SURVIVAL OF *SALMONELLA ENTERICA* AND SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* AND FLUCTUATIONS OF INDIGENOUS MICROBIOTA POPULATIONS THROUGH KOMBUCHA FERMENTATION

by

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(Under the Direction of Ynes Ortega)

ABSTRACT

Kombucha is a fermented, acidic beverage with a rapidly expanding market in the U.S. Four different kombucha home-fermentation starter kits were evaluated. Aerobic plate counts (APC), yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) were enumerated over 45 d. Survival of *Salmonella enterica* and Shiga toxin-producing *Escherichia coli* in kombucha were assessed over 14 d. Populations were enumerated (log CFU/mL) with surface plating techniques on selective media. All microbiota groups surmounted a significant population increase (1 – 1.5 log) by 2 d and did not significantly fluctuate throughout 3 – 14 days of fermentation. A 5-log reduction of *Salmonella* was observed by 5 – 10 d with an endpoint pH of 3.13 – 3.71. A 5-log reduction of STEC was observed by 7 – 14 d with an endpoint pH of 2.66 – 3.39. Variations may be attributed to differences in microbiota of starter cultures and types of base tea used for fermentation.

INDEX WORDS: kombucha, tea, fermentation, *Salmonella*, Shiga toxin-producing *Escherichia coli*, lactic acid bacteria, acetic acid bacteria

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DEDICATION

To my parents, Scottie and Tina Brewer.

It is their awe-inspiring work ethic and continued support that propelled me to where I am today.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 KOMBUCHA

Overview and description. Kombucha is an acidic, slightly sweet, carbonated beverage that involves the microbial fermentation of tea. Kombucha has been brewed for thousands of years in East Asia, reportedly with early beginnings in China and later spreading to Korea and Japan *(50)*. As trade routes expanded, kombucha made its way westward. Although reported timelines are conflicting, the Middle East, Russia, and Germany all have reports of kombucha consumption for centuries *(36, 112)*. After World War II, the consumption of the beverage expanded across Europe into France, Italy, and Switzerland *(50)*. Today, the beverage has popularity worldwide and can be purchased in supermarkets from commercial producers and through a number of online retailers *(50)*. Kombucha has especially seen recent growth in the United States, due in large part to media admiration for its health benefits *(112)*.

Kombucha has two general components, a fermented tea broth and a thick floating biofilm-like pellicle. This floating microbial mat is referred to by several names in the literature, including: pellicle, biofilm, SCOBY (Symbiotic Culture Of Bacteria and Yeasts), tea fungus, tea mushroom, and others *(36)*. This "tea fungus" was erroneously given a formal botanical name of *Medusomyces gisevii (50)*. However, it is not a single fungus or mushroom, but rather a floating consortium of bacteria and yeasts that is structured by a cellulose network *(2)*. This cellulose pellicle sets on top of the tea during the fermentation process and a new pellicle forms during each successive fermentation cycle *(27)*. While it is not a scientific term, "SCOBY" is the most commonly used phrase to describe this cellulose mat among kombucha producers and will be used for the remainder of this document. Contrary to popular belief, the SCOBY is not necessary for kombucha fermentation, but instead is a by-product of cellulose production

by acetic acid bacteria *Komagataeibacter xylinus* (previously *Acteobacter xylinum*) that is unique to kombucha *(2, 113)*.

Typical kombucha fermentation does not use pure cultures of bacteria and yeasts as is common with beer and yogurt production, but instead fermentation is induced by adding an undefined starter culture sample (SC) from another kombucha, a fermentation process often termed 'back slopping' *(16, 27)*. A sample of previously fermented kombucha as a starter culture (often referred to as "starter liquid") that contains the active microbiota necessary to begin fermentation is added at a 10 – 20 % (v/v) ratio to sweetened tea *(50, 71, 78)*. It is important that the tea used as the base for fermentation is acclimated to room temperature because if the tea is too hot, the microbiota in the SC will be killed off and if the tea is cold, it could send the microbiota into a state of temporary dormancy. The addition of the SC to the base tea will result in an immediate pH drop *(78)* and catalyzes the fermentation process. After the liquid SC is added and mixed with the sweetened tea, the SCOBY is placed on top, and the fermentation vessel is covered with thin material to allow air exchange but prevent environmental contamination. Cheesecloth, coffee filters, and paper towels are frequently used. The glass vessel containing the kombucha mixture is incubated at room temperature for 5 – 14+ days, allowing time for the fermentation process to occur *(78, 112)*. The end product is an effervescent, acidic, slightly alcoholic tea that many equate in similarity to apple cider *(42, 50, 60, 112)*. This unique flavor profile has led to a greater acceptance by consumers, and the beverage is widely consumed for its claimed health benefits.

Because of the simplicity of the process, kombucha may be produced for personal consumption by fermentation in the home *(15)*. The sugar content of the prepared tea usually varies from 5 - 10% and up to 20% for some commercial manufacturers *(50, 78)*. The type of sugar substrate can vary, as white sugar, honey, molasses, milk, wine, and even Coca-Cola have all been studied *(50, 65)*. Green or black tea is commonly used for the base tea, but any variety of tea type can be used, such as oolong or blue tea *(50)*. However, several publications report that black tea sweetened with sucrose is the most standard and purest form of kombucha *(50, 71, 112)*. Consumers who want to begin kombucha fermentation at home

may obtain their first starter culture from another local kombucha brewer *(17, 72)* or use kombucha starter kits that can be provided by internet purchase and mail order *(112)*. Given the recent rise in popularity of the product, there are numerous commercial suppliers that sell whole, mail-order kombucha starter kits with a SCOBY, liquid starter culture, tea blends, and specific instructions for consumer home brewing. It should be noted that while internet-purchased kombucha starter cultures may stem from a reputable commercial supplier, the starter cultures are still derived from the back-slopping style fermentation and the microbiota are generally undefined.

Proposed health benefits of kombucha. Many people drink kombucha not only for its unique taste but also for its claimed but unfounded health benefits. There are over 24 positive health effects associated with consuming kombucha that are noted in various studies and publications *(50, 53, 71, 75, 112)*. Dufresene and Farnworth provide a highly-cited review on the health effects of kombucha *(36)*. However, most of this scientific evidence is based on *in-vitro* cell lines or *in-vivo* rodent models. There are no human trials with kombucha and health effects to date that have a reputable study design *(50, 71)*. An epidemiological research group of the University of Missouri conducted a systematic review of literature regarding health effects in kombucha. While 310 articles of relevance were reviewed, only one study reported any empirical evidence of health benefits of kombucha in humans *(53)*. The single study identified reported daily consumption of kombucha was associated with normalized blood glucose levels in patients with non-insulin dependent diabetes mellitus and also referenced previous work that reported improvements with mild hypertension in individuals with the same treatment *(46)*. However, neither trial included a control group of human subjects for comparison. The epidemiological review stressed the need for human clinical trials to investigate kombucha health benefits especially in context of research advancements with human microbiome studies *(53)*.

While these reported health benefits lack evidence in human studies, some of the positive health effects that have numerous studies documenting the effects in rodent models include: blood glucose regulation *(3, 94, 96)*, cholesterol reduction *(3, 114)*, protection from heavy metal contamination *(34, 89)*,

hepatoprotection *(9, 80)*, and use as a probiotic *(59, 91)*. Kombucha is perhaps most well-known for its probiotic effects *(53, 59)*. Probiotic foods and dietary supplements deliver microorganisms that may help provide a balance in the gut microbiome and thereby, normalizing processes in the intestines and improving digestion *(85)*. Probiotics are also reported to boost the immune system, fight harmful bacterial overgrowths, and improve mood and overall well-being *(8, 112)*. It has also been suggested that the high nanocellulose content in kombucha may act as a dietary fiber prebiotic which expedites waste excretion of toxins and helps maintain a healthy microbiome *(59)*.

Indigenous microbiota of kombucha. Microbial communities in the environment are not static, and there exists an enormous diversity and complexity of species interactions *(2)*. Kombucha microbial community is particularly difficult to characterize because the process is generally artisanal *(78)*, each fermentation involves back-slopping of an undefined starter culture *(27)*, and seemingly minor changes in the environment can result in drastically different end result products *(42)*. One author noted that the exact microbial composition of kombucha simply cannot be characterized in its entirely because it varies so much *(50)*. The following are just some of the factors that alter the microbial community of kombucha: source of starter culture, type of base tea used, geographic location, climate, tap water source, type of sugar source, temperature, humidity, pH, amount of available oxygen, dissolved CO_2, and the size and shape of the fermentation vessel *(2, 27, 70, 72, 102, 112)*. The microbial community also differs significantly between the floating SCOBY and the liquid portion of kombucha *(27, 84)*. Swiss scientists produced one of the earliest reports of culturing and identifying microorganisms in kombucha in 1965 *(44)*, and since then there have been nearly 30 additional studies attempting to quantify and characterize the dynamic microbial community of kombucha *(2, 27)*. Most of these have been largely culture based which have several limitations *(64, 72, 95, 102)*. Recently, studies have characterized the microbial community of kombucha using metagenomic techniques and reported microbial diversity in terms of operational taxonomic units (OTUs) derived from the 16S rRNA ribosomal gene for bacteria and Internal Transcribed Spacer (ITS) and 26S rRNA ribosomal gene for fungi *(20, 27, 70, 84)*. Across these studies,

some consistencies become apparent regarding predominant bacteria genera but the overall microbial community varies considerably. This results of these metagenomic studies are summarized in Table 1.

It is well known that microbes present in kombucha are primarily acetic acid bacteria, lactic acid bacteria, and yeasts. Several studies report that yeasts outnumber bacterial cells *(51, 71, 112)*, even two fold higher in one study *(22)*. However, a high-throughput sequencing study involving industrial size kombucha brewing found that yeast and bacteria populations were not significantly different, and total mesophilic bacteria counts outnumbered yeasts in some cases *(27)*. That same study identified a much higher species richness of bacteria than yeasts *(27)*. On the other hand, a similar sequencing study found a higher species richness and higher alpha local diversity in yeasts as compared to bacteria *(20)*. As aforementioned, the SCOBY and liquid tea portion have different microbial communities. Some studies report finding higher cell counts *(27, 29)* and microbial diversity in the biofilm *(70)*; while others report finding higher cell counts *(22, 112)* and diversity in the liquid fermented tea *(20, 84)*.

A general consensus across studies is that acetic acid bacteria (AAB) predominate the bacterial community with lactic acid bacteria (LAB) taking up some smaller portion of the community. The reporting of AAB genera composition can be misleading across studies, in large part to simple taxonomic change. Numerous studies report *Acetobacter xylinum* as the dominating bacteria *(36, 65, 95)* which can be expected because it is primarily responsible for the production of cellulose and formation of the SCOBY *(8, 42, 71)*. However, *A. xylinum* was re-named as *Glucoacetobacter xylinus* in 2000 *(101)* and then re-classified as *Komagataeibacter xylinus* in 2012 *(113)* as more phenotypic and genetic diversity was reported. Because multiple reclassifications of this bacteria genus, most publications from the last 20 years may use any of the three genus terms to describe the same bacteria species, and several studies note this discrepancy in interrupting their sequencing results *(29, 70)*. Several studies report a combination of *Gluconobacter*, *Glucoacteobacter*, *Acetobacter*, or *Komagateibacter* as the dominating bacterial genera *(30, 50, 112)*, and various *Acetobacter*, *Gluconobacter*, and *Lyngbya* species as supporting but not dominating acetic acid bacteria genera *(2, 20, 84)*. The notable lactic acid genera seen across studies

include: *Lactobacillus*, *Leuconostoc*, *Bifidobacterium*, and *Oenococcus (2, 27, 84)*. Coton et al. reported green tea kombucha was dominated by lactic acid bacteria and black tea kombucha was dominated by acetic acid bacteria *(27)*. However, the same trend was not observed in the SCOBY. Over the course of fermentation, the community of both the green and black tea of the SCOBY was dominated by acetic acid bacteria *(27)*.

The yeast community of kombucha is far more inconsistent than the bacterial community. Although there are some yeast genera that consistently appear in these identification studies, the overall composition and proportion of yeast species can change drastically with environmental conditions. Some types of yeasts that have consistently been identified in kombucha through culture-based methods include: *Brettanomyces*, *Zygosaccharomyces*, *Saccharomyces*, *Pichia*, and *Candida (42-44, 64, 72, 95, 102)*. Since then, several other dominating yeast species have been identified in kombucha through the use of next-generation sequencing. Table 1 summarizes available studies that have analyzed the microbial community of kombucha with high-throughput sequencing technology.

TABLE 1. Survey of yeast and bacteria genera identified in metagenomic studies of kombucha microbial community

Publication	16S Bacterial IDs[1]	Dominating Bacteria Genera[2]	ITS Fungal IDs[1]	Dominating Fungi Genera[2]
A.J. Marsh et al. 2014 *(70)*	*Gluconacetobacter* *Lactobacillus* *Lactococcus* *Thermus*	*Gluconoacetobacter* *Lactobacillus*	*Leucosporidiella* *Pichia* *Zygosaccharomyces* *Dekkera*	*Zygosaccharomyces* *Dekkera*
Reva et al. 2015 *(84)*	*Herbaspirillum* *Halomonas* *Komagataeibacter* *Gluconobacter* *Acetobacter*	*Komagateibacter* *Gluconobacter*	*Dekkera* *Candida* *Pichia* *Saccharomyces*	*Pichia* *Saccharomyces*
S. Chakravorty et al. 2016 *(20)*	*Komagataeibacter* *Gluconobacter* *Lyngbya* *Bifdobacterium*	*Komagateibacter* *Gluconobacter*	*Candida* *Eremothecium* *Debaryomyces* *Meyerozyma* *Hanseniaspora* *Kazachstania* *Lachancea* *Starmera*	*Candida* *Lachancea*
Coton et al. 2017 *(27)*	*Cellulosimicrobium* *Enterobacter* *Gluconoacetobacter* *Gluconobacter* *Hydrogenophilus* *Kluyvera* *Lactobacillus* *Leuconostoc* *Oenococcus* *Pantoea* *Psuedochrobactrum* *Rothia*	*Gluconoacetobacter* *Oenococcus*	*Aspergillus* *Candida* *Clavispora* *Dekkera* *Hanseniaspora* *Kregervanrija* *Pichia* *Saccharomyces* *Torulapora* *Wickerhamomyces* *Zygoascus* *Zygotorulaspora*	*Dekkera* *Hanseniaspora*

[1] Only bacteria and yeast genera OTUs identified in metagenomic studies that compromised ≥ 1 % of the total community population are reported.

[2] "Dominating" bacteria and fungi genera refer to the top two genera that represented most of the microbial community.

1.2 FOOD SAFETY RISKS POSED WITH KOMBUCHA AND RELATED FOODS

Case studies of illnesses linked to kombucha. There have been documented cases of illnesses representing various symptoms associated kombucha although without conclusive links *(75)*. Most notably, there was a case in Iowa in 1995 where two female patients fell ill within a 7 day time period of each other after consuming kombucha *(17)*. One patient (59 years old) suffered from severe metabolic acidosis, went into cardiac arrest and later died. The cause of acidosis was never fully determined. The other patient (48 years old) suffered from respiratory distress and also went into cardiac arrest, but she was resuscitated, recovered, and eventually discharged from the hospital *(17)*. Both patients had reported drinking kombucha tea recently and had both received the kombucha starter culture from the same source. The CDC performed an epidemiologic analysis on the region and found that over 100 people had received a kombucha starter culture sample from the same source, but there were no other reported symptoms of disease or illness. Samples of the starter culture were sent to the FDA for testing, but no human pathogens or microbial toxins were detected *(17)*. Overall, kombucha was a primary suspect for the cause of disease but no conclusive links were established. The FDA has previously analyzed commercial kombucha samples and found no pathogenic organisms or chemical violations. The agency deemed it safe for human consumption but noted that in home preparations, *Aspergillus* mold contamination could be of concern *(17, 109)*.

Additionally, there have been some reported cases of illness in immunocompromised persons where kombucha was a suspected culprit. In 2008 in Los Angeles, California, there was a case of hepatoxicity in an HIV-positive student that reported recently drinking kombucha *(100)*. Again, at the South Dakota University Hospital in 2016, there was a case of hepatoxicity in a diabetic middle-aged woman who reported recently drinking kombucha *(39)*. At the Sheffield Teaching Hospital in the U.K. in 2017, there was a case of severe respiratory distress in a woman with a history of asthma who had been consuming kombucha. Her symptoms subsided after stopping consumption of the tea *(47)*. There are a collection other loosely linked reports of health issues in persons who had been drinking kombucha,

including: rash development, jaundice, GI distress, shortness of breath, and tachycardia *(37)*. All cases occurred in immunocompromised individuals, and kombucha was never conclusively linked as the sole cause *(37, 112)*. Murphy et al. provides a review of case studies of illness associated with loose links to kombucha and notes that consumption high doses of kombucha can lead to detrimental health impacts *(75)*. Notably, none of these illness case studies associated with kombucha consumption were reported to be associated with a foodborne pathogen.

Historical evidence of foodborne disease in tea. Considering its wide consumption, there have been relatively few bacterial foodborne disease outbreaks with commonplace, unadulterated tea. A 1981 publication by a member of the CDC assessing foodborne disease risk in food service establishments assigned tea a food-property risk factor coefficient of 1 which is assigned to "foods that are constitutionally incapable of supporting growth of pathogenic organisms because of their pH, water activity, or other growth limiting factors" *(12)*. Essentially, tea was one of the lowest risk foods for restaurants in the report.

Using the CDC National Outbreak Reporting System (NORS) database, a search was conducted for any outbreaks that had an association to tea *(19)*. A total of 20 foodborne outbreaks from 1998-2017 report a type of tea as a potential food vehicle or contaminated ingredient. Of those 20 outbreaks, only three are attributable to a bacterial pathogen. All three outbreaks in tea were associated with *Salmonella enterica (19)*. The first reported outbreak occurred in August 2006 at a college in North Carolina. Twenty-five illnesses were reported from the outbreak which was linked back to "iced tea" as the food vehicle for infection. *Salmonella enterica* serotype Bareilly was determined to be the culprit *(19)*. The next outbreak occurred a short time afterwards in September 2006 in Arizona. Fifty-two people reported illnesses, and the outbreak was linked back to a "hard iced tea" product from a restaurant in which *Salmonella enterica* serotype Oranienburg was reported as the etiology of disease *(19)*. The last and largest reported outbreak occurred in that same year in November 2006 in Texas, again at a university. Seventy-seven persons were reported with illness from the outbreak, and two persons were hospitalized.

The disease was suspect to have come from two local college cafeterias that were serving a late Thanksgiving meal *(105)*. The outbreak was traced back to either "turkey gravy" or "iced tea" with *Salmonella enterica* serotype Newport determined as the etiology of disease *(19, 105)*. In this case, tea was not absolutely determined to be the sole factor of disease, but it could also not be ruled out and should be considered.

Widescale outbreaks associated with tea leaves have been reported across Europe. From 2002-2003, a nationwide outbreak of *Salmonella enterica* serotype Agona occurred in Germany *(57)*. The outbreak primarily affected infants with 42 cases over the course of a few months. The outbreak was traced back to tea bags with aniseed. Subsequent testing of store products found that 11% of all tea products with aniseed were positive for *Salmonella (57)*. A similar occurrence happened in Serbia from March 2007-September 2008. There were 14 cases of infants falling ill with a *Salmonella enterica* serotype Seftenberg infection *(48)*. In both outbreaks, investigators and researchers concluded that the likely problem was that the tea leaves were not boiled for a sufficient amount of time, and hot water was being poured over the tea leaves instead of being boiled with the water *(48, 57)*. All of these cases could all lead to *Salmonella* still being present if the tea does not reach 55°C for 20-25 minutes *(7)*.

A study by Keller et al. demonstrated the viability of *Salmonella* on dried tea leaves throughout extended storage and subsequent transfer into tea brewed with the inoculated tea leaves *(54)*. The researchers found that *Salmonella* populations were still detectable on green tea leaves at 7 log (CFU/g) for greater than 6 months when stored at room temperature (25℃) and ≤ 30% relative humidity. Furthermore, *Salmonella* could be transferred into tea brewed from the inoculated tea leaves if the hot water poured over the tea leaves was not at a temperature of ≥ 75 ℃ and brewed for at least 1 minute *(54)*. When tea was brewed with *Salmonella* inoculated tea leaves and a water temperature of 55 ℃, less than a 2-log reduction of *Salmonella* was reported with an 8-minute brew *(54)*. This could potentially have implications for kombucha since green tea is commonly used for the base tea of the fermentation.

Tea could also serve as vehicle for foodborne pathogens after preparation by means of cross-contamination. When Zhao et al. analyzed iced tea from fast-food restaurants for presence of fecal coliforms, 92% of tea samples tested positive *(115)*. Additionally, species of *Klebsiella* and *Enterobacter* were isolated from the collections of fast-food iced tea, but no pathogenic *Escherichia coli* isolates were detectable *(115)*. While they ultimately concluded there was no imminent threat to human health, the researchers noted that coliform presence could be indicative of poor sanitation practices in the fast-food facilities and cross-contamination of the tea post-steeping preparation *(115)*. The aforementioned Keller et al. study found that when freshly brewed green tea was inoculated with *Salmonella* to give an initial population of 2 – 3 log (CFU/mL), *Salmonella* increased by additional 5 – 6 logs when the tea was stored at room temperature for 24 hours *(54)*. The researchers concluded that any antimicrobial factors in present in green tea were not effective against *Salmonella* as brewed-tea maintained populations in chilled conditions and populations increased exponentially in brewed-tea at room temperature *(54)*. If the base tea becomes contaminated by means of cross-contamination, this would have implications for kombucha especially considering the beverage is maintained at room temperature.

Food safety record of apple cider. As stated previously, many relate the taste and texture of kombucha to apple cider *(41, 50, 60)*. Although, the production of apple cider is quite different from kombucha, the products share some similarities. The term "apple cider" is somewhat ambiguous in the U.S. and varies according to different state regulations based on the production style and use of pasteurization *(81)*. Commercially available apple cider in interstate commerce usually refers to unfiltered apple juice that has been pasteurized (21 CFR 120.1) *(81)*. However, small cider mills may be exempt from federal food safety regulations *(111)* and produce a type of apple cider that is fermented with wild yeasts and sold unpasteurized. This style of fermented apple cider has similar pH values, sucrose content, and dissolved CO_2 levels to kombucha. Other food safety studies of apple cider report a range of 3.5 – 4.0 pH values and 10 – 12% sugar concentration *(87, 111, 116)* which is comparable to values typically seen in kombucha *(50, 71, 75, 78)*. Depending on the style of fermentation, apple cider is sometimes slightly

alcoholic as well *(60, 93)*. Outbreaks of foodborne disease with bacterial pathogens or parasites have been associated with types of ciders for decades *(107, 111)*. Therefore, historical record of foodborne outbreaks with apple cider may be relevant for kombucha, at least in terms of comparable biochemical components and microbiota composition.

Using the CDC National Outbreak Reporting System (NORS) database, a search query was conducted for any outbreaks that had an association to apple cider *(19)*. A total of 21 foodborne outbreaks from 1998-2017 report apple cider as a confirmed contaminated food vehicle *(19)*. Three of those outbreaks were associated with *Salmonella enterica*; two were attributed to *S. enterica* serotype Agona and the other was attributed to *S. enterica* serotype Typhimurium. Each outbreak caused a reported 8-10 illnesses. Ten apple cider outbreaks had a confirmed disease etiology of Shiga-toxin producing *Escherichia coli* (STEC). Seven of those outbreaks were reportedly caused by *E. coli* O157:H7. Two outbreaks were caused by *E. coli* O111:NM, and one was linked to *E. coli* O45. The outbreaks had a range of 2 - 56 reported illness cases. Four additional apple cider outbreaks had a confirmed etiology to the parasite *Cryptosporidium*. One outbreak that occurred in Ohio in 2003 had reported 144 illness cases and was confirmed to be caused by *Cryptosporidium parvum*. The three other outbreaks were traced back to *C. parvum, C. hominis*, or the species strain of *Cryptosporidium* was unconfirmed. Lastly, there were two outbreaks with apple cider that had a mixture of two confirmed disease agents, STEC and *C. parvum*. In each of these outbreaks, *E. coli* O111:NM and *Cryptosporidium parvum* were co-culprits.

A CDC report on pathogenic *E. coli* and *Cryptosporidium* outbreaks that occurred with apple cider in the fall of 1996 noted two major food safety risk points from the apple cider mills *(18)*. The mills were using dropped apples in processing which could have collected fecal material, pathogens, or other disease agents from the ground. Also, the mills had farm animals nearby and were washing the apples in well water. Parasites, coliforms, and other disease causing agents from the nearby cattle feces could contaminate a batch of apples in a number of ways, especially with an untreated water source, such as well water *(18)*. It should be stated that these potential routes of contamination for apple cider do not

necessarily resemble potential routes of cross-contamination for tea or kombucha. However, fermented, unpasteurized apple cider is one of the few beverages that resembles some of the unique qualities of kombucha and may hold some significance regarding viability of pathogens in terms of pH, sucrose content, and fermenting microbiota.

It had been previously thought that acidic and acidified foods (pH < 4.6) were safe from pathogen contamination because of their low pH *(55, 111)*. However, a 1991 outbreak of *E. coli* O157:H7 in apple cider *(6)* signaled that some foodborne pathogens could survive lower pH food matrices. Since then, there have been have been at least 26 reported outbreaks of foodborne disease in acidic beverages like juice and cider, with some outbreaks resulting in over 200 illness cases *(19)*. *Salmonella*, STEC, and *Cryptosporidium* are the contaminant pathogen in most of these acidic beverage outbreaks. It has been reported that STEC can survive at pH 3.56 for 42 days, and *Salmonella* can survive at pH 3.5 for 24 days *(111)*. Additionally, new research shows that both *E. coli* O157:H7 and some non-O157 STEC strains are able to adapt to acidic conditions and survive short-term exposure at very low pH levels such as 1.5 pH *(55)*. This extreme pH resistance is made possible by acid resistance genes that are upregulated in the microorganism *(55)*. *Cryptosporidium* can also survive at pH 3 – 4 for up to 4 weeks *(18)*. The large acid resistance profile of these foodborne pathogens suggests a potential for concern in the acidic product kombucha.

Concerns have been raised that fruit flies could be an issue kombucha when fermented in the home if the containers are not covered properly *(50)*, and it has been demonstrated that fruit files can carry pathogenic *E. coli* from one fruit to another and inoculate a fruit with an exposed wound *(49)*. Perhaps unlikely but noteworthy, this could introduce another pathway of contamination for kombucha home brewing because the fermentation vessels are exposed to the open-air space with only a thin covering to prevent environmental contamination.

1.3 FERMENTED FOODS AND FOOD SAFETY

History of fermenting practices and implications for food safety. Food fermentation processes have been used by humans as a means of food preservation for millennia, since at least the Neolithic period (~10,000 years BC) *(10, 76)*. Food fermentations can produce some unique and desirable organoleptic properties, but most importantly, fermentation (if handled properly) can yield food products that are generally devoid of pathogens and resistant to contamination by foodborne pathogen or spoilage organisms *(16, 98)*. There is a diverse array of microorganisms that utilize various fermentation metabolic pathways. Steinkraus groups fermented foods into seven major categories, but just three categories include the overwhelming majority of fermented foods and are most relevant here: lactic acid fermentation, alcoholic fermentation, and acetic acid fermentation *(98)*. While there are some rare examples of molds assisting in the breakdown of starches for fermentation, the microbial groups most responsible for these processes are: lactic acid bacteria (LAB), yeasts, and acetic acid bacteria (AAB), respectively *(98)*.

