Understanding the levels and behavior of *Salmonella* in naturally contaminated cashews and fermented cashew cheese analogs

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ABSTRACT

Between 2013 and 2021, six outbreaks of salmonellosis were associated with consumption of cashews and cashew cheese analogs, yet little is understood about the behavior of *Salmonella* in cashews or products made from them. The prevalence, levels, and types of *Salmonella* that may be associated with raw cashew kernels are largely unknown and growth of *Salmonella* has not been assessed during the fermentation of cashew cheese analogs. The nut-based dairy analog market is growing rapidly, and understanding the safety of nut-based products is crucial as consumers purchase and prepare fermented cheese analogs with increasing regularity.

In this thesis, the levels and distribution of *Salmonella* were determined in naturally contaminated cashew pieces used to prepare a cashew Brie analog associated with a 2020–2021 salmonellosis outbreak. Two unopened boxes from a single lot of cashew kernel pieces were each divided into seven approximately equal units and 10 50-g subsamples per unit were enriched for the presence of *Salmonella.* *Salmonella* was uniformly distributed throughout the boxes. *Salmonella* levels were 0.0023 MPN/g (95% confidence interval [0.0014, 0.0038]) using most probable number (MPN) estimations. Four *Salmonella* serovars were isolated from the cashew pieces: *Salmonella* Fresno, *Salmonella* Nima, *Salmonella* Urbana, and *Salmonella* Vinohrady. Two of these serovars, *Salmonella* Urbana and *Salmonella* Vinohrady, were in common with the outbreak-associated serovars linked to the 2020–2021 salmonellosis outbreak. *Salmonella* Fresno and *Salmonella* Nima were unique to the present study.

Following the recipe used by the implicated 2020–2021 cashew Brie analog producer, a fermented cashew cheese analog was prepared and *Salmonella* behavior was assessed. *Salmonella*-inoculated cashews were soaked under refrigeration for 24 h, then drained and blended to achieve a smooth paste. A commercial *Lactococcus lactis* starter culture and, in some cases, 1 or 2% NaCl or 0.5% citric acid were added to the cashew paste which was then held at ambient temperature for up to 72 h. Total aerobic bacteria counts, pH, and *Salmonella* levels were measured throughout the fermentation. Total aerobic bacteria (measured on M17 agar held at 30°C for 48 h) reached maximum levels of 9–9.5 log CFU/g after 48 h regardless of the presence of *Salmonella* or starter culture. The starting pH for the

cashew cheese analog was 6.0, with final pH between 4.5 and 5.0 by 72 h, regardless of the presence of starter culture. *Salmonella* levels increased significantly ($P \leq 0.05$) by 5.5–7.0 log when no starter culture was added to *Salmonella*-inoculated cashews. With starter culture, *Salmonella* levels increased significantly by 0.50–1.5 log; similar increases were observed when 1% or 2% NaCl was included. No significant change in *Salmonella* levels was observed with the addition of 0.5% citric acid. The findings from this work suggest that addition of starter culture and citric acid significantly inhibit the growth of *Salmonella* during cashew fermentation but additional control measures that exclude or reduce pathogens may be needed when preparing fermented cashew cheese analogs.

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Chapter 1: Introduction

Plant-based dairy analogs have risen in popularity over the past several years and represent a steadily growing industry, both globally and in the United States. Between 2021 and 2022, global plant-based cheese analog sales grew by 22% to a value of $869 million (Good Food Institute, 2022). This increase in plant-based cheese analog sales and consumption is driven by many factors that influence consumer decisions and behavior. The popularity of vegan and vegetarian diets, which may be followed for a variety of moral, health, environmental, or religious reasons (Rosenfeld & Burrow, 2017), plays a role in the increasing demand for plant-based alternatives. Despite the growing popularity of these types of products, research regarding the safety of plant-based cheese analogs is limited.

Tree nuts are a common raw ingredient used to prepare plant-based cheese analogs (Swinehart, Harris, Anderson, & Feng, 2023; Swinehart, Harris, Louvau, & Feng, 2023). Tree nuts, including almonds, cashews, pistachios, and walnuts, have been reportedly used by consumers as raw ingredients for preparation of nut-based cheese analogs, with cashews the most common (Swinehart, Harris, Anderson, & Feng, 2023). Cashews are an ideal ingredient for nut-based cheese analogs because of their mild flavor and creamy texture when soaked and blended. Cashew cheese analogs can be prepared with or without a fermentation step. Fermentations are characterized by the growth of desirable microorganisms and enzymatic conversion of the food components (Marco et al., 2021). This is often achieved by allowing a paste of cashews, water, and optional starter culture or added flavorings to ferment at room temperature or warmer for several hours up to several days (Swinehart, Harris, Louvau, & Feng, 2023). Many perceived health benefits are associated with fermented foods, so the creation of fermented nut-based cheeses that not only replicate the sensory profile of animal-based cheeses but have similar nutritional benefits is desirable. Fermented foods are believed to improve gut health, reduce disease risk, and enhance human health (Marco et al., 2021). Fermentation also extends the shelf-life of fresh ingredients and can improve flavor and texture. Because of the many benefits associated with fermented foods, creating fermented plant-based products is desirable for consumers and producers.

Although low moisture and fermented foods were once considered low risk as vehicles of foodborne illness, multiple outbreaks linked to tree nuts and tree nut products have been reported (Harris, Yada, Beuchat, & Danyluk, 2022), including six North American outbreaks linked to cashews between 2013 and 2021. In 2014–2015, cashew kernels were associated with an 18-case outbreak of *Salmonella* Stanley infections in six states (Centers for Disease Control and Prevention, National Outbreak Reporting System Dashboard). In 2015, a raw "sprouted" cashew-almond spread was the source of *Salmonella* Paratyphi B variant L(+) tartrate(+), which resulted in 13 reported cases of salmonellosis across 10 states (Centers for Disease Control and Prevention, 2016b; Heiman Marshall et al., 2018). In 2020, smoothies containing soaked cashews were linked to an 18-case outbreak of *Salmonella* Paratyphi B variant L(+) tartrate(+) illnesses in four states (Centers for Disease Control and Prevention, National Outbreak Reporting System Dashboard; Minnesota Department of Health, 2020).

Three salmonellosis outbreaks have been specifically linked to cashew cheese analogs. In 2013, a raw cashew cheese analog produced in California was associated with a 17-case outbreak of *Salmonella* Stanley infections (Centers for Disease Control and Prevention, 2014; Centers for Disease Control and Prevention, National Outbreak Reporting System Dashboard). In 2017, a fermented cashew nut-based cheese analog produced by a restaurant in British Columbia, Canada was associated with 23 cases of salmonellosis, with raw cashews identified as the source of the outbreak strain *Salmonella* Weltevreden (Schmitt, Yu, Greve, & McIntyre, 2018). In 2020–2021, 20 reported cases of salmonellosis (*Salmonella* Chester, Duisburg, Typhimurium, and Urbana) in four states were linked to consumption of a fermented cashew Brie analog produced in California from raw cashew pieces (Centers for Disease Control and Prevention, 2021; Lewis et al., 2023).

This thesis work focuses on the 2020–2021 salmonellosis outbreak linked to a cashew Brie analog. Raw cashew pieces associated with the outbreak were assessed for levels and distribution of *Salmonella* and several *Salmonella* serovars were identified. Separate cashews were purchased, inoculated with *Salmonella*, and used to prepare a fermented cashew cheese analog. The behavior of *Salmonella*, lactic acid bacteria starter culture, and pH were assessed.

Cashews

Over four million tons of cashews are produced annually worldwide, a portion of which are imported into the U.S. for direct consumption and further processing (International Nut and Dried Fruit Council, 2022; United Nations Conference on Trade and Development, 2021). Cashew fruit consists of the cashew apple, which is a fleshy stalk, and the cashew nut, which is attached to the lower end of the apple (Nogueira Oliveira, Gonçalves Mothé C., Gonçalves Mothé M., & Guimarães de Oliveira, 2019; Rosengarten Jr., 1984). When the cashew fruit is ripe, the fruit and nut fall from the tree to the ground, where the nuts are collected and separated from the fruit (Asogwa, Hammed, & Ndubuaka, 2008; Dendena & Corsi, 2014). Cashew nuts, which are surrounded by a testa, a papery skin, and a 5-mm thick shell, are then left to dry in the sun until a moisture content of approximately 8% is achieved (Fitzpatrick Jr., 2011; Asogwa, Hammed, & Ndubuaka, 2008; Azam-Ali & Judge, 2001; Rosengarten Jr., 1984). Following sun-drying, the cashew nuts are cleaned and sorted by size before the shelling and peeling processes begin. In-shell nuts are roasted, steam cooked, or immersed in a hot oil bath, depending on the production region, to remove toxic cashew nut shell liquid from the shell and for easier shell removal (Akinhami, Atasie, & Akintokun, 2008; Azam-Ali & Judge, 2001; Dendena & Corsi, 2014; Rosengarten Jr., 1984). The nuts are then shelled mechanically or by hand depending on the production region and facility size (Azam-Ali & Judge, 2001; Fitzpatrick, 2011; Rosengarten Jr., 1984).

The shelled cashews are dried again to loosen the testa, which is then removed by hand or using a mechanized system (Azam-Ali & Judge, 2001; Dendena & Corsi, 2014; Fitzpatrick, 2011). This drying process used to loosen the testa can involve dry heat or steam and may be referred to as blanching by cashew processors. Blanching is a validated antimicrobial control step for other tree nuts such as almonds when strict time and temperature guidelines are followed (Almond Board of California, 2007). However, for cashews, blanching is not intended as an antimicrobial step and is not a validated process control. Once the skins are removed, the kernels are sorted and graded based on size, quality, and color. Whole white cashew kernels are the highest quality cashews, whereas discolored or small pieces of broken

kernels are collected to be sold at a reduced price point (Dendena & Corsi, 2014; Fitzpatrick, 2011; Rosengarten Jr., 1984; United Nations Conference on Trade and Development, 2021).

Potential sources of bacterial contamination in the orchard include airborne dust, birds and domestic grazing animals, runoff water, fertilizer or soil amendments, and pathogen risk may increase with rainfall or due to cracking in the thin shell covering the kernel. With an intact shell, the kernels should be protected from contamination while on the tree, but once they fall to the orchard floor and the harvesting process begins, risks for contamination increase. In the processing facility, improper worker hygiene and surface sanitation may also lead to contamination (Wells, 2013).

Naturally contaminated low moisture foods

Salmonella outbreaks have been associated with several tree nuts and low moisture food sources, but naturally contaminated products are rarely available for evaluation. Levels of *Salmonella* have been determined in outbreak-associated low moisture foods, including alfalfa seeds (Inami and Moler, 1999), raw almonds (Danyluk et al., 2007), black pepper (Gustavsen and Breen, 1984), cake mix (Zhang et al., 2007), paprika (Lehmacher, Bockemühl, & Aleksic, 1995), and pine nuts (Wang et al., 2015), and, in all cases, were <0.1 MPN/g.

Populations of *Salmonella* were 0.028–0.093 MPN/g in naturally contaminated pine nuts associated with a 2011 salmonellosis outbreak when evaluated using five different pre-enrichment and four detection methods (Wang et al., 2015). *Salmonella* detection and isolation methods for recovery yield and *Salmonella* levels were evaluated for naturally contaminated alfalfa seeds linked to a 1999 salmonellosis outbreak (Inami and Moler, 1999; Inami, Lee, Hogue, & Brenden, 2001; Stewart, Reineke, Ulaszek, & Tortorello, 2001). *Salmonella* was isolated from naturally contaminated shredded alfalfa seeds at levels ranging from 0.00034–0.018 MPN/g (Inami, Lee, Hogue, & Brenden, 2001; Suslow, Wu, Fett, & Harris, 2002). Five *Salmonella* isolates were recovered from these naturally contaminated alfalfa seeds (*Salmonella* Cubana, Havana, Mbandaka, Newport, and Tennessee) (Inami, Lee, Hogue, & Brenden,

2001; Suslow, Wu, Fett, & Harris, 2002). Outbreak-associated cake mix was used to compare isolation procedures for *Salmonella* Typhimurium; levels of *Salmonella* were not determined (Zhang et al., 2007).

Preparation of cashew cheese analogs

Little is known about industrial-scale production of cashew cheese analogs, but some insight has been gained into consumer practices through consumer surveys and content analysis of online blogs and recipes (Swinehart, Harris, Anderson, & Feng, 2023; Swinehart, Harris, Louvau, & Feng, 2023). To prepare a fermented cashew cheese analog, cashews are first soaked in water to soften and hydrate. Cold or ambient temperature water is added to cover the nuts, which are then held at refrigeration or ambient temperatures for several hours to 1 day (Swinehart, Harris, Anderson, & Feng, 2023; Swinehart, Harris, Louvau, & Feng, 2023). Following soaking, the nuts are drained and blended to a smooth paste. At this step, a wider range of viable microorganisms or "starter cultures" can be added to the mixture, which is held, usually at ambient to warm temperatures, for several hours to days to ferment (BC Centre for Disease Control, 2017; Swinehart, Harris, Anderson, & Feng, 2023; Swinehart, Harris, Louvau, & Feng, 2023). Many consumer recipes recommend adding flavoring agents, such as sugar, salt, lemon juice, and apple cider vinegar, before or after fermentation. Online recipe blogs and videos provide a wide range of recommendations for soaking and fermenting nut-based cheeses (Swinehart, Harris, Anderson, & Feng, 2023; Swinehart, Harris, Louvau, & Feng, 2023). Variables such as time, temperature, ingredients, fermentation container, and storage conditions differ by recipe (BC Centre for Disease Control, 2017; Swinehart, Harris, Anderson, & Feng, 2023; Swinehart, Harris, Louvau, & Feng, 2023).

The small-scale producer implicated in the 2020–2021 salmonellosis outbreak linked with a cashew Brie analog soaked raw cashew pieces at refrigeration temperatures for 8 to 24 h, then drained the cashews and blended them to a smooth paste. A commercial freeze-dried mesophilic starter culture containing *Lactococcus lactis* strains was added to the paste, which was then held at ambient temperatures for 24 h to ferment. Following the bacterial fermentation, a commercial freeze-dried *Penicillium candidum* starter culture was added and the cheese analog was ripened under refrigeration for 1 week to

allow the mold rind to form. Cashew cheese analog preparation in Chapter 3 follows the bacterial fermentation process used by the implicated cashew Brie analog producer.

Starter cultures used for nut-based cheese fermentations

There is no standardized starter culture that is used for all nut-based cheese analog fermentations. Online recipes intended for consumers recommend a wide range of products including kefir, kombucha, commercially available lactic acid bacteria starter cultures, liquid from completed ferments, miso paste, probiotic capsules or powder, rejuvelac (a fermented liquid made by soaking seeds, such as wheat berries or quinoa, in water and fermenting for several days), and yogurt cultures (Swinehart, Harris, Anderson, & Feng, 2023; Swinehart, Harris, Louvau, & Feng, 2023). Very little is known regarding starter cultures used for industrial-scale production of fermented nut-based cheese analogs. Some plant-based cheese analog companies include live cultures in their ingredients list, which include rice miso, rejuvelac, and chickpea miso. A salmonellosis outbreak in 2018 was linked to a restaurant-prepared fermented cashew cheese which used rejuvelac as a source of live microorganisms (Schmitt, Yu, Greve, & McIntyre, 2018). These companies, and many others online, mention additional "cultures" in their ingredients that are not disclosed. It is difficult to know which types of microorganisms are being added to these fermented cheese analogs at an industrial scale. Some cheesemaking companies and starter culture companies offer vegan starter cultures for plant-based cheese and yogurt, which are made of bacterial strains grown on plant-based materials rather than lactose and other animal-derived ingredients. Starter cultures developed specifically for use with plant-based cheeses are not common and information is not available describing the formulation or efficacy of these cultures.

Many online consumer recipes recommend the use of probiotic capsules as a source of live microorganisms to start a nut-based cheese fermentation (Swinehart, Harris, Louvau, & Feng, 2023). Probiotic capsules available for purchase include mostly lactobacilli and bifidobacteria (Wang et al., 2020). Lactobacilli make up a large group of lactic acid bacteria that are commonly used in dairy cheese fermentations as either a starter culture or an adjunct. Lactic acid bacteria such as *Lactococcus lactis*,

Lacticaseibacillus casei, *Lactobacillus delbrueckii* subsp. *bulgaricus, Leuconostoc mesenteroides* subsp. *cremoris, Streptococcus thermophilus, Lactobacillus helveticus*, and *Pediococcus* species all can be used in dairy cheese fermentations and are common choices to create the desired sensory profile of a cheese (Boylston, Vinderola, Ghoddusi, & Reinheimer, 2004; Parente, Cogan, & Powell, 2017). Some of these strains, such as *L. casei*, are common in cheesemaking and can also be found in probiotic capsules (Wang et al., 2020). Bifidobacteria, the other type of bacteria commonly found in probiotic supplements, are anaerobes with a pH optimum of 6.5 to 7.0; they cannot survive well at pH below 4.5–5.0 (Boylston, Vinderola, Ghoddusi, & Reinheimer, 2004). Although *Bifidobacterium* has been added to fermented dairy products as a probiotic strain it is not commonly used as a starter culture for dairy cheeses. The environment must remain anaerobic and pH above 4.5–5.0 for the bifidobacteria to survive (Boylston, Vinderola, Ghoddusi, & Reinheimer, 2004; Shah, 2011).

