapter 3.

## **Preparation of samples**

For one replicate of the experiment, four uninoculated samples (negative controls) and ten inoculated samples were prepared for each formulation using the moisture-adjusted samples as described in Chapter 3. The sealed sample pouches were stored at 21 or 35°C in vacuum-

sealed desiccators containing the saturated solutions of either magnesium chloride hexahydrate or potassium chloride for various lengths of time. Samples used for 70°C experiments were stored in a preheated water bath.

### **Recovery of *Salmonella* spp.**

Sampling procedures previously described in Chapter 3 were used. Samples stored at 22°C were enumerated for *Salmonella* spp. on days 0 (immediately after inoculation and vacuum sealing), 14, 28, 84, and 168 of the study using the inoculated samples. Samples stored at 35°C were enumerated on days 0, 14, 28, 84, 140, and 168. For both temperature groups, controls were enumerated on days 3 and 168. Samples stored at 70°C were enumerated at 0 h (immediately after inoculation and vacuum sealing), 0.5, 1, 3, 10, and 24 h. Negative controls were enumerated at 0.5 and 24 h.

### **Measurement of water activity ( $a_w$ )**

The  $a_w$  of each formulation was measured at 25°C prior to mixing the sample with peptone or inoculum on day 0. For samples stored at 22 and 35°C, negative controls were used to measure  $a_w$  on days 3, 84, and 168 to observe any changes in  $a_w$  during storage. For samples stored at 70°C, negative controls were used to measure  $a_w$  at 0.5 and 24 h.

### **Statistical analysis**

The experiment was replicated three times. Final counts were analyzed after  $\log_{10}$  transformation using the analysis of variance (ANOVA) approach in Minitab 16 (Minitab Inc., State College, PA). Significant differences in means ( $p < 0.05$ ) were separated using Tukey's multiple comparison method.

## Results

### Analysis of raw materials and control samples

Samples of cocoa powder and cocoa butter did not test positive for the presence of *Salmonella* spp. Plating of all uninoculated control samples on days 3 and 168 of the experiment yielded levels of <100 CFU/g on non-selective media.

### Measurement of water activity ( $a_w$ )

The equilibrated  $a_w$  levels of uninoculated control samples are presented in Tables 4.2 – 4.4. At 70°C, samples previously adjusted to a target  $a_w$  of 0.33 had significantly lower measured  $a_w$  levels than those with a target  $a_w$  of 0.43 ( $p < 0.05$ ). At 22 and 35°C, no significant difference was observed between the two target  $a_w$  levels.

In regards to fat level, there was no difference in  $a_w$  between samples after 3, 28, and 168 days of storage at 22°C (Table 4.2). At 35°C, the 0% added fat samples had significantly lower  $a_w$  values than the 25 and 50% added fat samples on days 3 and 168 (Table 4.3). At 70°C, the 0% added fat samples had significantly lower  $a_w$  values than the 25 and 50% added fat samples after 24 h (Table 4.4).

### Enumeration of *Salmonella* spp. in inoculated samples

As shown in Tables 4.5 and 4.6, storage of *Salmonella* spp. in samples with different fat contents and target  $a_w$  levels for 168 days resulted in less than a 1 log unit reduction at 22°C when determined using non-selective and selective media. No difference in populations was observed between samples with 0, 25, and 50% added fat. However, when all time points were combined, populations were significantly higher overall in samples with target  $a_w$  levels of 0.33 compared to samples with target  $a_w$  levels of 0.43.

Tables 4.7 and 4.8 show a decline in *Salmonella* populations by approximately 1 log unit in all samples stored at 35°C for 168 days when determined using non-selective and selective media. No difference in populations was observed between samples with 0, 25, and 50% added fat. When all time points were combined, no population differences between samples with different target  $a_w$  levels were observed.

When all time points were combined at 70°C, *Salmonella* populations were significantly higher in samples with target  $a_w$  levels of 0.33 than in samples with target  $a_w$  levels of 0.43. Of the target 0.33  $a_w$  samples, significantly higher populations were observed in samples with 25 and 50% added fat than in those with 0% added fat using non-selective and selective media (Tables 4.9 and 4.10). However, no differences between *Salmonella* populations in the two higher fat level samples were observed. Differences in the level of uninjured cells recovered using selective media were observed after 0.5 h of storage, as *Salmonella* populations were significantly higher in samples containing 25 and 50% added fat than in those with 0% added fat (Table 4.10). For the target 0.43  $a_w$  samples, uninjured cell levels were only significantly different between the three samples after 0.5 and 24 h of storage (Table 4.10).