Historically, food fermentations occurred naturally through the activity of indigenous microbiota and prolonged environmental exposure of the food product. Alternatively, a small sample of previously fermented product might be used as a starter culture to inoculate the food with the microbiota needed to induce fermentation, through a process called back-slopping *(16)*. In the modern era of food industrialization, oftentimes the microorganism species and strain most desirable for the process have been isolated and are inoculated to the food product artificially via pure culture *(79)*. This is to create a systematic and controlled process to produce a safe and consistent product *(16, 79)*. In developing countries, where the proper water sanitation and food safety infrastructure might be lacking or the economic burden of storing and cooking food is too profound, fermented food products are still very common *(97, 98)*. These fermented foods may be unique to that geographic region and have a unique indigenous microbiota that is passed from person-to-person within families and local communities *(16)*.

These traditional fermented food products tend to be artisanal in nature and result in end products that have unique sensory characteristics that may differ from each batch *(16)*.

Overall, fermented foods have proven track record in terms of food safety *(77, 98)*. Three of the main principles of fermentation food safety, according to Steinkraus, are that lactic acid fermentation, ethanol fermentation, and acetic acid fermentation of are "generally safe" *(98)*. Indeed, fermented foods are only implicated (suspected or confirmed) as either a sole contaminated ingredient or part of contaminated food matrices in only 0.15% of foodborne outbreaks and 0.11% of all foodborne illness related cases in the past 19 years (1998-2017) reported to the CDC *(19)*.

However, those numbers may not adequately depict a true risk assessment of fermented food products. The U.S. population generally does not consume as many traditional, home-prepared fermented foods as developing countries because our food system is more industrialized *(16, 79)* and commercially fermented food products have very low safety risk *(15)*. However, in the last decade, there has been had increased interest in Europe and the U.S. for artisanal, 'cottage' foods *(16)* like those traditional fermented foods with an undefined starter culture *(15)*. This can be attributed in some part to changing consumer interests to gravitate toward more local *(15)* and 'natural' food products as well as a preference for unique, premium foods created in small batches that may have slightly different end products with each production cycle *(16)*.

This presents a unique challenge because many persons are beginning to ferment foods, including kombucha, at home with no prior experience *(15)*. While kombucha has seen an explosion in popularity in the last decade, many consumers see purchasing the pre-prepared, bottled kombucha in the store as cost prohibitive. Therefore, many consumers seek to ferment kombucha in their home for personal consumption. The University of Maine Food and Agriculture Cooperative Extension recently conducted a survey of Maine consumers of home-fermented foods *(15)*. Of those survey respondents who said they do currently ferment foods at home (n = 306), 32 % of those consumers report fermenting kombucha at

home (n = 98). Kombucha was the fifth most common home-fermented food product *(15)*. The assessment goes on to stress that more consumer education and food safety awareness of home-fermented foods is needed and specifically mentions a need for more research on kombucha *(15)*.

Home fermentation of kombucha presents a unique set of challenges in relation to other typically fermented foods. As described previously, kombucha is fermented by back-slopping with indigenous microbiota that are derived from the starter culture origin *(27, 50)*. Fermentation by means of an undefined starter culture may result in inconsistent fermentation timelines with inconsistent final products *(79)*. These inconsistencies can result in undesirable microorganisms taking hold *(4)*. Kombucha is often considered an artisanal process that is unique to an individual's palate and unyielding to standard protocols. These inconsistencies in fermentation time, quality, and safety are common among other artisanal food products fermented with undefined, indigenous microbiota such as African amasi *(74)*, Ethiopian ergo *(103)*, Mexican pulque *(40)*, traditional Greek olives *(4)*, South East Asian tempoyak *(25)*.

Viability of *Salmonella* and STEC during active fermentation. Properly fermented foods are considered "generally safe" *(98)* because they resist contamination and prevent pathogen populations becoming established. If pathogen populations are introduced – either by cross-contamination or artificial laboratory inoculation – growth is inhibited, and survival is minimal as populations will decline with storage *(1, 77)*. Fermented foods have a variety of pathogen-inhibitory qualities but the strongest are reduced pH, prescience of undissociated organic acids, and high population of active LAB microbiota *(1)*. However, if previously contaminated raw materials are used or fermentation parameters are weak because of an inadequate starter culture, improper storage, or ineffective fermentation maturation, concerns about the food safety of the final product come into question *(1, 77, 98)*. An overwhelming number of studies show that acid-resistant pathogens, most notably *Salmonella* and STEC, can survive for days, or up to weeks in some cases, in acidic products at refrigeration conditions (4 – 8°C) *(23, 61, 68, 92, 93, 107, 108, 116)*. However, most of those cases assume the point of contamination would be environmental interference just before packaging and chilled storage, or the product itself is harboring pathogen

survivors from the fermentation process. This point highlights why well-designed protocols with optimized control steps are needed for fermentation *(15)* and fermentation can only act as a supplement for good hygienic practices and not a replacement *(1, 77, 98)*.

The more pressing issue of fermented food safety risk is the point of contamination in which pathogens are introduced to the raw product food matrix before fermentation begins and are able survive throughout the process *(1, 77)*. Typically, pathogens inoculated as a pre-fermentation contaminant can survive for longer periods of time as opposed to a post-fermentation contaminant. When inoculated as a pre-fermentation contaminant in cassava dough, STEC and *Salmonella* survived 3 and 6 times longer, respectively, than when inoculated as post-fermentation contaminant *(68)*. When STEC was inoculated into previously fermented green datta a complete 6 log reduction and elimination occurred within 7 days, but when inoculated as a pre-fermentation contaminant, STEC populations only demonstrate a 3 log reduction over that same time period and then continued to sustain populations *(108)*. Similarly, when STEC and *Salmonella* were inoculated in previously-fermented amasi, a complete 7 log elimination occurred within 48 hours, but when inoculated as a pre-fermentation contaminant, both STEC and *Salmonella* populations actually grew and increased in first 24 hours and then declined by only 3 and 4 log within 48 hours, respectively *(74)*.

An over-arching conclusion of Adams and Nicholaides review of fermented food safety was that the survival of foodborne pathogens through fermentation was dependent on the dominance of LAB and other indigenous microflora in the initial stages of the process. They concluded that LAB inhibit the growth of most pathogens in raw product if the initial LAB population outnumbered the initial level of pathogen *(1)*. Another review of LAB inoculum levels and pathogen survival in meat products stated, more specifically, that a high inoculum of 6 – 9 log LAB was needed to inhibit pathogen survival *(86)*. Based on these findings, it can be concluded that pathogen risk for fermented foods is greatest when raw product is contaminated before fermentation, and the initial population of LAB and other indigenous microflora such as yeasts and AAB play a major role in the survival and sustainability of the pathogens.

There is a vast amount of literature describing the ability of *Salmonella* and STEC to survive specific pH levels of acidified media in laboratory conditions. Additionally, there are numerous pathogen challenge studies based on end-result fermented products or cases where the fermented product has been filtered or sterilized remove indigenous microbiota. None of those conditions closely reflect pathogen survival in a fermentation environment in which acid levels are gradually increasing because active microbiota (LAB, AAB, yeasts, etc.) populations within the food matrix are also increasing. To summarize some selected literature that would be particularly relevant, a survey of published data in which *Salmonella enterica* (Table 2) and pathogenic *Escherichia coli* (Table 3) are studied as a pre-fermentation contaminant in foods with an active microbiota population of LAB and/or yeasts throughout the course of fermentation at ambient temperature are summarized below. Perhaps because fermented food production in the Western world is very industrialized and specialized to select LAB cultures *(16)*, most of the studies included below are traditional fermented products endemic to specific locations in developing countries. In some cases, food products were naturally fermented with an undefined starter culture such as the case with pulque, amasi, tempoyak, cassava, and traditional Greek olives *(4, 25, 35, 40, 68)*. In most cases however, a sterilized or pasteurized food product base was deliberately inoculated with previously isolated, pure LAB cultures and or yeasts to induce fermentation. Additionally, because the initial indigenous microbiota is well documented as being important to the survival of pathogens in raw product *(1)*, the initial populations (if enumerated and reported) of LAB and yeasts are included with each study's findings.

In three selected cases reported in Table 2 and 3, the product was previously fermented, but the results may still be relevant. Regarding, the Zhao et al. study on the survival of STEC in unpasteurized apple cider, the apple cider had previously been fermented by the supplier and contained an active indigenous microbiota useful for fermentation. It is noteworthy that STEC survived as post-fermentation contaminant in acidic (pH < 4.0) apple cider for 3 – 6 d at ambient temperature *(116)*. However, it can be hypothesized that because the apple cider was frozen and then thawed, the microbiota necessary for

fermentation incurred a major population reduction and/or went into dormancy, and when the cider was thawed, it induced a sort of secondary renewal of fermentation cycle. This is supported by the fact that the pH of all ciders held at room temperature continued to decrease over a 3-day incubation at room temperature and even after freeze thaw, populations of APC and yeasts were active and reportable *(116)*. Regarding the Semancheck Golden 1996 publication, the food product in question was fresh, unpasteurized apple cider that contained relevant indigenous microbiota but had previously been fermented. In this case, to induce a secondary type of fermentation a high population of yeast was added to the product at time zero. Then the survival of STEC was enumerated in actively fermenting cider that had been supplemented with a boosted yeast population *(93)*. Lastly, regarding the Argyri et al. study, the olives had previously been fermented by an artificial inoculation of a *Lactobacillus* culture, but the olives were sealed in aerobic packing with brine to undergo a secondary fermentation with the indigenous microbiota of LAB and yeasts remaining on the olives *(4)*. The LAB and yeast populations are enumerated as well. However, the brine used in the study had not been fermented and the olives with their active indigenous population of LAB and yeasts served as the starter culture inoculum for the brine *(4)*.

While the level of initial pathogen inoculum, type of starter culture, and other parameters such as salt concentrations and ethanol content vary with each study, some overlying conclusions can be proposed. The studies of *Salmonella* survival as a pre-fermentation contaminant report a range of 3.8 – 4.3 pH end point at which a major reduction is observed. Most studies also report a 3.3 – 6 log reduction of *Salmonella* by 1 – 3 days. The fermentation of olives *(4)* and tempoyak *(25)* were exceptions with a 5 – 6.5 log reduction not occurring until 8 – 14 days. The studies of STEC survival as a pre-fermentation contaminant report a range of 3.48 – 4.4 pH end point at which a major reduction is observed. Most studies also report a 3 – 6 log reduction of STEC by 3 – 7 days. Because a longer fermentation time was typically required to observe a major reduction for STEC, this summary lends support to the idea the STEC is more acid resistant than *Salmonella (63, 90, 107)* and may be viable for a longer time window in fermenting products, like kombucha.

TABLE 2. Survey of *Salmonella enterica* challenge studies as a pre-fermentation contaminant in raw food products with an active LAB and/or yeast population

Survival of *Salmonella* in fermenting food products[1] and relevant characteristics observed when a major reduction in pathogen population is observed over the course of fermentation								
Food Product[2]	SC used	LAB initial population[3]	Yeast initial population[3]	Level of inhibition[4]	Time and Storage Conditions[5]	pH at inhibition (initial pH)[6]	Relevant biochemical compound concentrations	Reference
Pulque (Mexican fermented alcoholic beverage made from agave nectar)	traditional SC called seed pulque (SP) with natural microflora (LAB, yeasts, etc.) added to fresh nectar	Present in SC but pop. level not reported - 20% (v/v) addition of SP microbiota	Present in SC but pop. level not reported - 20% (v/v) addition of SP microbiota	3.3 log reduction	1 d at 22°C	4.0 (7.5)	5.4 % Ethanol	Gomez-Aldapa et al. 2011
Pulque (Mexican fermented alcoholic beverage made from agave nectar)	fresh nectar was allowed to naturally ferment with indigenous microflora present (LAB, yeasts, etc.)	Present in agave naturally but pop. level not reported	Present in agave naturally but pop. level not reported	3.3 log reduction	3 d at 22°C	4.0 (7.5)	0.3 % Ethanol	Gomez-Aldapa et al. 2011
Fermented olive fruits stored in 6% NaCl brine solution	*Lactobacillus pentosus* and natural microflora on olives	5.5 – 6.0	2.6 – 2.7	5.0 log reduction	8 d at 20°C	4.22 (relatively stable pH over storage)	6 % NaCl 0.1+ % lactic acid	Argyi et al. 2013
Brine for covering olives	natural microflora on olives and residual *Lactobacillus pentosus* pop. on olives	5.2 – 5.7	3.2 – 4.2	6.5 log reduction	14 d at 20°C	4.23 (6.08)	6 % NaCl 0.1+ % lactic acid	Argyi et al. 2013
Tempoyak (acidic, fermented condiment made from Durian fruit pulp)	fermented with natural indigenous microbiota	8.88	5.98	6.0 log reduction	8 d at 30°C	3.9 (7.1)	2.6 % titratable acidity 0.75% lactic acid 0.55% acetic acid	Chuah et al. 2016

							0.40% propionic acid	
Amasi (Zimbabwean fermented milk beverage)	*Lactobacillus diacetylactis* and *Candida kefyr* pure cultures	*8.9 – 9.9 (at 2 d, initial pop. not reported)	*7 – 7.9 (at 2 d, initial pop. not reported)	4 log reduction	2 d at 25°C	4.3 (6.8)	1.37 % titratable acidity (lactic acid)	Mufandaedza et al. 2006
Agbelima cassava (Ghanaian sour, fermented dough)	traditional SC called kudeme with natural microbiota (*Bacillus* spp., LAB, and yeasts)	Present in SC but pop. level not reported - 2.5% (m/m) addition of kudeme microbiota	Present in SC but pop. level not reported 2.5% (m/m) addition of kudeme	6 – 7 log reduction	1 d at ambient temperature	4.1 (5.7)	0.55 – 0.85 % titratable acidity (lactic acid)	Mante et al. 2003
Tempeh (acidified, cooked fermented soybeans)	*Lactobacillus plantarum* cells and *Rhizopus oligosporus* spores	2.84	N/A	2.47 log reduction	1.67 d (40 h) at 30°C	4.6 (5.8)	N/A	Ashenafi and Busse 1989
Borde (Ethiopian fermented beverage, made with maize flour and water)	mixture of LAB cultures previously isolated from Borde	3.0 - 3.4	N/A	~ 3.0 log reduction	1 d at ambient temperature	3.85 (4.7)	0.35% titratable acidity	Tadesse et al. 2005
Ergo (Ethiopian fermented dairy product)	mixture of LAB cultures previously isolated from Ergo and Brode	6.0 – 6.5	N/A	2.0 log reduction	2 d at ambient temperature	3.94 (6.5)	N/A	Tesfaye et al. 2011
Kimchi fermented cabbage (white radish, ginger, garlic, red pepper, sugar, salt)	Mixture of pure LAB cultures	4.2 – 4.5	N/A	4.8 log reduction	2 d at 25°C	4.1 (5.3)	N/A	Choi et al. 2018
Yogurt	*Streptococcus thermophilus Lactobacillus bulgaricus*	8.3-8.8	N/A	4.5 - 5.5 log reduction	2.5 d at 25°C	3.8 (4.5)	N/A	Savran et al. 2018
Brewer's wort	*Saccharomyces cerevisiae*	N/A	~ 5	5.69 log reduction	3 - 5 d at 25°C	4.3 (5.5)	2.5 – 5.1 % Ethanol (w/v)	Menz et al. 2010

(malt extract for beer fermentation)											

[1]Only studies that reported the survival of *S. enterica* as a pre-fermentation contaminant in raw product that is fermented by addition of an active indigenous microbiota or artificially added pure cultures of LAB and yeasts are included. The active fermentation process coincided with the survival/death of pathogen.

[2]Studies that examined food products with an active microbiota containing both LAB and yeasts are included first, followed by those with only LAB, and then only yeasts. These different subsections are noted with a dotted dividing line.

[3]Reported initial population values (log CFU/mL) for lactic acid bacteria (LAB) and/or yeasts are population ranges of the food product microbiota at the beginning of the challenge study when fermentation begins. This characteristic has been noted to play a major role in the viability of pathogens over the course of fermentation (Adams and Nicholaides 1997).

[4]The reported level of inhibition does not necessarily indicate the pathogen was completely undetectable at that point, but rather the level of inhibition corresponds largest reduction in pathogen population that was observed for those conditions for the length of the trial. Some pathogen challenge studies do not follow food product fermentation until complete elimination of pathogen is observed.

[5]The reported time and storage conditions are the length of time (days, d) and incubation conditions of the food product up to the point at which greatest pathogen inhibition was observed. It should be noted that the overwhelming majority of studies included in this survey were selected to reflect fermentation (incubation conditions) at or near room temperature.

[6]The reported pH is the specific pH value at which point greatest pathogen inhibition was observed; however, the decline of pH from initial food conditions is reported in parentheses.

TABLE 3. Survey of challenge studies with STEC as a pre-fermentation contaminant in raw food products with an active LAB and/or yeast population

Survival of STEC in fermenting food products[1] and relevant characteristics observed when a major reduction in pathogen population is observed over the course of fermentation								
Food Product[2]	SC used	LAB initial population[3]	Yeast initial population[3]	Level of inhibition[4]	Time and Storage Conditions[5]	pH at inhibition (initial pH)[6]	Relevant biochemical compound concentrations	Reference
Agbelima cassava (Ghanaian sour, fermented dough)	traditional starter culture called kudeme with natural microbiota (*Bacillus* spp., LAB, and yeasts)	Present but pop. level not reported 2.5% (m/m) addition of kudeme microbiota SC	Present but pop. level not reported 2.5% (m/m) addition of kudeme microbiota SC	6 log reduction	1 d at ambient temperature	4.1 (5.7)	0.55 – 0.85 % titratable acidity (lactic acid)	Mante et al. 2003
Awaze (Ethiopian fermented condiment, made from ripened red peppers and a variety of spices and salt)	a mixture of LAB and yeast cultures (previously isolated from Awaze)	6 – 6.5	~ 4	5 – 6 log reduction	7 d at ambient temperature	3.8 (4.9)	N/A	Tsegaye et al. 2004
Amasi from (Zimbabwean ferment milk beverage)	*Lactobacillus diacetylactis* and *Candida kefyr* pure cultures	*8.9 – 9.9 (at 2 d, initial pop. not reported)	*7 – 7.9 (at 2 d, initial pop. not reported)	3 log reduction	2 d at 25°C	4.3 (6.8)	1.37 % titratable acidity (lactic acid)	Mufandaedza et al. 2006
Amasi (South African fermented milk beverage)	traditional amasi starter culture with indigenous microbiota	6.4 – 7.5	4.1 – 4.5	6 log reduction	3 d at ambient temperature	4.0 (5.8)	N/A	Diamini et al. 2009
Fermented olive fruits stored in 6% NaCl brine solution	*Lactobacillus pentosus* and natural microflora on olives	5.2 – 5.7	3.2 – 4.2	6.5 log reduction	8 d at 20°C	4.23 (6.08)	6 % NaCl 0.1+ % lactic acid	Argyi et al. 2013
Brine for covering olives	natural microflora on olives and residual	5.5 – 6.0	2.6 – 2.7	5.0 log reduction	5 d at 20°C	4.22 (relatively stable pH	6 % NaCl 0.1+ % lactic acid	Argyi et al. 2013

	Lactobacillus pentosus pop. on olives					over storage)		
Apple Cider (unpasteurized, but had been frozen)	Indigenous microbiota post-thawing	(1 – 1.47 APC)[7]	1.18 – 1.60	5 log reduction	6 d at 25°C	3.1 – 3.7 (3.7 – 3.9)	N/A	Zhao, Doyle, and Besser 1993
Tempeh (acidified, fermented cooked soybeans)	*Lactobacillus plantarum* cells and *Rhizopus oligosporus* spores	2.84	N/A	2.47 log reduction	0.67 d (16 h) at 30°C	4.6 (5.8)	N/A	Ashenafi and Busse 1989
African yoghurt	*Lactobacillus bulgaricus* and *Streptococcus thermophilus* cultures added at 1% (v/v)	~ 6.0	N/A	8 log reduction	6 d at 25°C	3.57 (5.1)	N/A	Ogwaro et al. 2002
Green & Red Datta (Ethiopian fermented condiment, made from ripened chilies and a variety of spices and salt)	a mixture of LAB cultures (previously isolated from Datta)	6 – 6.5	N/A	2.5 – 3 log reduction	7 d at ambient temperature	4.4 (5.1)	N/A	Tsegaye et al. 2004
Borde (Ethiopian fermented beverage, made with maize flour and water)	a mixture of LAB cultures (previously isolated from Borde)	3.0 - 3.4	N/A	~ 1.7 log reduction	1d at ambient temperature	3.85 (4.7)	0.47% titratable acidity	Tadesse et al. 2005
Ergo (Ethiopian fermented dairy product)	mixture of LAB cultures (previously isolated from Ergo and Brode)	6.0 – 6.5	N/A	2.0 log reduction	2 d at ambient temperature	3.94 (6.5)	N/A	Tesfaye et al. 2011
Kimchi fermented cabbage (white radish, ginger, garlic, red pepper, sugar, salt)	mixture of LAB pure cultures	4.2 – 4.5	N/A	4.32 log reduction	2 d at 25°C	4.1 (5.3)	N/A	Choi et al. 2018

Apple Cider (unpasteurized, fresh/not frozen)	*S. cerevisiae* culture was added at 1% (v/v)	(2.4 APC)[7]	5.79	6.4 log reduction	3 d at 20°C	3.48 (3.62)	3.03 % (v/v) ethanol	Semanchek and Golden 1996
Brewer's wort (malt extract for beer fermentation)	*Saccharomyces cerevisiae*	N/A	~ 5	4 log reduction	3 - 5 d at 25°C	4.3 (5.5)	2.5 – 5.1 % Ethanol (w/v)	Menz et al. 2010

[1]Only studies that reported the survival of *E. coli* as a pre-fermentation contaminant in raw product that is fermented by addition of an active indigenous microbiota or artificially added pure cultures of LAB and yeasts are included. The active fermentation process coincided with the survival/death of pathogen.

[2]Studies that examined food products with an active microbiota containing both LAB and yeasts are included first, followed by those with only LAB, and then only yeasts. These different subsections are noted with a dotted dividing line.

[3]Reported initial population values (log CFU/mL) for lactic acid bacteria (LAB) and/or yeasts are population ranges of the food product microbiota at the beginning of the challenge study when fermentation begins. This characteristic has been noted to play a major role in the viability of pathogens over the course of fermentation (Adams and Nicholaides 1997).

[4]The reported level of inhibition does not necessarily indicate the pathogen was completely undetectable at that point, but rather the level of inhibition corresponds largest reduction in pathogen population that was observed for those conditions for the length of the trial. Some pathogen challenge studies do not follow food product fermentation until complete eliminatation of pathogen is observed.

[5]The reported time and storage conditions are the length of time (days, d) and incubation conditions of the food product up to the point at which greatest pathogen inhibition was observed. It should be noted that the overwhelming majority of studies included in this survey were selected to reflect fermentation (incubation conditions) at or near room temperature.

[6]The reported pH is the specific pH value at which point greatest pathogen inhibition was observed; however, the decline of pH from initial food conditions is reported in parentheses.

[7]LAB population was not measured but total aerobic plate count (APC) was enumerated. APC may be indicative LAB populations.

Acid tolerance response (ATR) and acid adaptation of *Salmonella* and STEC. The production of organic acids by microbiota through the process of food fermentations and thereby reduction of pH are known to be the major qualities *(1)* that give fermented foods a clean food safety record *(98)*. However, Gram-negative pathogens, primarily *Salmonella* and STEC, have been noted to display a resistance to increasing acidic parameters in some food matrices. This is particularly concerning because several studies have noted that increased acid resistance of pathogens also increases virulence of cells and lowers infectious dose *(14, 58, 63, 87, 107)* because the cells are able to survive the low pH levels of human gastric fluids *(55)*. In one of the first major publications to define these parameters, Chung and Goepfert reported that the minimum pH for growth of *Salmonella* for citric, gluconic, lactic, citric and acetic acids was 4.05, 4.20, 4.40, and 5.40, respectively *(26)*. They also noted that *Salmonella* appeared to be more tolerant of acidic conditions in ambient room temperature conditions (25 - 30°C). Later studies described variations in these minimum pH values under a variety of laboratory conditions. Jung and Beuchat reported a 5.0 log reduction of *Salmonella* in 10 h and 18 h for acetic and lactic acids when TSB was adjusted to pH 4.0 with each respective acid. The same study also reported that cell injury and inhibition of colony formation for *Salmonella* on TSA agar acidified with lactic acid at pH 4.4 and no growth was detectable at pH 4.0 *(52)*.

Foster et al. was among the first publications to define the acid tolerance response (ATR) of foodborne pathogens when in stationary phase at an extremely low pH *(63)*. The study used a type of "acid-shocked" inoculum and reported the minimum pH values for growth was 4.0 and 4.4 for *Salmonella* and STEC, respectively *(63)*. However, STEC was able to survive (not grow) at lower pH values of 2 – 2.5 pH than *Salmonella* at 3.0 pH in a complex medium *(63)*. In similar study, Leyer et al. used a type of "acid-shock" of STEC inoculum by exposing cells to TSB acidified to 5.0 pH with HCl for 5 h. They reported that acid-shocked STEC had significantly increased survival in TSB adjusted to pH 3.85 with lactic acid and in pasteurized apple cider at pH 3.46 *(62)*. Then a USDA research group (Buchanan and Edelson) published a highly cited method for inducing acid-adaptation (not acid-shock) in STEC by

adding 1% glucose to TSB *(14)*. STEC uses the additional glucose for a fermentation process of its own and produces lactic acid as by product. This gradually lowers the pH of TSB + 1% glucose from 7.2 to 4.5 within a 24 h incubation period. The end result was an STEC inoculum that had become acid adapted by means of this gradual lowering of pH, and this acid-adapted STEC survived acidic conditions in much higher numbers than its non-acid adapted comparison *(14)*.

A publication by Ryu and Beuchat clarified distinctions in these ATR methodologies. Acid-shock occurs when the cells are exposed to an abrupt shift from a higher pH to a lower pH, typically from neutral pH to 4.3 – 5.8 pH, depending on the study parameters. Acid adaptation occurs when there is a gradual decrease in environmental pH by means of fermentation and the cells undergo acid adaptation by gradually adjusting to the reduced pH of cell cytoplasm *(33, 87)*. The same publication reported that acid-adapted STEC populations had complete elimination in TSB adjusted to 3.9 pH in 18 h with acetic acid and 24 h with lactic acid. Furthermore, STEC was detectable up to 42 d in orange juice (pH 3.82 – 3.86) and up to 56 d in pasteurized apple cider (pH 3.56 – 3.98) *(87)*. In follow-up studies by the same research group, it was reported that STEC cells displayed acid injury and colony formation was inhibited on TSA acidified with acetic acid (pH 5.4), lactic acid (pH 4.5), and citric acid (pH 4.2). But when analyzing survival in acidified TSB, STEC populations plateaued and did not decrease at pH 4.5 with acetic and lactic acids. A pH of 3.9 was required of both lactic and acetic acids to eliminate STEC within 24 h. Conversely, STEC populations only remained constant were not eliminated at the lowest pH of 3.4 for citric and malic acids *(31, 88)*.

Samelis et al. expounded upon previously reported acid adaptation methods and reported that acid adaptation could also be induced for *Salmonella* and *Listeria* when 1% glucose is added to TSB *(90)*. They also reported that seemingly minute changes in pH of 3.5 to 3.7 resulted in significant differences between acid-adapted and non-acid adapted pathogen populations. STEC also demonstrated the greater acid resistance to pH values than *Salmonella* or *Listeria*. It could be concluded that bacterial pathogens can become naturally acid-adapted in the presence of glucose or other fermentable carbohydrates.