Common practices among home fermenters include the use of brine from completed fruit and vegetable fermentations as a method of introducing bacteria to nut-based cheese analogs (Swinehart, Harris, Anderson, & Feng, 2023). Vegetable fermentations are usually dominated by lactic acid bacteria, which are required to lower the pH through the formation of lactic acid. The primary species of lactic acid bacteria present in most vegetable fermentations include *Leuconostoc mesenteroides*, *Pediococcus pentosaceus, Pediococcus acidilactici*, *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, and *Lactiplantibacillus pentosus* (Medina-Pradas, Perez-Diaz, Garrido-Fernandez, & Arroyo-Lopez, 2017). Each of these organisms survives best in slightly different acidity and salt levels in the ferment. Other types of organisms, such as acetic acid bacteria, yeasts, and molds, may also be present in vegetable fermentations and can create off-flavors or undesirable characteristics (Medina-Pradas, Perez-Diaz, Garrido-Fernandez, & Arroyo-Lopez, 2017). Vegetable fermentations do not usually rely on starter cultures and use the native microorganisms present on the produce to begin fermentation. Thus, the types of organisms present, their levels, and survival throughout fermentation will vary among different types of vegetables or even among different batches from the same type of vegetable (BC Centre for Disease Control, 2017). Using fermented liquid to initiate nut-based cheese fermentations offers limited control

and understanding of the types of microorganisms present and therefore opens the potential for variability in flavor and texture profile. The lack of control and reproducibility may not consistently provide a sufficient pH decline during nut-based cheese analog fermentation, which increases the risk of pathogen growth and survival if present at the beginning of fermentation.

Rejuvelac is a fermented liquid made by soaking seeds, such as wheat berries or quinoa, in water and fermenting for several days (BC Centre for Disease Control, 2017; Schmitt, Yu, Greve, & McIntyre, 2018). The liquid is recommended as a starter for nut-based cheese analog fermentations in many online resources and has also been used commercially to ferment cashew cheese in restaurants and small-scale production (BC Centre for Disease Control, 2017; Schmitt, Yu, Greve, & McIntyre, 2018; Swinehart, Harris, Louvau, & Feng, 2023). Soaking low moisture foods, such as tree nuts, grains, and seeds, poses a risk for pathogen growth (Emch, Burroughs, Gaspar-Hernandez, & Waite-Cusic, 2021; Feng, Lieberman, Jung, & Harris, 2020; Lieberman et al., 2023). After 24 h of soaking at ambient temperature, *Salmonella* levels in buckwheat, millet, and quinoa increased by 5.0, 3.5, and 4.0 log, respectively (Emch, Burroughs, Gaspar-Hernandez, & Waite-Cusic, 2021). Introducing rejuvelac creates an additional risk for pathogen contamination and growth, given the inherent risks associated with its preparation.

Miso is a fermented food that is used both commercially and on online recipe blogs as a source of microorganisms for nut-based cheese fermentations (BC Centre for Disease Control, 2017; Swinehart, Harris, Louvau, & Feng, 2023). Miso is a traditional Japanese fermented food made from soybeans (Allwood, Wakeling, & Bean, 2021). Miso undergoes two fermentations, the first of which is started by the addition of the mold *Aspergillus oryzae.* The second fermentation relies on yeast and lactic acid bacteria to produce the expected miso flavor. The types of microorganisms present throughout a miso fermentation differ from those found in cheese fermentations, partly due to the different indigenous organisms on soybeans and partly because of the starter cultures used. The most common organisms in a finished miso include *Aspergillus oryzae*, *Zygosaccharomyces rouxii* (both fungal), *Enterococcus faecium, Staphylococcus gallinarum,* and *Tetragenococcus halophilis* (Allwood, Wakeling, & Bean,

2021). Other bacteria belonging to *Lactiplantibacillus*, *Leuconostoc*, *Pediococcus*, and *Weissella* have also been identified (Onda et al., 2002).

Fermentations may also be conducted without the addition of a starter culture. Raw tree nuts have a natural microflora potentially capable of fermenting in favorable conditions. *Bacillus*, *Xanthomonas*, *Achromobacter*, *Pseudomonas*, *Micrococcus*, and *Brevibacterium* were identified on almond kernels (King, Miller, & Eldridge, 1970). Microorganisms belonging to *Pediococcus*, *Weissella*, or *Leuconostoc* genera have been identified in a spontaneously fermented cashew cheese analog (Tabanelli et al., 2018). During room temperature soaking and fermenting, these microorganisms associated with tree nuts can spontaneously ferment and lower the pH of the cashew cheese analog (Tabanelli et al., 2018). Spontaneous fermentations are not typically conducted at industrial scale because of the lack of control associated with the variability of indigenous microorganisms. Some consumers reported spontaneous fermentations that did not include any starter culture (Swinehart, Harris, Anderson, & Feng, 2023).

After a 2017 salmonellosis outbreak associated with restaurant-prepared cashew cheese analog (Schmitt, Yu, Greve, & McIntyre, 2018), the BC Centre for Disease Control published a set of guidelines for these products based on information available at that time (BC Centre for Disease Control, 2017). The guidance specifically recommends the use of commercially available lactic acid bacteria starter cultures and discourages against the use of probiotics, rejuvelac, miso, kombucha, yogurt, and vegetable ferment brine as a starter culture for fermenting nut-based cheeses due to their unpredictability and lack of reproducibility. The guidance specifies that a pH decline must occur in the first 24 h, achieving 4.4 or lower by 48 h, to consider the fermentation successful. A pH of 4.4 or lower reduces the risk of *Listeria monocytogenes* growth but is not low enough to inhibit the growth of *Salmonella* (National Advisory Committee on Microbiological Criteria for Foods, 2009).

Fermentation – microbial progression

Little is known about the microbial progression during the fermentation of cashew cheese analogs. Tabanelli et al. (2018) analyzed a spontaneous fermentation of a home-made cashew cheese

analog and an industrial-scale fermentation using lactic acid bacteria to start the fermentation. A spontaneous home-made fermented cashew cheese was prepared and allowed to ferment for 48 h. Lactic acid bacteria dominated the fermentation and increased to approximately 8–9 log CFU/g, with lower levels (2–3 log CFU/g) of yeasts, *Enterobacteriaceae*, and staphylococci. The pH of the home-made cheese dropped from 5.94 to 4.64 over a 48-h fermentation due to the formation of lactic and acetic acids from 6.55 to 107.46 mM and 0.83 to 13.82 mM, respectively. Sixty isolates of lactic acid bacteria were identified in the home-made cheese, all of which belonged to *Pediococcus*, *Weissella*, or *Leuconostoc* genera. At the beginning of fermentation, *L. mesenteroides* was most prevalent, followed by *Pediococcus pentosaceus* and *Weissella* spp. During the first 24 h of fermentation, the cashew mixture was dominated by *P. pentosaceus* and *Weissella* spp. along with lower levels of *Pediococcus acidilactici* and *L. mesenteroides*. After 48 h, *Pediococcus acidilactici* became more prevalent and the *Pediococcus* species made up approximately half of the lactic acid bacteria population. *Pediococcus* was the only genera to persist throughout a 10-day storage period (Tabanelli et al., 2018).

Fermentation – lactic acid formation

The conversion of lactose to lactic acid by lactic acid bacteria is an important step in dairy cheese fermentation and is important in lowering the pH of cheese (McSweeney, 2007). Lactose is not available in tree nuts as a reliable sugar source, so lactic acid bacteria must utilize sucrose (or fructose and glucose) as alternative sugar sources (Wojdyło et al., 2022). Lactic acid bacteria can use sucrose as a carbon source to form lactic acid (Bonestroo, Kusters, de Wit, & Rombouts, 1992; Wang et al., 2021). The formation of lactic acid is essential for lowering pH and creates the acidic flavor associated with cheese.

Consumers and commercial producers often choose to add flavoring agents, such as lemon juice and salt, to their fermented nut-based cheeses to enhance the flavor and more closely mimic dairy cheese acidity and flavor profile (BC Centre for Disease Control, 2017; Swinehart, Harris, Anderson, & Feng, 2023). Sugar has also been reported as a common ingredient in cashew cheese analogs (Swinehart, Harris, Anderson, & Feng, 2023; Swinehart, Harris, Louvau, & Feng, 2023). The addition of sugars prior to

fermentation is a potential option to increase the amount of fermentable substrate for lactic acid bacteria and lead to higher lactic acid production, thereby lowering pH. Such addition may allow for enhanced growth of pathogens (faster and to higher final populations) if present . At present, there is no published research examining the impact of adding sugar to fermented nut-based cheese analogs.

Summary

Cashews are the most commonly used tree nut for the production of nut-based cheese analogs. Raw cashews have been known to be contaminated with *Salmonella* and have been identified as the source of six salmonellosis outbreaks, three of which were associated with cashew cheese analogs. Online recipes provide a wide range of different methods for preparing fermented nut-based cheese analogs, which include different soaking procedures, starter cultures, fermentation times and temperatures, added ingredients, and storage. Understanding the fermentation of nut-based cheeses and the behavior of *Salmonella* and other pathogens throughout the fermentation is important for developing scientifically-based recommendations for home- and industrial-scale plant-based dairy analog production..

In the following thesis, Chapter 2 focuses on understanding levels and distribution of *Salmonella* in naturally contaminated cashews associated with a salmonellosis outbreak. *Salmonella* isolates were recovered and one of those isolates was used in preparation of a cashew cheese analog, as discussed in Chapter 3. The results in Chapter 2 provide insight into the levels of *Salmonella* present on cashews and emphasize the risks of processing raw cashews into a fermented cashew cheese analog.

In Chapter 3, a fermented cashew cheese analog was prepared using *Salmonella*-inoculated cashews and a lactic acid bacteria starter culture. *Salmonella* behavior with and without added starter culture was evaluated. Total aerobic bacteria counts, pH, and water activity throughout the fermentation are also assessed. Recipes often call for the addition of other acid sources, such as lemon juice or apple cider vinegar, to add acidity to nut-based cheeses for flavor, but addition of acid also lowers pH which is an important factor when considering the safety of nut-based cheese analogs. The impact of adding salt and citric acid to cashew cheese analogs prior to fermentation is assessed in Chapter 3.

Chapter 4 focuses on future research topics beyond this thesis. There are several different cashew cheese recipes with soaking, time, and temperature instructions that differ from the ones evaluated in the present study. Several different starter cultures are used regularly by consumers, none of which have been evaluated for their efficacy or ability to lower the pH during fermentation. As the nut-based cheese analog industry grows, it is crucial that research continues to expand on the work presented in this thesis to understand the safety risks associated with different starter cultures, tree nuts, and production processes utilized by home consumers and industrial-scale producers.

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Chapter 2: Levels and distribution of *Salmonella* in naturally contaminated cashews

ABSTRACT

Raw materials associated with foodborne illness outbreaks are rarely available for evaluation. The levels and distribution of *Salmonella* were determined in naturally contaminated raw cashews linked to a salmonellosis outbreak associated with a fermented cashew cheese analog. Two unopened 22.7-kg boxes from a single lot of cashew kernel pieces were each divided into seven approximately equal units, 14 in total. Three 10-g subsamples per unit (n = 21) were evaluated for aerobic plate count (APC), coliform counts, and *Escherichia coli* counts, and 10 50-g subsamples per unit (n = 70) were enriched for the presence of *Salmonella*. Presumptive *Salmonella*-positive colonies were confirmed using CHROMagar Salmonella and real-time PCR (i*nvA*) and then serotyped using antigenic methods and genome sequencing prediction tools. APC and coliform counts ranged from 1.81 to 5.47 (mean 2.44 ± 0.63) log CFU/g and 0.60 to 5.20 (mean 1.74 ± 0.80) log CFU/g, respectively. *Salmonella* was recovered from four units in Box 1 and all seven units in Box 2. One of the 10 subsamples was positive in all but four of the positive units; one (Box 1) and three (Box 2) units had two positive subsamples. The level of *Salmonella* in the two boxes combined was 0.0023 most probable number/g (95% confidence interval [0.0014, 0.0038]). *Salmonella* Urbana was isolated from three of five positive subsamples in Box 1 and eight of 10 positive subsamples in Box 2. *Salmonella* Fresno and Vinohrady were unique to single subsamples from Box 1, and *Salmonella* Nima was isolated from two subsamples from Box 2. Of the four serovars recovered, *Salmonella* Urbana and *Salmonella* Vinohrady were in common with outbreak-associated clinical or product isolates. Understanding the distribution and concentration of *Salmonella* in naturally contaminated cashews provides important information for hazard analysis and risk assessments for soaked and fermented cashew products.

Introduction

Cashew nuts (*Anacardium occidentale* L.) are an important crop grown primarily in Côte d'Ivoire, India, Cambodia, and Vietnam, with smaller harvests coming from Brazil, Indonesia, and other countries in Eastern and Western Africa (International Nut and Dried Fruit Council, 2022). Over four million tons of cashews are produced annually worldwide, a portion of which are imported into the U.S. for direct consumption and further processing (International Nut and Dried Fruit Council, 2022; United Nations Conference on Trade and Development, 2021). From 2015 to 2019, the United States represented 28% of global cashew imports and 30% of global import value, exceeded only by the European Union. Vietnam exported the most cashews to the U.S. out of all cashew-producing regions, with 133,000 metric tons of cashew kernel imports to the U.S. in 2019. Cashew kernel imports from Africa represented an annual average of 6,862 metric tons from 2010 to 2019 (United Nations Conference on Trade and Development, 2021).

Cashew kernels or products made from them have been linked to several reported salmonellosis outbreaks in Australia, North America, and Vietnam (Harris, Yada, Beuchat, & Danyluk, 2022b). Six North American outbreaks have been linked to products that incorporated cashew kernels. In 2014–2015, *Salmonella* Stanley was associated with an 18-case salmonellosis outbreak in six states that was linked to cashew kernels (Centers for Disease Control and Prevention, National Outbreak Reporting System Dashboard). In 2015, a raw "sprouted" cashew-almond spread was the source of *Salmonella* Paratyphi B variant L(+) tartrate(+), which resulted in 13 reported cases of salmonellosis across 10 states (Centers for Disease Control and Prevention, 2016b; Heiman Marshall et al., 2018). In 2020, smoothies containing soaked cashews were linked to an 18-case outbreak of *Salmonella* Paratyphi B variant L(+) tartrate(+) illnesses in four states (Centers for Disease Control and Prevention, National Outbreak Reporting System Dashboard; Minnesota Department of Health, 2020).

Three North American salmonellosis outbreaks have been associated with cashew cheese analogs (i.e., nondairy products made from cashews soaked in water, sometimes with other ingredients added). In 2013, *Salmonella* Stanley was associated with a 17-case salmonellosis outbreak in three states linked to

consumption of raw cashew "cheese" produced in California (Centers for Disease Control and Prevention, 2014; Centers for Disease Control and Prevention, National Outbreak Reporting System Dashboard). In 2017, a fermented cashew nut "cheese" produced by a restaurant in British Columbia was associated with 23 cases of salmonellosis, with raw cashews identified as the source of the outbreak strain *Salmonella* Weltevreden (Schmitt, Yu, Greve, & McIntyre, 2018). In 2020–2021, 20 reported cases of salmonellosis (*Salmonella* Chester, Duisburg, Typhimurium, and Urbana) in four states were linked to consumption of a fermented cashew Brie analog produced commercially in California from raw cashew pieces (Centers for Disease Control and Prevention, 2021; Lewis et al., 2023).

Raw cashews were identified as the source of the 2020–2021 outbreak associated with the cashew Brie analog (U.S. Food and Drug Administration, 2021a). The focus of the present study was to assess levels and distribution of *Salmonella* in the naturally contaminated cashew pieces that were linked to this outbreak.

Materials and methods

Cashews

Two 22.7-kg boxes of raw blanched organic cashew pieces (small, white) linked to a 2020–2021 salmonellosis outbreak associated with a fermented cashew Brie analog (U.S. Food and Drug Administration, 2021a) were received from the implicated processor on May 27, 2021, in their original packaging; the cashews were in a hermetically sealed plastic bag within a sealed cardboard box. Both boxes were from the same lot, packed on May 5, 2020, in Côte d'Ivoire. Upon receipt, the original packaging was opened aseptically, and the contents of each box were then divided into seven approximately equal units (~3.2 kg each), 14 units in total. The boxes were not mixed before dividing into separate units; each unit represented a different area in the original packaging. Units were transferred aseptically to separate 40.64 × 40.64 cm Bitran specimen storage bags (Fisher Scientific), which were labeled, and then stored at 4°C. The processor had originally received the boxes of nuts from the supplier in late December 2020 and held them under unspecified ambient storage conditions.

Microbiological analysis of cashews

Each cashew unit was mixed with a sterile scoop in the bag prior to sampling, and then three 10-g subsamples per unit were removed to determine, in triplicate, aerobic plate count (APC), fecal coliform counts, and *Escherichia coli* counts. Each 10-g subsample of cashew pieces was transferred to one side of a double-sided 207-mL Whirl-Pak filter bag (Nasco), and 20 mL of Butterfield's phosphate buffer was added to the bag; subsamples were mixed by massaging the bag by hand for 30 s, shaking for 30 s, and massaging again for 30 s. The mixture was allowed to stand for 3–5 min and then the bag was massaged again briefly by hand to resuspend the cashews. From each bag, 1-mL aliquots of the undiluted liquid were transferred to separate sterile microcentrifuge tubes. Two 250-µL and two 50-µL aliquots of the undiluted liquid were each plated onto plate count agar (PCA) and CHROMagar ECC (DRG International) by using an automated spiral plater (Autoplate 4000) and then plates were incubated for 24 ± 2 h at 35 ± 2°C for PCA or 42 ± 2°C for CHROMagar ECC. Unless otherwise specified, culture media were Difco brand (BD).