For each storage temperature, non-selective media provided significantly higher *Salmonella* counts than did selective media ( $p < 0.05$ ). Sublethally injured cell populations are presented as a percentage of total recovered populations in Tables 4.11 – 4.13. At 22 and 35°C, no significant differences were observed in the percentage of sublethally injured cells in any of the samples (Tables 4.11 and 4.12). At 70°C, higher percentages of sublethally injured cells were observed in the 0% added fat sample at 10 h compared to the 25% added fat sample in the target 0.33  $a_w$  group. In the target 0.43  $a_w$  group, higher percentages of sublethally injured cells were observed at 3 and 10 h in the 0% added fat sample than in the 25 and 50% added fat samples.

## Discussion

This study investigated the role of added fat on the behavior of *Salmonella* spp. in cocoa-based foods at different  $a_w$  levels and storage temperatures. Inactivation and sublethal injury were evaluated using selective and non-selective media. Some differences in *Salmonella* behavior with time were observed.

*Salmonella* spp. can survive for long periods of time in low-moisture, high-fat foods such as chocolate and peanut butter, and a greater heat resistance in these products compared to higher moisture products has been established. The greater survival at 70°C of *Salmonella* populations in samples with 25 and 50% added fat compared to samples with 0% added fat indicates a protective effect of fat. The fact that no difference was observed between the 25 and 50% added fat samples is important because it suggests that while the presence of fat is significant at 70°C, the level of fat is not.

An influence of added fat level on *Salmonella* populations in samples stored at 22 and 35°C may be elucidated with additional storage time and sampling, but no significant influence could be established with these data. These findings are important because they show that *Salmonella* spp. behave similarly in low-moisture cocoa-based products over 24 weeks despite the addition or presence of fat. In other words, at 22 and 35°C high fat cocoa products are equally supportive of *Salmonella* spp. survival as low fat cocoa products.

Krapf and Gatenbein-Demarchi (10) reported a higher heat resistance of *Salmonella* cells in cocoa liquor and dark chocolate than in cocoa butter alone at 50 and 60°C for 23 h. This difference was attributed to the higher level of solutes in cocoa liquor and dark chocolate, which may have allowed the liquid inoculum to better dissolve in the matrices and experience a decline in  $a_w$ . In addition, the sugar content in dark chocolate was offered as an explanation for cells

exhibiting greater heat resistance in this product versus cocoa liquor, which contains only cocoa powder and cocoa butter. The strain of *Salmonella* used in the experiments was not reported. This study offers a possible explanation for why there were no differences between *Salmonella* populations in samples with 25 and 50% added fat and why both groups imparted a higher degree of protection to cells than cocoa powder (0% added fat) alone, suggesting that a combination of fat and cocoa solutes results in a higher rate of cell survival than fat or solutes alone.

The long term survival of *Salmonella* spp. in chocolate and chocolate products has been previously studied. Tamminga et al. (15) reported declines of approximately 7 log units of *S. Eastbourne* in dark chocolate and approximately 3 log units in milk chocolate after nine months of storage at 20°C. The milk chocolate contained 40.6% sucrose, 25.0% cocoa butter, 14.9% whole milk powder (25% fat), 9.9% skim milk powder (1.0% fat), 9.1% cocoa mass (54.5% fat), and 0.5% lecithin. The dark chocolate contained 49.4% sucrose, 39.5% cocoa mass, 10.5% cocoa butter, and 0.6% lecithin. The milk chocolate therefore contained approximately 14% more total fat than dark chocolate. The lower survival of *S. Eastbourne* in dark chocolate was attributed to the higher amount of non-fat cocoa solids, which suggests that a higher level of fat in the presence of solutes enhances survival. This was not observed at 70°C in the present study, but a direct comparison cannot be made because the study included sugar and different fat sources. The use of whole milk powder appeared to enhance survival in products that contained sucrose and cocoa fat. The  $a_w$  of the chocolate bars was reported to be between 0.30 and 0.51, similar to the levels chosen for this study. Chocolate samples were melted at a temperature of approximately 40°C, inoculated with 24 h broth culture, mixed for 15 min, cast in molds, cooled at 4°C for 30 min, and wrapped before storage and sampling.