These studies demonstrate that there are a variety of laboratory methodologies for studying the ATR of foodborne pathogens. Because of the diverse array of studies published on this topic, the results of pH limitations for pathogen survival also vary considerably as well. The methodology used for acid-adaptation, type of organic acid, time of acid exposure, food matrix, carbohydrate substrate, and others conditions act as confounding variables to compare results *(58)*. However, there are some overreaching conclusions regarding ATR of these pathogens.

It is generally accepted that acetic acid is more inhibitory than lactic acid for both *Salmonella (26, 90)* and STEC *(31, 33)*. Acetic acid is more inhibitory than lactic acid because it is a weaker acid with a higher pKa *(104)* and lower molecular weight *(1, 33)*. Because of the higher pKa value, the percentage of acetic acid ($pK_a = 4.76$) in its undissociated form will be greater than lactic acid ($pK_a = 3.86$) at moderately acidic pH ranges. Acids in their undissociated form are more inhibitory to cells because they will pass through the cell membrane and dissociate once inside the cell and decrease the cytoplasm pH *(1, 33)*. However, the concentrations of each of these acids plays a big role. When equal concentrations of lactic and acetic acids are compared, lactic acid is more inhibitory because lactic acid will reduce the environmental pH more so overall *(58, 90)*. Because lactic acid is the stronger acid and will reduce pH, this also increases the percentage of undissociated acetic acid; therefore, mixtures of acetic acid and lactic acid create a synergy of pathogen inhibition *(1)*. Heterofermentative LAB are capable of producing both acids which initiates this synergy in some fermented foods *(1, 38)*. Although variations occur in studies, citric and malic acids are usually less inhibitory than both lactic and acetic acid *(31, 38, 52, 88)*. Breidt et al. reported that gluconic acid had no significant inhibitory effect on STEC and could instead be used as a negative control, non-inhibitory buffer *(11)*. The concentrations of these organic acids will undoubtedly play a major role on the inhibitory role of kombucha fermentation.

CHAPTER 2

MATERIALS AND METHODS

Pathogen strains used. Three *Salmonella enterica* subspecies *enterica* serotypes were used: Baildon (serogroup D_2) (from a patient in an outbreak of salmonellosis associated with diced tomatoes), Newport SN78 (serogroup C2) (bovine fecal isolate), and Oranienburg 002/SD48 (serogroup C_1) (source unknown). Three Shiga toxin-producing *Escherichia coli* strains were used: O157:H7 SEA13B88 (serogroup E) (from patient in an outbreak associated with unpasteurized apple cider), O111:NM CFS4 (serogroup B_1) (bovine fecal isolate), and O45:H2 MI05-14 (serogroup B_2) (from a child HUS patient, Michigan Health Department). Cultures were provided by the University of Georgia Center for Food Safety (Griffin, GA) culture collection library. All strains were preserved at -20℃ in tryptic soy broth (TSB, pH 7.3 ± 0.2) (Neogen, Lansing, MI) supplemented with 15% (v/v) glycerol.

Inoculum preparation. Stock cultures were revived and maintained in 10 mL of TSB (Neogen) in sterile 16 x 125 mm-screw cap glass test tubes (Pyrex No. 9825) (Corning, Corning, NY) at 4℃. Before inoculum preparation, each culture was subject to three successive transfers. For the first transfer, a loop inoculum was streaked onto selective agars to ensure proper colony formation. *Salmonella enterica* subspecies *enterica* (hereafter referred to as "*Salmonella*") serotypes were streaked onto xylose-lysine-tergitol 4 agar (XLT4, pH 7.4 ± 0.2) (Neogen, Lansing, MI) *(73)* and Shiga producing-*Escherichia coli* (hereafter referred to as "STEC") strains were streaked onto MacConkey agar with sorbitol (SMAC, pH: 7.1 ± 0.2) (Neogen, Lansing, MI) *(69)*. Each agar type was incubated at 37°C for 24 h. For the second transfer, a colony was selected from each selective agar then streaked for isolation onto Tryptic Soy Agar (TSA, pH 7.3 ± 0.2) (Neogen, Lansing, MI) supplemented with 0.1 mg/mL Ampillicin (Sigma-Aldrich, St. Louis, MO) and then incubated at 37°C for 24 h. For the third transfer, a colony was selected from TSA-Amp agar and a loop inoculum was transferred to 10 mL of TSB and incubated at 37°C for 24 h.

After incubation, cultures were washed with an addition of 30mL of sterile, E-Pure™ ultrapure water (Thermo Fisher Scientific, Waltham, MA) and centrifuged at 10,000 x *g* for 15 minutes. The supernatant was aspirated, and each culture was subject to two additional washes and centrifugations. Each pellet was re-suspended in approximately 10 mL of E-Pure™ ultrapure water. Equal volumes of the approx. 10 mL cell suspension of each serotype were combined and mixed via vortex to give approximately 30 mL of the three-serotype mixture inoculum for each trial. The inoculum preparations of *Salmonella* and STEC were created separately and experimentally tested in separate trials.

After preparation of the combined serotype inoculum and before inoculating treatments, the mixed-serotype inoculum population was calculated with spread plating. A 100 µL (0.1 mL) volume of the mixed-serotype cell suspension was serially diluted (1:10) into 900 µL (0.9 mL) of sterile, 0.1% peptone water (BD Bacto™ Peptone, Fisher Scientific, Hampton, NH) in microcentrifuge tubes. Serially diluted samples of inoculum were surface plated in duplicate (0.1 mL) onto selective agar. Mixed-serotype suspensions of *Salmonella* were plated onto XLT4 agar and STEC onto SMAC agar. Plates were incubated at 37℃ for 24 h before enumeration of presumptive colonies. Stock inoculum concentrations of *Salmonella* ranged from 8.00 (SD ± 0.18) to 9.84 (SD ± 0.30) log CFU/mL. Stock inoculum concentrations of STEC ranged from 8.89 (SD ± 0.32) to 9.18 (SD ± 0.15) log CFU/mL.

Acquisition of kombucha starter kits and culture activation. Four different commercially available kombucha starter kits (Brands A, B, C, & D) were ordered through internet purchase and delivered by mail. Each kit included specific instructions for brewing tea suitable for that kombucha culture as well as instructions for kombucha brewing and fermentation. Supplier instructions were carefully followed to mimic kombucha home brewer practices. Starter kit packages of Brands B, C, & D included a kombucha pellicle ("SCOBY") and approximately 200-350 mL of liquid kombucha starter culture in a vacuum sealed plastic package. The instructions mandated an initial two cycles of a 2 – 3 weeks fermentation to activate the cultures before brewing kombucha that is suitable for consumption. Brand A package included only a dehydrated kombucha pellicle ("SCOBY") and no liquid starter culture.

Instead, Brand A instructions mandated three separate brewing cycles of 30 d with decreasing concentrations of a distilled white vinegar (5% acetic acid strength) (Kroger Co., Cincinnati, OH) and tea. For the first cycle, 16.7% (v/v) of vinegar was added to the tea and the dehydrated pellicle. For the second and third cycles, 8.3% (v/v) of vinegar and 8.3% (v/v) of liquid starter culture from the previous batch was added to the tea and pellicle. After the third cycle of fermentation with tea and vinegar, Brand A kombucha cultures were suitable for brewing as the pellicle no longer had a dehydrated appearance, and the kombucha was effervescent.

Preparation of sterile base tea for kombucha. Kombucha starter kits included specific instructions for tea leaf mass and sucrose concentrations for preparation of tea to be used as a base for kombucha starter culture inoculation. Because previous studies have noted that residual elements in tap water can affect the end result of kombucha, Barnstead™ E-Pure™ ultrapure water (Thermo Fisher Scientific) (resistivity (ΩM): 16.73 ± 0.13 at 25°C) (TOC: > 10 ppb) which is essentially devoid of ions was used to brew the tea. All tea blends were reportedly organically grown as is common for kombucha practices. Granulated white sugar (sucrose) (Kroger Co., Cincinnati, OH) was purchased at a local retailer and used as the sweetener for tea brewing. Brand A did not supply tea or offer any unaltered, tea blends for purchase, but instead, recommended the consumer select a green tea of their choice. For Brand A kombucha, a popular type of kombucha tea, Yogi Kombucha Green Tea (Springfield, Oregon) was used as the base tea. Brand A recommended 14 g of loose tea and 100 g of sugar (10.0% sucrose) per liter. Brands B, C, & D each supplied initial quantities of tea and recommended their own specific tea blend for kombucha brewing. Brand B tea was a mix of black and green loose tea leaves (2 : 3, black tea : green tea ratio) and recommended 5.2 g of loose leaf tea and 70 g of sugar (7.0% sucrose) per liter. Brand C tea was a mix of black and green loose tea leaves and recommended 4.0 g of loose leaf tea and 61 g of sugar (6.1% sucrose) per liter. Brand D tea was a mix of black and oolong loose tea leaves and recommended 3.0 g of loose leaf tea and 61 g of sugar (6.1% sucrose) per liter. This information is summarized below in Table 4.

Recommended quantities of each tea blend and sucrose were added to sterilized, glass 2L flasks with a sterile stir bar. Boiling E-Pure™ ultrapure water was added to each flask containing the tea and sugar and filled to the appropriate volume. Each flask was vigorously stirred for 3 – 5 minutes on a stir plate to ensure adequate mixing and dissolution of the sucrose. Then flasks were placed in an ice water bath for cooling. Tea was steeped for 15 – 20 minutes in the ice water bath. Tea was strained with a sterile metal strainer into a separate sterile, glass bottle. The bottles containing the strained tea were returned to the ice water bath for 1- 2 hours for proper cooling.

Once the strained tea had cooled to room temperature, the tea was filter-sterilized to remove tea sediments and remaining microbiota that could interfere with brewing. A 5 mL serological, aspirating pipet was connected with sterile rubber tubing to a 0.1-micron, hollow fiber, Sawyer MINI water filter (Sawyer Products, Safety Harbor, FL) for entry flow of the tea. The exit flow from the filter was connected with sterile tubing to another 5 mL serological, aspirating pipet inside a 2L sterile, glass flask with a spout for vacuum attachment. Sterile rubber tubing was also used to connect the flask spout to the vacuum. Under vacuum pressure, the strained tea flowed through the tubing to the Sawyer filter and the 0.1 µm filter-sterilized tea exited the filter via tubing into the filtration flask. The filter-sterilized tea was used immediately or sealed and stored overnight at 4℃ for kombucha preparation the following day. If the tea was cooled to 4℃ in overnight storage, the containers were set out to warm to room temperature before kombucha preparation.

Stock kombucha preparation and maintenance. The four kombucha brands were brewed and maintained separately in glass jars for the “stock kombucha.” These preparations most closely replicate consumer home brewing practices. Stock kombuchas were never intentionally introduced to a pathogen and served as the source of starter cultures for experimental trials. For kombucha preparation, various liquid apportionments of stock kombucha jars from a previous batch were collected in a sterile, glass bottle and mixed with a sterile stir bar to create a pooled starter culture of each brand. The starter culture is a pool from the jars to create a diverse, composite microbiota inoculum for fermenting since previous

studies have noted that slight alterations such as geometric dimensions of the brewing container *(66)* can affect the end result kombucha. The pooled kombucha starter culture was added at 10% (v/v) to filter-sterilized tea in a sterile, glass container and mixed for 10 – 15 minutes with a sterile stir bar to ensure a homogenous distribution of kombucha starter culture microbiota and tea. This fresh kombucha preparation was distributed to glass jars. Two sterile, quart Ball® Mason jars (Ball Corporation, Broomfield, Colorado) and two sterile pint Ball® Mason jars (Ball Corporation) were assigned to each of the four kombucha brands. Quart jars received approximately 650 mL of kombucha tea and pint jars received approximately 300 mL of kombucha tea. Pellicles ("SCOBYs") from previous jar preparations were carefully set a top the liquid kombucha in the jar. Each jar was covered with a dry sterile square of a folded paper towel singlet (17.7 cm x 14.0 cm) and secured with a sterile metal O-ring. Stock kombuchas were changed out with fresh tea using this protocol every 2 to 3 weeks for maintenance to maintain active cultures. Some SCOBY's were retained from each kombucha transfer process to add to the new batch of stock kombucha jars.

In preparation for experimental trials, stock kombucha jars were transferred 6 – 10 days before the first day of the trial. This was carefully planned so that the starter culture for each trial was at approximately the same fermentation stage and therefore similar acidity and microbial activity.

Preparation of kombucha and base tea for pathogen challenge studies. There were twelve different treatment groups for each pathogen trial: kombucha + pathogen (K + *S. e.* & K + *E. c.*), base tea + pathogen (BT + *S. e.* & BT + *E. c.*), and kombucha with no pathogen inoculum (NK) for each of the four brands (× 4). BT served as a positive control to track pathogen populations in tea without the variable of kombucha starter culture addition. Triplicate test tubes of each K and BT (+ pathogen) treatment were prepared for each time point. Sterile base tea was prepared as previously described and dispensed (10.0 mL) to sterile 16 x 125 mm-screw cap glass test tubes (Pyrex, Corning). The remaining bulk volume of sterile base tea measured using a sterile, 1 L graduated cylinder and transferred to a new sterile container. Pooled kombucha starter culture (6 – 10 d fermentation) was added at a 10% (v/v) ratio corresponding to

the base tea measurement volume and mixed thoroughly with a sterile stir bar. The fresh kombucha preparation was dispensed (10.0 mL) to sterile 16 x 125 mm-screw cap glass test tubes (Pyrex, Corning). Test tubes containing 10.0 mL treatments of K and BT were inoculated with 100 µL (0.1 mL) of the combined-serotype inoculum for a 1:100 (v/v) dilution for the initial 0 h time point. After inoculation, test tubes were sealed with the screw cap and vortexed to ensure homogenous mixing of the pathogen throughout. Screw caps of test tubes were significantly loosened to allow air exchange, as aerobic fermentation is necessary for kombucha fermentation. Treatments were incubated at ambient air room temperature (20.9℃ ± 1.5) on the benchtop for a 14 d fermentation cycle.

Preparation of kombucha for indigenous microbiota examination. Fresh kombucha was prepared as previously described and withheld from pathogen inoculation (NK). Kombucha was dispensed (30.0 mL) to sterile 25 x 150 mm-screw cap glass test tubes (Pyrex No. 9826-25) (Corning, Corning, NY). NK was prepared and dispensed at the same time as pathogen trial preparation. Measurements of microbiota enumeration for NK were taken in tandem with pathogen trials; however, only single samples measured. Treatments were incubated at ambient air room temperature (20.9℃ ± 1.5) on the benchtop for a 14 – 45 d fermentation cycle.

Microbiological analysis for *Salmonella* and STEC. Samples were taken from kombucha (K) and base tea (BT) treatment test tubes at specific time points throughout a monitored 14 d fermentation cycle. Upon each time point sample harvest, selected test tubes were screwed tightly and vigorously vortexed because the fermentation process can result in settling of microbes. Samples pulled from K and BT treatment tubes were also manually pipetted up and down to ensure mixing of sample before dilution of sample. First, kombucha (K) samples were initially subject a 1:1 dilution (0.85 mL : 0.85 mL) into sterile, 2 × concentrated D/E Neutralizing Broth with Tween (pH: 7.6 ± 0.2) (Neogen, Lansing, MI) *(32)*. D/E Neutralizing Broth with Tween (hereafter referred to as "D/E") was used to partially neutralize the acidity of kombucha before plating. The initial dilution of kombucha samples into D/E changed from a purple hue to bright yellow hue as the acidity of kombucha increased with fermentation time. Preliminary

method development trials reported a 1 – 2 log higher enumeration of *Salmonella* populations over a longer time period when an initial 1:1 dilution with 2 × D/E was used which is similar to previous reported studies *(21, 32, 99)*. After the initial 1:1 dilution of kombucha into D/E, samples (0.1 mL) were serially diluted in (0.9 mL) 0.1% peptone water in sterile microcentrifuge tubes. Base tea samples (0.1 mL) did not receive an initial dilution with D/E and were directly serially diluted in (0.9 mL) 0.1% peptone water.

Populations of *Salmonella* were monitored by surface plating samples on XLT4 agar (pH 7.4 ± 0.2) *(73)* (quadruplicate, 0.1 mL or 0.25 mL) after 0.25 (6 h), 1, 2, 3, 5, 7, 10, and 14 days. Plates were incubated at 37℃ for 24 – 36 h before presumptive colonies were counted. Populations of STEC were monitored by surface plating samples onto SMAC agar (pH: 7.1 ± 0.2) *(69, 82)* (quadruplicate, 0.1 mL or 0.25 mL) after 6 h and 1, 2, 3, 5, 6, 7, 10, and 14 days. Plates were incubated at 37℃ for 24 – 36 h before presumptive colonies were counted. There were triplicate sample measurements of each treatment for each time point, and two replicate trials conducted for each *Salmonella* and STEC challenge study.

Preparation of media used to enumerate indigenous microbiota of kombucha. Four distinct microbial groups from the indigenous microbiota in kombucha were monitored: aerobic plate counts (APC), yeasts (Y), lactic acid bacteria (LAB), and acetic acid bacteria (AAB). Microbial groups were enumerated on non-selective and selective media for each of the four kombucha treatments. Aerobic plate counts populations were enumerated on BD Difco™ plate count agar (PCA) (pH: 7.0 ± 0.2) (Fisher Scientific, Hampton, NH). Yeast populations were enumerated on BD Difco™ dichloran rose bengal chloramphenicol agar (DRBC) (pH: 5.6 ± 0.2).

Media used to enumerate LAB and AAB required supplements of sodium benzoate and citric acid to lower pH and inhibit yeast colony formation. A filter-sterilized, stock concentrated solution of 100,000 ppm (0.1 g/mL) of sodium benzoate (pH: 6.8 ± 0.2) dissolved in E-pure water was used. A heat-sterilized, stock concentration of 50 % (m/v) (pH: 1.1 ± 0.2) citric acid was used. A liquid addition of citric acid was

used to lower the pH and then a 350ppm concentration of sodium benzoate was added to inhibit yeast growth. Volumes were aseptically added to molten agar after cooling to 60℃. Lactic acid bacteria (LAB) populations were enumerated on lactobacilli MRS (deMan, Rogosa and Sharpe) agar (pH: 6.5 ± 0.2) (Neogen, Lansing, MI) supplemented with 350 ppm of sodium benzoate and 1% (v/v) of aqueous citric solution (final pH: 4.8 ± 0.1).

Acetic acid bacteria (AAB) populations were enumerated Hestrin & Schramm (HS) *(45)* agar modified to incorporate a variety of other carbon sources that AAB have been reported to use, including mannitol, sucrose, and ethanol *(67)*. The HS-modified agar medium is defined as follows (%, w/v): 1% D-glucose (VWR), 1% sucrose (granulated white sugar) (Kroger Co.), 1% D-mannitol (Sigma-Aldrich), 0.5% (v/v) pure, 200 proof ethanol (EtOH) (Sigma-Aldrich), 0.5% Bacto™ peptone, 0.5% yeast extract (VWR), 0.27% sodium phosphate dibasic, anhydrous (Na_2HPO_4) (Fisher Scientific), 0.115% citric acid, anhydrous (Sigma-Aldrich) and 1.5% agar (Neogen). Liquid, pure ethanol was aseptically added to the molten agar after it had cooled to 60℃. HS-modified agar (pH: 5.9 ± 0.03) was supplemented with 350 ppm of sodium benzoate and 0.6% (v/v) of concentrated, liquid citric acid solution (final pH: 4.0 ± 0.2).

Microbiological analysis for indigenous microbiota populations of kombucha. Samples were taken from the negative control kombucha (NK) that was not inoculated. Measurements were taken at various time points over a 45 d fermentation cycle and with the first 14 d of incubation occurring in tandem (alongside) the pathogen trials. Samples were not taken from kombucha inoculated with pathogen (K+ *S. e.* & K + *E. c.*) because the pathogen population would become apparent on the non-selective media used for these experiments and interfere with measuring distinct microbial groups themselves. Upon each time point, selected test tubes were screwed tightly and vigorously vortexed and sample volumes were also manually pipetted up and down to ensure mixing of sample before dilution of sample. All samples (NK) were initially subject a 1:1 dilution (0.85 mL : 0.85 mL) into sterile, 2 × concentrated D/E and then serially diluted (0.1 mL) in sterile, 0.1% peptone water (0.9 mL) in sterile microcentrifuge tubes.

Aerobic plate count populations were monitored by surface plating (0.1 mL, quadruplicate) on PCA and incubated at 30℃ for 3 – 4 d before colonies were counted. Yeast populations were monitored by surface plating (0.1 mL, quadruplicate) on DRBC agar and incubated at 25℃ for 7 d before colonies were counted. Lactic acid bacteria populations were monitored by surface plating (0.1 mL, quadruplicate) on MRS agar supplemented with 350 ppm of sodium benzoate and 1% (v/v) aqueous citric acid solution and incubated at 30℃ for 5 d before colonies were counted. Acetic acid populations were monitored by surface plating (0.1 mL, quadruplicate) on HS and HS-modified agar supplemented with 350 ppm of sodium benzoate and 0.6% (v/v) aqueous citric acid solution and incubated at 30℃ for 5 d before colonies were counted.

Measurements of the four distinct microbial groups were taken at: 6 h, and 1, 2, 3, 5, 7, 10, 14, 21, 30, and 45 d. The starter culture for each kombucha trial preparation was also enumerated to create the 0 h time point measurement based on the 10% (v/v) dilution of starter culture in base tea. There were only single samples for measurement at each time point but six separate replicate experiment trials were conducted to collect variants in measurement. Four experimental trials of microbiota enumeration were in-tandem with pathogen survival in kombucha challenge studies, and two additional trials of microbiota enumeration took place to collect additional samples for even analysis.

Chemical analysis (pH, Brix, specific gravity, and ethanol). Samples from each treatment type (K+ *S. e.*, K + *E. c.*, BT + *S. e.*, BT + *E. c.*, and NK) were taken throughout each experimental trial. Triplicate test tube samples of K and BT used for pathogen enumeration were combined to create a composite sample for measurement at each sampling time. Approximately 10 - 15 mL volume of each sample was passed through a sterile, syringe 0.45 µm cellulose acetate membrane filter (VWR) to remove microorganisms and kombucha sediment (bacteria cellulose clumps). The filtered samples were stored in sterile, brown polypropylene, 17 mm x 120 mm, screw cap, conical Cellstar® tubes (VWR). Tubes were tightly sealed and stored at 4℃ until chemical analysis. Sample pH was measured using a Corning 430 pH meter (Cole Palmer, Vernon Hills, IL). Sample Brix (°Bx) and specific gravity was measured using an

Anpro® Dual Scale Specific Gravity/Brix Beer Wort Refractometer (Anpro-tek, Shenzhem, China). Liquid ethanol content was measured using an enzymatic assay with alcohol-dehydrogenase (ADH) test, Enzytec™*Liquid* Ethanol (R-Biopharm AG, Darmstadt, Germany) (AOAC® Official Method 2017.07) *(60)*. Enzymatic ethanol content measurements were taken only from pathogen treatment samples (K + *S. e*. & K + *E. c*.) and at 7 days. For comparison, percent alcohol by volume (ABV %) was also calculated from changes specific gravity for all sample time points using two different mathematical formulas for calculating ABV% in beer and wine.

Statistical analysis. Challenge studies of *Salmonella* and STEC were each replicated twice. Triplicate samples, each consisting of 10 mL of inoculated kombucha (K) or base tea (BT) representing each of the four brands, were analyzed at each sampling time in each replicate trial. Experimental trials to indigenous microbiota enumeration were replicated six times, with four trials in-tandem with pathogen challenge studies. Single samples, each consisting of 30 mL of negative control kombucha (NK) were analyzed at each sampling time in each replicate trial. Data were analyzed with a general linear model using JMP Pro® software (version 13.2, SAS Institute, Cary, NC). Statistical significance ($P \leq 0.05$) of differences between mean values was determined using analysis of variance (two-way ANOVA) test followed by Tukey's HSD (honestly significant difference) paired means comparison test.

TABLE 4. Tea blends and sucrose concentrations for each type of base tea

Brand	Tea Leaf Blend	Tea Quantity (g/L)	Sucrose Conc. (%)
A	Green	8	10
B	Green/black mix	5.2	7.0
C	Green/black mix	4.0	6.1
D	Oolong/black mix	3.0	6.1

CHAPTER 3

RESULTS AND DISCUSSION

Survival of *Salmonella* and STEC in kombucha and base tea. As expected, the surviving populations of *Salmonella* and STEC were significantly higher in base tea (n = 216) (n = 215) than kombucha (n = 248) (n = 278) ($P < 0.0001$) (Figure 1). This first establishes that kombucha fermentation had a significant inhibitory effect on pathogen populations over the course of the 14 days of the study.

To assess viability between the two pathogens, surviving populations of *Salmonella* and STEC in kombucha and base tea were compared. There was a significant difference between *Salmonella* (n = 248) and STEC (n = 278) surviving populations in kombucha ($P < 0.0001$) when values from the four kombucha brands were combined. The mean value of surviving STEC populations was significantly larger than the mean value of *Salmonella* populations (Figure 2A) in kombucha. Interestingly, a significant difference was also apparent between *Salmonella* (n = 216) and STEC (n = 215) surviving populations in base tea ($P = 0.0054$) when values from the four tea brands were combined. The mean value of surviving STEC populations was significantly larger than the mean value of *Salmonella* populations (Figure 2B) in base tea as well. These findings confer that STEC survived longer with larger populations than *Salmonella* in kombucha but also in the positive control, base tea. While kombucha had much more inhibitory effect on both pathogens, it could be reasoned that conditions of the type of base tea used for kombucha fermentation play a role in the survival of these pathogens as well.

To further extrapolate these comparisons, the surviving populations of *Salmonella* and STEC were also compared between the four different brands of kombucha and base tea. When data sets of each brand were compared as a whole, there was not a statistical significant difference in *Salmonella* surviving populations between the four kombucha brands (A, n = 54) (B, n =54) (C, n = 70), (D, n = 70) ($P = 0.1013$) (Figure 3A). This finding can be attributed in some part to the wide variation of *Salmonella*

surviving populations in Kombucha B (Figure 10). However, it is noteworthy that there was not a significant difference ($P > 0.05$) of *Salmonella* populations of Kombucha A, C, or D either, using Tukey paired mean comparisons. Similarly, when data sets of each brand were compared as a whole, there was not a significant difference in STEC surviving populations between the four kombucha brands (A, n = 70) (B, n =70) (C, n = 68), (D, n = 70) ($P = 0.0610$) (Figure 3B). STEC exhibited greater survival with larger population values over time in Kombucha C than Kombucha D. However, there was not a significant difference ($P > 0.05$) of STEC populations between any other kombucha brands with Tukey mean comparisons.

To assess the viability of the two pathogens in each brand, *Salmonella* and STEC surviving populations in each kombucha brand were compared (Figure 3C). There was a significant difference between *Salmonella* and STEC survival for Kombucha A ($P = 0.0009$) and Kombucha C ($P = 0.0046$), but these differences were not significant for Kombucha B ($P = 0.5493$) and Kombucha D ($P = 0.1085$). The distribution of STEC surviving populations was greater than *Salmonella* surviving populations in Kombucha A and C, but the same cannot be concluded for Kombucha B and D (Figure 3C). Furthermore, when each kombucha treatment (K + *S. e*. and K + *E. c*. of Brands A, B, C and D) were compared, the survival of STEC in Kombucha A and C was significantly greater than the survival of *Salmonella* in Kombucha A ($P = 0.0199$ and 0.0003, respectively) and Kombucha D ($P = 0.0494$ and 0.0007, respectively), but all other surviving populations of each treatment were not statistically different from each other ($P > 0.05$) with Tukey mean comparisons (Figure 3C).