Enrichment for Salmonella

A modified U.S. Food and Drug Administration *Bacteriological Analytical Manual* (FDA BAM) method for nuts and nut meats was used to enrich *Salmonella* (Andrews et al., 2022). For each unit, cashew pieces were mixed with a sterile scoop, and then 10 50-g subsamples were aseptically transferred to one side of separate double-sided 1,630-mL Whirl-Pak filter bags (Nasco), and 450 mL of Universal preenrichment broth was added to each bag. Subsamples were mixed by hand massaging each bag for 60 s and then incubated at 35 ± 2°C for 24 ± 2 h.

After incubation, each preenriched subsample was hand massaged for 60 s, and then 1 mL and 0.1 mL were transferred to 10 mL of tetrathionate broth (TT) (Oxoid) and 10 mL of Rappaport-Vassiliadis broth (RV) (Oxoid), respectively. The tubes were vortexed and then incubated at 35 ± 2°C and 42 ± 2°C for TT and RV, respectively, for 24 ± 2 h. The enrichments were streaked onto Hektoen enteric agar (HE), xylose lysine desoxycholate agar (XLD), and bismuth sulfite agar (BSA) and incubated at 35 ±

2°C for 24 ± 2 h (HE and XLD plates) or at 35 ± 2°C for 48 ± 2 h (BSA plates). If present, two typical *Salmonella* colonies were selected from each enrichment and media combination (TT/HE, TT/XLD, TT/BSA, RV/HE, RV/XLD, RV/BSA), restreaked onto CHROMagar Salmonella plates (DRG International), and incubated at 37 ± 2°C for 24 ± 2 h.

Presumptive *Salmonella* isolates from the CHROMagar Salmonella plates were confirmed using real-time polymerase chain reaction (qPCR) with *invA* probe (6-FAM/TGGAAGCGCTCGCATTGTGG/8-QSY7) (Andrews et al., 2022; Rahn et al., 1992). Primers used were *SalINVF* (5'-AACGTGTTTCCGTGCGTAAT-3') and *SalINVR* (5'-TCCATCAAATTAGCGGAGGC-3'). An oligo stock assay was prepared using 18 µL of 100 µM *SalINVF* and *SalINVR*, 5 µL of 100 µM *invA* probe, and 59 µL of sterile ultrapure water (Milli-Q Advantage A10, MilliporeSigma). Each reaction included 8 µL of sterile ultrapure water, 1 µL of the prepared oligo stock, 10 µL of Luna Universal Probe qPCR Master Mix (New England BioLabs), and 1 µL of DNA. The qPCR run included a denaturation step at 95°C for 30 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s.

Due to limited time and resources, only those cashew units that yielded two positive subsamples in the first sampling (four in total) were reevaluated a second time at 24 weeks from the original enrichment date for that unit using the same procedure described above (n = 40).

Identification of Salmonella isolates

Confirmed *Salmonella* isolates were stored at –80°C in 15% glycerol and tryptic soy broth (EMD Millipore). Two isolates for each positive subsample were selected from different secondary enrichment and plating combinations. When available, isolates originating from TT/HE and RV/HE were serotyped. For five subsamples, all six isolates from each enrichment media and plate combination were serotyped. Selected *Salmonella* isolates were submitted to the California Animal Health and Food Safety Laboratory System (Davis, CA) for classical antigenic serotyping.

Further confirmation of *Salmonella* serovars was performed using the SeqSero2 serotype prediction tool (Zhang et al., 2019). One *Salmonella* isolate per positive subsample from the TT/HE combination was streaked onto tryptic soy agar (TSA: tryptic soy broth [TSB] plus 1.5% granulated agar), then incubated at 37°C for 24 ± 2 h. One colony per plate was inoculated into 2 ml of TSB and held at 37°C for 24 ± 2 h. *Salmonella* cells were collected by centrifugation at 18,210 ×*g* for 2 min. The supernatant was discarded and the DNA was extracted with the QIAamp DNA minikit (Qiagen) following the manufacturer's directions. DNA samples were sent to the UC Davis Genome Center DNA Technologies and Expression Analysis Core Laboratory (Davis, CA) for genome sequencing. The resulting sequencing reads were input into the GalaxyTrakr SeqSero2 v1.1.1 collection workflow, and *Salmonella* serotypes of each isolate were predicted (Morin et al., 2021). Sequences were submitted to he National Center for Biotechnology Information under BioProject ID PRJNA945503 (Supplemental Table S2.1).

Data analysis

A total of 10 50-g subsamples of cashews from each of the seven units from each box were analyzed during initial sampling (140 50-g subsamples). Four units, those which yielded two *Salmonella*-positive subsamples during the initial sampling, were reevaluated a second time at 24 weeks from the original enrichment date for that unit (40 50-g subsamples).

For APC and coliform counts, appropriate dilutions for each 10-g subsample were plated in duplicate at 50 µL and 250 µL. The plating volume that resulted in counts between 25 and 250 colonies was selected for use in population count analysis. When counts for both plates were below this range but countable, the count for the 250-µL plate was used to represent the greater volume of sample plated. Student's unpaired *t* test was performed for the APC and coliform counts between the two boxes.

Prevalence confidence intervals (95%) were calculated using the Clopper-Pearson exact model, available at https://epitools.ausvet.com.au/ciproportion. Populations of *Salmonella* were estimated using

the most-probable-number online calculator (https://mpncalc.galaxytrakr.org/), as advised in the FDA BAM (Blodgett, 2020; Ferguson & Ihrie, 2019).

Results

Microbial populations on cashews

The APC in Box 1 ranged from 1.94 to 5.47 log CFU/g (mean 2.56 ± 0.84 log CFU/g) and coliform counts ranged from 0.60 to 5.20 log CFU/g (mean 1.94 ± 0.98 log CFU/g) (Fig. 2.1). The APC in Box 2 ranged from 1.81 to 2.93 log CFU/g (mean 2.33 ± 0.28 log CFU/g) and coliform counts ranged from 0.60 to 2.74 log CFU/g (mean 1.54 ± 0.53 log CFU/g). Both boxes originated from the same lot, giving a combined mean APC of 2.44 ± 0.63 log CFU/g and mean coliform counts of 1.74 ± 0.80 log CFU/g. No significant difference was observed between the APC (P = 2.245) and coliform counts (P = 0.107) between Box 1 and Box 2. None of the subsamples were below the limit of detection for all plating volumes; however, two subsamples (one from Box 1 and one from Box 2) had counts at the limit of detection (0.60 log CFU/g) (Fig. 2.1). The high and low outliers for both APC and coliform counts were associated with different subsamples. *E. coli* levels were below the limit of detection by plating (0.60 log CFU/g) for all 42 samples evaluated.

Prevalence and levels of Salmonella on cashews

Salmonella was isolated from 11 of the 14 units (78.6%) from Boxes 1 and 2 (Fig. 2.2). Four out of the seven units (57%) in Box 1 yielded *Salmonella* isolates; one unit contained two *Salmonella*-positive subsamples while three units contained one positive subsample. All seven units in Box 2 (100%) yielded at least one *Salmonella*-positive isolate; three of the seven units contained two positive subsamples (Fig. 2.2). *Salmonella* was isolated from 5 of 70 (7.1%, with 95% confidence interval (CI) [2.4, 15.9]) subsamples from Box 1 and from 10 of 70 (14.3%, 95% CI [7.1, 24.7]) subsamples from Box 2, for a combined prevalence of 10.7% (95% CI [6.1, 17.1]; 15 positive of 140 subsamples).

Out of the 11 units that yielded positive subsamples during the initial sampling, the four units that yielded two positive subsamples were analyzed a second time at 24 weeks after the initial sampling date. Of these four units ([Box-Unit] 1-7, 2-1, 2-2, 2-5), two yielded no *Salmonella*-positive subsamples out of 10 ([Box-Unit] 1-7 and 2-5) and two yielded one *Salmonella*-positive subsample out of 10 ([Box-Unit] 2-1 and 2-2), for a total of two positive subsamples out of 40 total (5.0%, 95% CI [0.6, 16.9]; Fig. 2.2).

Salmonella was not recovered from all combinations of broth and plating media in all samples (Table 2.2). The TT/HE and TT/BSA combinations yielded *Salmonella* isolates for all 17 *Salmonella*-positive subsamples (15 from initial sampling and two from 24-week sampling). *Salmonella* isolates were obtained from all the media combinations containing TT broth, except for TT/XLD from Unit 6-Subsample 5 (1 out of 17, 5.9%). RV broth did not consistently yield *Salmonella* isolates. Five positive subsamples (29.4%) did not yield isolates from the RV/HE and RV/XLD media combinations ([Unit-Subsample] 3-5, 4-8, 5-1, 5-4, 6-5); four subsamples (23.5%) did not yield isolates from RV/BSA ([Unit-Subsample] 4-8, 5-1, 5-4, 6-5).

Levels of *Salmonella* in the naturally contaminated cashew pieces were estimated to be 0.0015 MPN/g (95% CI [0.00063, 0.0036]) in Box 1, and 0.0031 MPN/g (95% CI [0.0017, 0.0057]) in Box 2 for a combined level of 0.0023 MPN/g (95% CI [0.0014, 0.0038]) for the lot (Table 2.1). *Salmonella* levels at 24 weeks after the initial sampling date were 0.0014 MPN/g (95% CI [0.00035, 0.0056]) in Box 2 and 0.0011 MPN/g (95% CI [0.00026, 0.0042]) combined for Box 1 and Box 2.

Serotyping of Salmonella isolates

Four *Salmonella* serovars were isolated from the naturally contaminated cashew pieces (Table 2.2; Fig. 2.2). For each positive subsample, at least two isolates from different enrichment broth and media combinations were serotyped (Table 2.2). *Salmonella* serovars Fresno, Nima, Urbana, and Vinohrady were isolated. *Salmonella* Fresno and *Salmonella* Vinohrady were identified in one unit each (9.1%) from Box 1 and *Salmonella* Nima in two units (18.2%) from Box 2 (Fig. 2.2). *Salmonella* Urbana was identified in 10 of 11 positive units (90.9%) from Boxes 1 and 2. *Salmonella* Urbana was identified

in three of four positive units in Box 1; each of the seven positive units in Box 2 had at least one isolate from one subsample that was identified as *Salmonella* Urbana.

Six isolates (one from each enrichment/plating medium combination) were serotyped for three of the *Salmonella*-positive subsamples from Box 1 (subsample 2-3, 4-6, and 7-1). All six isolates were identified as *Salmonella* Urbana for Box 1-Unit 2 and Box 1-Unit 7 and as *Salmonella* Fresno for Box 1-Unit 4. Two units (Box 2-Unit 1 and Box 2-Unit 2), sampled after 24 weeks of storage, yielded one *Salmonella*-positive subsample each. All six isolates were serotyped for each unit and all were identified as *Salmonella* Urbana (Table 2.2 and Fig. 2.2).

Discussion

The present study evaluated cashew pieces linked to the 2020–2021 salmonellosis outbreak associated with a cashew Brie analog. Four *Salmonella* serovars were linked with outbreak-associated cases: *Salmonella* Chester, Duisburg, Urbana, and Typhimurium (Centers for Disease Control and Prevention, 2021; Lewis et al., 2023; U.S. Food and Drug Administration, 2021b) (Supplemental Table S2.1). *Salmonella* Chester and Urbana were recovered from several unopened packages of finished "cheese" that were clinically linked to the outbreak cases by whole-genome sequencing (Centers for Disease Control and Prevention, 2021; U.S. Food and Drug Administration, 2021b). *Salmonella* Urbana was the only serovar recovered from raw cashew pieces used to make the product (U.S. Food and Drug Administration, 2021b). *Salmonella* Vinohrady was isolated from products aging at the food production facility and in product packages of "cheese" purchased from retail locations but not from reported clinical isolates (U.S. Food and Drug Administration, 2021b). *Salmonella* Leiden was isolated from one of 51 environmental swabs collected from the processing facility but was not linked to any clinical isolates (U.S. Food and Drug Administration, 2021b).

Four *Salmonella* serovars were isolated from the naturally contaminated cashews in the present study: *Salmonella* Fresno, Nima, Urbana, and Vinohrady. *Salmonella* Urbana was the only serovar in common between the reported outbreak-associated serovars (Supplemental Fig. S2.1); *Salmonella*

Vinohrady was the serovar in common with product isolates (Supplemental Fig. S2.2). *Salmonella* Urbana and *Salmonella* Vinohrady collected during the current study were uploaded to the National Center for Biotechnology Information pathogen detection database and clustered with their corresponding outbreak-associated clinical and product isolates. *Salmonella* Fresno was in common with a clinical isolate of unknown source (Supplemental Fig. S2.3). During U.S. retail surveys of raw tree nuts carried out from 2014–2017, *Salmonella* serovars Brunei (2015), Give (2015), Mbandaka (2015), Nima (2014), and Weltevedren (2014) were isolated from raw cashews, with *Salmonella* Nima in common with the present study (Zhang et al., 2017; Zhang et al., 2021).

Multiple serovar outbreaks are less common than outbreaks linked to single *Salmonella* serovars. However, other tree nut-associated outbreaks have been linked to multiple serovars, including a 2009 pistachio-associated outbreak that was linked to *Salmonella* Montevideo, Newport, and Senftenberg (Centers for Disease Control and Prevention, 2009; Whitham et al., 2021) and a 2016 pistachio-associated outbreak that was linked to *Salmonella* Montevideo and Senftenberg (Centers for Disease Control and Prevention, 2016a).

Salmonella was isolated from one or two 50-g subsamples from 11 of 14 units in the two boxes of cashews evaluated in the present study, consistent with a uniform distribution of the organism. Of the 140 50-g subsamples (7,000 g in total), 15 were positive for *Salmonella*, a 10.7% defective rate among the 50-g subsamples analyzed. A calculated population of 0.0023 MPN/g translates to approximately two cells (1.73 cells) in a 750-g sample. Nuts are categorized as a Category II food, which requires 30 individual 25-g analytical units as a representative sample or two 375-g composites for a total of 750 g (Andrews and Hammack, 2022). Presuming a uniform and random distribution of *Salmonella* among the cashew pieces, a sample size of 750 g of cashews would have been at the limit of detection for *Salmonella*.

Following the modified FDA BAM (Andrews et al., 2022), two secondary enrichment broths were used in combination with three selective agars. Despite unequal isolation of *Salmonella* from the different media combinations (Table 2.2), the different enrichment media types did not select for different *Salmonella* serovars within a single subsample, likely due to the estimated low level of contamination.

Two colonies were selected from each enrichment combination, which lowers the chances of detecting multiple serovars from a single positive subsample; however, at a contamination level of 0.0023 MPN/g (one cell in every 10 50-g subsamples), more than one serovar per subsample would not be predicted (Table 2.2).

Low levels of *Salmonella* are consistent across all analyzed tree nuts (Harris, Yada, Beuchat, & Danyluk, 2022a). The mean level of *Salmonella* observed in the naturally contaminated raw cashew pieces in the present study is similar to *Salmonella* levels found in other retail surveys and outbreak-associated tree nuts. In two U.S retail surveys that included raw cashew kernels, *Salmonella* prevalence was 0.20% in 350-g samples with levels of <0.003 MPN/g (Zhang et al., 2021), and 0.55% with combined levels ranging from <0.003 to 0.092 MPN/g for cashews, hazelnuts, macadamia nuts, pecans, pine nuts, and walnuts (Zhang et al., 2017); levels of *Salmonella* in individual nut types were not reported. Where MPN estimates for *Salmonella* could be calculated in other raw tree nuts, levels have ranged from 0.008 to 0.18 MPN/g for almonds (Bansal et al., 2010; Danyluk et al., 2007), <0.01 to 0.23 MPN/g for Brazil nuts (Little, Rawal, de Pinna, & McLauchlin, 2010), 0.00092 to 0.037 MPN/g for in-shell hazelnuts (Letchworth, 2020), <0.003 to 0.75 MPN/g for macadamia nuts (Zhang et al., 2021), <0.0047 to 0.39 MPN/g for in-shell pecans (Brar et al., 2016), <0.003 to 0.43 MPN/g for shelled and in-shell pistachios (Harris et al., 2016; Zhang et al., 2021), and <0.003 to 0.092 MPN/g for in-shell walnuts (Davidson et al., 2015).

Recalled raw almonds associated with a 2000–2001 salmonellosis outbreak were evaluated for the presence of *Salmonella* in 100-g samples from 50 unopened 22.7-kg boxes of almonds from four production lots; the outbreak strain of *Salmonella* was recovered from all four lots at levels of 0.085 ± 0.013 MPN/g (Danyluk et al., 2007). Levels of *Salmonella* in the almonds at the time of the outbreak were estimated to be approximately 1 MPN/g (Lambertini et al., 2012) using predicted ambient temperature declines and assuming 6 months of storage.