Jujena and Eblen (7) reported a protective effect of increasing levels of fat during thermal treatments of *Salmonella* Typhimurium DT104 in beef, with deviations from the first order kinetics. Based on their findings, the authors suggested that in order to achieve a 7 log unit reduction, contaminated products should be heated to an internal temperature of 58°C and that products with 7% fat be held for 53.5 min, while samples with 24% fat should be held for 208.1 min. The increase in heat resistance was attributed to the reduction of  $a_w$  in beef as fat content increased. Although the  $a_w$  of beef is much higher than the range used in the current study, results at 70°C followed a similar trend. Populations of *Salmonella* were higher when  $a_w$  was lower, and populations were higher overall in the two added fat samples compared to the 0% added fat samples.

*Salmonella* spp. do not grow in products with  $a_w$  levels below 0.93 (12), but may survive for long period of time in low-moisture environments. The enhanced heat resistance of *Salmonella* spp. with decreasing moisture levels has been well documented. Barrile and Cone (2) reported that the survival of *S. Anatum* in milk chocolate at 70°C significantly decreased with added moisture (1 to 4%) and Goepfert et al. (4) observed an enhanced heat resistance of eight *Salmonella* strains at 57.2°C with decreasing  $a_w$  levels of the heating menstruum ( $a_w$  0.87 to 0.99). The results at 70°C are in agreement with this trend, indicating significantly higher *Salmonella* populations in samples with target  $a_w$  levels of 0.33 than in samples with target  $a_w$  levels of 0.43.

It was necessary to analyze the  $a_w$  values of uninoculated control samples to determine if the samples with different target  $a_w$  levels had statistically different  $a_w$  values. The observed behavior of *Salmonella* spp. may be misleading if target  $a_w$  levels were not achieved in the samples. This may help explain the results at 35°C, where no statistical difference was observed

between the *Salmonella* populations in the target  $a_w$  0.33 and 0.43 samples. In this case, no significant difference could be established between the  $a_w$  levels of the uninoculated control groups and a conclusion on the effect of  $a_w$  cannot be drawn. It should be noted that the  $a_w$  at 35°C was lower for the controls containing 0% added fat than for controls with 25 and 50% added fat, which may have enhanced survival. At 22°C, although no statistical difference was observed in  $a_w$  levels between the uninoculated samples, *Salmonella* survival was greater in the lower target  $a_w$  samples. It is possible that the  $a_w$  levels were significantly different in the inoculated samples and influenced survival as expected, but in this case a conclusion cannot be made regarding the role of  $a_w$  at 22°C. At 70°C, *Salmonella* populations were influenced by  $a_w$ , which was significantly different in the controls as expected.

The desiccation of *Salmonella* cells prior to inoculation and storage in this study may explain the slow decline in population for each treatment. The history of inocula can affect subsequent cell behavior. For instance, Kirby and Davies (9) reported that dehydration of *S. Typhimurium* LT2 cells on hydrophobic membranes at 57% equilibrium relative humidity at 37°C for 48 h increased their resistance to heat treatments at 135°C. In another study (6), salmonellae were dried at 35°C for 24 h on paper discs and stored at various temperatures. At 4°C, cells experienced only a 0.54 log unit reduction after 22-24 months, demonstrating a high resistance in refrigerated storage temperatures. Gruzdev and Sela (5) reported an increase in resistance of desiccated *S. Typhimurium* to different stresses, including exposure to NaCl, bile salts, and ethanol. In particular, desiccated cells were more resistant than nondesiccated cells when exposed to dry heat. They experienced no significant population reduction when exposed to 60°C for 1 h, a 1.5 log unit reduction at 80°C for 1 h, and a 3.1 log unit reduction at 100°C for

1 h. On the other hand, desiccated cells declined as much as 3 log units at 60°C and 8 log units at 80 and 100°C.