Overall, accompanying data supports the conclusion that STEC survives longer with greater population values in kombucha than *Salmonella* during kombucha fermentation, across kombucha brands. However, when analyzing differences between each kombucha brand of pathogen survival, these findings become more complex. Generally, Kombucha C demonstrated the most profound survival of populations of both pathogens, and Kombucha D permitted the least survival of pathogens ($P = 0.0199$), with Kombucha A and B being of intermediate of these (C > B & A > D) ($P = 0.0303$) but variations occur.

While differences in surviving *Salmonella* and STEC populations between brands were not statistically significant (Figure 3 A – C), overall results cannot be lumped together. Significant changes in survival of *Salmonella* and STEC between kombucha brands are significant when specific time points in the 14-day fermentation period are compared.

Regarding the positive control base tea treatments, *Salmonella* had a significantly lower surviving population in Brand D base tea ($P < 0.0001$) with a population mean 1.47 – 1.85 log (CFU/mL) lower than the other three base tea brands (Figure 4A). While Brand C base tea technically had a significantly lower *Salmonella* population than Brand A ($P = 0.0480$), the mean population difference was only 0.39 log (CFU/mL) and probably holds little practical significance. A similar finding was supported for STEC surviving populations in base tea. Populations of STEC in Brand D were significantly lower ($P < 0.0001$) with a population mean 0.58 – 1.32 log (CFU/mL) lower than the other three brands (Figure 4B). Regarding differences in *Salmonella* and STEC populations within each brand of base tea, STEC had a significantly greater population mean than *Salmonella* in Brand A ($P = 0.0344$) and Brand D ($P < 0.0001$) but no significant difference was observed between pathogens for Brand B and C (Figure 4C). Generally, Base Tea A supported the greatest population growth of *Salmonella* and STEC, and Base Tea D permitted the least growth and survival of pathogens ($P < 0.0001$), with Base Tea B and C being of intermediate of these (A > B & C > D) ($P < 0.0001$).

Populations of *Salmonella* and STEC were enumerated from samples of four different brands of kombucha and base tea at 10 different sampling points over a 14-day fermentation period. Survival curves of *Salmonella* and STEC in kombucha and base tea, when brands are combined, are shown in Figure 5. Specific population values of pathogens, when brands are combined, are listed in Table 5 as well. The first significant decrease in *Salmonella* populations occurred by 1 d followed by the next significant decrease at 3 d and then 5 d. When data from the four brands were combined, the mean population log reduction of *Salmonella* (n = 28) in kombucha was 4.96 log (CFU/mL) at 5 d and 5.45 log (CFU/mL) at 7 d. STEC populations in kombucha remained more stagnant in the early days of fermentation. The first

significant decrease in STEC populations occurred at 3 d, followed by significant decreases in a stair-step like decline with each sampling time point until 10 d. When the four brands were combined, the mean population log reduction of STEC ($n \geq 28$) in kombucha was 2.50 log (CFU/mL) at 5 d and 4.42 log (CFU/mL) at 7 d. Overall, a 5.0+ log reduction of *Salmonella* and STEC populations was observed, on average, at 7 days and 10 days, respectively.

While not the survival of *Salmonella* and STEC was not statistically significant between brands when compared as whole, the survival of pathogens between brands has notable variations of statistical significance when comparing populations over time. Survival curves for *Salmonella* and STEC in different kombucha brands are shown in Figure 6 and Figure 7, respectively. Specific population values of each pathogen are also listed in Table 6 and Table 7. *Salmonella* populations fell most rapidly in Kombucha D followed by Kombucha A and C. While data reports a prolonged survival of *Salmonella* in Kombucha B, it is difficult to draw firm conclusions because of extreme variations in trials. As demonstrated in Figure 10, there were wide variations in the survival of *Salmonella* between the two replicate trials in Kombucha B. There are numerous confounding variables that might explain the variations between trials, but the presence of nematode contaminant (*Panagrellus redivivus*) was confirmed in Kombucha B which may explain some considerable differences. When the two replicate trials are combined, a 5.0+ log reduction of *Salmonella* population was observed, on average, at 7 d for Brand A, at 10 d for Brand B, at 7 d for Brand C, and 5 d for Brand D (Table 6) (Figure 6). However, this should be interpreted with some caution because *Salmonella* populations were still detectable with spread plating by the endpoint of the trial at 14 days for single trials of Brand B and C Kombucha.

STEC populations fell most rapidly in Kombucha D followed by Kombucha A and B, with populations demonstrating a prolonged presence up to 14 d in Kombucha C (Figure 7). The wide variation in pathogen decline for Kombucha B was not observed for STEC trials. While the mean populations are low (< 1 log), it should be noted that STEC populations were still detectable with spread plating at the end point of the trial at 14 days for single trials of Brand A and Brand C (Table 7). When

the two replicate trials are combined, a 5.0+ log reduction of STEC population was observed, on average, at 10 d for Brand A, at 10 d for Brand B, at 14 d for Brand C, and 7 d for Brand D (Table 7) (Figure 7).

Populations of *Salmonella* and STEC in the four base tea brands over the 14 day incubation period are also show in Figure 8 and 9, respectively. For Brands A, B, and C, populations of both pathogens increased by 1 - 2 log (CFU/mL) in 24 h and generally plateaued and continued to maintain that population throughout the 14-day trial (Figure 8 & 9). Brand C appeared to support a slight gradual decline of STEC over time (Figure 9). Both pathogens proliferated most readily in Brand A tea. Interestingly, neither pathogen had significant growth in Brand D but instead a gradual decline of pathogen populations. In fact, *Salmonella* populations decreased by 2.58 log (CFU/mL) in Brand D by 10 days (Figure 8).

Because all teas were filter-sterilized before inoculation, there must some unique chemical characteristics to Brand D to inhibit pathogen growth. Notably, Brand A was a green tea and Brand D was a mix of oolong and black tea (Table 4). These findings are contrary to some previous studies *(5)* that report tea has some inherent antimicrobial properties. Except for Brand D base tea, pathogen populations not only survived by actually grew and increased by 1 – 2 log (CFU/mL) throughout the duration of the trial and maintained a 7 – 8 log population for 14 days. Kim et al. reported that *Listeria monocytogenes* and *Staphylococcus aureus* populations were inhibited by green tea, but none of the types of tea used in the study (black, green, jasmine, oolong) inhibited growth of STEC and *Salmonella (56)*. Chou et al. reported that green tea had the most inhibitory effects on STEC and *Salmonella* populations and black tea was the least inhibitory *(24)*. However, oolong tea leaves harvested in the summer were more inhibitory than all other treatments *(24)*. These findings are at least partially supported by our data. Brand D was a black/oolong tea mix and was significantly most inhibitory to pathogen growth, but Brand A was a green tea and typically supported the highest number of pathogen populations. Brands B and C were a mix of green and black tea and supported growth and sustained populations of the pathogens throughout 14 days.

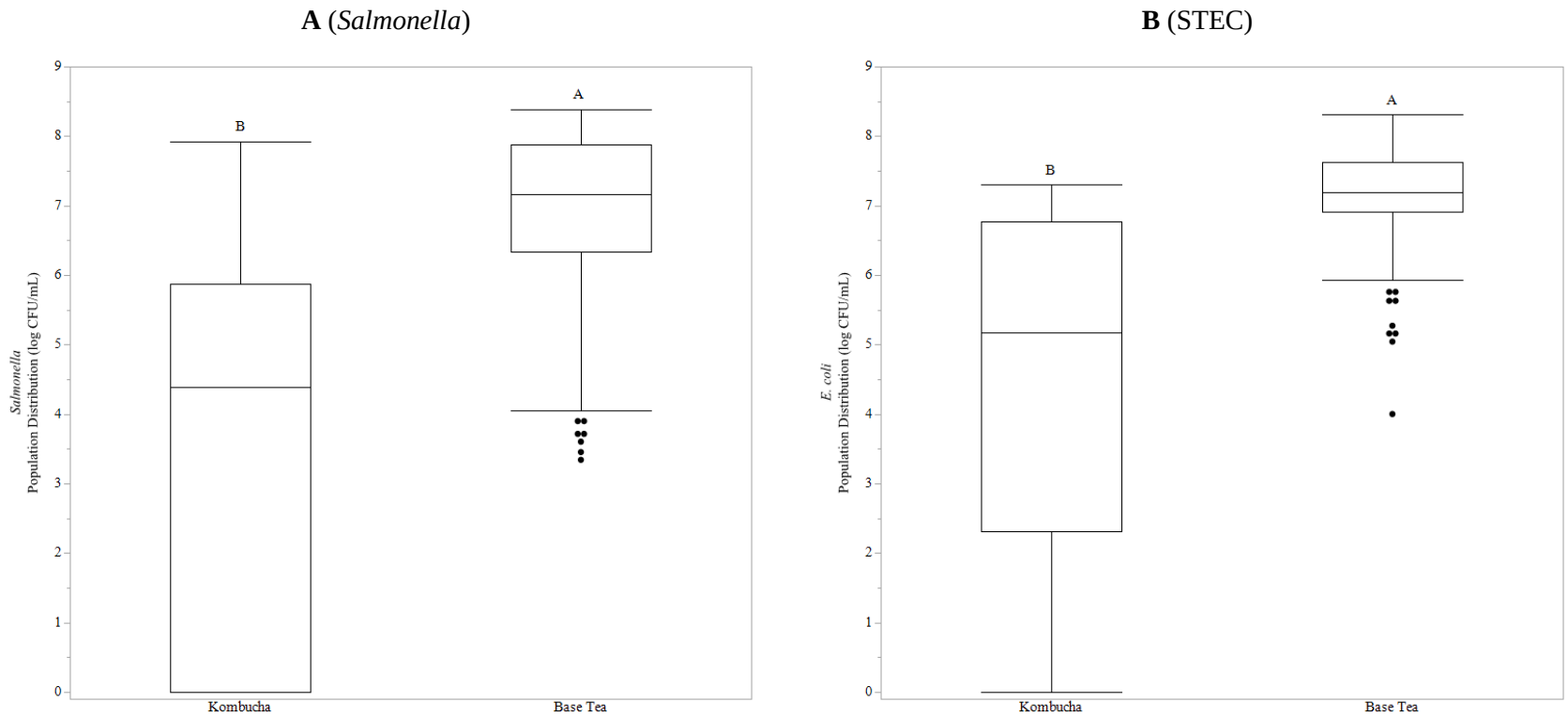


FIGURE 1 (A & B). Comparisons of pathogen population distributions recovered from kombucha and the positive control base tea of *Salmonella* ($n \geq 216$) ($n = 248$ and 216, respectively) (**A**), and of STEC ($n \geq 215$) ($n = 278$ and 215, respectively) (**B**) over a 14 day fermentation cycle incubated at room temperature (20.9°C ± 1.5). Boxplot distributions represent median, first quartile, third quartile, maximum, minimum, and outlier values. Boxplots annotated with different uppercase letters are significantly different ($P \leq 0.05$) based on a pooled t-test.

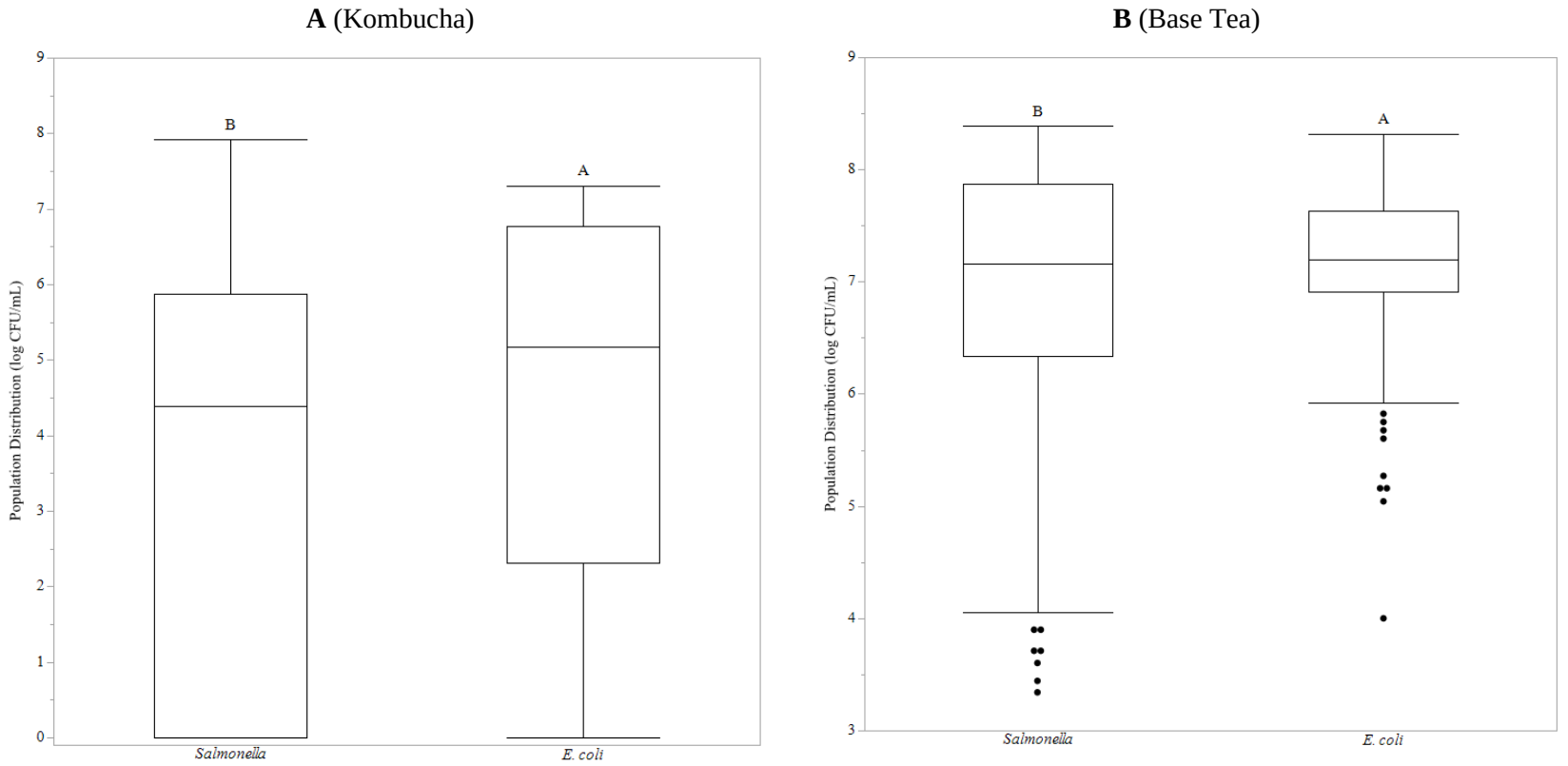
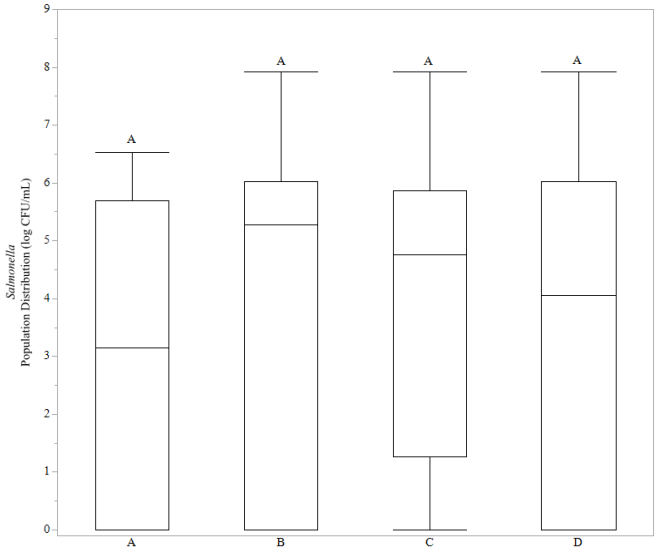


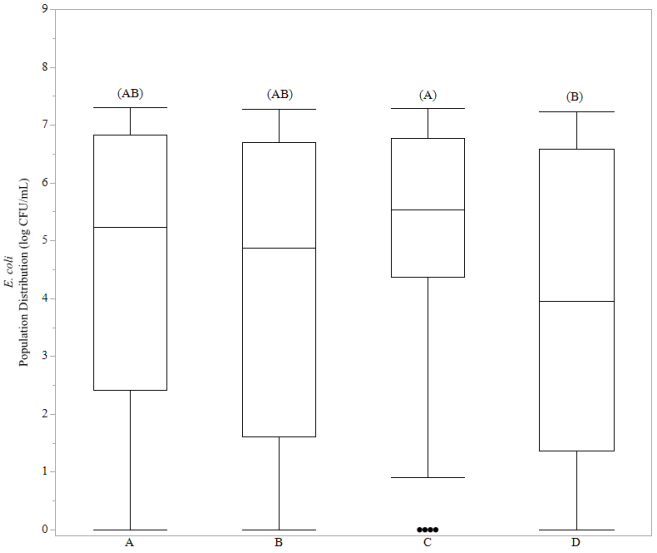
FIGURE 2 (A & B). Comparisons of *Salmonella* and STEC population distributions recovered from kombucha (n ≥ 248) (n = 248 and 278, respectively) (**A**), and positive control base tea (n ≥ 215) (n = 215 and 216, respectively) (**B**), recovered over a 14 day fermentation cycle incubated at room temperature (20.9°C ± 1.5). Boxplot distributions represent median, first quartile, third quartile, maximum, minimum, and outlier values. Boxplots annotated with different uppercase letters are significantly different ($P \leq 0.05$) based on a pooled t-test.

FIGURE 3 (A - C). Comparisons of pathogen population distributions recovered from the four different kombucha brands for *Salmonella* ($n \geq 54$) (**A**), and for STEC ($n \geq 68$) (**B**), and for all kombucha pathogen treatments (*Salmonella* and STEC) ($n \geq 54$) (**C**) over a 14 day fermentation cycle incubated at room temperature (20.9°C ± 1.5). Boxplot distributions represent median, first quartile, third quartile, maximum, minimum, and outlier values. Boxplots annotated with different uppercase letters are significantly different ($P \leq 0.05$) based on Tukey mean comparisons following a two-way ANOVA. For Figure 1.3B, annotations are contained within parentheses ( ) because ANOVA results were nearly significant ($P = 0.0610$) and thus a slightly larger threshold ($\alpha = 0.07$) was used for Tukey mean comparisons to distinguish differences. For Figure 1.3C, for the pair of pathogen treatments within each kombucha brand, *Salmonella* and STEC population distributions that are significantly different ($P \leq 0.05$) from each other are denoted with an asterisk (*), based on a pooled t-test.

A (Kombucha + *Salmonella*)



B (Kombucha+ STEC)



C (All Kombucha + Pathogen Treatments)*

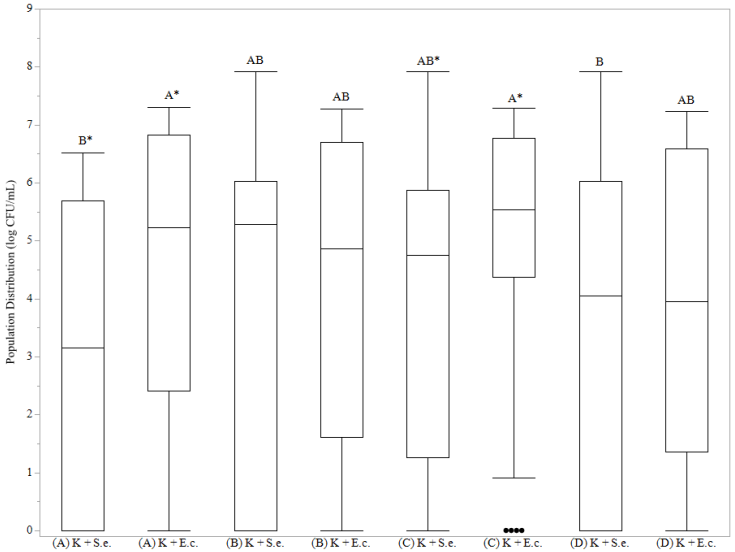
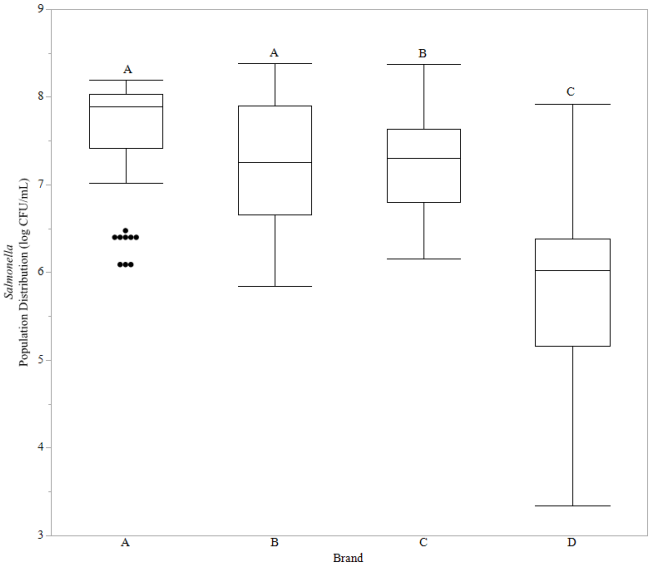
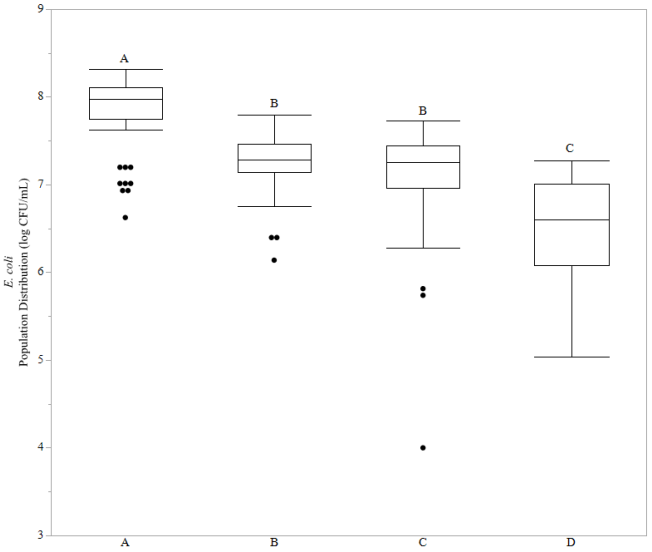


FIGURE 4 (A - C). Comparisons of pathogen population distributions recovered from the four different base tea brands (positive controls) for *Salmonella* (n = 54) (**A**), and for STEC (n = 54) (**B**), and for all base tea pathogen treatments (*Salmonella* and STEC) (n = 54) (**C**) over a 14 day fermentation cycle incubated at room temperature (20.9℃ ± 1.5). Boxplot distributions represent median, first quartile, third quartile, maximum, minimum, and outlier values. Boxplots annotated with different uppercase letters are significantly different ($P \leq 0.05$) based on Tukey mean comparisons following a two-way ANOVA. For Figure 1.3C, for the pair of pathogen treatments within each base tea brand, *Salmonella* and STEC population distributions that are significantly different ($P \leq 0.05$) from each other are denoted with an asterisk (*), based on a pooled t-test.

A (Base Tea + *Salmonella*)



B (Base Tea + STEC)



C (All Base Tea + Pathogen Treatments)*

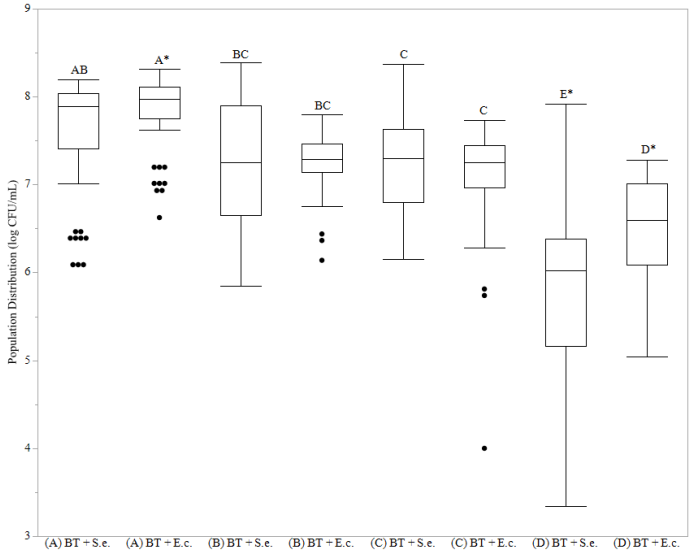


FIGURE 5. Survival curves for *Salmonella* (red) and STEC (blue) in kombucha (solid line) and positive control base tea (dotted line) over a 14-day fermentation cycle incubated at room temperature (20.9°C ± 1.5) when data from the four brands are combined. Values are population means with error bars representing standard error of the mean for at least two independent, replicate trials with triplicate samples ($n \geq 6$) for each treatment. In this figure, the reported values of *Salmonella* and STEC populations are calculated from an increased sample size ($n \geq 24$), because data from the four brands of kombucha and base tea are combined. Initial populations of *Salmonella* ranged from 6.10 (SD ± 0.18) to 7.84 (SD ± 0.30) log CFU/mL. Initial populations of STEC ranged from 6.77 (SD ± 0.32) to 7.20 (SD ± 0.15) log CFU/mL. Asterisk (*) annotations correspond to the day at which a 5.0+ log reduction of *Salmonella* or STEC in kombucha is reported from a dataset of combined kombucha brands and combined replicate trials. The detection limit (dashed line) was 0.301 log CFU/mL calculated from quadruplicate plates with direct spread plating of 0.25 mL of kombucha with 1:1 D/E dilution.