Naturally contaminated outbreak-associated products are rarely available for analysis of levels and prevalence. Dry ingredients are more likely to be available during or after an outbreak investigation,

given their typically extended shelf life. *Salmonella* survives well in dried ingredients or foods including tree nuts (Podolak et al., 2010; Santillana Farakos, Frank, & Schaffner, 2013), especially when products are refrigerated or frozen (Beuchat and Mann, 2010; Brar et al., 2015; Kimber et al, 2012; Uesugi, Danyluk, & Harris, 2006), and thus the data collected, even weeks or months later, may inform exposure assessment (Lambertini et al., 2012; Santillana Farakos et al., 2017). In the present study, the cashews were packaged in May 2020 and held in ambient conditions until receipt in May 2021, after which the cashews were held at 4°C. The 24-week analysis was conducted nearly 2 years after the initial packing date of the cashews. *Salmonella* was detected in two of 10 subsamples in each of the four units initially and in one of 10 subsamples in two of the four units after 24 weeks of storage at 4°C. Although the prevalence of *Salmonella* was lower upon retesting, the sample size was insufficient to determine whether *Salmonella* levels had declined during refrigerated storage. There are currently no published data on the survival of *Salmonella* in cashews.

Large ranges in aerobic plate count and coliform counts were observed among subsamples from the same units and same boxes. The product evaluated in the present study was labeled as raw blanched organic cashew pieces (small, white) and contained different sizes of cashew kernels, broken pieces, and smaller nut pieces that looked like dust. During collection of this type of product, kernels are sorted manually based on size (Fitzpatrick, 2011; Rosengarten Jr., 1984). The extended collection period and numerous raw material sources that can contribute to a single lot of cashew pieces might explain the variability in background microbiota populations. This also might explain why multiple *Salmonella* serovars were isolated from the cashews, both in the present study and during the FDA outbreak investigation, despite low levels of *Salmonella* present.

Cashew fruits consist of an accessory fruit, the cashew apple, and the cashew nut, which is attached to the lower end of the apple (Nogueira Oliveira, Gonçalves Mothé, C., Gonçalves Mothé, M., & Guimarães de Oliveira, 2019; Rosengarten Jr., 1984). When the cashew fruit is ripe, the apple and nut fall to the ground, where they are dried in the sun before the nuts are manually separated from the apple and collected (Asogwa, Hammed, & Ndubuaku, 2008; Azam-Ali & Judge, 2001; Dendena & Corsi, 2014;

Fitzpatrick, 2011). In-shell nuts are roasted, steam cooked, or immersed in a hot oil bath, depending on region, to remove toxic cashew nut shell liquid from the shell and for easier shell removal (Akinhami, Atasie, & Akintokun, 2008; Azam-Ali & Judge, 2001; Dendena & Corsi, 2014; Rosengarten Jr., 1984). The nuts are then shelled mechanically or by hand depending on the production region and facility size (Azam-Ali & Judge, 2001; Fitzpatrick, 2011; Rosengarten Jr., 1984). The shelled cashews are dried to loosen the testa, the papery skin covering the kernel, and then the testa is removed by hand or using a mechanized system (Azam-Ali & Judge, 2001; Dendena & Corsi, 2014; Fitzpatrick, 2011). The process of drying the cashews to loosen the testa can involve dry heat or steam and may be referred to as blanching by cashew processors. Once the skins are removed, the kernels are sorted and graded based on size, quality, and color (Dendena & Corsi, 2014; Fitzpatrick, 2011; Rosengarten Jr., 1984; United Nations Conference on Trade and Development, 2021).

The raw cashew pieces used to prepare the cashew Brie analog associated with the 2020–2021 salmonellosis outbreak were labeled "blanched." The process of blanching cashews is not typically intended as an antimicrobial step, although thermal blanching procedures may be validated as a pasteurization process (Ivarsson & Napasol, 2013). Although the outbreak-associated cashews were labeled "blanched," product specifications stated that the raw cashews were not subject to validated interventions to eliminate microbiological hazards. The implicated cashew cheese analog processor did not use any pasteurization or pathogen reduction control steps to treat the raw cashews before proceeding with soaking and fermentation steps (U.S. Food and Drug Administration, 2021b). Tree nut processors or suppliers should appropriately communicate whether *Salmonella* control measures have been applied to the product. Terms such as "blanched" should be well defined to avoid misunderstandings that could lead to improper handling and treatment of raw ingredients.

A typical serving size for cashews is 28 g (1 oz). At the *Salmonella* levels identified, it is expected that one serving of cashews would contain 0.0644 cells. At this level, there is a low per-serving probability of illness occurring. However, there are several steps that have the potential to increase *Salmonella* populations in cashew cheese analog production. The process of making cashew "cheese"

involves an initial soaking step followed by an ambient temperature fermentation, both of which provide an opportunity for the amplification of low levels of pathogens (Emch, Burroughs, Gaspar-Hernandez, & Waite-Cusic, 2021; Feng, Lieberman, Jung, & Harris, 2020; Savran, Pérez-Rodríguez, & Halkman, 2017; Silva de Nascimento et al., 2013). Soaking provides an opportunity for *Salmonella* distribution and multiplication. *Salmonella* levels increased by more than 5 log CFU/g when cashews were soaked for 24 h at ambient temperature (Emch, Burroughs, Gaspar-Hernandez, & Waite-Cusic, 2021). Similarly, *Salmonella* populations increased by ~4 log CFU/g during a 24-h ambient soak of almonds and walnuts (Feng, Lieberman, Jung, & Harris, 2020; Lieberman et al., 2023). In making cashew dairy analogs, soaked cashews are often blended, ground, or processed to achieve a paste consistency, increasing the potential for spreading and distribution of microorganisms. The outbreak-associated cheese analog also had a secondary mold ripening step that included fungal starter cultures and several days of incubation to result in the classically molded rind that is typical of Brie cheese. There is currently no published data on the growth of *Salmonella* during cashew cheese analog fermentation. Levels of *Salmonella* in the implicated cheese analog associated with the outbreak were not reported (Centers for Disease Control and Prevention, 2021; Lewis et al., 2023; U.S. Food and Drug Administration, 2021a).

Additional information is needed on the survival of *Salmonella* in cashews under different storage conditions and on the behavior of *Salmonella* during the production of cashew cheese analogs. In the short term, raw cashews should be assumed to be contaminated with *Salmonella* and production of cashew dairy analogs should include nuts that have been treated using a validated process that appropriately reduces populations of *Salmonella*. Additional control measures should include soaking the nuts under refrigeration. Also, good manufacturing practices, including facility and equipment sanitation and human hygiene, should be followed through all stages of production to lower the risk of in-process contamination and amplification of pathogens during soaking and fermentation.

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Table 2.1

Levels of *Salmonella* (MPN/g) and 95% confidence intervals determined from two 22.7-kg boxes of cashew pieces from the same lot.

Sample event	Box[a]	50-g subsamples no. positive/no. sampled	MPN/g	95% confidence interval (MPN/g)
Initial	1	5/70	0.0015	0.00063–0.0036
	2	10/70	0.0031	0.0017–0.0057
	Combined	15/140	0.0023	0.0014–0.0038
24 weeks[b]	1	0/10	N/A	N/A
	2	2/30	0.0014	0.00035–0.0056
	Combined	2/40	0.0011	0.00026–0.0042

[a] Each box was divided into seven units; 10 50-g subsamples were enriched for each unit.
[b] Four units (one from Box 1 and three from Box 2) were retested (10 50-g subsamples each) 24 weeks after the initial sampling of that unit.

Table 2.2

Salmonella serovars isolated from two 22.7-kg boxes of cashew pieces, serotyped using antigenic methods [a]

Sampling Point	Box	Unit-Subsample	Media combination[b]					
			TT/HE	TT/XLD	TT/BSA	RV/HE	RV/XLD	RV/BSA
Initial	1	2-3	Urbana[c]	Urbana	Urbana	Urbana	Urbana	Urbana
	1	4-6	Fresno[c]	Fresno	Fresno	Fresno	Fresno	Fresno
	1	5-5	Urbana[c]	+	+	Urbana	+	+
	1	7-1	Urbana[c]	Urbana	Urbana	Urbana[c]	Urbana	Urbana
	1	7-6	Vinohrady[c]	+	+	Vinohrady	+	+
	2	1-3	Urbana[c]	+	+	Urbana	+	+
	2	1-7	Nima[c]	+	+	Nima	+	+
	2	2-2	Urbana[c]	+	+	Urbana	+	+
	2	2-3	Nima[c]	+	+	Nima	+	+
	2	3-5	Urbana[c]	+	+	-	-	Urbana
	2	4-8	Urbana[c]	Urbana	+	-	-	-
	2	5-1	Urbana[c]	Urbana	+	-	-	-
	2	5-4	Urbana[c]	Urbana	+	-	-	-
	2	6-5	Urbana[c]	-	Urbana	-	-	-
	2	7-10	Urbana[c]	+	+	Urbana	+	+
24 weeks[d]	2	1-3	Urbana	Urbana	Urbana	Urbana	Urbana	Urbana
	2	2-6	Urbana	Urbana	Urbana	Urbana	Urbana	Urbana

[a] When available, isolates originating from both TT/HE and RV/HE media combinations[b] were serotyped. Subsamples for which RV/HE isolates were not available are indicated in the table and the alternative isolate is shown. For five subsamples, all six isolates were serotyped. Presumptive *Salmonella*-positive colonies not serotyped (+) and *Salmonella*-negative (-) results are also shown.

[b] BSA, bismuth sulfite agar; HE, Hektoen enteric agar; RV, Rappaport-Vassiliadis broth; TT, tetrathionate broth; XLD, xylose lysine desoxycholate agar.

[c] Serotype confirmed by sequencing.

[d] Four units (one from Box 1 and three from Box 2) were retested (10 50-g subsamples each) 24 weeks after the initial sampling of that unit, two of which yielded *Salmonella*-positive subsamples.

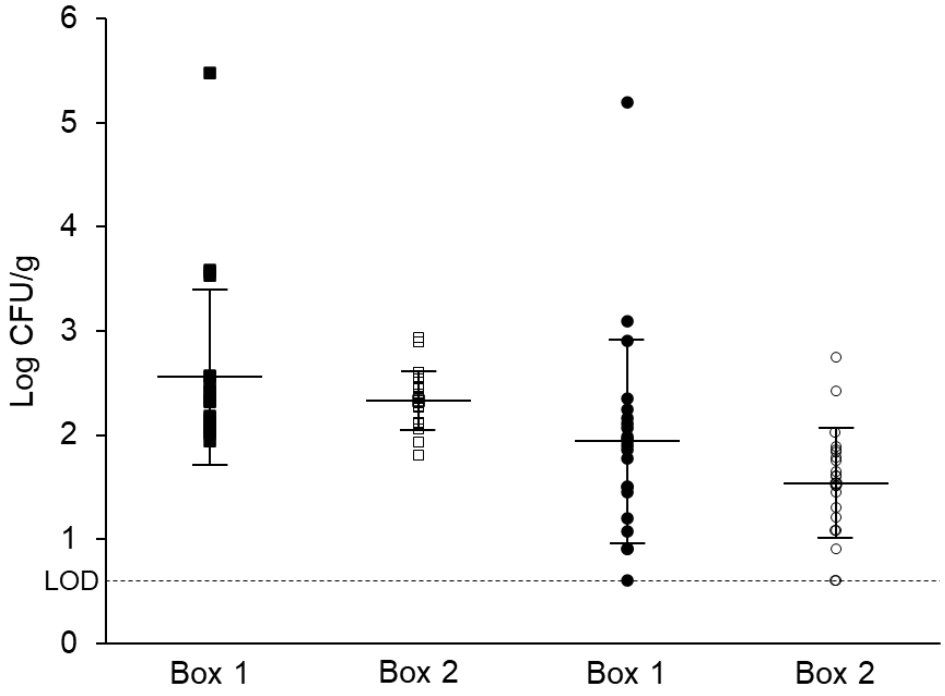


Figure 2.1. Aerobic plate counts (squares) and coliform counts (circles) for subsamples from two 22.7-kg boxes of cashew pieces obtained from the same lot (n = 21 for each box). No significant difference was observed for aerobic plate counts and coliform counts (t-test; P = 0.245 and 0.107, respectively) between Boxes 1 and 2. *E. coli* was not detected in any subsample by plating (LOD, limit of detection 0.60 log CFU/g).

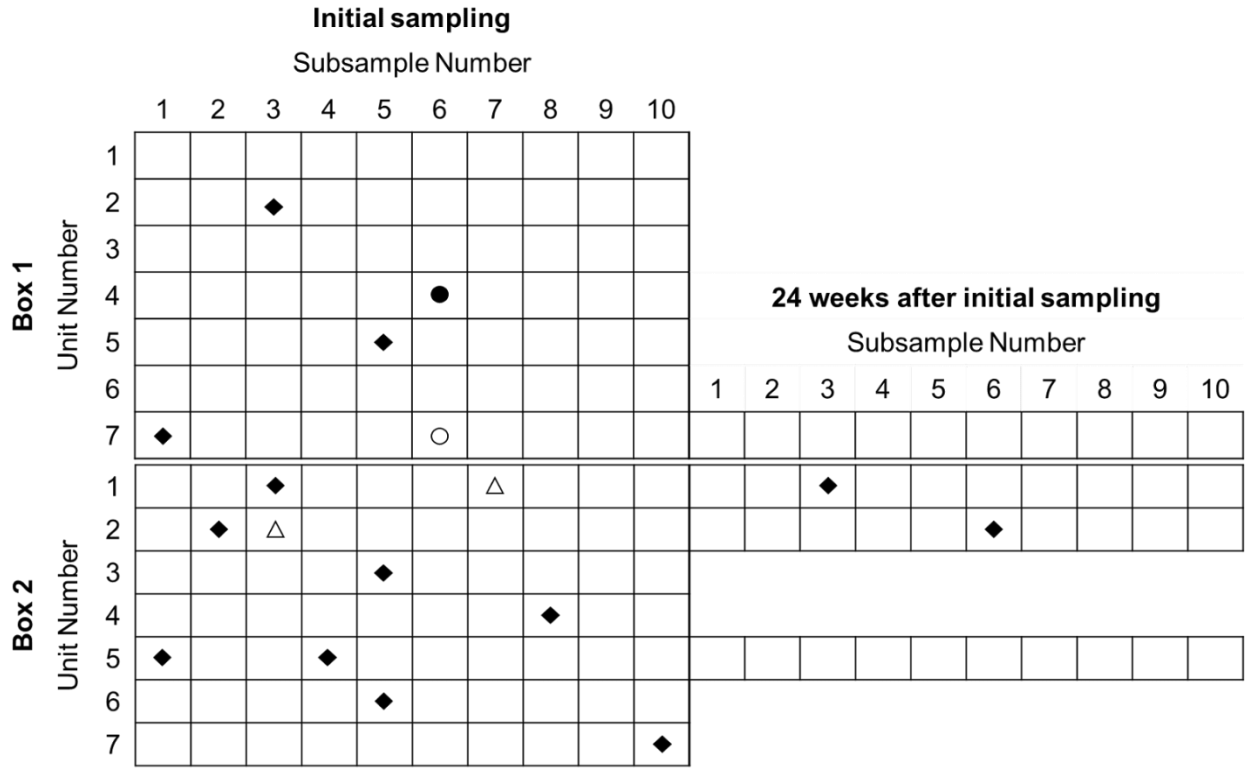


Figure 2.2. Distribution of *Salmonella* recovered from two 22.7-kg boxes of cashew pieces from the same lot (Boxes 1 and 2). Each box was divided into seven units and 10 50-g subsamples from each unit were enriched for *Salmonella*. *Salmonella*-positive subsamples are indicated with symbols representing the four serovars (Fresno [closed circle], Nima [open triangle], Urbana [closed diamond], and Vinohrady [open circle]) that were recovered. Four units yielded two positive subsamples during initial sampling (Box 1 Unit 7; Box 2 Units 1, 2, and 5) and were resampled 24 weeks after the initial sampling date for that unit. Cashews were stored at 4°C.

Supplemental Table S2.1

Salmonella serovar information from outbreak investigation and current study. Sequences were submitted to the National Center for Biotechnology Information under BioProject ID PRJNA945503.