Some *Salmonella* survival studies using chocolate and other low-moisture foods have utilized liquid inocula (15, 10, 8), in which initial rapid declines of the bacteria were attributed to osmotic shock. Studies have been conducted using lyophilized cells in an attempt to limit this effect (11, 2). The history of the inoculum should be taken into consideration before making a direct comparison between studies. The choice to stress cells by desiccation prior to inoculation was made to more closely resemble real world scenarios in which *Salmonella* spp. may become dehydrated in the environment and contaminate dry foods. This may occur in chocolate processing and packaging plants, which are low moisture environments where the cells may reside on equipment surfaces.

Processes aimed to reduce *Salmonella* spp. in food can produce cells with no injury, sublethal injury, or lethal injury (17). The lower populations of *Salmonella* spp. in selective media for all treatments in this study indicate that a significant number of survivors at each treatment were sublethally injured due to membrane damage. The highest percentages of injured cells were observed at 70°C, which may be attributed to irreversible membrane damage. Ribosomal injury has been reported as one major mechanism of inactivation at high temperatures and is mitigated by low  $a_w$  levels (1).

The amount of fat added to the cocoa samples did not affect *Salmonella* spp. at 22, 35, or 70°C. However, the combination of some fat and cocoa powder was observed to protect *Salmonella* at 70°C compared to cocoa powder alone, suggesting a synergistic role of fat and solutes. These results are significant for processors of chocolate and other low-moisture foods because they demonstrate the ability of *Salmonella* spp. to survive for long periods of time in

products in both low and high fat cocoa products, as well as their increased resistance to heat treatments such as conching in high fat products.

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Table 4.1. Sample formulations used in this study, expressed as percent of total weight

Sample	Cocoa Powder (%)	Cocoa Butter (%)
0% added fat	100	0
25% added fat	75	25
50% added fat	50	50

Table 4.2. Water activity of uninoculated control samples stored at 22°C<sup>a</sup>

Sample	Target $a_w = 0.33$			Target $a_w = 0.43$		
	Storage time (days)					
	3	28	168	3	28	168
0% added fat	0.32 ± 0.05	0.37 ± 0.05	0.35 ± 0.06	0.38 ± 0.01	0.33 ± 0.04	0.35 ± 0.02
25% added fat	0.31 ± 0.04	0.38 ± 0.03	0.40 ± 0.02	0.43 ± 0.03	0.32 ± 0.05	0.41 ± 0.03
50% added fat	0.29 ± 0.07	0.39 ± 0.02	0.41 ± 0.01	0.40 ± 0.01	0.32 ± 0.05	0.37 ± 0.01

<sup>a</sup>Mean ± SD, n = 3. Mean values in the same column are not significantly different ( $p < 0.05$ ).

Table 4.3. Water activity of uninoculated control samples stored at 35°C<sup>a</sup>

Sample	Target a <sub>w</sub> = 0.33			Target a <sub>w</sub> = 0.43		
	Storage time (days)					
	3	28	168	3	28	168
0% added fat	0.32 ± 0.01 b	0.32 ± 0.04 a	0.33 ± 0.04 b	0.36 ± 0.02 b	0.32 ± 0.03 a	0.33 ± 0.02 b
25% added fat	0.41 ± 0.02 a	0.36 ± 0.05 a	0.42 ± 0.02 a	0.43 ± 0.01 a	0.37 ± 0.06 a	0.43 ± 0.00 a
50% added fat	0.40 ± 0.00 a	0.36 ± 0.04 a	0.39 ± 0.00 ab	0.41 ± 0.01 ab	0.38 ± 0.04 a	0.40 ± 0.01 a

<sup>a</sup>Mean ± SD, n = 3. Mean values in the same column are significantly different (p < 0.05) if the letters that follow are different.

Table 4.4. Water activity of uninoculated control samples stored at 70°C<sup>a</sup>

Sample	Target a <sub>w</sub> = 0.33		Target a <sub>w</sub> = 0.43	
	Storage time (h)		Storage time (h)	
	0.5	24	0.5	24
0% added fat	0.33 ± 0.01 a	0.32 ± 0.01 b	0.36 ± 0.00 b	0.34 ± 0.01 b
25% added fat	0.36 ± 0.02 a	0.38 ± 0.00 a	0.38 ± 0.01 b	0.41 ± 0.01 a
50% added fat	0.39 ± 0.03 a	0.37 ± 0.02 a	0.42 ± 0.02 a	0.41 ± 0.02 a

<sup>a</sup>Mean ± SD, n = 3. Mean values in the same column are significantly different (p < 0.05) if the letters that follow are different.