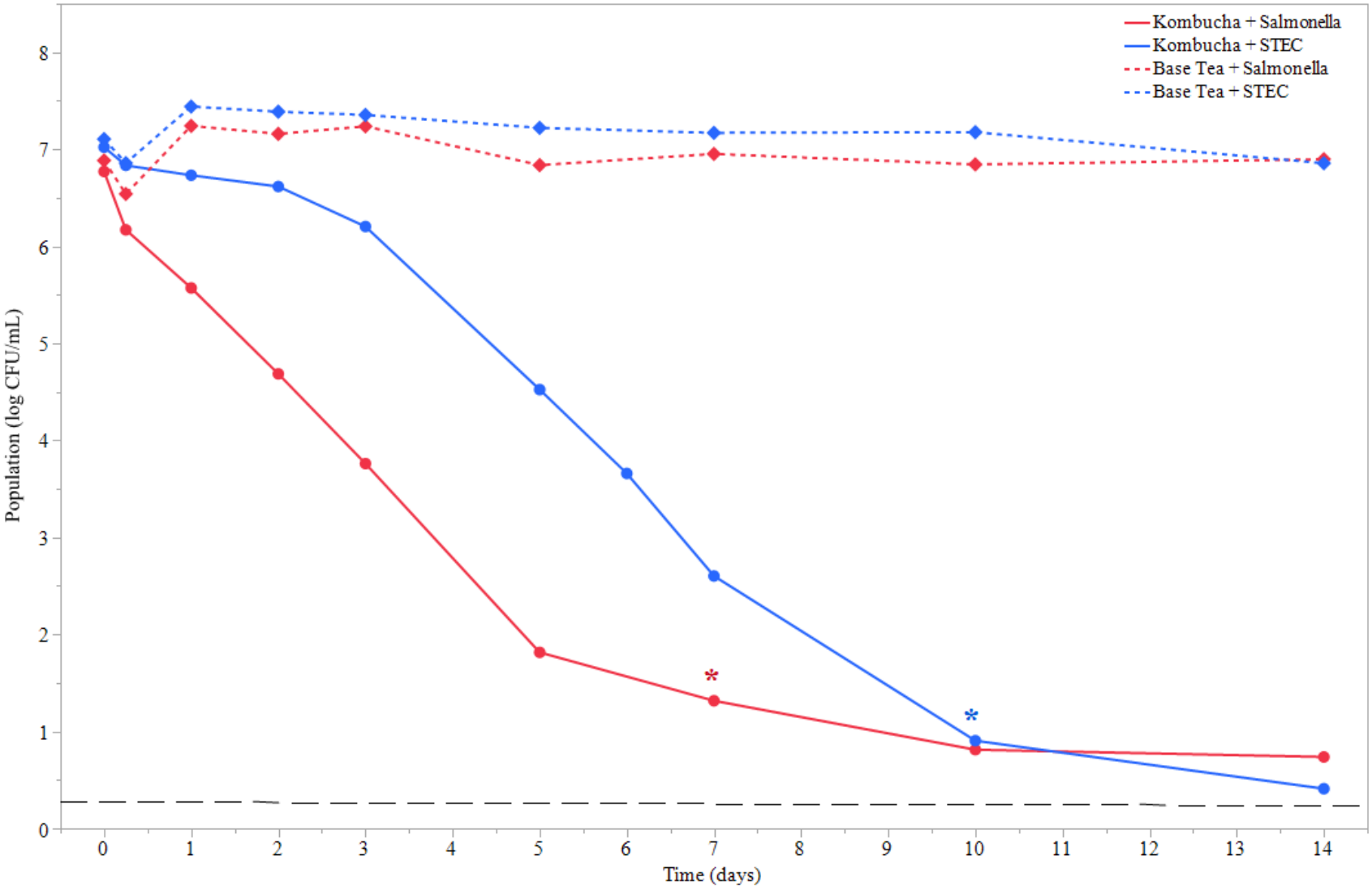

Kombucha + Salmonella
Kombucha + STEC
Base Tea + Salmonella
Base Tea + STEC
Population (log CFU/mL)
Time (days)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
*
*

FIGURE 6. Survival curves for *Salmonella* in four different brands of kombucha A (red), B (green), C (blue), and D (orange) over a 14-day fermentation cycle incubated at room temperature (20.9℃ ± 1.5). Values are population means with error bars representing standard error of the mean for at least two independent, replicate trials with triplicate samples ($n \geq 6$) for each treatment. Initial populations of *Salmonella* ranged from 6.10 (SD ± 0.18) to 7.84 (SD ± 0.30) log CFU/mL. Asterisk (*) annotations correspond to the day at which a 5.0+ log reduction of *Salmonella* is reported for each brand of kombucha when data from the two trials is combined. The detection limit (dashed line) was 0.301 log CFU/mL calculated from quadruplicate plates with direct spread plating of 0.25 mL of kombucha with 1:1 D/E dilution on XLT4 agar.

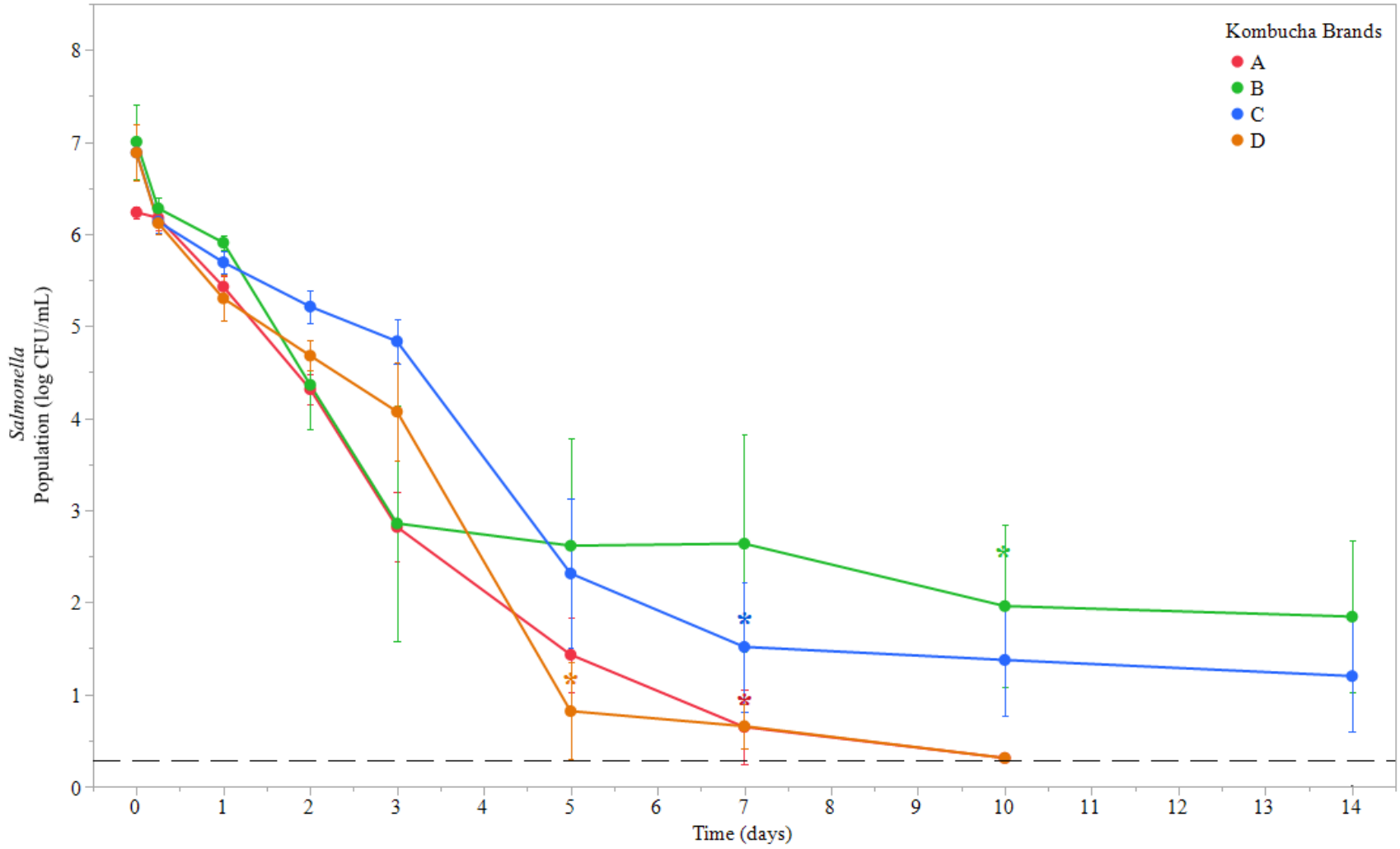

Kombucha Brands
A
B
C
D
Salmonella
Population (log CFU/mL)
Time (days)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14

FIGURE 7. Survival curves for STEC in four different brands of kombucha A (red), B (green), C (blue), and D (orange) over a 14-day fermentation cycle incubated at room temperature (20.9℃ ± 1.5). Values are population means with error bars representing standard error of the mean for at least two independent, replicate trials with triplicate samples ($n \geq 6$) for each treatment. Initial populations of STEC ranged from 6.77 (SD ± 0.32) to 7.20 (SD ± 0.15) log CFU/mL. Asterisk (*) annotations correspond to the day at which a 5.0+ log reduction of STEC is reported for each brand of kombucha when data from the two trials is combined. The detection limit (dashed line) was 0.301 log CFU/mL calculated from quadruplicate plates with direct spread plating of 0.25 mL of kombucha with 1:1 D/E dilution on SMAC agar.

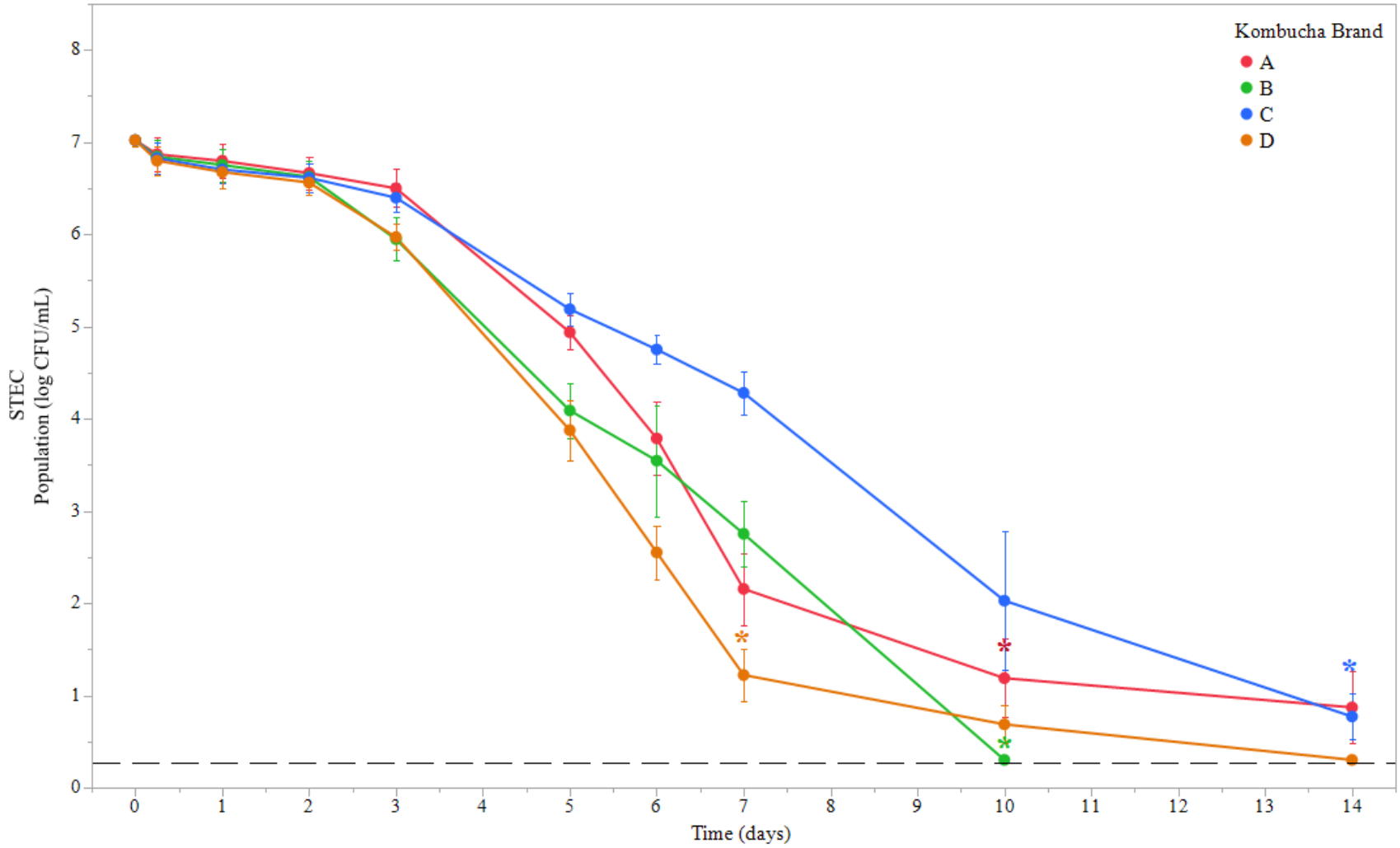

Kombucha Brand
A
B
C
D
STEC
Population (log CFU/mL)
Time (days)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14

FIGURE 8. Populations of *Salmonella* in four different brands of base tea A (red), B (green), C (blue), and D (orange) over a 14-day fermentation cycle incubated at room temperature (20.9°C ± 1.5). Values are population means with error bars representing standard error of the mean for two independent, replicate trials with triplicate samples (n = 6) for each treatment. Initial populations of *Salmonella* ranged from 6.10 (SD ± 0.18) to 7.84 (SD ± 0.30) log CFU/mL.

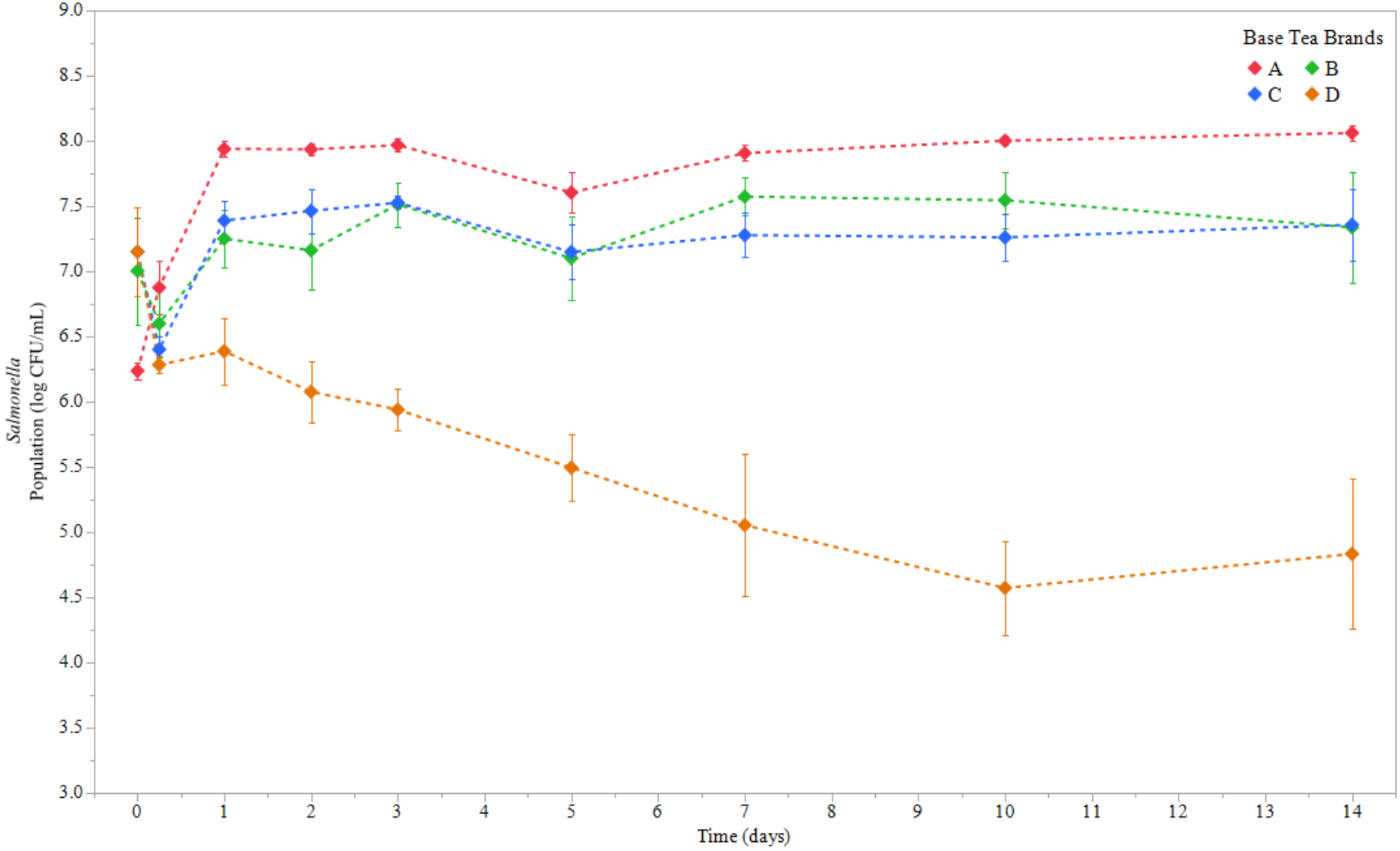

Base Tea Brands
A
B
C
D
Salmonella
Population (log CFU/mL)
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Time (days)

FIGURE 9. Populations of STEC in four different brands of base tea A (red), B (green), C (blue), and D (orange) over a 14-day fermentation cycle incubated at room temperature (20.9°C ± 1.5). Values are population means with error bars representing standard error of the mean for two independent, replicate trials with triplicate samples (n = 6) for each treatment. Initial populations of STEC ranged from 6.77 (SD ± 0.32) to 7.20 (SD ± 0.15) log CFU/mL.

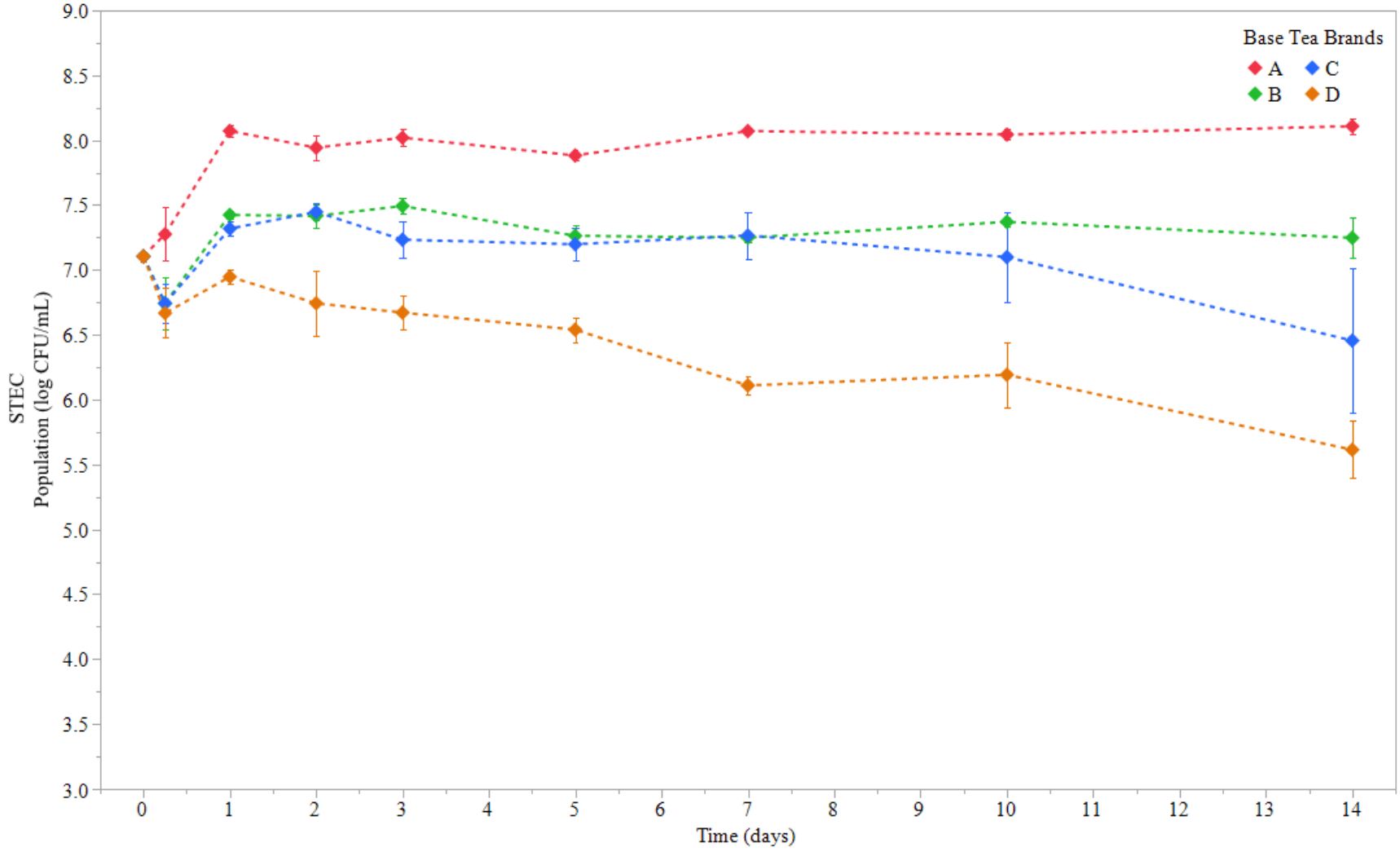

Base Tea Brands
A
C
B
D
STEC
Population (log CFU/mL)
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Time (days)

FIGURE 10. Survival curves for *Salmonella* in Kombucha B in Trial 1 (squares) and Trial 2 (triangles) over a 14-day fermentation cycle incubated at room temperature (20.9°C ± 1.5). Values are population means with error bars representing standard deviation of the mean with each trial data having triplicate samples (n = 3) for each sampling time. Initial populations of *Salmonella* ranged from 6.10 (SD ± 0.18) to 7.84 (SD ± 0.30) log CFU/mL. The detection limit (dashed line) was 0.301 log CFU/mL calculated from quadruplicate plates with direct spread plating of 0.25 mL of kombucha with 1:1 D/E dilution on XLT4 agar.

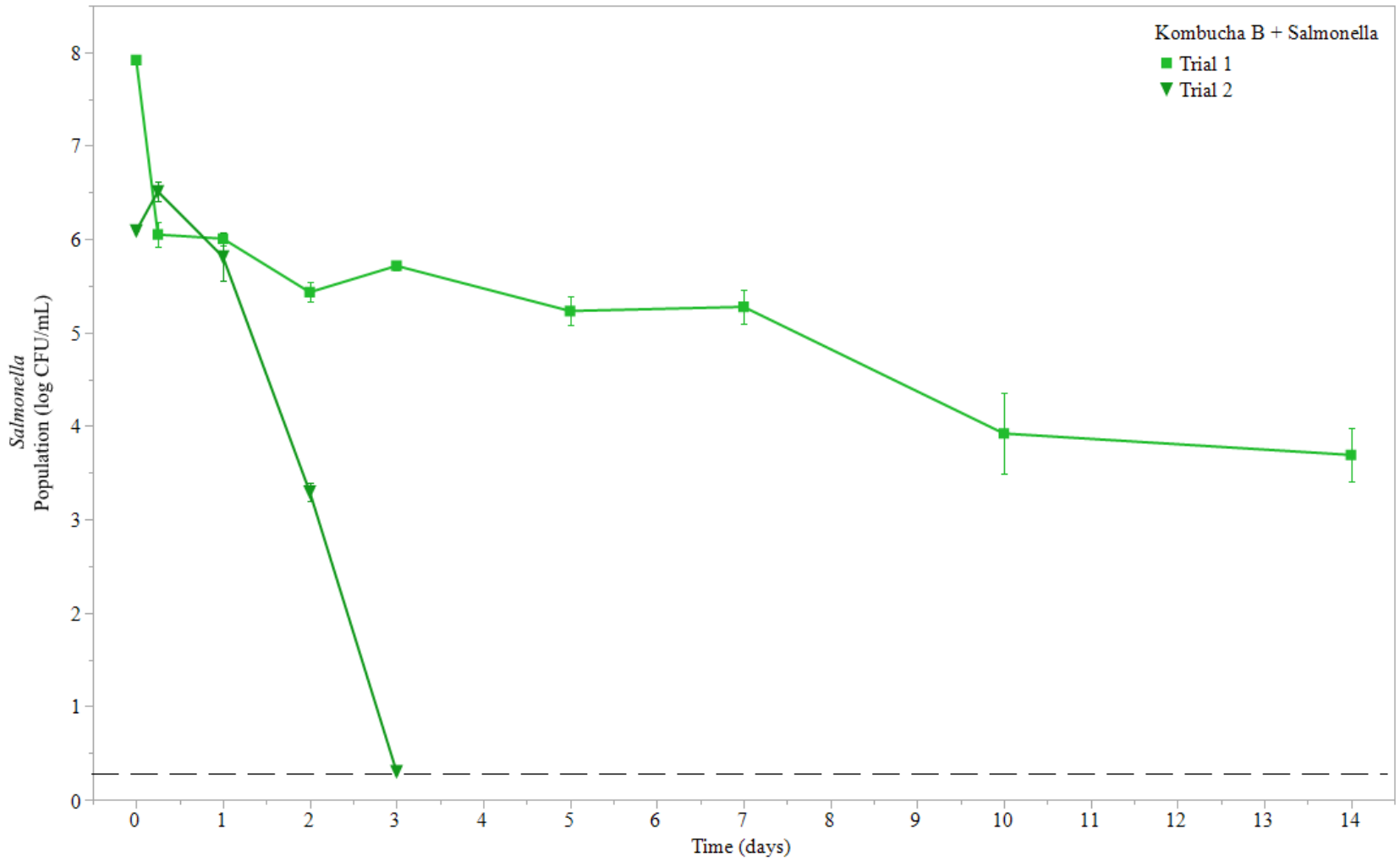

Kombucha B + Salmonella
Trial 1
Trial 2
Salmonella
Population (log CFU/mL)
0
1
2
3
4
5
6
7
8
Time (days)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14

TABLE 5. Recovery of pathogens from kombucha (brands combined) fermented for 14 days at room temperature

Time[d] (days)	*Salmonella*[a] Population Recovered (log CFU/mL)[b] Mean[c]	*Salmonella*[a] 95% CI	STEC[a] Population Recovered (log CFU/mL)[b] Mean[c]	STEC[a] 95% CI
0	6.77 $_{A\,a}$	(6.27, 7.28)	7.02 $_{A\,a}$	(6.71, 7.33)
0.25	6.17 $_{AB\,a}$	(5.67, 6.67)	6.83 $_{AB\,b}$	(6.48, 7.19)
1	5.57 $_{BC\,a}$	(5.07, 6.08)	6.73 $_{AB\,b}$	(6.37, 7.09)
2	4.69 $_{CD\,a}$	(4.18, 5.19)	6.62 $_{AB\,b}$	(6.26, 6.98)
3	3.76 $_{D\,a}$	(3.26, 4.27)	6.20 $_{B\,b}$	(5.85, 6.56)
5	1.82 $_{E\,a}$	(1.28, 2.35)	4.52 $_{C\,b}$	(4.21, 4.83)
6	N/A[e]	N/A	3.66 $_{D}$	(3.35, 3.97)
7	**1.32** $_{E\,a}$	**(0.80, 1.83)**	2.60 $_{E\,b}$	(2.29, 2.91)
10	0.81 $_{E\,a}$	(0.31, 1.32)	**0.91** $_{F\,a}$	**(0.58, 1.23)**
14	0.74 $_{E\,a}$	(0.23, 1.24)	0.41 $_{F\,a}$	(0.05, 0.77)

[a] Survival of pathogens was analyzed in four different kombucha brands and these values are the combined population means of the four brands. Values are population means and 95% confidence intervals ($\alpha = 0.05$) of at least two independent, replicate trials with triplicate samples ($n \geq 6$) for each treatment. The reported values of *Salmonella* and STEC populations are calculated from an increased sample size ($n \geq 24$), because the four kombucha brands combined.

[b] Populations of pathogens recovered on selective agars from kombucha inoculated at the start (0 d) of fermentation. Initial populations of *Salmonella* ranged from 6.10 (SD ± 0.18) to 7.84 (SD ± 0.30) log CFU/mL. Initial populations of STEC ranged from 6.77 (SD ± 0.32) to 7.20 (SD ± 0.15) log CFU/mL. The detection limit was 0.301 log CFU/mL calculated from quadruplicate plates with direct spread plating of 0.25 mL of kombucha with 1:1 D/E dilution.

[c] Within each pathogen, population mean values followed with different uppercase letters are significantly different ($P \leq 0.05$). This corresponds a significant drop in pathogen populations over time. Those values in bold correspond to the point at which a 5.0+ log reduction of the pathogen is recorded.

[d] Within each sampling time, population mean values followed with different lowercase letters are significantly different ($P \leq 0.05$). This corresponds significant difference in Salmonella and STEC populations at that time.

[e] Populations of *Salmonella* were not sampled and enumerated at 6 d.

TABLE 6. Recovery of *Salmonella* populations in four kombucha brands (A, B, C and D) fermented for 14 days at room temperature.