Salmonella serovar	Outbreak isolation source[a]	Present study isolation source	Accession number | LJH number (for sequenced isolates in present study)
Chester[b]	Clinical (1, 2, 3), Brie, CDPH (3) Truffle Brie, CDPH (1)		
Duisburg	Clinical (1, 2)		
Fresno		Box 1, Unit 4-Subsample 6	(SAMN33786943) | LJH1798
Leiden	Environmental swab, FDA (3)		
Nima		Box 2, Unit 1-Subsample 7	(SAMN33786949) | LJH1796
		Box 2, Unit 2-Subsample 3	(SAMN33786951) | LJH1899
Typhimurium	Clinical (1)		
Urbana[c]	Clinical (1, 2, 3), Brie, CDPH (3), Truffle Brie, CDPH and TN DOH (1) Raw cashews from facility, FDA (1, 2, 3)		
		Box 1, Unit 2-Subsample 3	(SAMN33786942) | LJH1894
		Box 1, Unit 5-Subsample 5	(SAMN33786944) | LJH1895
		Box 1, Unit 7-Subsample 1	(SAMN33786945) | LJH1789
		Box 2, Unit 1-Subsample 3	(SAMN33786948) | LJH1897
		Box 2, Unit 2-Subsample 2	(SAMN33786950) | LJH1898
		Box 2, Unit 3-Subsample 3	(SAMN33786952) | LJH1900
		Box 2, Unit 4-Subsample 8	(SAMN33786954) | LJH1901
		Box 2, Unit 5-Subsample 1	(SAMN33786953) | LJH1902
		Box 2, Unit 5-Subsample 4	(SAMN33786955) | LJH1903
		Box 2, Unit 6-Subsample 5	(SAMN33786956) | LJH1904
		Box 2, Unit 7-Subsample 10	(SAMN33786957) | LJH1905
Vinohrady[d]	Brie, CDPH (3), Black Garlic Brie, FDA (3), Truffle Brie, TN DOH (3)		
		Box 1, Unit 7-Subsample 6	(SAMN33786947) | LJH1797

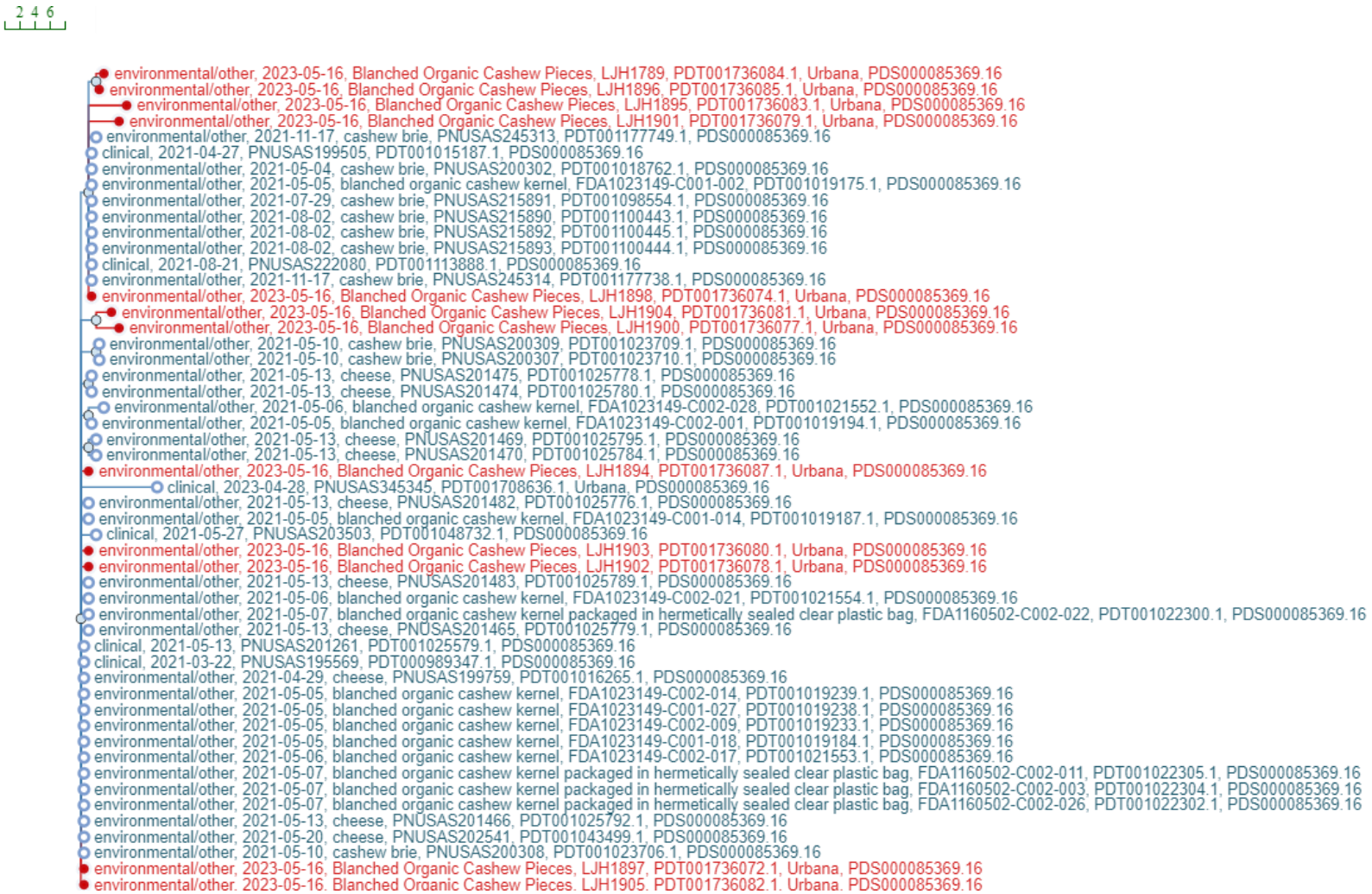
[a] Numbers in table indicate which reference mentions this isolate and from which source. Note that some of the isolates are from the U.S. Food and Drug Administration (FDA), others from California Department of Public Health (CDPH), and others from the Tennessee Department of Health (TN DOH).

(1) Centers for Disease Control and Prevention (2021). Investigation details: *Salmonella* outbreak linked to Jule's Cashew Brie. Available at: https://www.cdc.gov/salmonella/duisburg-04-21/details.html. Accessed June 6, 2023.
(2) U.S. Food and Drug Administration (2021a). Outbreak investigation of *Salmonella*: Jule's Cashew Brie (April 2021). Available at: https://www.fda.gov/food/outbreaks-foodborne-illness/outbreak-investigation-salmonella-jules-cashew-brie-april-2021. Accessed June 6, 2023.
(3) U.S. Food and Drug Administration (2021b). Warning letter: Jule's Foods. Available at: https://www.fda.gov/inspections-compliance-enforcement-and-criminal-investigations/warning-letters/jules-foods-615218-10192021. Accessed June 6, 2023.

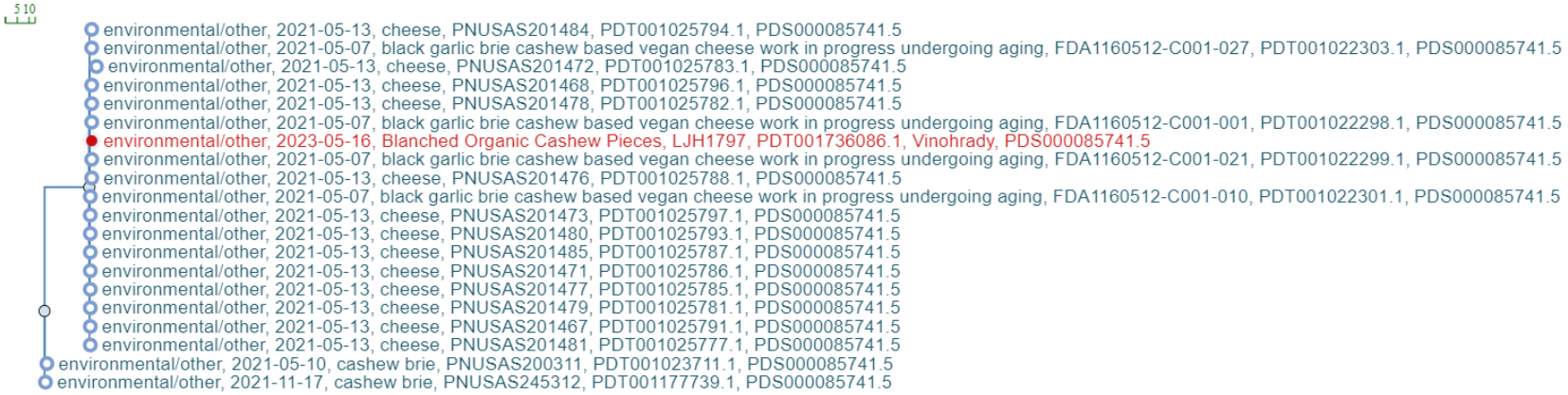
[b] Whole genome sequencing confirmed that the isolate found in the Brie is genetically identical to four clinical isolates (*3*).

[c] Whole genome sequencing confirmed all Brie, Truffle Brie, and raw cashew isolates isolated CDPH, FDA, and TN DOH are genetically identical to each other and to two clinical isolates (*3*).

[d] Brie, Black Garlic Brie, and Truffle Brie isolates are all genetically identical by whole genome sequencing.



Supplemental Figure S2.1. Single-nucleotide polymorphism (SNP) tree for *Salmonella* Urbana isolates collected during current study (in red) and product isolates from outbreak analysis (in blue). SNP tree generated using the National Center for Biotechnology Information pathogen detection database (average 3 SNPs, maximum 16 SNPs).



Supplemental Figure S2.2. Single-nucleotide polymorphism (SNP) tree for *Salmonella* Vinohrady isolates collected during current study (in red) and product isolates from outbreak analysis (in blue). SNP tree generated using the National Center for Biotechnology Information pathogen detection database (average 5 SNPs, maximum 23 SNPs).

Supplemental Figure S2.3. Single-nucleotide polymorphism (SNP) tree for *Salmonella* Fresno isolates collected during current study (in red) and a clinical isolate (in blue). SNP tree generated using the National Center for Biotechnology Information pathogen detection database (3 SNPs).

Chapter 3: Behavior of *Salmonella* during preparation of a fermented cashew cheese analog

ABSTRACT

Between 2013 and 2021 there were three reported salmonellosis outbreaks in North America linked to consumption of cashew cheese analogs that were prepared from soaked and fermented cashews. The behavior of *Salmonella* was evaluated during fermentation of cashews to better understand the risks associated with plant-based fermentations. Single or seven-strain rifampin-resistant *Salmonella*-inoculated cashews (1 to 2 log CFU/g) were soaked 1:1 (w/v) in water at 4°C for 24 ± 1 h, drained, and then blended with water. *Salmonella*-inoculated or uninoculated cashews with or without added commercial *Lactococcus lactis* starter culture, NaCl (1% and 2% w/w), citric acid (0.5% w/w), or a combination of NaCl and citric acid, were held at 24.21 ± 0.77 °C for up to 72 h. The pH, aerobic plate counts (M17 agar), and *Salmonella* levels (CHROMagar Salmonella supplemented with rifampin at 50 µg/mL) were measured in duplicate at 0, 24, 48, and 72 h in replicate experiments (n = 4). The pH decreased significantly from initial levels of ~6 to 4.5–5 at 24 or 48 h in the presence or absence of the starter culture, respectively. When starter culture was present, aerobic bacteria counts increased from an initial ~8 log CFU/g to ~9 log CFU/g after 24 h. Populations of *Salmonella* increased significantly ($P \leq 0.05$) by 6–7 log after 24 h in the absence of starter culture. In some cases, *Salmonella* levels increased significantly by 0.5–1.6 log after 24 h in the presence of starter culture, with or without added NaCl. No significant change in *Salmonella* populations was observed over 72 h when citric acid was added in the presence of starter culture. Addition of a starter culture or starter culture with citric acid inhibited growth but did not reduce initial populations of *Salmonella* during cashew cheese analog fermentation; additional control measures may be needed to further enhance the safety of plant-based fermented products.

Introduction

Globally, plant-based cheese analogs have gained popularity, with 22% growth between 2021 and 2022 to $869 million; Europe and North America make up 95% of sales (Good Food Institute, 2022). Plant-based cheese analog sales in the United States were $233 million in 2022, representing a 51% growth in sales since 2019. Plant-based cheese analogs can be made with or without a fermentation step, characterized by desirable growth of microorganisms and enzymatic conversions of the food components (Marco et al., 2021). Legumes, plant starches, and plant-derived oils are commonly found in cheese analogs that use alternative (non-fermentation) processing methods (Grossman & McClements, 2021). Tree nuts are a common base ingredient, with almonds, cashews, pistachios, and walnuts among the most popular used to prepare fermented cheese analogs in the home (Swinehart, Harris, Anderson, & Feng, 2023).

To prepare a fermented nut-based cheese analog, nuts are usually soaked, drained, blended, and mixed with a starter culture before fermenting at ambient temperature for ≥24 h (BC Centre for Disease Control, 2017; Swinehart, Harris, Anderson, & Feng, 2023; Swinehart, Harris, Louvau, & Feng, 2023). Lactic acid bacteria-initiated fermentations result in the production of lactic acid and other flavor compounds that can be found in dairy cheeses. Commonly used bacterial starter cultures in homemade cheese analogs include probiotic capsules, commercial *Lactococcus lactis*, or rejuvelac (a soaked wheat berry liquid) (Swinehart, Harris, Anderson, & Feng, 2023). Mold starter cultures, often *Penicillium candidum*, also may be added to the blended and fermented tree nuts, followed by an aging process (~1 week) at refrigeration temperatures to replicate rind-ripened cheeses (e.g., Brie-style). Lemon juice, salt, or flavorings may be added at several points during the preparation (BC Centre for Disease Control, 2017).

There is limited data on the microbiology of fermented nut-based cheese analogs. A cashew cheese analog was prepared by soaking cashews at room temperature for 8 h, draining and rinsing the soaked cashews, then blending them with fresh water and fermenting at 22°C for 48 h (Tabanelli et al., 2018). Lactic acid bacteria isolated during the spontaneous fermentation were used to inoculate a pilot-

scale cashew cheese analog. During both fermentations, populations of lactic acid bacteria (as determined on MRS agar) increased from 8 to 9 log CFU/g between 0 and 24 h and the pH decreased from 5.9 to 4.8. The behavior of foodborne pathogens during the cashew cheese analog fermentation was not assessed (Tabanelli et al., 2018).

Three salmonellosis outbreaks in Canada and the United States have been associated with consumption of cheese analogs, all of which were made with cashews. In 2013, *Salmonella* Stanley was associated with a 17-case salmonellosis outbreak in three states that was linked to consumption of raw cashew cheese analog produced in California (Centers for Disease Control and Prevention, 2014; Centers for Disease Control and Prevention, National Outbreak Reporting System Dashboard). In 2017, a fermented cashew cheese analog produced by a restaurant in British Columbia was associated with 23 cases of salmonellosis, with raw cashews identified as the source of the outbreak strain *Salmonella* Weltevreden (Schmitt, Yu, Greve, & McIntyre, 2018). In 2020–2021, 20 reported cases of salmonellosis in four states were linked to consumption of a fermented cashew Brie analog produced commercially in California from raw cashew pieces (Centers for Disease Control and Prevention, 2021). Food safety issues with cashew-based cheese analogs have not been limited to North America. In 2022–2023, a plant-based cheese analog made in France from almond milk and organic cashew nuts was recalled due to *Listeria monocytogenes* contamination (Santé publique France, 2023).

Populations of *Salmonella* in naturally contaminated raw tree nuts are generally very low (0.0008–2.4 most probable number [MPN]/g) (Harris, Yada, Beuchat, & Danyluk, 2022). *Salmonella* levels of <0.003 MPN/g were determined in raw cashews collected from U.S. retail markets (Zhang et al., 2021). The *Salmonella* levels in naturally contaminated raw cashews associated with a 2020−2021 salmonellosis outbreak linked to a cashew Brie analog was 0.0023 MPN/g (95% confidence interval [0.0014, 0.0038]) (Louvau & Harris, 2023). *Salmonella* is known to survive on low-moisture foods, including tree nuts, for extended periods of time (Podolak et al., 2010; Santillana Farakos, Frank, & Schaffner, 2013). Ambient temperature soaking of cashews and other nuts provides an opportunity for low levels of *Salmonella* to increase by 2 to >5 log within 24 h (Emch, Burroughs, Gaspar-Hernandez, &

Waite-Cusic, 2021; Feng, Lieberman, Jung, & Harris, 2020; Lieberman et al., 2023), and the subsequent blending step increases the potential for distribution of pathogens prior to fermentation. Little is known about the impact of a fermentation step on survival or growth of foodborne pathogens in nut-based cheese analogs. The objectives of the present study were to understand the impact of the addition of lactic acid bacteria starter culture, salt, and citric acid on the behavior of *Salmonella* during the fermentation of a cashew cheese analog.

Materials and methods

Cashew kernels

Organic cashew pieces (raw) purchased online (nuts.com) were held in cold storage (4°C) up to 6 months prior to use. Two lots of cashews were used throughout the present study; lot numbers and associated experimental trials are shown in Table 3.1.

Bacterial cultures

A single strain of rifampin-resistant *Salmonella* Enteritidis phage type (PT) 30 (parent strain LJH608 isolated from almonds associated with a raw almond outbreak) was used in early trials (Table 3.1). After work with the single strain inoculum, additional *Salmonella* strains originating from several outbreak-associated low moisture food sources became available and were used to prepare a seven-strain *Salmonella* cocktail inoculum (Supplemental Table S3.1). The isolates used in the seven-strain cocktail included *Salmonella* Enteritidis PT 30 and *Salmonella* Urbana, isolated from naturally contaminated cashews associated with a 2020−2021 cashew Brie analog salmonellosis outbreak (parent strain LJH1789) (Louvau & Harris, 2023). Additional parent strains in the cocktail received from the Illinois Institute of Technology Institute for Food Safety and Health (Bedford Park, IL), were *Salmonella enterica* subsp. *enterica* serovars Agona LJH1784 (toasted oats cereal), Mbandaka LJH1786 (tahini sesame paste), Montevideo LJH1785 (black and red pepper), Reading LJH1788 (laboratory strain), and Tennessee LJH1783 (peanut butter). Rifampin-resistant mutants of each parent strain were isolated using a single-

step procedure (Ruiz et al., 2008). All isolates were stored at −80°C in tryptic soy broth (TSB; BD) supplemented with 15% glycerol.

A freeze-dried commercial *Lactococcus lactis* starter culture (Choozit MM100, Danisco), which is a mixture of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, and *Lactococcus lactis* subsp. *lactis* biovar diacetylactis, was used in selected cashew cheese analog fermentations (Table 3.1). The starter culture was stored at −20°C.