Table 4.5. *Salmonella* spp. recovered using non-selective media<sup>a</sup> in products stored at 22°C<sup>b</sup>

Storage time (days)	Sample, a <sub>w</sub> , and population (log <sub>10</sub> CFU/g sample)					
	Target a <sub>w</sub> = 0.33			Target a <sub>w</sub> = 0.43		
	0% added fat	25% added fat	50% added fat	0% added fat	25% added fat	50% added fat
0	9.38 ± 0.01	9.32 ± 0.05	9.38 ± 0.10	9.31 ± 0.08	9.28 ± 0.14	9.23 ± 0.19
14	9.14 ± 0.03	9.14 ± 0.04	9.24 ± 0.08	9.07 ± 0.06	9.14 ± 0.18	9.03 ± 0.08
28	9.15 ± 0.19	9.24 ± 0.16	9.05 ± 0.06	9.08 ± 0.09	8.96 ± 0.12	8.98 ± 0.15
84	9.00 ± 0.05	8.92 ± 0.06	8.92 ± 0.13	9.03 ± 0.25	8.69 ± 0.16	8.82 ± 0.12
168	9.20 ± 0.06	9.09 ± 0.02	9.00 ± 0.12	8.95 ± 0.07	8.70 ± 0.16	8.76 ± 0.08

<sup>a</sup>Trypticase soy agar modified with yeast extract, ammonium iron (III) citrate, and sodium thiosulfate

<sup>b</sup>Mean ± SD, n = 3. Mean values in the same row (within the same a<sub>w</sub>) are not significantly different (p < 0.05).

Table 4.6. Uninjured *Salmonella* spp. recovered using selective media<sup>a</sup> in products stored at 22°C<sup>b</sup>

Storage time (days)	Sample, a <sub>w</sub> , and population (log <sub>10</sub> CFU/g sample)					
	Target a <sub>w</sub> = 0.33			Target a <sub>w</sub> = 0.43		
	0% added fat	25% added fat	50% added fat	0% added fat	25% added fat	50% added fat
0	9.04 ± 0.06	8.97 ± 0.24	8.98 ± 0.14	8.80 ± 0.08	8.78 ± 0.06	8.87 ± 0.14
14	8.73 ± 0.12	8.76 ± 0.03	8.83 ± 0.12	8.64 ± 0.05	8.64 ± 0.21	8.51 ± 0.04
28	8.62 ± 0.17	8.83 ± 0.09	8.61 ± 0.11	8.75 ± 0.03	8.74 ± 0.10	8.72 ± 0.16
84	8.72 ± 0.08	8.62 ± 0.04	8.64 ± 0.23	8.36 ± 0.17	8.32 ± 0.21	8.46 ± 0.05
168	8.95 ± 0.21	8.71 ± 0.13	8.67 ± 0.25	8.59 ± 0.08	8.48 ± 0.35	8.40 ± 0.07

<sup>a</sup>Trypticase soy agar modified with yeast extract, ammonium iron (III) citrate, sodium thiosulfate, and sodium desoxycholate

<sup>b</sup>Mean ± SD, n = 3. Mean values in the same row (within the same a<sub>w</sub>) are not significantly different (p < 0.05).

Table 4.7. *Salmonella* spp. recovered using non-selective media<sup>a</sup> in products stored at 35°C<sup>b</sup>