	Salmonella populations recovered (log CFU/mL) in kombucha brands at fermentation time[a]							
Time[d]	Kombucha A[c]		Kombucha B[c]		Kombucha C[c]		Kombucha D[c]	
(days)	Mean[b]	95% CI	Mean[b]	95% CI	Mean[b]	95% CI	Mean[b]	Mean CI
0	6.24 $_{A\,a}$	(5.75, 6.73)	7.00 $_{A\,a}$	(5.32, 8.69)	6.89 $_{A\,a}$	(5.97, 7.80)	6.89 $_{A\,a}$	(6.32, 7.45)
0.25	6.18 $_{A\,a}$	(5.69, 6.67)	6.28 $_{AB\,a}$	(4.60, 7.96)	6.13 $_{AB\,a}$	(5.22, 7.05)	6.12 $_{AB\,a}$	(5.56, 6.68)
1	5.43 $_{AB\,a}$	(4.94, 5.92)	5.91 $_{AB\,a}$	(4.23, 7.59)	5.69 $_{AB\,a}$	(4.78, 6.60)	5.30 $_{BC\,a}$	(4.74, 5.87)
2	4.32 $_{B\,a}$	(3.83, 4.81)	4.36 $_{ABC\,a}$	(2.68, 6.05)	5.21 $_{AB\,a}$	(4.30, 6.12)	4.68 $_{C\,a}$	(4.12, 5.24)
3	2.82 $_{C\,a}$	(2.33, 3.31)	2.86 $_{BC\,a}$	(1.17, 4.54)	4.84 $_{B\,a}$	(3.93, 5.75)	4.07 $_{C\,a}$	(3.51, 4.64)
5	1.43 $_{D\,a}$	(0.94, 1.92)	2.62 $_{BC\,a}$	(0.93, 4.30)	2.31 $_{C\,a}$	(1.34, 3.29)	**0.82** $_{D\,a}$	**(0.17, 1.47)**
7	**0.65** $_{DE\,a}$	**(0.16, 1.14)**	2.64 $_{BC\,a}$	(0.96, 4.32)	**1.52** $_{C\,a}$	**(0.54, 2.49)**	0.65 $_{D\,a}$	(0.09, 1.22)
10	BD[e] $_{E\,b*}$	(0.00, 0.49)	**1.96** $_{C\,a*}$	**(0.28, 3.64)**	1.37 $_{C\,ab*}$	(0.46, 2.29)	BD $_{D\,b*}$	(0.00, 0.56)
14	BD $_{E\,b*}$	(0.00, 0.49)	1.85 $_{C\,a*}$	(0.16, 3.53)	1.20 $_{C\,ab*}$	(0.29, 2.11)	BD $_{D\,b*}$	(0.00, 0.56)

[a] Surviving populations of *Salmonella* recovered (log CFU/mL) on XLT4 at fermentation time (day) from kombucha inoculated at the start (0 d) of fermentation. Initial populations of *Salmonella* ranged from 6.10 (SD ± 0.18) to 7.84 (SD ± 0.30) log CFU/mL.

[b] Values are population means and 95% confidence intervals ($\alpha = 0.05$) of at least two independent, replicate trials with triplicate samples ($n \geq 6$) for each treatment.

[c] Within each kombucha brand, *Salmonella* population mean values followed by different uppercase letters ($_{A, B, C, D, E}$) are significantly different ($P \leq 0.05$). This corresponds to a significant drop in *Salmonella* populations over time. Those values in bold correspond to the point at which a 5.0+ log reduction of the pathogen is recorded.

[d] Within each sampling time (day), *Salmonella* population values followed by different lowercase letters ($_{a, b}$) are significantly different ($P \leq 0.05$). This corresponds to a significant difference in *Salmonella* populations between brands at that sampling time.

[e] Populations reported with "BD" indicate an instance when zero colonies were recovered on XLT4 and the value is below detection. The detection limit was 0.301 log CFU/mL calculated from quadruplicate plates with direct spread plating of 0.25 mL of kombucha with 1:1 D/E dilution.

[*] Analysis of variance statistical test reported a significant difference between mean values ($P \leq 0.05$) but did not report significant differences in individual means comparisons. In these cases, *Salmonella* population values followed by different lowercase letters ($_{a*, b*}$) were nearly significant ($\alpha = 0.10$) ($P \leq 0.10$).

TABLE 7. Recovery of STEC populations in four kombucha brands (A, B, C and D) fermented for 14 days at room temperature.

	STEC populations recovered (log CFU/mL) in kombucha brands at fermentation time[a]							
Time[d]	Kombucha A[c]		Kombucha B[c]		Kombucha C[c]		Kombucha D[c]	
(days)	Mean[b]	95% CI	Mean[b]	95% CI	Mean[b]	95% CI	Mean[b]	95% CI
0	7.02 $_{A\ a}$	(6.46, 7.59)	7.02 $_{A\ a}$	(6.47, 7.58)	7.02 $_{A\ a}$	(6.53, 7.52)	7.02 $_{A\ a}$	(6.61, 7.44)
0.25	6.87 $_{A\ a}$	(6.22, 7.52)	6.84 $_{A\ a}$	(6.20, 7.48)	6.83 $_{A\ a}$	(6.25, 7.40)	6.80 $_{AB\ a}$	(6.32, 7.28)
1	6.80 $_{A\ a}$	(6.15, 7.45)	6.75 $_{A\ a}$	(6.11, 7.39)	6.70 $_{A\ a}$	(6.13, 7.28)	6.68 $_{AB\ a}$	(6.20, 7.16)
2	6.67 $_{A\ a}$	(6.01, 7.32)	6.62 $_{A\ a}$	(5.99, 7.26)	6.61 $_{A\ a}$	(6.04, 7.19)	6.56 $_{AB\ a}$	(6.08, 7.04)
3	6.50 $_{A\ a}$	(5.85, 7.15)	5.95 $_{A\ a}$	(5.31, 6.59)	6.40 $_{AB\ a}$	(5.83, 6.97)	5.97 $_{B\ a}$	(5.49, 6.45)
5	4.94 $_{B\ ab}$	(4.38, 5.50)	4.09 $_{B\ bc}$	(3.54, 4.64)	5.19 $_{BC\ a}$	(4.69, 5.69)	3.88 $_{C\ c}$	(3.46, 4.29)
6	3.79 $_{B\ ab}$	(3.22, 4.35)	3.55 $_{BC\ ab}$	(2.99, 4.10)	4.75 $_{C\ a}$	(4.26, 5.25)	2.55 $_{D\ b}$	(2.14, 2.97)
7	2.16 $_{C\ bc}$	(1.59, 2.72)	2.75 $_{C\ b}$	(2.20, 3.31)	4.28 $_{C\ a}$	(3.79, 4.78)	**1.22** $_{E\ c}$	**(0.81, 1.64)**
10	**1.19** $_{C\ ab}$	**(0.62, 1.75)**	**BD**[e] $_{D\ b}$	**(0.00, 0.55)**	2.03 $_{D\ a}$	(1.46, 2.60)	0.69 $_{EF\ ab}$	(0.27, 1.10)
14	0.87 $_{C\ a*}$	(0.22, 1.52)	BD $_{D\ b*}$	(0.00, 0.64)	**0.77** $_{D\ ab*}$	**(0.20, 1.34)**	BD $_{F\ b*}$	(0.00, 0.48)

[a] Survival of STEC recovered (log CFU/mL) on SMAC at fermentation time (day) from kombucha inoculated at the start (0 d) of fermentation. Initial populations of STEC ranged from 6.77 (SD ± 0.32) to 7.20 (SD ± 0.15) log CFU/mL.

[b] Values are population means and 95% confidence intervals ($\alpha = 0.05$) of at least two independent, replicate trials with triplicate samples ($n \geq 6$) for each treatment.

[c] Within each kombucha brand, STEC population mean values followed by different uppercase letters ($_{A, B, C, D, E, F}$) are significantly different ($P \leq 0.05$). This corresponds to a significant drop in STEC populations over time. Those values in bold correspond to the point at which a 5.0+ log reduction of the pathogen is recorded.

[d] Within each sampling time (day), STEC population values followed by different lowercase letters ($_{a, b, c}$) are significantly different ($P \leq 0.05$). This corresponds to a significant difference in STEC populations between brands at that sampling time.

[e] Populations reported with "BD" indicate an instance when zero colonies were recovered on SMAC and the value is below detection. The detection limit was 0.301 log CFU/mL calculated from quadruplicate plates with direct spread plating of 0.25 mL of kombucha with 1:1 D/E dilution.

[*] Analysis of variance statistical test reported a significant difference between mean values ($P \leq 0.05$) but did not report significant differences in individual means comparisons. In these cases, STEC population values followed by different lowercase letters ($_{a*, b*, c*}$) were nearly significant ($\alpha = 0.10$) ($P \leq 0.10$).

Fluctuations of indigenous microbiota populations through kombucha fermentation. Given the findings in the previous section, the kombucha fermentation process induced when starter culture (SC) microbiota is added to base tea (BT), plays the primary role in inhibition of pathogen survival. The indigenous microbiota in kombucha, whether by microbial activity or lowering pH alone, are the drivers of fermentation and pathogen inhibition. Thus, enumerating the microbiota in kombucha over the course of fermentation may shed some light on these different inhibitory effects.

When the population distributions of four microbiota groups enumerated in kombucha (APC, yeasts, LAB, and AAB) ($n \geq 242$) were compared as a whole, yeast populations were the only significantly different group ($P < 0.0001$) (Figure 11). Yeast populations were significantly lower than bacterial populations. While it initially appears perplexing that the three bacterial groups were not significantly different, aerobic plate counts (APC) are used as an indicator of microbial growth in a variety of food productions *(83, 106)*, and if APC is a reliable indicator for kombucha fermentation, APC populations should not differ from other bacteria populations. Although lactic acid bacteria (LAB) and acetic acid bacteria (AAB) populations were not significantly different when compared as a whole, some variations in LAB and AAB populations were significantly different when compared over time (Table 11 & 12). When the four microbiota groups were combined and kombucha brands compared, the summation of indigenous microbiota enumerated was significantly different between brands and each kombucha brand was significantly different from each other ($P < 0.0001$). While combining all these four different microbial groups for comparison might have minimal conclusive inference, the data demonstrates that sum total indigenous microbiota of each brand was significantly different from each other and suggests that there are microbiota differences between brands (Figure 12).

Kombucha Brands B and C had significantly higher APC populations than Brand D, and Brand A has the lowest APC populations (Figure 13A). Kombucha Brand B had the highest yeast populations and kombucha Brands D and A had the lowest yeast populations (Figure 13B). Kombucha Brand B had the highest LAB populations with Brand D and C having intermediate populations, and Brand A had the

lowest LAB populations (Figure 13C). Kombucha Brand B also had the highest AAB populations, and Brands D and A had the lowest AAB populations (Figure 13D). Across all microbial groups, Brand B consistently had the highest microbiota populations, and Brands C and D had intermediate levels of microbiota. Brand A consistently had the lowest microbiota populations across microbial groups.

The fluctuations of these microbiota group populations (brands combined) over the 14 day fermentation period are displayed in Figure 14 and specific population values are reported in Table 8. The first significant increase in APC populations occurred by 2 d of fermentation; then APC populations generally plateaued with a gradual decrease after 7 days. The first significant increase in yeast populations also occurred by 2 days fermentation with a peak at 7 days. The first significant increase in LAB populations occurred, slightly later, by 3 days with population peaks at 3 and 5 days. The first significant increase in AAB population was at 2 days fermentation with a peak in population at 7 days. While there were variations in peaks and falls and significant points of change for these microbial groups, the populations did not significantly change (< 1.5 log) between 3 – 14 days for any microbiota group. Overall, all microbiota surmounted a significant population increase by 2 – 3 days and then remained relatively consistent throughout fermentation.

The fluctuations of these microbial groups were also extrapolated across brands. Fluctuations of APC populations between brands over 14 days are displayed in Figure 15 and Table 9. Fluctuations of yeast populations between brands over 14 days are displayed in Figure 16 and Table 10. Among all brands, yeast populations tended to reach a significant increase by 2 – 3 days but then populations oscillate without significant increases or decrease throughout the 45-day fermentation period observed (Table 10). Fluctuations of LAB populations between brands over 14 days are displayed in Figure 17 and Table 11. LAB populations peaked for at 3 days fermentation for Brand A and D and 5 days fermentation for Brand B and C. Fluctuations of AAB populations between brands over 14 days are displayed in Figure 18 and Table 12. Notably, AAB populations of Brand D peaked very early at 1 day of fermentation while other brands did not reach a peak AAB population until 3 or 7 days.

Because these microbiota enumerations were taken from the negative control kombucha without pathogen inoculation (NK), there are limitations when making any conclusions about the influence of these microbiota groups on pathogen populations. Accepting that caveat, some observations are made. It is notable that Brand C kombucha harbored pathogens for a longer amount of time and reached a peak LAB population later than the other kombucha brands. It could be hypothesized that because lactic acid is an inhibitory organic acid, if the LAB population tracks with lactic acid production, Brand C may not have demonstrated inhibitory characteristics until later in fermentation because of lower lactic acid levels. It is also notable that Brand D was the most inhibitory to pathogens and reported a peak of AAB populations much earlier than the other brands. Because acetic acid is the among the most inhibitory organic acids, it could be hypothesized that Brand D demonstrated early pathogen decline because acetic acid concentrations are higher earlier in fermentation. However, these microbiota population values did not always align with all pathogen survival characteristics. Kombucha Brand A had the lowest population values for every microbial group in every comparison (Figure 12 & 13); therefore, it would be reasonable to predict that Brand A would be least inhibitory to pathogens because of lower populations of microbiota, but that is not the case. Brand A was intermediately inhibitory to *Salmonella* and STEC, in between Brand D and C, respectively (Figures 6 & 7). As described in the methods section, Brand A kombucha arrived from the commercial supplier in the form of a dehydrated SCOBY and was "activated" through two 30 d cycles of fermentation where a mixture of green tea and distilled white vinegar was added. It is likely this unusual dehydrated starter culture and addition of large volumes of acetic acid played a role in the decreased microbiota populations of Brand A.

These kombucha microbiota values are generally comparable to those reported by Coton et al. over an 8 day fermentation period *(27)*. But Coton et al. reports that kombucha of green tea base support more growth of LAB while a black tea base support more growth of AAB. Our data does not support that. The only brand of kombucha with a pure green tea base was kombucha Brand A, and in fact, Brand A had a significantly lower LAB population than the other brands (Figure 12C).

Considering acetic acid is one of the most inhibitory organic acids in fermentation *(1, 26, 104)*, it would be suspected that AAB populations would track with pathogen decline, but that proposition was not clear in this data because changes in AAB populations did not necessarily correlate to reductions of pathogen populations. Kombucha microbiota is typically dominated by populations of *Gluconobacter* and *Gluconacteobacter* spp. (Table 1) and it has been reported that gluconic acid is not inhibitory to Gram-negative pathogens *(11)*. It could be hypothesized that these *Gluconobacter* and *Gluconoacetobacter* bacteria are included in major segments of the AAB population values enumerated on HS-modified agar and while these bacterial genera are a type of acetic acid bacteria, they are do not produce primarily acetic acid, and the production of gluconic acid is actually not inhibitory to *Salmonella* and STEC.

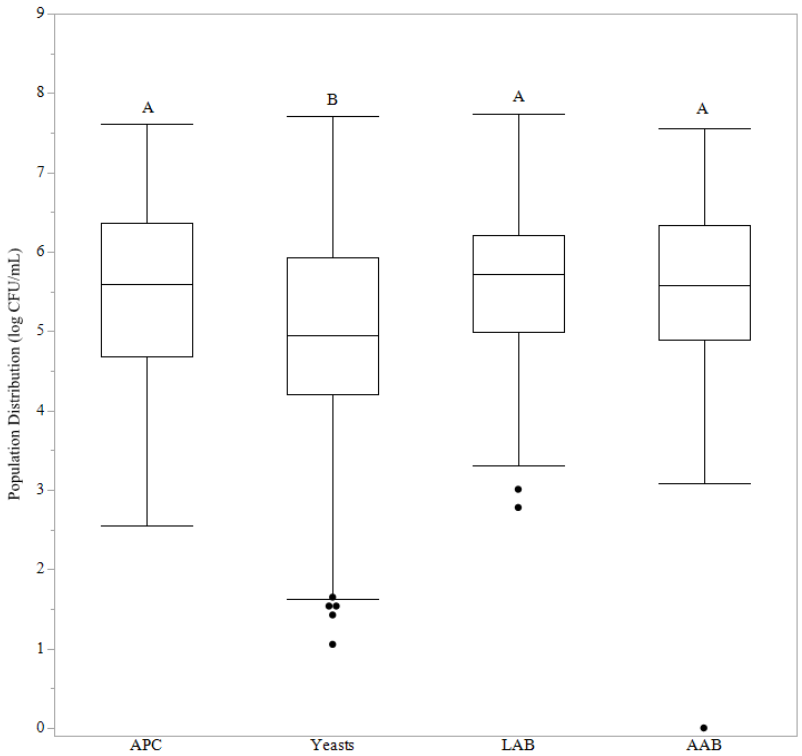


FIGURE 11. Comparisons of kombucha indigenous microbiota population distributions for aerobic plate counts (APC) (n = 243), yeasts (n = 243), lactic acid bacteria (LAB) (n = 247), and acetic acid bacteria (AAB) (n = 242) enumerated from negative control kombucha (NK) over 45 days of fermentation incubated at room temperature (20.9°C ± 1.5) when data from the four different brands of kombucha are combined. Boxplot distributions represent median, first quartile, third quartile, maximum, minimum, and outlier values. Boxplots annotated with different uppercase letters are significantly different ($P \leq 0.05$) based on Tukey mean comparisons following a two-way ANOVA.

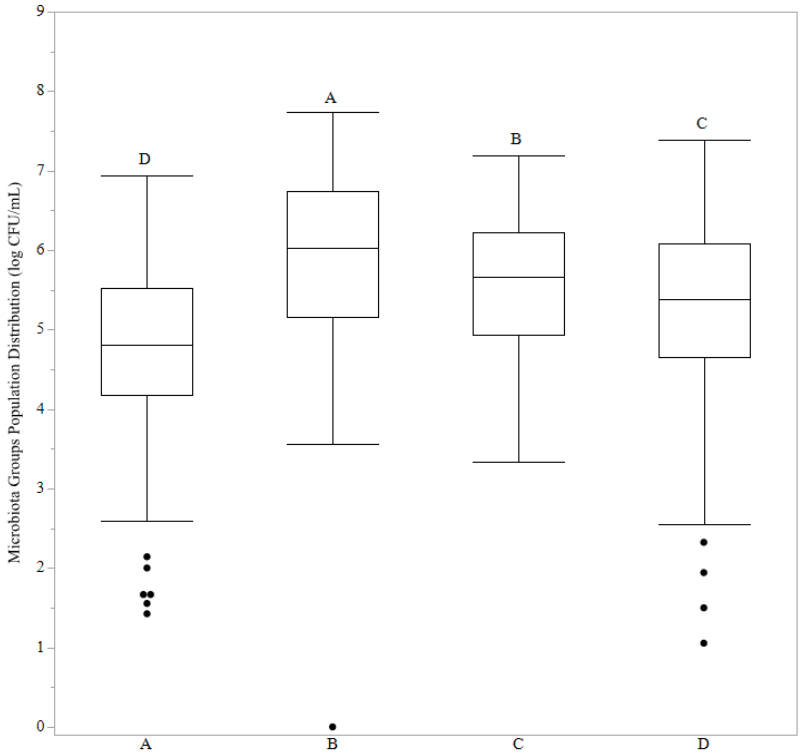
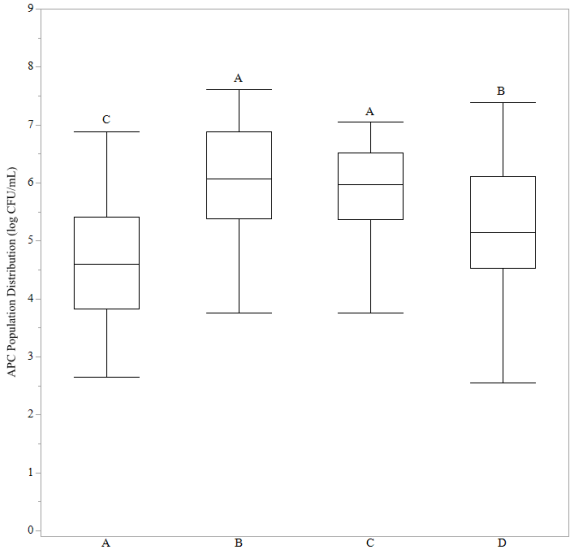


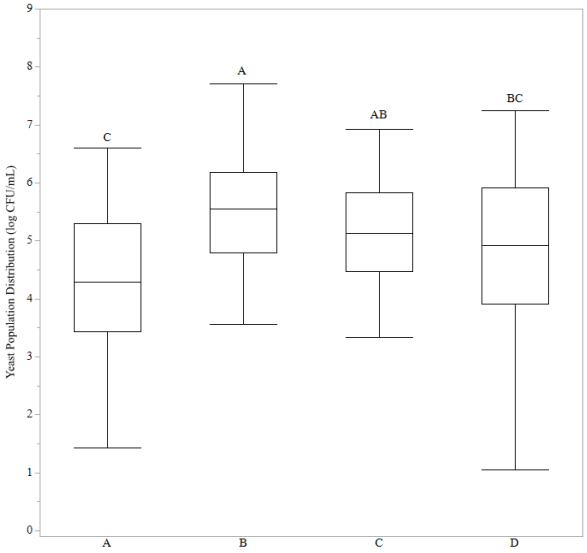
FIGURE 12. Comparisons of kombucha indigenous microbiota population distributions for the four different brands, A (n = 214), B (n = 237), C (n = 265), and D (n = 259) enumerated from negative control kombucha (NK) over 45 days of fermentation incubated at room temperature (20.9°C ± 1.5) when data from the four different microbial groups (APC, yeasts, LAB, and AAB) are combined. Boxplot distributions represent median, first quartile, third quartile, maximum, minimum, and outlier values. Boxplots annotated with different uppercase letters are significantly different ($P \leq 0.05$) based on Tukey mean comparisons following a two-way ANOVA.

FIGURE 13 (A - D). Comparisons of kombucha indigenous microbiota population distributions between four different brands, A, B, C, and D for aerobic plate counts (APC) ($n \geq 60$) (**A**), yeasts ($n \geq 60$) (**B**), lactic acid bacteria (LAB) ($n \geq 60$) (**C**), and acetic acid bacteria (AAB) ($n \geq 60$) (**D**) enumerated from negative control kombucha (NK) over 45 days of fermentation incubated at room temperature (20.9°C ± 1.5) for at least five independent, replicate trials. Boxplot distributions represent median, first quartile, third quartile, maximum, minimum, and outlier values. Boxplots annotated with different uppercase letters are significantly different ($P \leq 0.05$) based on Tukey mean comparisons following a two-way ANOVA.

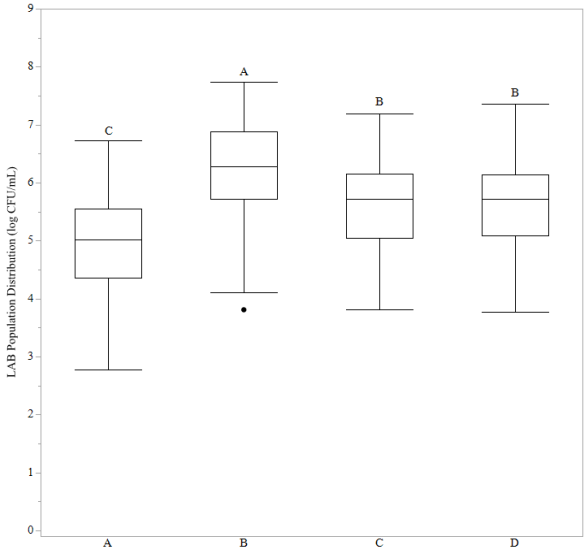
A (APC Populations Across Brands)



B (Yeast Populations Across Brands)



C (LAB Populations Across Brands)



D (AAB Populations Across Brands)

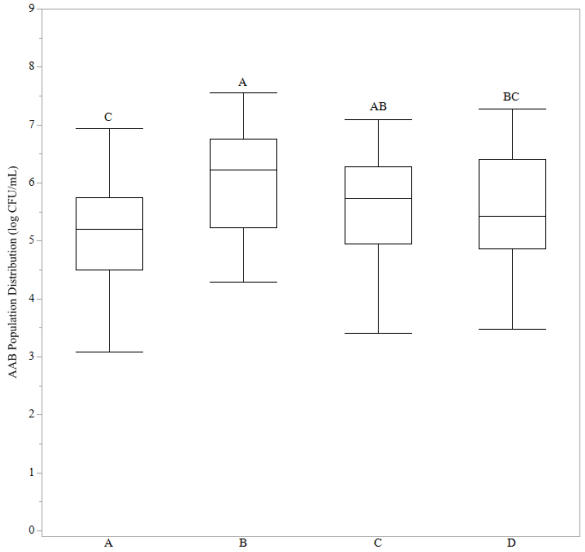


FIGURE 14. Populations of indigenous microbiota in kombucha, including aerobic plate counts (APC) (black), yeasts (purple), lactic acid bacteria (LAB) (dark yellow), and acetic acid bacteria (AAB) (magenta) over a 14-day fermentation cycle incubated at room temperature (20.9℃ ± 1.5) when data from the four brands are combined. Values are population means with error bars representing standard error of the mean for at least five independent, replicate trials with single samples ($n \geq 5$) for each treatment and sampling time. In this figure, the reported microbiota group population values are calculated from an increased sample size ($n \geq 20$), because data from the four brands of kombucha are combined. While samples for indigenous microbiota enumeration were taken from negative control kombucha without pathogen (NK), the preparation and sampling times of each NK trial coincided with each pathogen challenge study trial. Enumerations of indigenous microbiota continued up to 45 days but only data for the first 14 days of fermentation are included in this figure.