Preparation of inoculum

Frozen cultures of *Salmonella* were streaked onto tryptic soy agar (TSA; TSB plus 1.5% granulated agar [BD]) supplemented with rifampin (DRG International) at 50 µg/mL (TSAR) and incubated at 37°C for 24 h. A single colony from each strain was transferred twice successively into 10 mL TSB and incubated at 37°C for 24 h (Feng, Lieberman, Jung, & Harris, 2020). The second overnight culture (1 mL) was spread over a large (150 × 15 mm; single strain inoculum) or a small TSAR plate (100 × 15 mm; cocktail inoculum) and incubated at 37°C for 24 h to produce a bacterial lawn. Lawns of each strain were collected in 9 mL of sterile 0.1% peptone (large plate; single strain) or 4 mL of sterile 0.1% peptone (small plate; cocktail strains). For each organism, the resulting cell suspension was mixed by vortexing, and equal volumes of each strain (2 mL) were combined. The single strain suspension and the *Salmonella* cocktail were each diluted to 6 log CFU/mL with 0.1% peptone.

Inoculation procedure

Cashew kernels were inoculated as described by Kimber et al. (2012). Liquid inoculum (25 mL) was added to 400 g of cashews in 30.5 × 30.5 cm plastic zipper bags (Bitran, Fisher Scientific). Bags were sealed and then shaken by hand for 2 min to evenly distribute the inoculum over the cashews. The inoculated cashews were then spread in a single layer onto two sheets of 46 × 57 cm filter paper (Qualitative P5, Fisher Scientific) that were folded in half and placed on a wire drying rack within a lidded plastic bin; the lid was left partially open (by 7 to 10 cm), covered by mesh, to allow for air flow.

The cashews were allowed to dry at ambient conditions for 6 days (single strain inoculum; 21.5 ± 0.4 °C, 31.0% ± 5.3% relative humidity [RH]) or 5 to 7 days (cocktail inoculum; 21.9 ± 0.4 °C, 36.1% ± 5.3% RH and 22.2 ± 0.3 °C, 44.3% ± 7.3% RH) until they returned to pre-inoculation moisture levels of ~4%. *Salmonella* populations decreased by 1 log CFU/g during drying, achieving target final populations of 3 to 4 log CFU/g on the dried cashews.

Cashew cheese analog preparation and fermentation

Salmonella-inoculated cashews (~3 log CFU/g; 50 or 100 g) were mixed with uninoculated cashews (450 or 400 g, respectively) to achieve a target *Salmonella* population of 1–2 log CFU/g (Table 3.1); control batches of cashews were prepared with 500 g of uninoculated cashews. Each 500-g batch of inoculated or uninoculated cashews was added to 500 mL of sterile ultrapure water (Milli-Q Advantage A10, MilliporeSigma) in a 30.5 × 30.5 cm plastic zipper bag and held at 4°C for 24 h.

After 24 h, the soaked cashews were drained by aseptically cutting a corner of the bag and carefully pouring all soaking liquid into a disposable plastic bottle (1 L; Fisher Scientific). Half of the soaked cashews were transferred to a sterile metal blender jar (946 mL capacity; Waring) along with 50 mL of sterile ultrapure water. The cashews were blended on low speed for 15 s, mixed with a sterile silicone spatula, then blended again for 15 s. Another 30 mL of sterile ultrapure water was added to the blender jar, and the cashews were blended for two additional 15-s intervals; between each interval the spatula was used to scrape down the inside of the blender jar and mix the cashews. The blended cashews were transferred to a sterile metal bowl (1.4 L). The same process was repeated for the second half of the soaked cashews, and the blended cashews were added to the same metal bowl.

Starter culture (0.2 g, based on recommendations from the commercial seller's website), sodium chloride (NaCl; 1% and 2% w/w [weight of NaCl/weight of drained soaked cashews])] or citric acid (CA; 0.5% w/w [weight of CA/weight of drained soaked cashews]) (Table 3.1) were added, as appropriate, to the blended *Salmonella*-inoculated or control cashews in the bowl and mixed with a sterile silicone spatula for 1 min to fully incorporate. The blended cashews for all batches were transferred into separate

17 × 20 cm plastic zipper bags (Bitran). Bags were sealed and then held in a covered secondary container (6.8 L, Sterilite) for 72 h in an incubator set to 25°C (24.21 ± 0.77 °C).

Microbial enumeration

At each sampling time, the closed bag of blended cashews was massaged by hand to mix without incorporating oxygen. Duplicate 10-g samples were transferred into one side of double-sided 207-mL Whirl-Pak filter bags (Nasco) using a sterile plastic spatula. For each 10-g sample, 20 mL of 0.1% peptone was added. When appropriate, samples were diluted with 0.1% peptone. Samples were plated in duplicate by spiral plating 50 and 250 µL (Autoplate 4000, Advanced Instruments) onto M17 agar supplemented with 10% lactose (BD) and onto CHROMagar Salmonella supplemented with rifampin at 50 µg/mL (CHROMagar-SR) and incubated at 30°C for 48 h or 37°C for 24 h, respectively. When counts were anticipated to be low, duplicate 250 µL samples of the initial dilution were plated by hand.

Temperature, water activity, and pH

Incubator temperature was recorded using data loggers (TempTale 4, Sensitech Inc.). The pH of all batches was measured with a pH meter (InLab® Viscous Pro-ISM, Mettler-Toledo); triplicate readings were taken and averaged. The water activity of batches prepared without *Salmonella* was measured with a water activity meter (Aqualab model 4TE, Decagon Devices) (Table 3.1); single measurements were recorded for each sample.

Statistical analysis

All experiments were replicated twice, with two samples per batch ($n = 4$). Each sample was plated in duplicate. Samples were taken at 0 and 24 h and, in some cases, 12, 48, and 72 h (Table 3.1). Colonies on the duplicate plates were counted and averaged and the log CFU/g was calculated using the appropriate plating volume and dilution (limit of detection LOD 0.48 log CFU/g). Bacterial populations for each of the two samples per batch in both replicate trials were averaged to achieve the final log CFU/g

for each batch. Final levels in log CFU/g were averaged across both replicate trials and standard deviation was determined, then analysis of variance (ANOVA) followed by Tukey-Kramer tests were performed; differences between mean values were considered significant at $P \leq 0.05$. Significance tests were conducted using JMP 16.0 software (SAS Institute Inc.).

Results

Cashew cheese analog fermentation using single strain inoculum

A low but measurable level of *Salmonella* in the soaked and blended cashews was targeted using a mixture of inoculated and uninoculated cashews. An initial batch of cheese was made using 50 g of single strain *Salmonella* Enteritidis PT 30–inoculated cashews with 450 g of uninoculated cashews (Trial 1; Table 3.1). *Salmonella* levels were 10.73 ± 0.09 log CFU/mL in the inoculum and 4.23 ± 0.13 log CFU/g on the cashews immediately after inoculation. Following 6 days of drying at ambient conditions *Salmonella* levels on the inoculated cashews decreased by approximately 1 log to 3.32 ± 0.01 log CFU/g. *Salmonella* levels were 1.22 ± 0.16 log CFU/g when the inoculated (50 g) and uninoculated (450 g) cashews were combined, which was close to the LOD (0.48 log CFU/g) for the plating method. For this reason, all subsequent experiments (Trials 2–4) used a ratio of 100 g of inoculated cashews to 400 g of uninoculated cashews to achieve initial *Salmonella* levels in the blended cashews that were closer to 2 log CFU/g (1.79 ± 0.24 log CFU/g).

In Trial 1, three batches of cheese analog were prepared: raw cashews (lot A) inoculated with either a starter culture or a single strain *Salmonella* Enteritidis PT 30 inoculum, or a combination of both (Table 3.1). When cashews were inoculated with starter culture only, aerobic bacteria counts, as determined using M17 agar incubated at 30°C for 48 h, were 8.14 ± 0.21 log CFU/g at 0 h and significantly increased ($P \leq 0.05$) by 1.32 log to 9.46 ± 0.16 at 24 h; further significant population change was not observed at 48 or 72 h (Table 3.2). No colonies were observed at the lowest dilution on CHROMagar-SR.

Broth cultures of *Salmonella* formed colonies on the M17 agar (data not shown). For *Salmonella*-inoculated cashews without starter culture, populations measured on M17 agar significantly increased by 5.96 log from an initial 1.96 ± 0.08 log CFU/g to 7.92 ± 0.45 log CFU/g at 24 h. A further significant increase of 1.54 log occurred at 48 h; there was no significant change in populations at 72 h (Table 3.2). *Salmonella* levels measured on CHROMagar-SR significantly increased by 5.66 log from 1.44 ± 0.20 log CFU/g at 0 h to 7.10 ± 0.21 log CFU/g at 24 h, and then increased further by 1.39 log to 8.49 ± 0.38 log CFU/g at 48 h; no significant further change was observed at 72 h.

Microbial counts on M17 agar were consistently higher by 0.5–0.8 log at 0 and 24 h or 0.95–1 log at 48 and 72 h compared to those on CHROMagar-SR for cashew cheese analogs prepared with *Salmonella* and no starter culture (Table 3.2). A mixture of colony types was observed on M17 agar, suggesting that natural microbiota present on the raw cashews multiplied during the fermentation. *Salmonella* formed uniform, smooth, round colonies on M17 agar; the natural microbiota did not form colonies on CHROMagar Salmonella with added rifampin (data not shown).

Initial M17 agar counts of 8.21 ± 0.14 at 0 h were observed for cashews inoculated with both single strain *Salmonella* Enteritidis PT 30 and starter culture (Table 3.2). Populations significantly increased by 1.17 log at 24 h; no additional significant change in population was observed at 48 or 72 h (Table 3.2). *Salmonella* levels significantly increased by 1.74 log at 24 h, from an initial 1.07 ± 0.36 log CFU/g to 2.81 ± 0.18 log CFU/g. Populations at 72 h (2.46 ± 0.02 log CFU/g) were significantly lower than those at 48 h but still significantly higher than those observed at 0 h. In the presence of starter culture, *Salmonella* population increases at 24, 48, and 72 h were significantly lower than that observed without the addition of starter culture (e.g., increases at 48 h of 1.8 log compared to 7.0 log, respectively).

Cashew cheese analog fermentation with added NaCl

NaCl (0, 1, or 2% w/w) was added to cashews after soaking and blending (Table 3.1). Counts on M17 agar significantly increased from 8.2 to 9.1 log CFU/g at 24 h in cashews with starter culture and 2%

NaCl (w/w) (Trial 2; Table 3.2). This increase of 0.90 log was similar to that observed in Trial 1 cashews without starter culture or NaCl (1.3 log) (Table 3.2).

In Trial 2, cashews were inoculated with single strain *Salmonella* Enteritidis PT 30 at a ratio of 100 g of inoculated cashews to 400 g of uninoculated cashews to achieve a starting *Salmonella* level of 1.79 ± 0.24 log CFU/g prior to soaking. In the presence of starter culture and NaCl, counts on M17 agar increased significantly by 0.9–1.0 log at 24 h. *Salmonella* levels of 1.7–1.8 log CFU/g were observed at 0 h in *Salmonella*-inoculated cashews with 0, 1, and 2% NaCl (Table 3.2); populations significantly increased by 1.4–1.6 log to 3.1–3.4 log CFU/g after 24 h.

Cashew cheese analog fermentation using cocktail inoculum

In Trial 3, raw cashews (lot B) were inoculated with starter culture, a seven-strain *Salmonella* cocktail, or a combination of both (Table 3.1). *Salmonella* levels in the seven-strain *Salmonella* cocktail inoculum were 10.61 ± 0.01 log CFU/g. *Salmonella* levels on the cashews were 4.25 ± 0.17 log CFU/g after inoculation and 3.51 ± 0.06 log CFU/g after 5 or 7 days of drying at ambient conditions. The combination of 100 g of *Salmonella* cocktail-inoculated cashews with 400 g of uninoculated cashews resulted in a final population of 2.13 ± 0.21 log CFU/g before soaking.

In the control cashew cheese analog with cashews and starter culture, initial counts observed on M17 agar of 8.3 log CFU/g significantly increased by 0.8 log at 12 h; no further significant changes in aerobic bacteria populations were observed at 24, 48, or 72 h (Trial 3; Table 3.3). The aerobic bacteria counts in cashews from lots A and B with only starter culture were similar (Tables 3.1 and 3.2), indicating that the cashew lot did not impact fermentation with starter culture.

Salmonella cocktail–inoculated cashews in the absence of starter culture had initial aerobic bacteria counts of 2.0 at 0 h, which increased significantly by 3.4 log at 12 h, and further significant increases of 2.9 and 0.98 log at 24 and 48 h, respectively; no significant change occurred between 48 and 72 h (Table 3.3). *Salmonella* levels significantly increased from 1.8 log CFU/g by 2.1 and 3.2 log at 12 h

and 24 h, respectively, after which no significant change occurred. Maximum *Salmonella* levels achieved were 8.05 ± 0.36 log CFU/g at 48 h.

When cashews were inoculated with *Salmonella* cocktail and with added starter culture, aerobic bacteria counts of 8.2 log CFU/g were observed at 0 h. Significant increases of 0.9 log to 9.1 log CFU/g were observed at 12 h (Table 3.3). Aerobic bacteria levels were 9.3 log CFU/g at 24, 48, and 72 h. *Salmonella* levels increased significantly by 0.7 log from 1.9 to 2.6 log CFU/g at 12 h, then decreased by 0.5 log to 2.0 log CFU/g at 48 h. A further significant 1.0 log decrease at 72 h to 0.9 log CFU/g resulted in *Salmonella* levels that were significantly lower than the initial populations (Table 3.3).

Cashew cheese analog fermentation with added citric acid and NaCl

In Trial 4, the impact of both citric acid and NaCl were investigated. Counts on M17 agar were 8.0 at 0 h and decreased significantly by 0.9 log after 24 h in the control cheese analog including raw cashews (lot B) inoculated only with starter culture and 0.5% citric acid (w/w); no further significant change in population was observed at 48 or 72 h (Table 3.3). Aerobic bacteria levels were similar between the cashews prepared with only starter culture (Trials 1 to 3) and those with citric acid (Trial 4).

Salmonella cocktail-inoculated cashews with starter culture had aerobic bacteria counts of 8.2 log CFU/g at 0 h, which increased significantly by 1.2 log to 9.4 log CFU/g at 24 h (Table 3.3). *Salmonella* levels did not change significantly at 24 or 48 h from an initial 2.1 log CFU/g, but significantly decreased by 1.2 log at 72 h to a final population of 1.2 log CFU/g.

When 0.5% citric acid (w/w) was added to cocktail-inoculated cashews with starter culture, a significant increase in aerobic bacteria counts of 1.1 log occurred between 0 and 24 h, after which no significant population change was observed (Table 3.3). Similarly, in *Salmonella*-inoculated cashews with starter culture, 0.5% citric acid, and 1% NaCl, populations of aerobic bacteria significantly increased by 0.96 log at 24 h. No significant change in *Salmonella* populations was observed over the 72-h fermentation when citric acid alone, or in combination with NaCl, was added.

Change in pH during fermentation

The initial pH of the soaked and blended cashews was 5.99–6.35 for all cashew cheese analogs prepared without 0.5% citric acid (Tables 3.2 and 3.3). The pH of control batches prepared with cashews from lots A and B and starter culture followed similar trends throughout the fermentation (Tables 3.2 and 3.3). For cheese analogs prepared with a starter culture with or without *Salmonella*-inoculated cashews and with or without NaCl, the pH significantly declined to 4.6–5.1 at 24 h (Tables 3.2 and 3.3); in Trial 3 (Table 3.3), similar declines were observed at 12 h. In contrast, for cheese analogs prepared with *Salmonella*-inoculated cashews without starter culture, no significant declines in pH were observed at 12 h (Trial 2; Table 3.3). No significant decline in pH at 24 h was observed with single-strain *Salmonella* inoculated cashews in Trial 1 (pH 6.0; Table 3.2) but a significant decline to pH 5.8 was observed with cocktail-inoculated cashews in Trial 3 (Table 3.3).

When 0.5% citric acid was added to the blended cashews the initial pH was 5.26–5.36 (Table 3.3); significantly lower by approximately 1.0 compared to batches that did not include citric acid (pH 6.4; Table 3.3). After 24 h, the pH had declined to 4.7–4.8, which was not always significantly different from the initial pH (Trial 4, Table 3.3).

The final pH for all batches in Trials 1 through 4 was 4.4–4.9, regardless of the presence of *Salmonella* or starter culture, or the addition of NaCl or citric acid. Within each trial, the final pH at 72 h for each batch in Trials 1, 2, and 3 were statistically the same.

Water activity

The water activity of raw cashews before soaking was 0.5 (Table 3.4). After soaking and during fermentation, water activity was recorded only for controls prepared without *Salmonella*. At 0 h, the water activity was 0.99–1.0 for all cheese analogs except when 2% NaCl (w/w) was added. For these batches, the water activity was significantly lower (0.97). For cheese analogs prepared with raw cashews (lots A and B) or with citric acid, the water activity decreased by <0.01 at 24 h (0.99). Likewise, no significant change in water activity was observed during 24 h fermentation when NaCl was added to the cashews.