Storage time (days)	Sample, a <sub>w</sub> , and population (log <sub>10</sub> CFU/g sample)					
	Target a <sub>w</sub> = 0.33			Target a <sub>w</sub> = 0.43		
	0% added fat	25% added fat	50% added fat	0% added fat	25% added fat	50% added fat
0	9.22 ± 0.08	9.24 ± 0.13	9.27 ± 0.06	9.28 ± 0.14	9.21 ± 0.12	9.25 ± 0.07
14	8.93 ± 0.03	8.91 ± 0.06	8.88 ± 0.14	8.85 ± 0.08	8.77 ± 0.11	8.92 ± 0.04
28	8.84 ± 0.13	8.70 ± 0.03	8.72 ± 0.09	8.88 ± 0.15	8.55 ± 0.43	8.75 ± 0.24
84	8.38 ± 0.24	8.41 ± 0.48	8.42 ± 0.37	8.37 ± 0.29	8.26 ± 0.51	8.32 ± 0.51
140	8.55 ± 0.30	8.51 ± 0.43	8.46 ± 0.23	8.36 ± 0.33	8.26 ± 0.53	8.48 ± 0.20
168	8.27 ± 0.43	8.12 ± 0.56	8.38 ± 0.15	8.08 ± 0.35	7.86 ± 0.63	8.06 ± 0.29

<sup>a</sup>Trypticase soy agar modified with yeast extract, ammonium iron (III) citrate, and sodium thiosulfate

<sup>b</sup>Mean ± SD, n = 3. Mean values in the same row (within the same a<sub>w</sub>) are not significantly different (p < 0.05).

Table 4.8. Uninjured *Salmonella* spp. recovered using selective media<sup>a</sup> in products stored at 35°C<sup>b</sup>

Storage time (days)	Sample, a <sub>w</sub> , and population (log <sub>10</sub> CFU/g sample)					
	Target a <sub>w</sub> = 0.33			Target a <sub>w</sub> = 0.43		
	0% added fat	25% added fat	50% added fat	0% added fat	25% added fat	50% added fat
0	8.82 ± 0.08	8.85 ± 0.06	8.73 ± 0.07	8.81 ± 0.08	8.76 ± 0.06	8.70 ± 0.03
14	8.49 ± 0.20	8.31 ± 0.18	8.32 ± 0.14	8.45 ± 0.28	8.16 ± 0.13	8.37 ± 0.17
28	8.40 ± 0.15	8.33 ± 0.13	8.29 ± 0.05	8.56 ± 0.07	8.24 ± 0.44	8.30 ± 0.11
84	7.88 ± 0.34	7.87 ± 0.60	7.90 ± 0.43	7.56 ± 0.34	7.63 ± 0.63	7.67 ± 0.59
140	7.89 ± 0.35	7.82 ± 0.69	7.73 ± 0.33	7.45 ± 0.70	7.51 ± 0.86	7.72 ± 0.38
168	7.86 ± 0.53	7.83 ± 0.43	7.97 ± 0.21	7.53 ± 0.51	7.48 ± 0.67	7.80 ± 0.53

<sup>a</sup>Trypticase soy agar modified with yeast extract, ammonium iron (III) citrate, sodium thiosulfate, and sodium desoxycholate

<sup>b</sup>Mean ± SD, n = 3. Mean values in the same row (within the same a<sub>w</sub>) are not significantly different (p < 0.05).

Table 4.9. *Salmonella* spp. recovered using non-selective media<sup>a</sup> in products stored at 70°C<sup>b</sup>

Storage time (h)	Sample, a <sub>w</sub> , and population (log <sub>10</sub> CFU/g sample)					
	Target a <sub>w</sub> = 0.33			Target a <sub>w</sub> = 0.43		
	0% added fat	25% added fat	50% added fat	0% added fat	25% added fat	50% added fat
0	9.63 ± 0.23 a	9.65 ± 0.14 a	9.71 ± 0.17 a	9.43 ± 0.07 a	9.46 ± 0.11 a	9.48 ± 0.19 a
0.5	8.91 ± 0.04 b	9.51 ± 0.06 a	9.30 ± 0.12 a	8.65 ± 0.01 a	8.91 ± 0.16 a	8.98 ± 0.17 a
1	8.65 ± 0.13 b	9.09 ± 0.09 a	9.08 ± 0.14 a	8.60 ± 0.08 a	8.64 ± 0.32 a	8.92 ± 0.15 a
3	7.66 ± 0.28 b	8.41 ± 0.37 ab	8.67 ± 0.21 a	7.71 ± 0.25 a	8.11 ± 0.35 a	8.44 ± 0.20 a
10	6.99 ± 0.05 a	7.77 ± 0.51 a	7.83 ± 0.14 a	6.65 ± 0.30 a	7.49 ± 0.63 a	7.41 ± 0.58 a
24	5.22 ± 0.73 b	6.91 ± 0.38 a	6.64 ± 0.11 ab	5.29 ± 0.21 a	6.74 ± 0.41 a	6.55 ± 0.73 a

<sup>a</sup>Trypticase soy agar modified with yeast extract, ammonium iron (III) citrate, and sodium thiosulfate

<sup>b</sup>Mean ± SD, n = 3. Mean values in the same row (within the same a<sub>w</sub>) are significantly different if the letters that follow are different (p < 0.05).