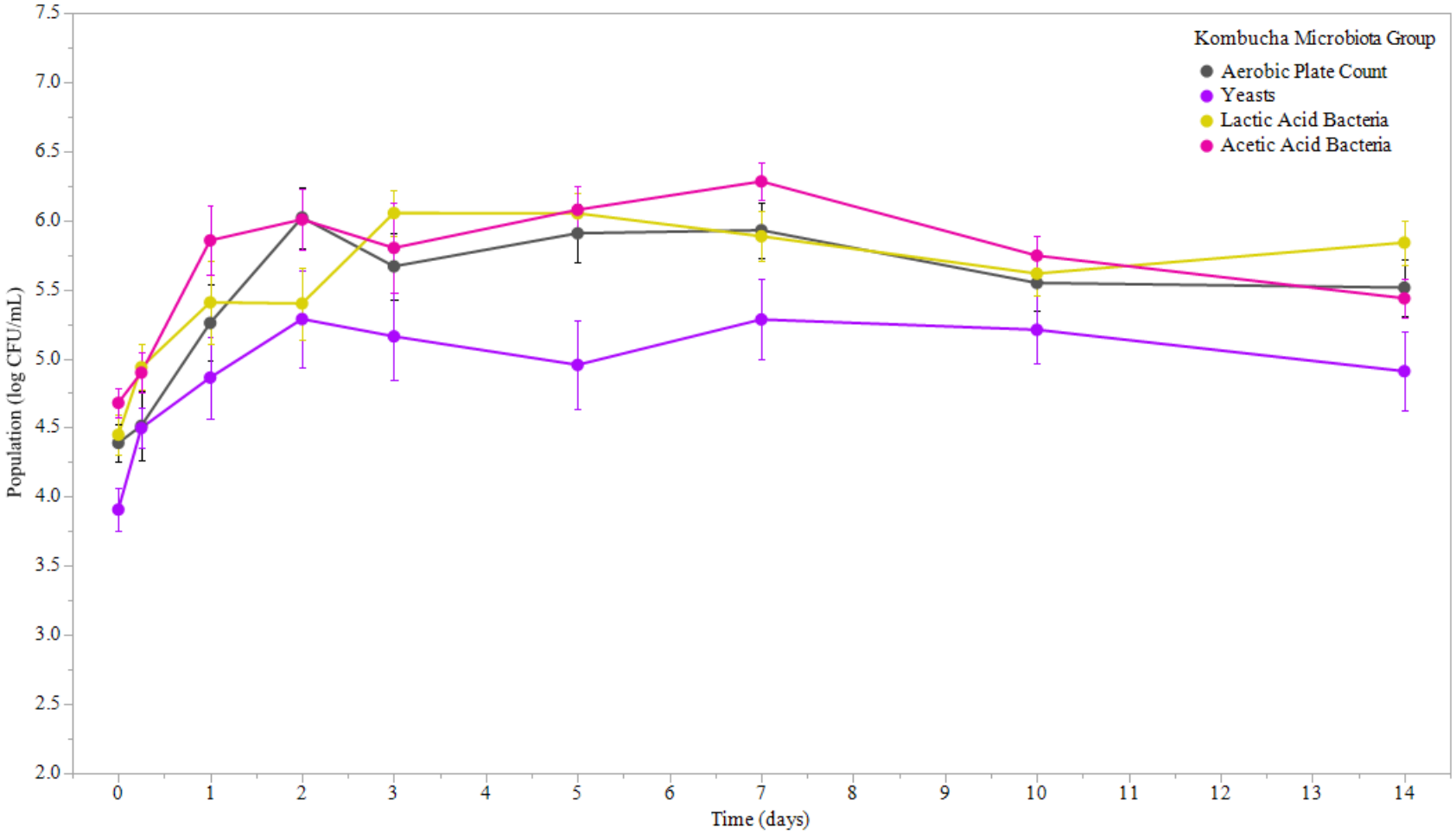

Kombucha Microbiota Group
Aerobic Plate Count
Yeasts
Lactic Acid Bacteria
Acetic Acid Bacteria
Population (log CFU/mL)
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Time (days)

FIGURE 15. Populations of aerobic plate counts (APC) (squares) in four different brands of kombucha A (red), B (green), C (blue), and D (orange) over a 14-day fermentation cycle incubated at room temperature (20.9°C ± 1.5). Values are population means for at least five independent, replicate trials with single samples ($n \geq 5$) for each treatment and sampling time. While samples for APC enumeration were taken from negative control kombucha without pathogen (NK), the preparation and sampling times of each NK trial coincided with each pathogen challenge study trial. Enumerations of APC continued up to 45 days but only data for the first 14 days of fermentation are included in this figure.

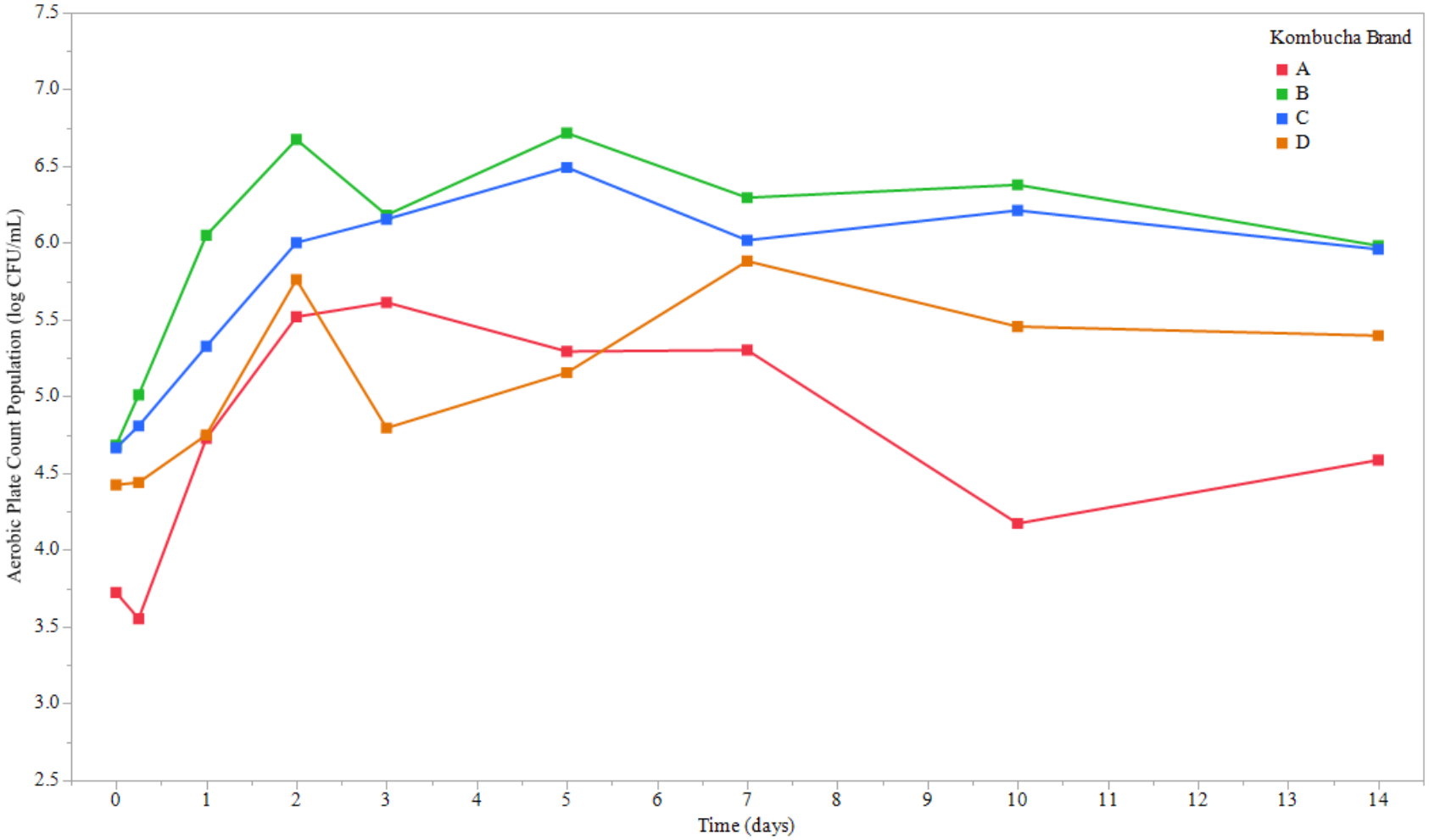

Kombucha Brand
A
B
C
D
Aerobic Plate Count Population (log CFU/mL)
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Time (days)

FIGURE 16. Populations of yeast (asterisks) in four different brands of kombucha A (red), B (green), C (blue), and D (orange) over a 14-day fermentation cycle incubated at room temperature (20.9℃ ± 1.5). Values are population means for at least five independent, replicate trials with single samples ($n \geq 5$) for each treatment and sampling time. The extreme difference between 3 and 5 d of yeast population for brand A probably have little significance as the values have a wide standard deviation and are not significantly different from each other ($P = 0.3781$). While samples for yeast enumeration were taken from negative control kombucha without pathogen (NK), the preparation and sampling times of each NK trial coincided with each pathogen challenge study trial. Enumerations of yeast populations continued up to 45 days but only data for the first 14 days of fermentation are included in this figure.

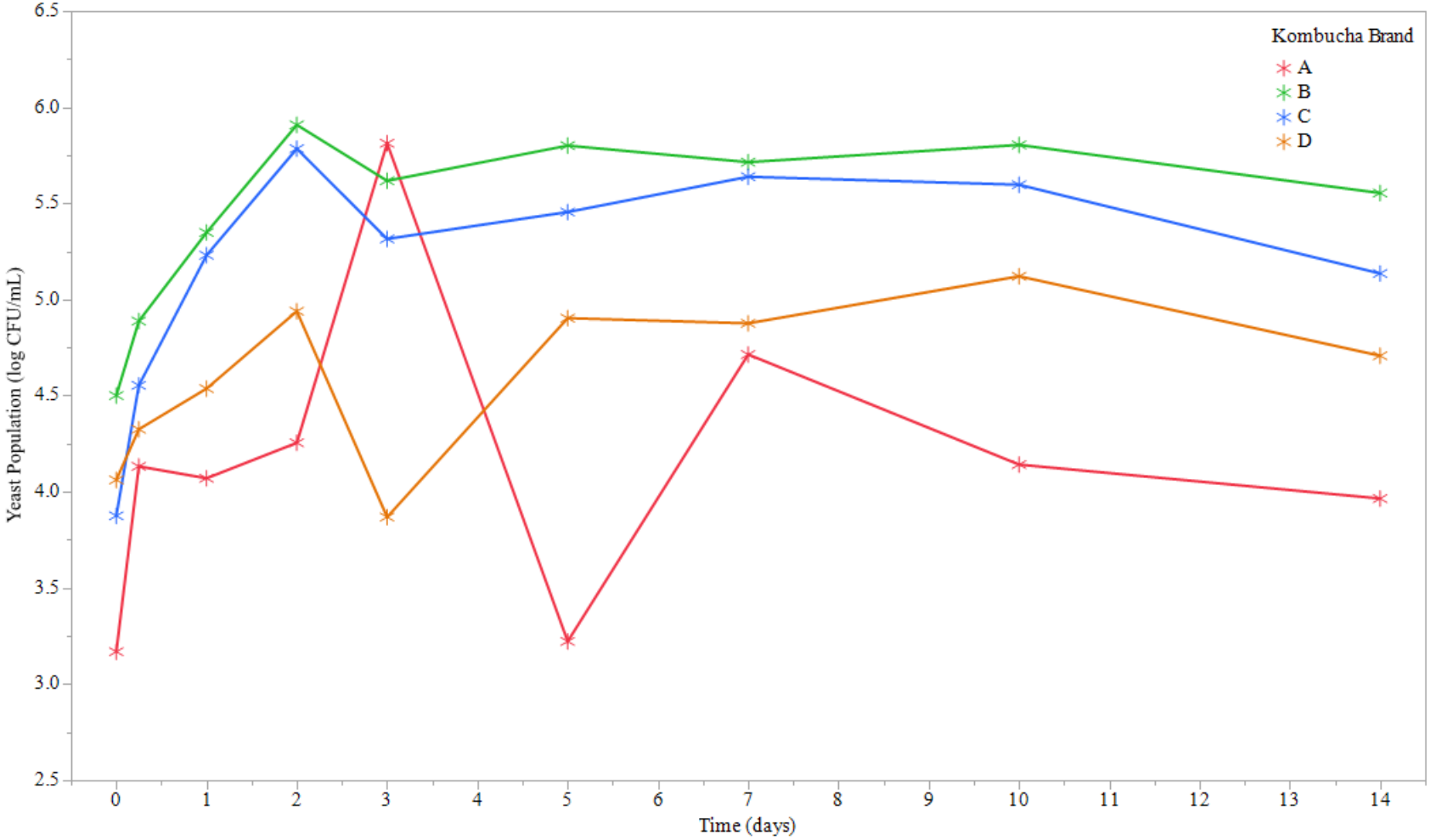

Kombucha Brand
A
B
C
D
Yeast Population (log CFU/mL)
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Time (days)

FIGURE 17. Populations of lactic acid bacteria (LAB) (diamonds) in four different brands of kombucha A (red), B (green), C (blue), and D (orange) over a 14-day fermentation cycle incubated at room temperature (20.9℃ ± 1.5). Values are population means for at least five independent, replicate trials with single samples ($n \geq 5$) for each treatment and sampling time. Asterisk (*) annotations correspond to the day at which the peak of LAB population is recorded for each brand of kombucha when data from all trials are combined. While samples for LAB enumeration were taken from negative control kombucha without pathogen (NK), the preparation and sampling times of each NK trial coincided with each pathogen challenge study trial. Enumerations of LAB population continued up to 45 days but only data for the first 14 days of fermentation are included in this figure.

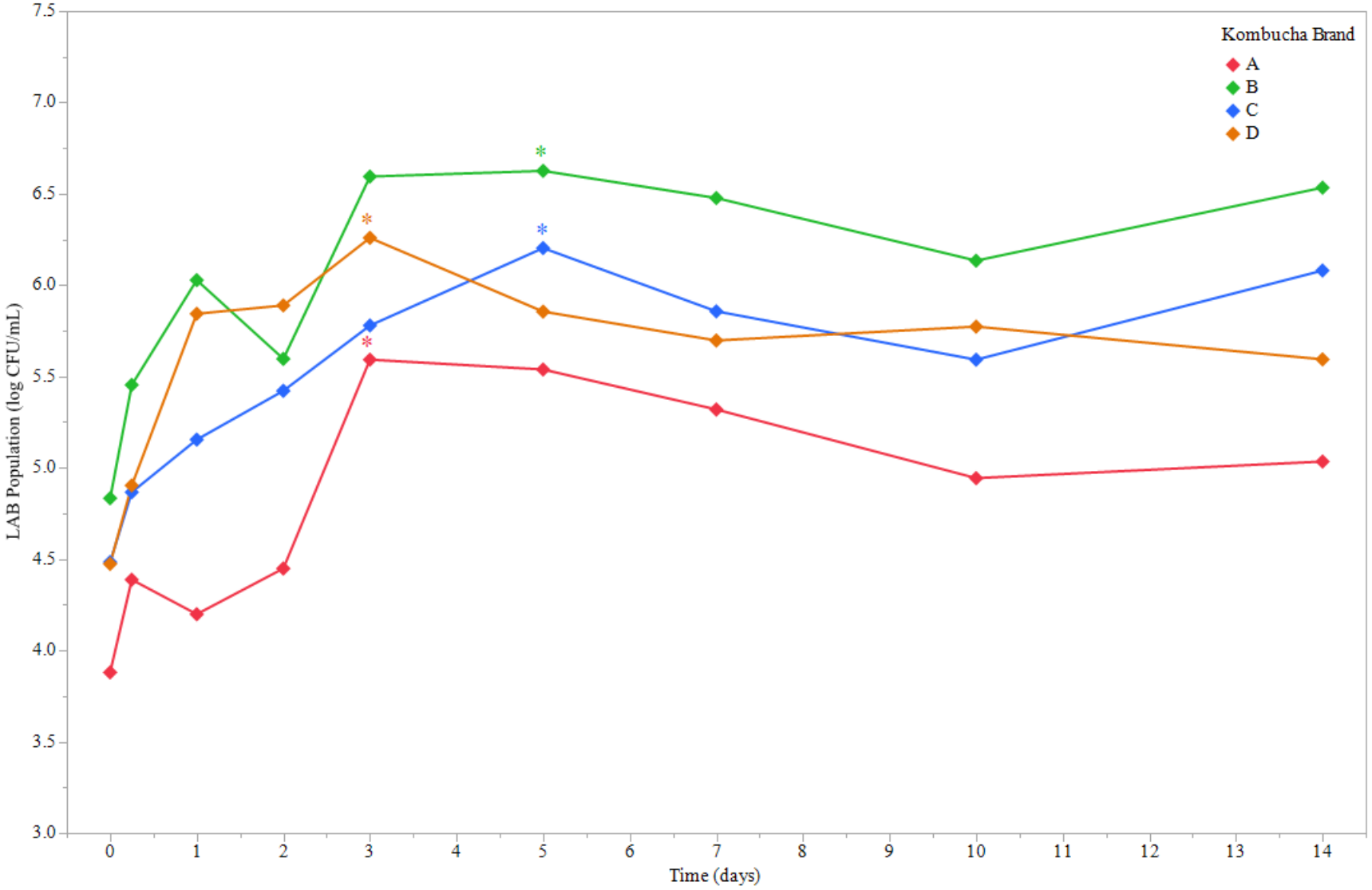

Kombucha Brand
A
B
C
D
LAB Population (log CFU/mL)
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Time (days)

FIGURE 18. Populations of acetic acid bacteria (triangles) in four different brands of kombucha A (red), B (green), C (blue), and D (orange) over a 14-day fermentation cycle incubated at room temperature (20.9°C ± 1.5). Values are population means for at least five independent, replicate trials with single samples ($n \geq 5$) for each treatment and sampling time. Asterisk (*) annotations correspond to the day at which the peak of AAB population is recorded for each brand of kombucha when data from all trials are combined. While samples for AAB enumeration were taken from negative control kombucha without pathogen (NK), the preparation and sampling times of each NK trial coincided with each pathogen challenge study trial. Enumerations of AAB population continued up to 45 days but only data for the first 14 days of fermentation are included in this figure.

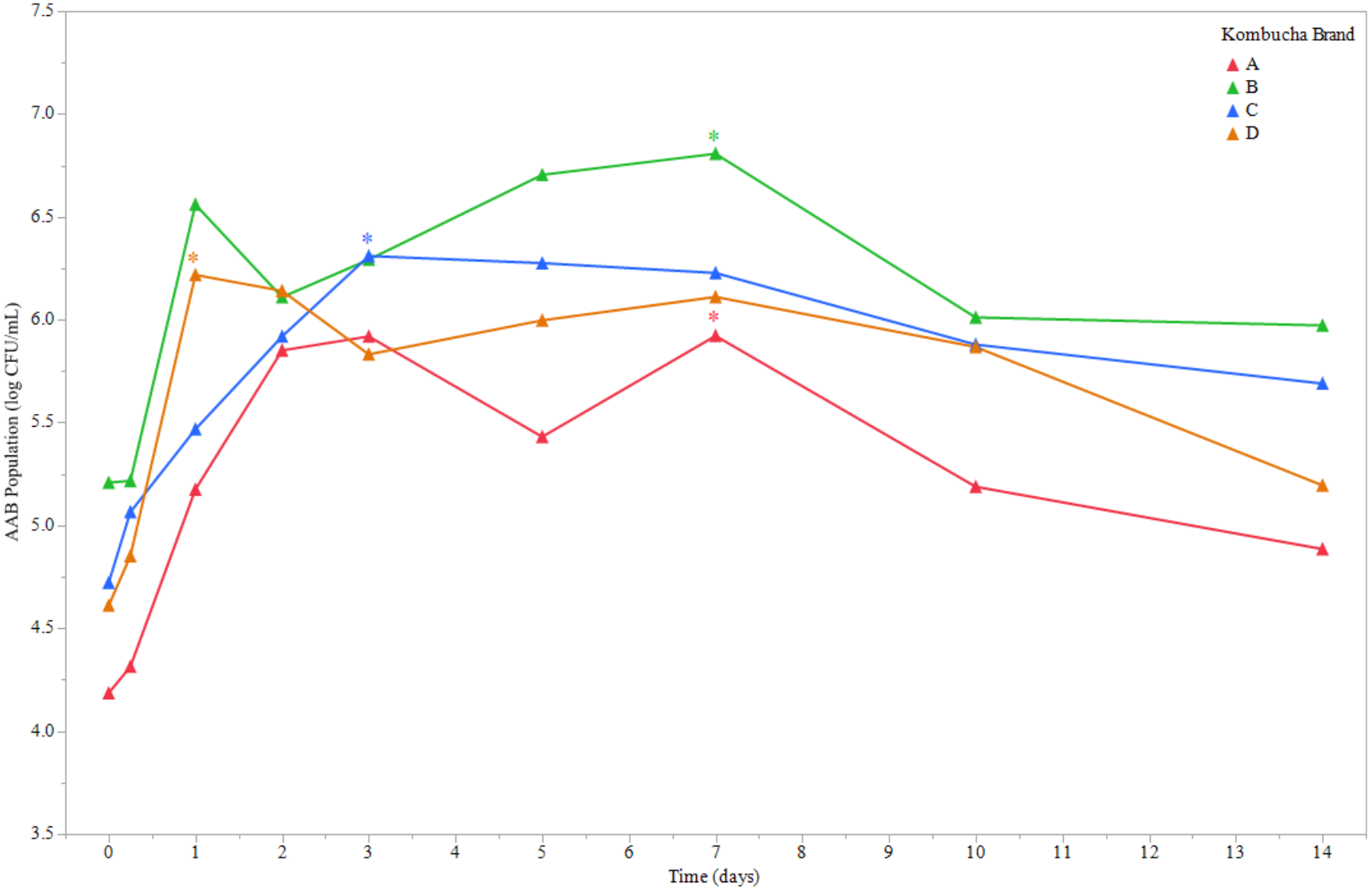

Kombucha Brand
A
B
C
D
AAB Population (log CFU/mL)
Time (days)
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14

TABLE 8. Fluctuations of indigenous microbiota populations (APC, Y, LAB, & AAB) in kombucha (brands combined) over the course of fermentation up to 45 days at room temperature.

	Indigenous microbiota population (log CFU/mL) enumerated from kombucha (brands combined) at fermentation time											
Time	APC Populations			Yeast populations			LAB populations			AAB populations		
(days)	Mean		95% CI	Mean		95% CI	Mean		95% CI	Mean		95% CI
0	4.39	C	(3.98, 4.80)	3.91	B	(3.40, 4.41)	4.45	C	(4.10, 4.80)	4.68	E	(4.33, 5.03)
0.25	4.51	BC	(4.01, 5.01)	4.50	AB	(3.89, 5.11)	4.94	BC	(4.53, 5.35)	4.90	DE	(4.47, 5.33)
1	5.26	ABC	(4.67, 5.84)	4.86	AB	(4.15, 5.57)	5.41	ABC	(4.93, 5.89)	5.86	ABCDE	(5.27, 6.44)
2	6.02	A	(5.52, 6.52)	5.29	A	(4.68, 5.90)	5.40	AB	(4.99, 5.81)	6.01	ABC	(5.56, 6.45)
3	5.67	A	(5.25, 6.08)	5.16	AB	(4.60, 5.72)	6.05	A	(5.71, 6.39)	5.80	ABCD	(5.45, 6.15)
5	5.91	A	(5.49, 6.32)	4.95	AB	(4.44, 5.47)	6.05	A	(5.69, 6.41)	6.08	AB	(5.71, 6.44)
7	5.93	A	(5.51, 6.35)	5.28	A	(4.78, 5.79)	5.88	A	(5.54, 6.22)	6.28	A	(5.94, 6.63)
10	5.55	AB	(5.21, 5.88)	5.21	A	(4.80, 5.62)	5.62	AB	(5.34, 5.89)	5.74	ABCD	(5.46, 6.03)
14	5.51	AB	(5.14, 5.89)	4.91	AB	(4.46, 5.35)	5.84	A	(5.54, 6.14)	5.44	BCDE	(5.11, 5.76)
21	5.77	A	(5.29, 6.26)	5.21	A	(4.67, 5.75)	5.86	AB	(5.49, 6.22)	5.95	ABC	(5.55, 6.34)
30	5.68	A	(5.27, 6.08)	5.05	AB	(4.56, 5.55)	5.74	AB	(5.41, 6.07)	5.24	BCDE	(4.90, 5.59)
45	5.34	ABC	(4.86, 5.83)	5.28	A	(4.69, 5.87)	5.81	AB	(5.41, 6.21)	5.03	CDE	(4.61, 5.44)

[a] Four types of microbiota were enumerated: aerobic plate counts (APC), yeasts (Y), lactic acid bacteria (LAB), and acetic acid bacteria (AAB). These groups were analyzed in four different kombucha brands, and these values are the combined population means of the four brands over 45 days of fermentation. Values are population means and 95% confidence intervals ($\alpha = 0.05$) of at least five independent, replicate trials with single samples ($n \geq 5$) for each treatment. The reported microbiota group populations are calculated from an increased sample size ($n \geq 20$), because the four kombucha brands combined. While samples were taken from negative control kombucha without pathogen (NK), the preparation and enumerations of each NK trial corresponded to each pathogen challenge study trial as all treatments were prepared and sampled in the same time frame.

[b] Populations of microbiota groups enumerated from kombucha samples by surface plating on selective agars. Time zero (0 d) corresponds to the moment starter culture (SC) was added to base tea (BT) at 10 % (v/v) ratio to induce the start of kombucha fermentation. Time zero (0 d) values are calculated fractions of SC populations to reflect this (0.1 x SC pop. = 0 d). The detection limit was 1.30 log CFU/mL or 20 CFU/mL (surface plating of 0.1 mL of kombucha with 1:1 DE dilution).

[c] Within each type of microbiota group, population mean values followed with different uppercase letters ($_{A, B, C, D, E}$) are significantly different ($P \leq 0.05$). This corresponds a significant change in that microbiota population over time.

[d] Values in bold are peak population values of that microbiota group during the course of 0 – 45 d fermentation time frame analyzed.

TABLE 9. Fluctuations of Aerobic Plate Counts (APC) (aerobic mesophile populations) in four different kombucha brands over the course of fermentation up to 45 days at room temperature.

	APC populations (log CFU/mL) enumerated from kombucha brands at fermentation time											
Time	Kombucha A			Kombucha B			Kombucha C			Kombucha D		
(days)	Mean		95% CI	Mean		95% CI	Mean		95% CI	Mean		95% CI
0	3.72	B b	(3.02, 4.43)	4.68	B a	(3.93, 5.44)	4.67	C a	(4.24, 5.09)	4.43	A ab	(3.55, 5.30)
0.25	3.55	B a	(2.65, 4.46)	5.01	AB a	(4.17, 5.86)	4.81	BC a	(4.29, 5.33)	4.44	A a	(3.36, 5.52)
1	4.73	AB a	(3.62, 5.84)	6.05	AB a	(5.07, 7.03)	5.33	ABC a	(4.72, 5.93)	4.75	A a	(3.51, 5.99)
2	5.52	AB a	(4.61, 6.43)	6.67	A a	(5.83, 7.52)	6.00	AB a	(5.48, 6.52)	5.76	A a	(4.69, 6.84)
3	5.61	A a	(4.91, 6.32)	6.18	AB a	(5.43, 6.94)	6.16	A a	(5.73, 6.58)	4.80	A a	(3.92, 5.67)
5	5.29	AB bc	(4.59, 6.00)	6.72	A a	(5.96, 7.47)	6.49	A ab	(6.07, 6.92)	5.16	A c	(4.28, 6.04)
7	5.30	AB a	(4.52, 6.09)	6.30	AB a	(5.60, 6.99)	6.02	A a	(5.59, 6.44)	5.88	A a	(4.92, 6.85)
10	4.17	AB b	(3.62, 4.73)	6.38	AB a	(5.74, 7.02)	6.21	A a	(5.86, 6.56)	5.46	A a	(4.74, 6.17)
14	4.59	AB b	(3.95, 5.23)	5.98	AB a	(5.29, 6.67)	5.96	A a	(5.59, 6.33)	5.40	A ab	(4.58, 6.21)
21	4.34	AB b	(3.56, 5.13)	6.56	AB a	(5.71, 7.41)	6.23	A a	(5.71, 6.75)	5.96	A a	(4.88, 7.04)
30	4.83	AB b	(4.19, 5.47)	6.13	AB ab	(5.37, 6.89)	6.20	A a	(5.78, 6.63)	5.62	A ab	(4.74, 6.50)
45	3.95	AB b	(3.17, 4.74)	5.97	AB a	(5.12, 6.81)	5.87	AB a	(5.35, 6.39)	5.58	A a	(4.51, 6.66)

[a] Aerobic plate counts (APC) populations were enumerated on PCA from four separate kombucha brands (A, B, C & D) over 45 days of fermentation. While samples were taken from negative control kombucha without pathogen (NK), the preparation and enumerations of each NK trial corresponded to each pathogen challenge study trial as all treatments were prepared and sampled in the same time frame. Initial APC population (0 d) values correspond to the moment starter culture (SC) was added to base tea (BT) at 10 % (v/v) ratio to induce the start of kombucha fermentation. Initial population (0 d) values are calculated fractions of SC populations to reflect this (0.1 x SC pop. = 0 d). The detection limit was 1.30 log CFU/mL or 20 CFU/mL (surface plating of 0.1 mL of kombucha with 1:1 DE dilution).