Discussion

Fermentation practices for preparing nut-based cheese analogs include several steps, beginning with soaking at refrigeration to room temperature (BC Centre for Disease Control, 2017; Swinehart, Harris, Anderson, & Feng, 2023; Swinehart, Harris, Louvau, & Feng, 2023). *Salmonella* growth in tree nuts soaked at ambient temperature (~23°C) begins after ~8 h, and after a 24-h soak increases by ~4 log CFU/g on almonds (Feng, Lieberman, Jung, & Harris, 2020), ~5.5 log CFU/g on cashews (Emch, Burroughs, Gaspar-Hernandez, & Waite-Cusic, 2021), and ~2 log CFU/g on walnuts (Lieberman et al., 2023). Soaking at refrigeration temperatures is recommended to inhibit the growth of *Salmonella* and other foodborne pathogens. Following the soaking step, tree nuts are usually drained, blended, and then fermented either with addition of starter culture or by reliance on spontaneous fermentations. The most common starter cultures include probiotic powders or capsules, lactic acid bacteria cultures, rejuvelac (a fermented wheat berry beverage), miso, yogurt cultures, completed vegetable or fruit fermentations (such as sauerkraut or kimchi), kefir, and kombucha (BC Centre for Disease Control, 2017; Swinehart, Harris, Anderson, & Feng, 2023; Swinehart, Harris, Louvau, & Feng, 2023). Ingredients are often added to the cheese analogs prior to fermentation, with the most popular being salt, sugar, and lemon juice (Swinehart, Harris, Louvau, & Feng, 2023). Fermentation temperatures and time differ depending on the recipe and range from <20°C to >35°C and for 1 h up to 5 weeks. Blog and video recipes most commonly suggest fermentation temperatures and times between 20–35°C and 12–48 h (Swinehart, Harris, Louvau, & Feng, 2023).

In the present study, the fermentation protocol approximated the first step in the production of the cashew Brie analog recipe implicated in the 2020–2021 *Salmonella* outbreak (personal communication). This included a refrigerated soak, blending of the soaked cashews, addition of a commercial freeze dried mesophilic *L. lactis* starter culture, and holding at ambient temperature for 24 h. Additional steps were included in the production of the Brie analog including addition of a *P. candidum* mold starter culture and a refrigerated ripening period to form the typical mold rind; these steps were omitted in the present study. In dairy cheeses, aging for 60 days at ≥1.67°C is considered a reliable control measure to reduce the risk

of pathogen survival (21 CFR 133). Aging of nut-based cheeses has not been assessed, and the impact on safety and quality of this type of refrigerated ripening or aging is not understood. The impact on pathogen behavior following the addition of a mold starter culture to cashew cheese analogs also has not been evaluated.

A spontaneous cashew cheese analog was previously prepared under household conditions (Tabanelli et al., 2018). Populations of lactic acid bacteria reached 9.34 ± 0.21 log CFU/g at the end of the 48 h fermentation, which are comparable to levels of aerobic bacteria observed in the present study. The pH decreased by 1.3, from 5.94 ± 0.09 at 0 h to 4.64 ± 0.05 at 48 h. The initial pH as well as the pH at 48 h were similar to results in the present study at 48 h for all cashew cheese analogs, both with and without commercial *L. lactis* starter culture. In the present study, total aerobic bacteria levels increased from approximately 2 log CFU/g to >9 log CFU/g by 48 h for all cheese analogs prepared with *Salmonella*-inoculated cashews without added starter culture, regardless of inoculum. With starter culture, aerobic bacteria counts started at approximately 8 log CFU/g and increased to >9 log CFU/g by 24 h for all cheese analogs, regardless of the lot of cashews used, or the presence of *Salmonella*, added citric acid, or NaCl. All fermentations in the present study started with an initial pH of ~6 except for those with added citric acid which was ~pH 5.3. The final pH at 72 h was between 4.4 and 4.9 at 72 h for all prepared cheese analogs, regardless of the addition of *Salmonella*, starter culture, citric acid, or NaCl. These results suggest that the presence of *Salmonella*, NaCl, or citric acid did not impact the growth of the starter culture or the ability of the starter culture to lower pH, and that *Salmonella* and/or the natural microbiota present on raw cashews were able to lower the pH in 72 h under fermentation conditions in the absence of an added starter culture. The use of added 1% NaCl, 2% NaCl, or 0.5% citric acid did not have an impact on the final pH reached by the end of fermentation.

Use of pH has been recommended as a tool to reduce the risk of *L. monocytogenes* growth in fermented cashew cheese analogs (BC Centre for Disease Control, 2017). No cheese analog outbreaks involving *L. monocytogenes* have occurred to date, but one recall of a French plant-based cheese analog occurred in early 2023 (Santé publique France, 2023). A pH of 4.4 or lower is recommended for cashew

cheese analogs by the end of fermentation to reduce the risk of the growth of *L. monocytogenes*, although this pH is not low enough to inhibit the growth of *Salmonella*, which can grow in pH as low as 3.7 (National Advisory Committee on Microbiological Criteria for Foods, 2009). In the present study, pH did not consistently reach levels at or below 4.4 after 72 h, even with citric acid added prior to fermentation. The addition of sugar may lead to greater and more rapid reductions in pH during fermentation, but was not assessed in the present study.

Typical *Salmonella* levels recovered from naturally-contaminated raw tree nuts are usually <0.5 MPN/g. *Salmonella* levels in almonds, shelled and in-shell pistachios, and in-shell walnuts have been reported as 0.008 to 0.18 MPN/g (Bansal et al., 2010; Danyluk et al., 2007), <0.003 to 0.43 MPN/g (Harris et al., 2016; Zhang et al., 2021), and <0.003 to 0.092 (Davidson et al., 2015), respectively. Naturally contaminated cashews associated with a 2020−2021 salmonellosis outbreak in a cashew Brie analog had *Salmonella* levels of 0.0023 MPN/g (Louvau & Harris, 2023). For this reason, target initial populations of *Salmonella* in the cashews used for fermentation were 1−2 log CFU/g, to represent low level but measurable contamination. The LOD using plating methods was 0.48 log CFU/g in the present study. Populations of *Salmonella* increased by ~5.5 log in 24 h when no starter culture was present, very similar to the increase observed during a 24 h ambient temperature soak of whole cashews in water (Emch, Burroughs, Gaspar-Hernandez, & Waite-Cusic, 2021).

Little is known about the behavior of *Salmonella* during food fermentations, and no literature is available focused specifically on plant-based dairy alternatives. *Salmonella* Enteritidis and *Salmonella* Typhimurium levels increased by ~0.50 log CFU/g during a 4.5-day fermentation of dairy yogurt at 44°C for three initial inoculum levels (3, 5, and 7 log CFU/g), where the pH decreased from 6 to 4 over the fermentation period (Savran, Pérez-Rodríguez, & Halkman, 2017). During a 7-day cocoa bean fermentation, *Salmonella* growth of 1–2 log CFU/g was observed during the final 24 h of fermentation when the pH was greater than 4 (Silva de Nascimento et al., 2013). *Salmonella* levels in the present study increased significantly by approximately 5.5 log at 24 h for cashew cheese analogs prepared with *Salmonella*-inoculated cashews and no starter culture. In the presence of starter culture, *Salmonella* levels

increased significantly by approximately 1.7 log (single strain inoculum) or 0.5 log (cocktail inoculum). *Salmonella* growth was suppressed in the presence of starter culture, but not inhibited.

Salt and citric acid (in the form of lemon juice) are commonly added to fermented cashew cheeses, based on a consumer survey and online content analysis (Swinehart, Harris, Anderson, & Feng, 2023; Swinehart, Harris, Louvau, & Feng, 2023), and were chosen as additional ingredients to assess in the present study due to their prevalence in consumer recipes as well as their use in other fermented foods. Salt added at 10% inhibited the growth of *Salmonella* on almonds during a 24-h ambient temperature soak, while concentrations of 20% reduced *Salmonella* levels (Emch, Burroughs, Gaspar-Hernandez, & Waite-Cusic, 2021). Salt is added to cabbage at concentrations of 2.25 to 2.50% for sauerkraut fermentations (UC ANR, 2020). Salt concentrations in finished dairy cheeses often range from 1 to 5% salt by weight (Guinee & Fox, 2007). In the present study, cashew cheese analogs with 1% and 2% NaCl added prior to fermentation achieved the same levels of *Salmonella* growth compared to batches with only *Salmonella*-inoculated cashews and starter culture, suggesting that the combination of starter culture and NaCl is not a sufficient control to inhibit *Salmonella* growth during a 24-h fermentation. Citric acid is the primary acid in lemon juice, which is a commonly added ingredient in fermented nut-based cheese analogs (Swinehart, Harris, Anderson, & Feng, 2023; Swinehart, Harris, Louvau, & Feng, 2023). When citric acid was added at 1% and 1.4% (w/w) to tabbouleh salad with *Salmonella* Typhimurium stored at 21°C, no change in *Salmonella* levels were observed until 72 h when levels decreased by ~2 log (Al-Rousan et al., 2018). Tahini inoculated with *Salmonella* Typhimurium and 0.5% citric acid was held at 21°C (Al-Nabulsi et al., 2014). After 24 h, a decrease in *Salmonella* populations was observed In the present study, the addition of 0.5% citric acid inhibited *Salmonella* growth during a 72 h cashew cheese analog fermentation but did not result in a significant change in population.

Currently, a single guidance document for preparation of fermented nut-based cheese analogs in commercial operations is available from British Columbia Centre for Disease Control (BC Centre for Disease Control, 2017). A hurdle approach was suggested, which includes purchasing cashews treated with a validated antimicrobial process, the use of commercial starter culture, refrigerated soaking

conditions, and monitoring of pH as methods to mitigate the risk of *Salmonella* growth associated with fermented cashew cheese analog production.

It is recommended that producers purchase cashews treated with a validated antimicrobial control step from a reliable distributor prior to soaking and fermentation, and that clear communication about the treatments is prioritized. If treated cashews are not available for purchase, producers should apply a kill step that is validated for a 5-log reduction in *Salmonella* populations before using the raw ingredient in any further soaking or fermentation steps (BC Centre for Disease Control, 2017).

In the present study, *Salmonella* populations increased significantly in the absence of a starter culture, and growth was significantly suppressed, but not completely prevented by the addition of the commercial mesophilic *L. lactis* starter culture used. This single commercial starter culture was used at a single dose. It is not known if the growth of *Salmonella* would have been similarly impacted with the use of other starter cultures or if different initial levels of *L. lactis* were applied. Although some commercial starter cultures are marketed for production of plant-based fermentations, there are no published data on their efficacy. It is recommended that producers do not rely on a spontaneous fermentation and use a commercial starter culture when preparing this type of product to reduce *Salmonella* growth during fermentation (BC Centre for Disease Control, 2017).

Previous literature has highlighted the risk of pathogen growth during ambient temperature soaking (Emch, Burroughs, Gaspar-Hernandez, & Waite-Cusic, 2021; Feng, Lieberman, Jung, & Harris, 2020; Lieberman et al., 2023); cashew cheese analog producers should soak cashews under refrigeration. pH is recommended as an indicator of progress during fermentation, but a change in pH should not be relied on to control *Salmonella* levels. The pH achieved during cashew cheese analog fermentation often is not low enough to inhibit *Salmonella* growth.

There is currently no published literature evaluating the growth of *Salmonella* during cashew cheese analog fermentations. The present study demonstrated that use of a starter culture significantly reduced *Salmonella* growth, while addition of 0.5% citric acid prevented growth. Further research is needed to better understand *Salmonella* behavior during different cashew cheese analog fermentations and

to further support current recommendations. The use of starter culture is important for mitigating the risk of *Salmonella* growth during fermentation. The present study evaluated the use of one commercially available *L. lactis* starter culture, however, several other types of starter cultures have been reportedly used by consumers and at an industrial scale and should be assessed for their impact on *Salmonella* behavior and pH. The present study followed the recipe implicated in the 2020–2021 cashew Brie analog-associated *Salmonella* outbreak. Additional fermentation times and temperatures, aging processes, and storage conditions (time, temperature, humidity) should be considered. Two lots from a single source of raw cashew pieces were used in the present study; fermentations made with other varieties of cashews from different growing regions might provide insight into the impact of different raw ingredients on the fermentation profile, final pH, and *Salmonella* behavior. Similarly, other added ingredients, such as sugar, acetic acid, or flavorings, may be added to fermentations and should be evaluated.

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Table 3.1

Cashew lots and associated experimental batches and sampling used in the study.

Trial	Cashew lot	*Salmonella* inoculation type	Inoculated-to-uninoculated ratio[a]	Cashew cheese analog batch[b]	Sampling schedule
1	A	Single strain (s)	0:500 g	LAB A	Plating, pH, water activity: 0, 24, 48, 72 h
			50:450 g	sSAL	Plating, pH: 0, 24, 48, 72 h
			50:450 g	sSAL+LAB	Plating, pH: 0, 24, 48, 72 h
2	A		0:500 g	LAB 2% NaCl	Plating, pH, water activity: 0, 24 h
			100:400 g	sSAL+LAB	Plating, pH: 0, 24 h
			100:400 g	sSAL+LAB 1% NaCl	Plating, pH: 0, 24 h
			100:400 g	sSAL+LAB 2% NaCl	Plating, pH: 0, 24 h
3	B	Cocktail (c)	0:500 g	LAB B	Plating, pH, water activity: 0, 12 (no water activity), 24, 48, 72 h
			100:400 g	cSAL	Plating, pH: 0, 12, 24, 48, 72 h
			100:400 g	cSAL+LAB	Plating, pH: 0, 12, 24, 48, 72 h
4	B		0:500 g	LAB+CA	Plating, pH, water activity: 0, 24, 48, 72 h
			100:400 g	cSAL+LAB	Plating, pH: 0, 24, 48, 72 h
			100:400 g	cSAL+LAB+CA	Plating, pH: 0, 24, 48, 72 h
			100:400 g	cSAL+LAB+CA+NaCl	Plating, pH: 0, 24, 48, 72 h

[a] *Salmonella*-inoculated cashews (SAL), either single strain or seven-strain cocktail, were mixed with uninoculated cashews to achieve target populations of 1–2 log CFU/g.

[b] LAB A or B, uninoculated cashews with starter culture from lot A or B, respectively; SAL, inoculated cashews and no starter culture; SAL+LAB, inoculated cashews with starter culture; LAB 2% NaCl, uninoculated cashews with starter culture and 2% NaCl (w/w); SAL+LAB 1% NaCl or SAL+LAB 2% NaCl, inoculated cashews with starter culture and 1% or 2% NaCl (w/w); LAB+CA, uninoculated cashews with starter culture and 0.5% citric acid (w/w); SAL+LAB+CA, inoculated cashews with starter culture and 0.5% citric acid (w/w); SAL+LAB+CA+ NaCl, inoculated cashews with starter culture, 0.5% citric acid (w/w), and 1% NaCl (w/w).

Table 3.2

Populations of *Salmonella* and total aerobic bacteria and pH measured during fermentation of *Salmonella* Enteritidis PT 30–inoculated cashews with and without starter culture and NaCl [a].

Trial	Batch [b]	Fermentation time (h)	Aerobic bacteria [c] (log CFU/g)	*Salmonella* [d] (log CFU/g)	pH
1	LAB A	0	8.14 ± 0.21 [A]	N/A	6.12 ± 0.10 [A]
		24	9.46 ± 0.16 [B]	N/A	4.85 ± 0.06 [B]
		48	9.36 ± 0.09 [B]	N/A	4.74 ± 0.10 [B]
		72	9.17 ± 0.04 [B]	N/A	4.84 ± 0.14 [B]
	sSAL	0	1.96 ± 0.08 [A]	1.44 ± 0.20 [A]	6.19 ± 0.25 [A]
		24	7.92 ± 0.45 [B]	7.10 ± 0.21 [B]	6.03 ± 0.13 [A]
		48	9.46 ± 0.09 [C]	8.49 ± 0.38 [C]	4.98 ± 0.31 [B]
		72	9.35 ± 0.07 [C]	8.40 ± 0.33 [C]	4.90 ± 0.23 [B]
	sSAL+LAB	0	8.21 ± 0.14 [A]	1.07 ± 0.36 [A]	6.12 ± 0.24 [A]
		24	9.38 ± 0.08 [B]	2.81 ± 0.18 [BC]	4.87 ± 0.10 [B]
		48	9.37 ± 0.11 [B]	2.87 ± 0.04 [B]	4.74 ± 0.17 [B]
		72	9.23 ± 0.06 [B]	2.46 ± 0.02 [C]	4.90 ± 0.11 [B]
2	LAB 2% NaCl	0	8.23 ± 0.03 [A]	N/A	5.99 ± 0.22 [A]
		24	9.13 ± 0.05 [B]	N/A	4.87 ± 0.14 [B]
	sSAL+LAB 0% NaCl	0	8.30 ± 0.04 [A]	1.75 ± 0.24 [A]	6.25 ± 0.05 [A]
		24	9.33 ± 0.04 [B]	3.37 ± 0.03 [B]	4.97 ± 0.11 [B]
	sSAL+LAB 1% NaCl	0	8.27 ± 0.05 [A]	1.70 ± 0.26 [A]	6.16 ± 0.21 [A]
		24	9.29 ± 0.09 [B]	3.14 ± 0.34 [B]	4.93 ± 0.19 [B]
	sSAL+LAB 2% NaCl	0	8.29 ± 0.04 [A]	1.82 ± 0.20 [A]	6.03 ± 0.20 [A]
		24	9.18 ± 0.17 [B]	3.37 ± 0.23 [B]	5.06 ± 0.13 [B]

[a] Values are means ± standard deviation ($n = 4$); within columns for each batch, means with different uppercase letters are significantly different ($P \leq 0.05$) across fermentation timepoints.
[b] LAB A, uninoculated cashews from lot A with starter culture; sSAL, inoculated cashews and no starter culture; sSAL+LAB, inoculated cashews with starter culture; LAB 2% NaCl, uninoculated cashews with starter culture and 2% NaCl (w/w); sSAL+LAB 1% NaCl or sSAL+LAB 2% NaCl, inoculated cashews with starter culture and 1% or 2% NaCl (w/w).
[c] Plated on M17 agar and held at 30°C for 48 h.
[d] Plated on CHROMagar Salmonella and held at 37°C for 24 h.