Table 4.10. Uninjured *Salmonella* spp. recovered using selective media<sup>a</sup> in products stored at 70°C<sup>b</sup>

Storage time (h)	Sample, a <sub>w</sub> , and population (log <sub>10</sub> CFU/g sample)					
	Target a <sub>w</sub> = 0.33			Target a <sub>w</sub> = 0.43		
	0% added fat	25% added fat	50% added fat	0% added fat	25% added fat	50% added fat
0	9.09 ± 0.11 a	9.15 ± 0.07 a	9.11 ± 0.08 a	9.01 ± 0.11 a	8.99 ± 0.15 a	8.95 ± 0.11 a
0.5	8.35 ± 0.18 b	8.86 ± 0.10 a	8.85 ± 0.18 a	8.04 ± 0.06 b	8.50 ± 0.22 a	8.53 ± 0.01 a
1	8.04 ± 0.12 b	8.65 ± 0.08 a	8.56 ± 0.21 a	8.03 ± 0.14 a	8.14 ± 0.38 a	8.40 ± 0.17 a
3	6.85 ± 0.41 b	7.76 ± 0.31 ab	8.11 ± 0.09 a	6.93 ± 0.32 a	7.67 ± 0.36 a	7.91 ± 0.29 a
10	5.79 ± 0.23 b	7.15 ± 0.57 a	7.10 ± 0.12 a	5.60 ± 0.16 a	6.97 ± 0.67 a	6.95 ± 0.56 a
24	4.27 ± 0.51 b	6.12 ± 0.52 a	5.71 ± 0.09 a	4.09 ± 0.15 b	6.03 ± 0.30 a	5.78 ± 0.90 ab

<sup>a</sup>Trypticase soy agar modified with yeast extract, ammonium iron (III) citrate, sodium thiosulfate, and sodium desoxycholate

<sup>b</sup>Mean ± SD, n = 3. Mean values in the same row (within the same a<sub>w</sub>) are significantly different if the letters that follow are different (p < 0.05).

Table 4.11. Sublethally injured populations of *Salmonella* spp. expressed as percentage of entire population at 22°C<sup>a</sup>

Storage time (days)	Target a <sub>w</sub> = 0.33			Target a <sub>w</sub> = 0.43		
	Sample			Sample		
	0% added fat	25% added fat	50% added fat	0% added fat	25% added fat	50% added fat
0	53.2 ± 6.8	50.5 ± 21.5	58.9 ± 13.9	68.4 ± 5.5	66.6 ± 10.7	55.7 ± 7.9
14	59.9 ± 7.6	58.9 ± 0.8	60.2 ± 7.2	62.5 ± 2.8	67.2 ± 8.4	68.5 ± 7.5
28	70.2 ± 8.3	60.1 ± 11.3	63.4 ± 4.2	52.8 ± 5.6	39.6 ± 4.1	44.2 ± 12.9
84	46.7 ± 7.3	49.9 ± 3.2	46.2 ± 12.3	75.1 ± 13.6	55.7 ± 12.0	56.1 ± 9.0
168	58.8 ± 14.7	57.1 ± 10.1	52.1 ± 13.7	56.2 ± 6.1	32.2 ± 32.6	55.0 ± 10.6

<sup>a</sup>Mean ± SD, n=3. Mean values in the same row (within the same a<sub>w</sub>) are not significantly different (p < 0.05).