Table 3.3

Populations of *Salmonella* and total aerobic bacteria and pH during fermentation of *Salmonella* cocktail–inoculated cashews with and without starter culture, citric acid, and NaCl [a,b].

Trial	Batch [c]	Fermentation time (h)	Aerobic bacteria [d] (log CFU/g)	*Salmonella* [e] (log CFU/g)	pH
3	LAB B	0	8.32 ± 0.11 [A]	N/A	6.07 ± 0.10 [A]
		12	9.11 ± 0.23 [B]	N/A	4.88 ± 0.15 [B]
		24	9.32 ± 0.11 [B]	N/A	4.64 ± 0.16 [BC]
		48	9.37 ± 0.04 [B]	N/A	4.50 ± 0.10 [C]
		72	9.30 ± 0.01 [B]	N/A	4.56 ± 0.06 [C]
	cSAL	0	2.02 ± 0.05 [A]	1.82 ± 0.17 [A]	6.13 ± 0.16 [A]
		12	5.44 ± 0.55 [B]	3.94 ± 0.28 [B]	6.08 ± 0.10 [AB]
		24	8.34 ± 0.22 [C]	7.17 ± 0.18 [C]	5.82 ± 0.20 [B]
		48	9.32 ± 0.02 [D]	8.05 ± 0.36 [C]	4.81 ± 0.20 [C]
		72	9.28 ± 0.03 [D]	7.52 ± 0.89 [C]	4.62 ± 0.14 [C]
	cSAL+LAB	0	8.20 ± 0.03 [A]	1.88 ± 0.17 [A]	6.09 ± 0.12 [A]
		12	9.13 ± 0.24 [B]	2.55 ± 0.22 [B]	4.90 ± 0.12 [B]
		24	9.32 ± 0.03 [B]	2.45 ± 0.09 [B]	4.64 ± 0.07 [C]
		48	9.34 ± 0.02 [B]	1.99 ± 0.06 [A]	4.55 ± 0.08 [C]
		72	9.26 ± 0.04 [B]	0.93 ± 0.17 [C]	4.42 ± 0.17 [C]
4	LAB+CA	0	8.01 ± 0.10 [A]	N/A	5.30 ± 0.31 [A]
		24	8.87 ± 0.33 [B]	N/A	4.80 ± 0.23 [B]
		48	9.08 ± 0.03 [B]	N/A	4.44 ± 0.06 [B]
		72	9.18 ± 0.16 [B]	N/A	4.42 ± 0.07 [B]
	cSAL+LAB	0	8.15 ± 0.04 [A]	2.14 ± 0.21 [A]	6.35 ± 0.41 [A]
		24	9.37 ± 0.01 [B]	2.60 ± 0.35 [A]	4.95 ± 0.30 [B]
		48	9.35 ± 0.04 [B]	2.36 ± 0.06 [A]	4.58 ± 0.05 [B]
		72	9.13 ± 0.19 [C]	1.15 ± 0.63 [B]	4.46 ± 0.05 [B]
	cSAL+LAB+CA	0	8.02 ± 0.07 [A]	2.08 ± 0.08 [A]	5.36 ± 0.44 [A]
		24	9.10 ± 0.10 [B]	1.65 ± 0.65 [A]	4.81 ± 0.33 [AB]
		48	9.09 ± 0.02 [B]	1.94 ± 0.28 [A]	4.47 ± 0.04 [B]
		72	9.07 ± 0.05 [B]	1.27 ± 0.35 [A]	4.41 ± 0.06 [B]
	cSAL+LAB+CA+NaCl	0	8.02 ± 0.15 [A]	2.06 ± 0.19 [A]	5.26 ± 0.42 [A]
		24	8.98 ± 0.08 [B]	1.92 ± 0.51 [A]	4.71 ± 0.30 [AB]
		48	8.96 ± 0.04 [B]	2.13 ± 0.14 [A]	4.45 ± 0.04 [B]
		72	8.90 ± 0.08 [B]	1.94 ± 0.22 [A]	4.35 ± 0.05 [B]

[a] Values are means ± standard deviation (n = 4); within columns for each batch, means with different uppercase letters are significantly different ($P \leq 0.05$) across fermentation timepoints.
[b] Cashews were inoculated with a seven-strain cocktail of *Salmonella*.
[c] LAB B, uninoculated cashews from lot B with starter culture; cSAL, inoculated cashews and no starter culture; cSAL+LAB, inoculated cashews with starter culture; LAB+CA, uninoculated cashews with starter culture and 0.5% citric acid (w/w); cSAL+LAB+CA, inoculated cashews with starter culture and 0.5% citric acid (w/w); cSAL+LAB+CA+NaCl, inoculated cashews with starter culture, 0.5% citric acid (w/w), and 1% NaCl (w/w).

[d] Plated on M17 agar and held at 30°C for 48 h.
[e] Plated on CHROMagar Salmonella and held at 37°C for 24 h.

Table 3.4

Water activity of cashew cheese analogs prepared with a lactic acid bacteria starter culture with and without the addition of NaCl or citric acid [a]

Trial	Batch [b]	Fermentation time (h) [c]	Water activity [d]
1	LAB A	0	0.9921 ± 0.0007 [Aa]
		24	0.9886 ± 0.0006 [Ba]
		48	0.9915 ± 0.0018 [Aa]
		72	0.9901 ± 0.0006 [ABa]
2	LAB 2% NaCl	0	0.9736 ± 0.0014 [Ab]
		24	0.9743 ± 0.0002 [Ab]
3	LAB B	0	1.0009 ± 0.0051 [Aa]
		24	0.9922 ± 0.0019 [Ba]
		48	0.9915 ± 0.0018 [Ba]
		72	0.9931 ± 0.0035 [Ba]
4	LAB+CA	0	0.9982 ± 0.0041 [Aa]
		24	0.9959 ± 0.0014 [Ac]
		48	0.9942 ± 0.0017 [Aa]
		72	0.9964 ± 0.0024 [Aa]

[a] Water activity of raw cashews was 0.5045 ± 0.0077.
[b] LAB A or LAB B, uninoculated cashews from lot A or B, respectively, with starter culture; LAB 2% NaCl, uninoculated cashews from lot A with starter culture and 2% NaCl (w/w); LAB+CA, uninoculated cashews from lot B with starter culture and 0.5% citric acid (w/w).
[c] Fermentation time refers to hours after soaking, draining, and blending the cashews with added water.
[d] Values are means ± standard deviation (n = 4); within columns for each batch, means with different uppercase letters are significantly different ($P \leq 0.05$) across fermentation timepoints; for each fermentation time, means with different lowercase letters are significantly different across all batches.

Supplemental Table S3.1

Strain designations and sources for bacteria used in the present study.

Salmonella serovar	Original strain designation[a]	Harris culture collection | rifampin-resistant strain designation	Isolation source (original strain source)	Reference
Tennessee	K4643	LJH 1783 | LJH 1790	Isolated from peanut butter associated with 2007 outbreak (provided by Dr. L. Beuchat, University of Georgia (Athens, GA))	CDC, 2007
Agona	447967	LJH 1784 | LJH 1791	Isolated from toasted oats cereal associated with 1998 outbreak (provided by FDA, ORA Arkansas Regional Lab (Jefferson, AR))	CDC, 1998
Montevideo	488275	LJH 1785 | LJH 1792	Isolated from black and red pepper associated with 2009 outbreak (provided by FDA, ORA Arkansas Regional Lab (Jefferson, AR))	CDC, 2010
Mbandaka	698538	LJH 1786 | LJH 1793	Isolated from tahini sesame paste associated with 2013 outbreak (provided by FDA, ORA Arkansas Regional Lab (Jefferson, AR))	CDC, 2013
Enteritidis PT[b] 30		LJH 608 | LJH636	Isolated from raw almonds associated with 2000 to 2001 outbreak (provided by Silliker laboratories). Deposited to ATCC by Dr. L. J. Harris as ATCC BAA-1045	Chan et al., 2002; Isaacs et al., 2005
Reading	Moff 180418	LJH 1788 | LJH 1794	Laboratory strain (provided by FDA, CFSAN (Bedford Park, IL))	N/A
Urbana	N/A	LJH 1789 | LJH 1795	Environmental isolate from raw cashew pieces	Louvau and Harris, 2023

[a] All original isolates were received from the U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition (Bedford Park, IL).
[b] PT, phage type.

Chapter 4: Summary and future research

Summary

Nut-based cheese analogs are part of a quickly expanding market, but little information is available to understand the fermentation process or safety related to these types of products. The work in this thesis centered around a 2020–2021 salmonellosis outbreak linked to a cashew Brie analog. Chapter 2 assessed the levels and distribution of *Salmonella* in naturally contaminated cashews, finding low levels (0.0023 MPN/g) of *Salmonella* uniformly distributed throughout. Four *Salmonella* serovars were identified during the analysis, Fresno, Nima, Urbana, and Vinohrady, with the latter two being in common with the outbreak investigation. In Chapter 3, a fermented cashew cheese analog was prepared and *Salmonella* behavior was assessed. One recipe was used to prepare the cashew cheese analog, using two lots of cashews from one source, a single starter culture, one fermentation temperature, and addition of salt or citric acid. The research demonstrated that *Salmonella* grows in soaked and ground cashews at typical fermentation temperatures. The addition of a starter culture significantly reduced the overall population increase of *Salmonella* during fermentation but did not completely prevent growth. Addition of salt had no impact on the behavior of *Salmonella*, while citric acid prevented growth but did not decrease *Salmonella* levels. However, there are many factors that potentially impact the safety of nut-based cheese fermentations that remain unexplored.

Cashews

This research used one source of cashews to prepare the fermented cashew cheese analog. Cashew pieces were purchased from an online retailer; two lots were used over the course of the experiments and all the cashews originated from Vietnam. Many countries outside of Vietnam are major cashew producing regions; many varieties of cashews are grown. The final pH after fermentation did not reach the recommended 4.4 after 72 h of fermentation. Future work should consider cashew variety, growing region, and form (whole kernel or pieces). Compositional analysis of the raw cashews might also

provide information that could be used to predict the final pH. The pH of batches prepared with *Salmonella* but without starter culture dropped to levels that were similar to those with starter culture after 48 h, indicating that the combination of *Salmonella* and background microorganisms present on the cashew pieces lower the pH. Determining the pH drop in fermented cashews without either *Salmonella* or starter culture would be useful to understand the influence of *Salmonella* and background microbiota on the final pH. Cashews originating from different growing regions and different producers may have different background microorganism compositions that can impact the final pH achieved throughout fermentation. An analysis of the microbiome of the fermentation may yield useful information on the types of microorganisms present and that dominate after soaking and fermentation.

There was a unique opportunity to have naturally contaminated cashews available for this study (Chapter 2). The cashews linked to the 2020–2021 Jule's cashew Brie analog salmonellosis outbreak originated in the Ivory Coast. They were stored under refrigerated temperatures since May 2021. The implicated cashew cheese analog producer reportedly used pH 4.4 as a target for the end of fermentation. Comparing the fermentation characteristics of these cashews with those used in Chapter 3 may yield additional useful information.

Starter culture

The starter culture used in this study was a single commercial mixed-strain *Lactococcus lactis* mesophilic starter culture. This starter culture was used by the implicated producer in the 2020–2021 cashew Brie analog outbreak and is intended for use in dairy cheese fermentations. The results of this study clearly show the positive impact of using a starter culture in suppressing the growth of *Salmonella* during fermentation and provide data to support current recommendations from the British Columbia Centre for Disease Control for use of lactic acid bacteria starter cultures when fermenting cashew cheeses (BC Centre for Disease Control, 2017). However, several other starter cultures are used by consumers or recommended on online blogs and recipes (Swinehart, Harris, Anderson, & Feng, 2023; Swinehart, Harris, Louvau, & Feng, 2023). These starter cultures include probiotic capsules and powders, rejuvelac,

miso, yogurt cultures, completed vegetable or fruit fermentations (such as sauerkraut or kimchi), kefir, and kombucha. No published research using any of these types of starter cultures for fermenting cashew cheese analogs is available. Future research should evaluate some of the more common starter cultures to understand their impact on the fermentation and on *Salmonella* growth and survival compared to the lactic acid bacteria starter culture used in Chapter 3. It would be helpful to have a better understanding of the range of starter cultures that are used in commercial practice.

Recipe modifications

Fermentation times and temperatures

The protocol used in Chapter 3 was based closely on the recipe used by Jule's to create their cashew Brie analog associated with the 2020–2021 outbreak (Centers for Disease Control and Prevention, 2021; Lewis et al., 2023). This protocol included a refrigerated 24 h soak, followed by inoculation with starter culture and a 24 h ambient temperature fermentation. Consumers use a wide variety of fermentation times and temperatures when preparing cashew cheese analogs (Swinehart, Harris, Anderson, & Feng, 2023). Fermentations at refrigerated temperatures or at temperatures much warmer than ambient temperature would likely impact the overall fermentation, final pH, and behavior of *Salmonella*. Fermentation times ranged from less than 12 h up to several days depending on the recipe. The results from Chapter 3 showed that the main bacterial growth and pH drop during an ambient temperature fermentation occur in the first 12 to 24 h. Future research should consider conducting fermentations at different temperatures and times to better understand the impact on *Salmonella* behavior and the progression of the fermentation.

Ripening and storage

The cashew Brie analog associated with the 2020–2021 *Salmonella* outbreak included a week-long refrigerated ripening step to form a mold rind. Chapter 3 focused only on the bacterial fermentation step of the cashew cheese analog preparation, but future work including addition of a *Penicillium*

candidum mold starter culture and subsequent rind formation during ripening would shed further insight into pH changes and *Salmonella* behavior during an extended ripening period.

The shelf life of cashew cheese analogs has not been assessed, particularly with regards to the behavior of *Salmonella.* Given that these types of products can be kept at refrigerated temperatures for extended periods of time by at-home producers and consumers, it would be beneficial to assess *Salmonella* behavior during storage.

Added ingredients

The current work evaluated the addition of salt and citric acid prior to the fermentation of a cashew cheese analog. Salt was added at concentrations of 1% and 2% (w/w) based on concentrations often added to other types of fermented vegetables and dairy cheeses. These concentrations of salt did not inhibit *Salmonella* growth. Future work might consider addition of salt at higher concentrations, but the sensory impact of higher salt concentrations would need to also be assessed. Citric acid was added at 0.5% and inhibited *Salmonella* growth. Future work may consider increasing the concentration of citric acid or addition of other acids such as acetic acid which was reported as a common ingredient by consumers (usually in the form of apple cider vinegar; Swinehart, Harris, Anderson, & Feng, 2023).

Sugar is an ingredient commonly mentioned in online blogs and recipes that was not assessed in the current study. Sugar (which can be added in many forms including table sugar, honey, maple syrup, agave, etc.) could impact both the fermentation and *Salmonella* behavior greatly. Sugar would provide additional carbohydrate sources, compared to what is naturally available in cashews, to the bacteria present during the fermentation. This might give an advantage to the starter culture to grow more quickly and lower the pH earlier on during the fermentation. Additional sugar might also give *Salmonella* an advantage and allow for more *Salmonella* growth given that there is no longer a limited food supply for which to compete with the starter culture. Understanding the impact of sugar as an added ingredient would provide important information about the fermentation progression and *Salmonella* behavior. It would also prove useful to understand consumer acceptance of sugar as an added ingredient, as the

consumer base for plant-based cheese products might reject products that have added sugar listed as an ingredient.

Metagenomic analysis

Samples from each fermentation in Chapter 3 were frozen at –20° for future metagenomic analysis. Metagenomic analysis would provide insight into the microbial progression throughout the fermentation. This would be useful to understand which microorganisms–the starter culture, background microorganisms, and *Salmonella*–dominate at each stage of the fermentation and would provide more insight into understanding the fermentation process.

All *Salmonella* enrichments from the naturally contaminated cashews in Chapter 2 have been sent to the U.S. FDA CFSAN laboratory for metagenomic analysis with a goal to understand the microbial composition that results for each type of enrichment media, and between *Salmonella*-positive and negative enrichments. These enrichments will also be used to evaluate the robustness of *Salmonella* serovar detection methods used by the FDA.

Other tree nuts and pathogens

This thesis focused entirely on cashews and cashew cheese analogs. Other tree nuts have been reported as raw ingredients for nut-based cheeses, including almonds, pistachios, and walnuts. Although outbreaks related specifically to nut-based cheese analogs have exclusively been linked to cashew-based products, almonds, pistachios, and walnuts have been linked to other *Salmonella* (almonds and pistachios) and *Escherichia coli* (walnuts) outbreaks in North America (Harris & Yada, 2022). Behavior of *Salmonella* was assessed in the current study, but it would also be valuable to evaluate the behavior of *Escherichia coli* and *Listeria monocytogenes* during nut-based cheese analog fermentations. A recall of cashew cheese analog in France due to *L. monocytogenes* contamination occurred in January 2023 (Santé publique France, 2023). Similarly, pathogenic strains of *Escherichia coli* have been associated with hazelnuts and walnuts (Harris, Yada, Beuchat, & Danyluk, 2022). Understanding the fermentation

characteristics for different types of tree nuts and pathogen behavior during such fermentations would be beneficial for broadening safety and understanding of nut-based cheese analogs.

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