Table 4.12. Sublethally injured populations of *Salmonella* spp. expressed as percentage of entire population at 35°C<sup>a</sup>

Storage time (days)	Target a <sub>w</sub> = 0.33			Target a <sub>w</sub> = 0.43		
	Sample			Sample		
	0% added fat	25% added fat	50% added fat	0% added fat	25% added fat	50% added fat
0	57.8 ± 13.2	58.2 ± 6.9	71.2 ± 3.0	65.2 ± 7.0	64.2 ± 6.2	71.7 ± 2.0
14	60.9 ± 15.2	73.2 ± 10.6	71.8 ± 7.9	53.8 ± 24.35	74.8 ± 4.7	69.0 ± 11.7
28	62.0 ± 10.3	55.5 ± 9.0	62.8 ± 3.1	51.0 ± 12.4	50.0 ± 10.4	63.0 ± 9.9
84	67.2 ± 8.9	70.1 ± 8.3	69.4 ± 4.7	84.2 ± 2.3	74.3 ± 11.8	75.4 ± 11.4
140	77.2 ± 5.4	76.1 ± 11.4	80.2 ± 8.2	82.9 ± 11.4	77.3 ± 12.2	80.5 ± 9.2
168	58.8 ± 15.6	43.6 ± 24.6	60.3 ± 10.1	69.7 ± 11.9	57.2 ± 7.8	12.6 ± 82.1

<sup>a</sup>Mean ± SD, n=3. Mean values in the same row (within the same a<sub>w</sub>) are not significantly different (p < 0.05).

Table 4.13. Sublethally injured populations of *Salmonella* spp. expressed as percentage of entire population at 70°C<sup>a</sup>

Storage time (h)	Target a <sub>w</sub> = 0.33			Target a <sub>w</sub> = 0.43		
	Sample			Sample		
	0% added fat	25% added fat	50% added fat	0% added fat	25% added fat	50% added fat
0	70.2 ± 7.8 a	68.2 ± 6.3 a	74.3 ± 5.1 a	60.4 ± 10.0 a	65.4 ± 7.4 a	70.2 ± 4.8 a
0.5	68.6 ± 16.5 a	76.2 ± 7.8 a	63.1 ± 9.2 a	75.4 ± 4.0 a	60.7 ± 7.4 a	61.6 ± 14.7 a
1	75.6 ± 2.6 a	63.9 ± 2.5 a	68.9 ± 7.4 a	71.5 ± 8.6 a	67.6 ± 6.5 a	70.1 ± 4.0 a
3	83.3 ± 5.3 a	77.4 ± 2.8 a	71.2 ± 8.6 a	83.3 ± 3.3 a	62.9 ± 1.2 b	70.3 ± 6.0 b
10	92.8 ± 3.0 a	74.5 ± 7.1 b	81.4 ± 2.6 ab	90.1 ± 4.0 a	69.1 ± 4.0 b	65.5 ± 3.0 b
24	87.2 ± 6.8 a	82.9 ± 5.2 a	88.0 ± 1.4 a	91.9 ± 5.8 a	80.0 ± 5.3 a	82.1 ± 6.1 a

<sup>a</sup>Mean ± SD, n=3. Mean values in the same row (within the same a<sub>w</sub>) are significantly different if the letters that follow are different (p < 0.05).

## CHAPTER 5

### SUMMARY AND CONCLUSIONS

The objective of this work was to evaluate the effect of different lipids on the behavior of *Salmonella* spp. in low-moisture cocoa-based products at 22°C, as well as to determine the influence of fat level on *Salmonella* spp. at various  $a_w$  levels and storage temperatures. Samples were formulated with different lipid components, inoculated with desiccated *Salmonella* cells, and stored at 22, 35, or 70°C. *Salmonella* populations were enumerated over time.

Results demonstrate the long term survival of *Salmonella* spp. in low-moisture, high-fat foods, showing some cell inactivation by monolaurin but no effects by other acylglycerols of various chain lengths and molecular weights. A synergistic protective effect of cocoa fat and solutes on *Salmonella* populations was observed at 70°C and  $a_w$  0.33 and 0.43, but no effect of added fat could be established at lower temperatures.

Future studies should focus on extended storage periods to observe potential effects of treatments as prolonged dehydration stress may produce increased inactivation of *Salmonella* spp. These data are helpful in performing risk assessments and ultimately improving mitigation strategies designed to reduce the persistence of *Salmonella* spp. in low-moisture, high-fat foods; however, they may not be applied to different *Salmonella* strains or to products with different compositions.