[b] Values are population means and 95% confidence intervals ($\alpha = 0.05$) of at least five independent, replicate trials with single samples ($n \geq 5$) for each treatment.

[c] Within each kombucha brand and APC population, values followed by different uppercase letters ($_{A, B, C}$) are significantly different ($P \leq 0.05$). This corresponds a significant change in APC populations over time.

[d] Within each sampling time (day) and APC population, values followed by different lowercase letters ($_{a, b, c}$) are significantly different ($P \leq 0.05$). This corresponds significant difference in APC populations between brands at that corresponding time point.

TABLE 10. Fluctuations of yeast populations in four different kombucha brands over the course of fermentation up to 45 days at room temperature.

Yeast populations (log CFU/mL) enumerated from kombucha brands at fermentation time												
Time	Kombucha A			Kombucha B			Kombucha C			Kombucha D		
(days)	Mean		95% CI	Mean		95% CI	Mean		95% CI	Mean		95% CI
0	3.17	A b	(1.97, 4.37)	4.50	A a	(3.57, 5.43)	3.88	B ab	(3.20, 4.55)	4.06	A ab	(2.95, 5.17)
0.25	4.13	A a	(2.58, 5.68)	4.89	A a	(3.85, 5.92)	4.55	AB a	(3.72, 5.39)	4.33	A a	(2.96, 5.69)
1	4.07	A a	(2.17, 5.97)	5.35	A a	(4.15, 6.55)	5.23	AB a	(4.27, 6.19)	4.54	A a	(2.97, 6.11)
2	4.26	A a	(2.71, 5.80)	5.91	A a	(4.87, 6.94)	5.78	A a	(4.95, 6.62)	4.94	A a	(3.58, 6.30)
3	5.81	A a	(4.27, 7.36)	5.62	A a	(4.69, 6.54)	5.32	AB a	(4.64, 5.99)	3.87	A a	(2.51, 5.23)
5	3.22	A b	(1.88, 4.56)	5.80	A a	(4.87, 6.73)	5.46	AB ab	(4.78, 6.13)	4.90	A ab	(3.79, 6.01)
7	4.71	A a	(3.37, 6.05)	5.71	A a	(4.87, 6.56)	5.64	A a	(4.96, 6.32)	4.88	A a	(3.76, 5.99)
10	4.14	A a	(3.13, 5.18)	5.80	A a	(5.07, 6.54)	5.60	A a	(5.04, 6.15)	5.12	A a	(4.21, 6.03)
14	3.97	A a	(2.77, 5.20)	5.55	A a	(4.77, 6.34)	5.14	AB a	(4.55, 5.72)	4.71	A a	(3.75, 5.67)
21	4.84	A a	(3.50, 6.18)	5.69	A a	(4.76, 6.61)	4.99	AB a	(4.25, 5.74)	5.26	A a	(4.04, 6.48)
30	4.39	A b	(3.29, 5.48)	5.95	A a	(5.02, 6.87)	4.73	AB ab	(4.05, 5.41)	5.30	A ab	(4.19, 6.41)
45	4.82	A a	(3.48, 6.16)	5.87	A a	(4.83, 6.90)	5.11	AB a	(4.28, 5.94)	5.33	A a	(3.96, 6.69)

[a] Yeast populations were enumerated on DRBC from four separate kombucha brands (A, B, C & D) over 45 days of fermentation. While samples were taken from negative control kombucha without pathogen (NK), the preparation and enumerations of each NK trial corresponded to each pathogen challenge study trial as all treatments were prepared and sampled in the same time frame. Initial yeast population (0 d) values correspond to the moment starter culture (SC) was added to base tea (BT) at 10 % (v/v) ratio to induce the start of kombucha fermentation. Initial population (0 d) values are calculated fractions of SC populations to reflect this (0.1 x SC pop. = 0 d). The detection limit was 1.30 log CFU/mL or 20 CFU/mL (surface plating of 0.1 mL of kombucha with 1:1 DE dilution).

[b] Values are population means and 95% confidence intervals ($\alpha = 0.05$) of at least five independent, replicate trials with single samples ($n \geq 5$) for each treatment.

[c] Within each kombucha brand and Yeast population, values followed by different uppercase letters ($_{A, B}$) are significantly different ($P \leq 0.05$). This corresponds a significant change in Yeast populations over time.

[d] Within each sampling time (day) and Yeast population, values followed by different lowercase letters ($_{a, b}$) are significantly different ($P \leq 0.05$). This corresponds significant difference in Yeast populations between brands at that corresponding time point.

TABLE 11. Fluctuations of lactic acid bacteria (LAB) populations in four different kombucha brands over the course of fermentation up to 45 days at room temperature.

	LAB populations (log CFU/mL) enumerated from kombucha brands at fermentation time											
Time	Kombucha A			Kombucha B			Kombucha C			Kombucha D		
(days)	Mean		95% CI	Mean		95% CI	Mean		95% CI	Mean		95% CI
0	3.88	B a	(3.09, 4.67)	4.83	B a	(4.11, 5.56)	4.48	C a	(4.04, 4.93)	4.47	B a	(3.85, 5.10)
0.25	4.39	AB a	(3.47, 5.30)	5.45	AB a	(4.65, 6.26)	4.87	BC a	(4.33, 5.41)	4.90	AB a	(4.14, 5.66)
1	4.20	AB a	(3.08, 5.32)	6.03	AB a	(5.10, 6.96)	5.15	ABC a	(4.53, 5.78)	5.84	AB a	(4.96, 6.72)
2	4.45	AB a	(3.53, 5.37)	5.60	AB a	(4.79, 6.40)	5.42	ABC a	(4.88, 5.96)	5.89	AB a	(5.13, 6.65)
3	5.59	A a	(4.88, 6.30)	6.59	A a	(5.87, 7.32)	5.78	AB a	(5.34, 6.22)	6.26	A a	(5.64, 6.88)
5	5.54	AB b	(4.74, 6.33)	6.81	A a	(6.12, 7.50)	6.20	A ab	(5.76, 6.65)	5.86	AB ab	(5.23, 6.48)
7	5.32	AB a	(4.53, 6.11)	6.48	A a	(5.82, 7.14)	5.86	AB a	(5.42, 6.30)	5.70	AB a	(5.08, 6.32)
10	4.94	AB b	(4.38, 5.51)	6.13	A a	(5.56, 6.70)	5.59	AB ab	(5.23, 5.95)	5.77	AB ab	(5.27, 6.28)
14	5.03	AB c	(4.39, 5.68)	6.53	AB a	(5.92, 7.14)	6.08	A ab	(5.70, 6.46)	5.59	AB bc	(5.06, 6.13)
21	5.08	AB a	(4.28, 5.87)	6.16	AB a	(5.44, 6.88)	6.00	AB a	(5.52, 6.48)	6.04	AB a	(5.35, 6.72)
30	5.07	AB b	(4.42, 5.72)	6.80	A a	(6.07, 7.52)	5.70	AB b	(5.26, 6.14)	5.56	AB b	(4.94, 6.18)
45	5.20	AB b	(4.41, 6.00)	6.55	AB a	(5.74, 7.36)	5.60	ABC ab	(5.06, 6.15)	5.88	AB ab	(5.12, 6.64)

[a] LAB populations were enumerated on MRS supplemented with sodium benzoate and citric acid from four separate kombucha brands (A, B, C & D) over 45 days of fermentation. While samples were taken from negative control kombucha without pathogen (NK), the preparation and enumerations of each NK trial corresponded to each pathogen challenge study trial as all treatments were prepared and sampled in the same time frame. Initial LAB population (0 d) values correspond to the moment starter culture (SC) was added to base tea (BT) at 10 % (v/v) ratio to induce the start of kombucha fermentation. Initial population (0 d) values are calculated fractions of SC populations to reflect this (0.1 x SC pop. = 0 d). The detection limit was 1.30 log CFU/mL or 20 CFU/mL (surface plating of 0.1 mL of kombucha with 1:1 DE dilution).

[b] Values are population means and 95% confidence intervals ($\alpha = 0.05$) of at least five independent, replicate trials with single samples ($n \geq 5$) for each treatment.

[c] Within each kombucha brand and LAB population, values followed by different uppercase letters ($_{A, B, C}$) are significantly different ($P \leq 0.05$). This corresponds a significant change in LAB populations over time.

[d] Within each sampling time (day) and LAB population, values followed by different lowercase letters ($_{a, b, c}$) are significantly different ($P \leq 0.05$). This corresponds significant difference in LAB populations between brands at that corresponding time point.

TABLE 12. Fluctuations of acetic acid bacteria (AAB) populations in four different kombucha brands over the course of fermentation up to 45 days at room temperature.

	AAB populations (log CFU/mL) enumerated from kombucha brands at fermentation time											
Time	Kombucha A			Kombucha B			Kombucha C			Kombucha D		
(days)	Mean		95% CI	B mean		B 95%CI	C mean		C 95%CI	D mean		D 95%CI
0	4.19	C b	(3.61, 4.76)	5.21	B a	(4.50, 5.92)	4.72	C ab	(4.15, 5.30)	4.61	B ab	(3.98, 5.24)
0.25	4.31	BC a	(3.57, 5.05)	5.22	AB a	(4.42, 6.01)	5.07	ABC a	(4.36, 5.77)	4.85	AB a	(4.08, 5.63)
1	5.17	ABC a	(4.27, 6.08)	6.56	AB a	(5.44, 7.68)	5.47	ABC a	(4.47, 6.46)	6.22	AB a	(5.12, 7.31)
2	5.85	AB a	(5.11, 6.59)	6.11	AB a	(5.32, 6.90)	5.92	ABC a	(5.21, 6.62)	6.14	AB a	(5.25, 7.04)
3	5.92	A a	(5.35, 6.49)	6.29	AB a	(5.50, 7.09)	6.31	A a	(5.73, 6.89)	5.83	AB a	(5.20, 6.47)
5	5.43	ABC b	(4.86, 6.00)	6.70	AB a	(5.91, 7.50)	6.28	AB ab	(5.70, 6.85)	6.00	AB ab	(5.36, 6.63)
7	5.92	A a	(5.35, 6.49)	6.81	A a	(6.16, 7.45)	6.23	AB a	(5.65, 6.80)	6.11	A a	(5.48, 6.74)
10	5.19	ABC a	(4.74, 5.64)	6.01	AB a	(5.45, 6.57)	5.88	ABC a	(5.41, 6.35)	5.87	AB a	(5.35, 6.38)
14	4.89	ABC b	(4.36, 5.41)	5.97	AB a	(5.33, 6.62)	5.69	ABC ab	(5.16, 6.22)	5.20	AB ab	(4.61, 5.78)
21	5.30	ABC a	(4.66, 5.94)	6.28	AB a	(5.57, 6.99)	5.98	ABC a	(5.35, 6.61)	6.13	AB a	(5.36, 6.91)
30	5.09	ABC a	(4.57, 5.62)	5.63	AB a	(4.92, 6.34)	5.28	ABC a	(4.71, 5.86)	5.04	AB a	(4.41, 5.67)
45	4.70	ABC a	(4.06, 5.34)	5.72	AB a	(4.93, 6.52)	4.81	BC a	(4.10, 5.51)	4.88	AB a	(4.10, 5.65)

[a] AAB populations were enumerated on HS-modified supplemented with sodium benzoate and citric acid from four separate kombucha brands (A, B, C & D) over 45 days of fermentation. While samples were taken from negative control kombucha without pathogen (NK), the preparation and enumerations of each NK trial corresponded to each pathogen challenge study trial as all treatments were prepared and sampled in the same time frame. Initial AAB population (0 d) values correspond to the moment starter culture (SC) was added to base tea (BT) at 10 % (v/v) ratio to induce the start of kombucha fermentation. Initial population (0 d) values are calculated fractions of SC populations to reflect this (0.1 x SC pop. = 0 d). The detection limit was 1.30 log CFU/mL or 20 CFU/mL (surface plating of 0.1 mL of kombucha with 1:1 DE dilution).

[b] Values are population means and 95% confidence intervals ($\alpha = 0.05$) of at least five independent, replicate trials with single samples ($n \geq 5$) for each treatment.

[c] Within each kombucha brand and AAB population, values followed by different uppercase letters ($_{A, B, C}$) are significantly different ($P \leq 0.05$). This corresponds a significant change in AAB populations over time.

[d] Within each sampling time (day) and AAB population, values followed by different lowercase letters ($_{a, b, c}$) are significantly different ($P \leq 0.05$). This corresponds significant difference in AAB populations between brands at that corresponding time point.

Changes in biochemistry of kombucha and base tea through fermentation. There was an immediate decline of pH immediately after starter culture is added to base tea (Table 13). Six hours (0.25 d) after adding SC to BT, early kombucha pH was significantly lower ($P \leq 0.0001$) than the pH of the base tea across brands with a mean pH drop of -1.28 reduction after SC was added to tea. Across all trials and brands, in only one instance, did the pH of kombucha not fall ≤ 4.2 pH within the first six hours (Table 13). This near immediate decline to ≤ 4.2 pH after adding SC to the tea means that kombucha would be considered a non-PHF (potentially hazardous food) under the FDA *Food Code (78, 110)* before even 24 h of fermentation. However, pathogen populations (Tables 5 – 7) persist for a much longer time period. There was not a significant difference ($P \geq 0.05$) between SC or 6 h pH values between brands. The pH of Brand D base tea was significantly lower than Brand A ($P = 0.0459$) but the other base tea brands were not significantly different from each other. The base teas were mildly acidic (pH < 5.5) in their own inherent nature and might even fall under acid-shock conditions depending on what methodology is used as a comparison *(63, 88)*.

As expected, pH values of kombucha treatments (NK, K + *S. e.* & K + *E. c.*) were each significantly ($P \leq 0.0001$) lower than pH values of base tea + pathogen treatments (BT + *S. e.* & BT + *E. c.*). The fermentation of kombucha by indigenous microbiota significantly lowered the pH compared to the base tea. Pathogen inoculation was not a confounding factor because the pH negative control kombucha (NK) was also significantly lower than pH values of base tea inoculated with pathogens, and NK pH values tracked alongside the kombucha + pathogen treatments. The pathogen kombucha treatment pH values were not significantly different ($P \geq 0.05$) from the pH values of the negative control kombucha. This suggests that the pathogen growth in kombucha did not significantly alter the pH of the kombucha.

Changes in pH and Brix values for all kombucha and base tea treatments are reported in Tables 14 – 19. The significant changes in pH of K + *S. e.* samples over time were as follows: 0.25 d, 10 d, and 14 d. The significant changes in pH of K + *E. c.* samples over time were as follows: 0.25 d, 5 d, 7 d, 10 d

and 14 d. The significant changes in pH of K + *E. c*. over time, generally correspond to significant reductions in STEC populations and suggest that reduction of pH may be the primary reason STEC populations declined with fermentation time. On the other hand, the first significant change in pH of K + *S. e*. samples did not occur until 10 days, but *Salmonella* populations significantly declined at earlier time points of 2, 3, and 5 days. This may suggest that there are some other inhibitory factors in kombucha fermentation that significantly reduced *Salmonella* populations. It is also possible that the one trial of Brand B Kombucha + *Salmonella* that had prolonged *Salmonella* populations (Figure 10) and the higher pH values artificially weighed down the dataset to reduce significance of time points.

When brands were combined, there was not a ($P \geq 0.05$) significant difference in pH over time for the base tea + pathogen treatments (BT + *S. e*. and BT + *E. c*.). But when this comparison was extrapolated to each brand of base tea, Brand D had a significantly lower pH after 24 h of incubation for both *Salmonella* ($P = 0.0392$) and STEC trials ($P = 0.0114$), but the other brands did not have a significant change in pH. While the change may not be statistically significant, there was an unexpected decrease in pH of all base tea samples after 24 h of incubation with pathogen (Tables 14 & 18 – 19) (Figure 19 & 20). A potential explanation for this finding is that *Salmonella* and STEC were actively fermenting the sugars derived from sucrose dissolved in the tea and were thereby producing organic acids which lower the pH. This would mean that methods described by Buchanan and Edelson and Samelis et al. to induce acid-adaptation in *Salmonella (90)* and STEC *(14)* by adding 1% glucose to TSB was naturally occurring in the base tea because of the excess sucrose present. While it also seems unusual that the pH base tea + *Salmonella* samples increased in pH from 2 – 14 days, a similar finding was reported by Samelis et al. when analyzing pH changes in TSB+glucose with *Salmonella* over time. That study reported that TSBG + *Salmonella* had a 0.43 pH increase from 7 days to 14 days *(90)*.

Although there is a gradual decrease in sugar content (Brix, °Bx) in all kombucha samples over time (Tables 14 – 17) these changes were not significant ($P \geq 0.05$). Most kombucha treatments had decreased by less than 1% sugar (°Bx) by the end of the pathogen trials at 14 days. Table 20 reports

changes in specific gravity and calculated ABV (% ethanol) over time. It is well documented that traditional ethanol measurements used for beer and wine making are not sufficient for measuring the small amounts of alcohol in kombucha *(15, 51, 60, 71, 75)*. However, two types of ABV values were calculated for kombucha samples based on changes in specific gravity using two different formulas for ABV. These ABV values were calculated to analyze if these formula-derived ABV values show any potential use regarding changes in our kombucha samples over time. Overall, the values in Table 20 support other findings that these formula calculations for ABV are practically useless for kombucha. Some time points report negative ABV values which is not possible and most reported ABV values were nearly negligible. It is worth noting that when data from all types of kombucha treatments (NK, K + *S. e*., & K + *E. c*.) were pooled ($n \geq 68$), these ABV calculations report an approximate values of 0.139 – 0.231% ABV and 0.155 – 0.241% ABV for 7 days and 14 days of fermentation, respectively.

FIGURE 19. Changes in pH of the five different treatment groups: kombucha (solid line) inoculated with *Salmonella* (K + *S. e.*) (red), inoculated with STEC (K + *E. c.*) (blue) and non-inoculated negative control (green) as well as the positive control base tea (dotted line) inoculated with *Salmonella* (BT + *S. e.*) (red) and inoculated with STEC (BT + *E. c.*) (blue) over a 14-day fermentation cycle incubated at room temperature (20.9°C ± 1.5) when data from the four brands are combined. Reported are mean pH values with error bars representing standard error of the mean for at least two independent, replicate trials with single samples ($n \geq 2$) for each pathogen treatment, and at least four independent, replicate trials with single samples ($n \geq 4$) for negative control kombucha. For each pathogen treatment, the triplicate samples used for enumeration of pathogen survival were combined to create a composite sample for pH measurement, and the single sample pH values for each trial are representative of the combined triplicate test tubes. In this figure, the reported pH values are calculated from an increased sample size ($n \geq 8$) for pathogen treatments and ($n \geq 16$) for negative control kombucha, because data from the four brands of kombucha and base tea are combined.

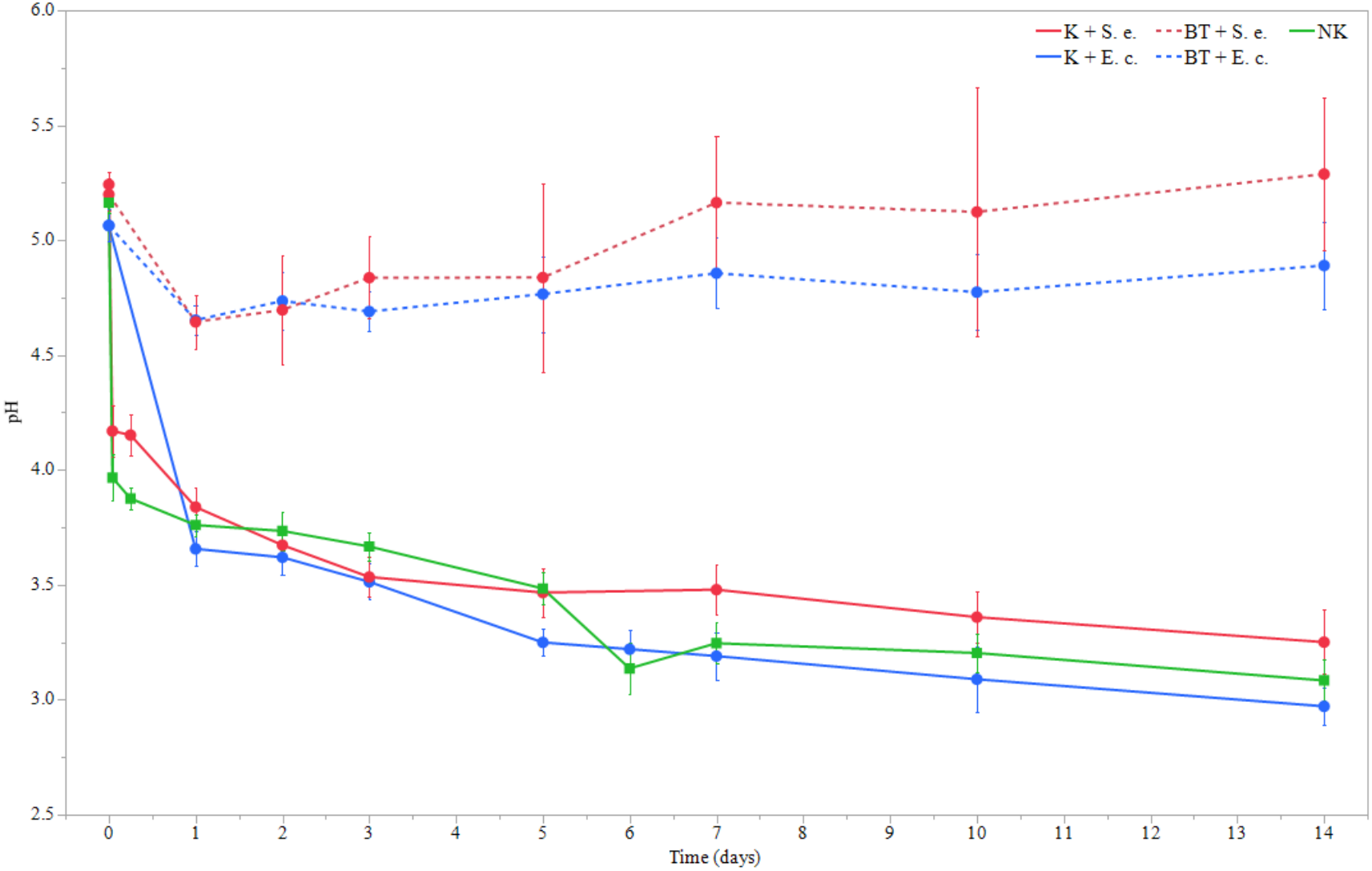

K + S. e.
BT + S. e.
NK
K + E. c.
BT + E. c.
pH
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Time (days)

FIGURE 20. Changes in pH of kombucha (solid line) and base tea (dotted line) inoculated with *Salmonella* (K + *S. e*.) (BT + *S. e*.) for the four different brands A (red), B (green), C (blue), and D (orange) over a 14-day fermentation cycle incubated at room temperature (20.9℃ ± 1.5). Reported are mean pH values with error bars representing standard error of the mean for at least two independent, replicate trials with single samples ($n \geq 2$) for each treatment. The triplicate samples used for enumeration of *Salmonella* surviving populations were combined to create a composite sample for pH measurement, and the single sample pH values for each trial are representative of the combined triplicate test tubes.

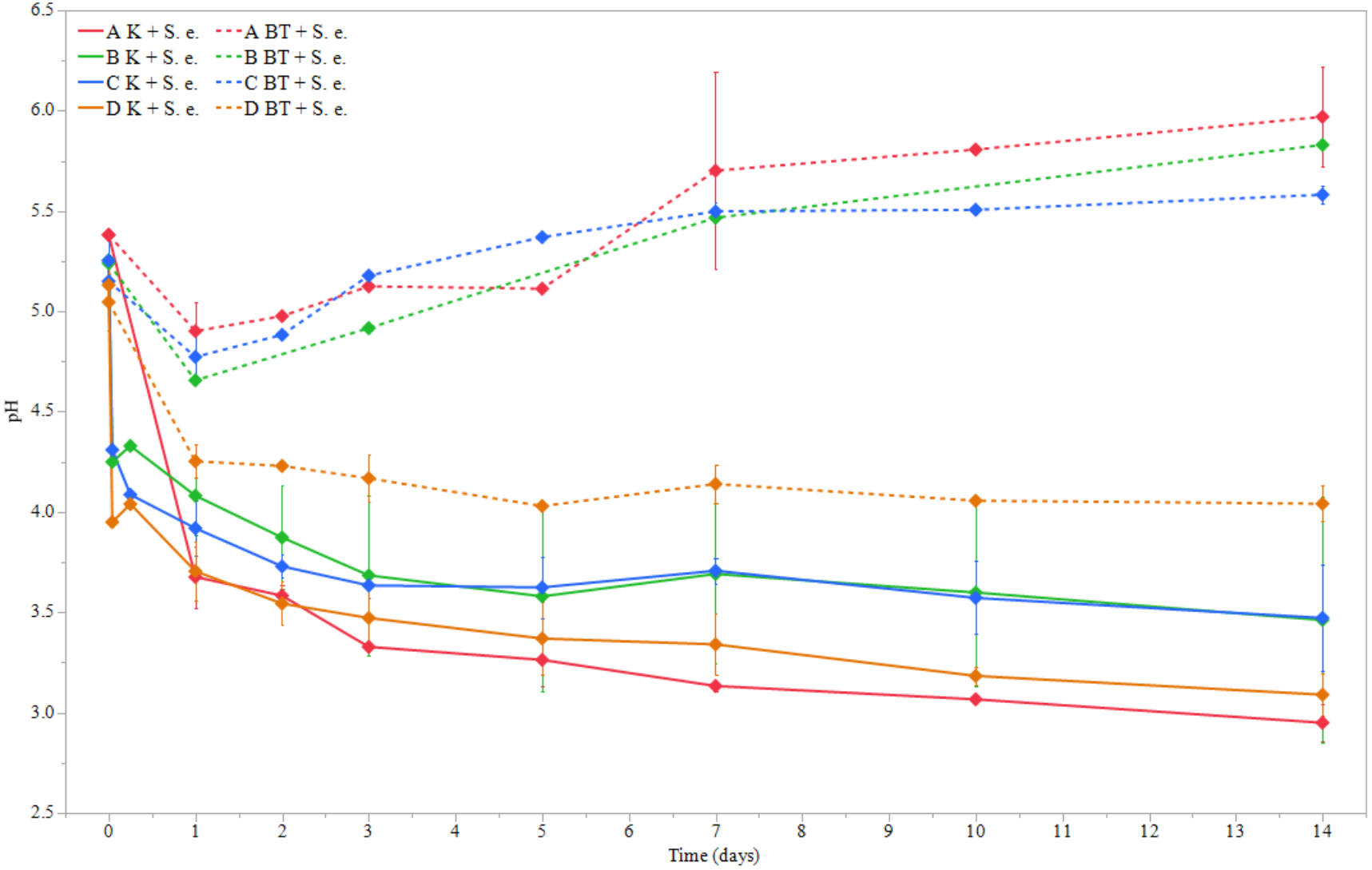

A K + S. e.
A BT + S. e.
B K + S. e.
B BT + S. e.
C K + S. e.
C BT + S. e.
D K + S. e.
D BT + S. e.
pH
Time (days)
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14

FIGURE 21. Changes in pH of kombucha (solid line) and base tea (dotted line) inoculated with STEC (K + *E. c*.) (BT + *E. c*.) for the four different brands A (red), B (green), C (blue), and D (orange) over a 14-day fermentation cycle incubated at room temperature (20.9℃ ± 1.5). Reported are mean pH values with error bars representing standard error of the mean for at least two independent, replicate trials with single samples ($n \geq 2$) for each treatment. The triplicate samples used for enumeration of STEC surviving populations were combined to create a composite sample for pH measurement, and the single sample pH values for each trial are representative of the combined triplicate test tubes.

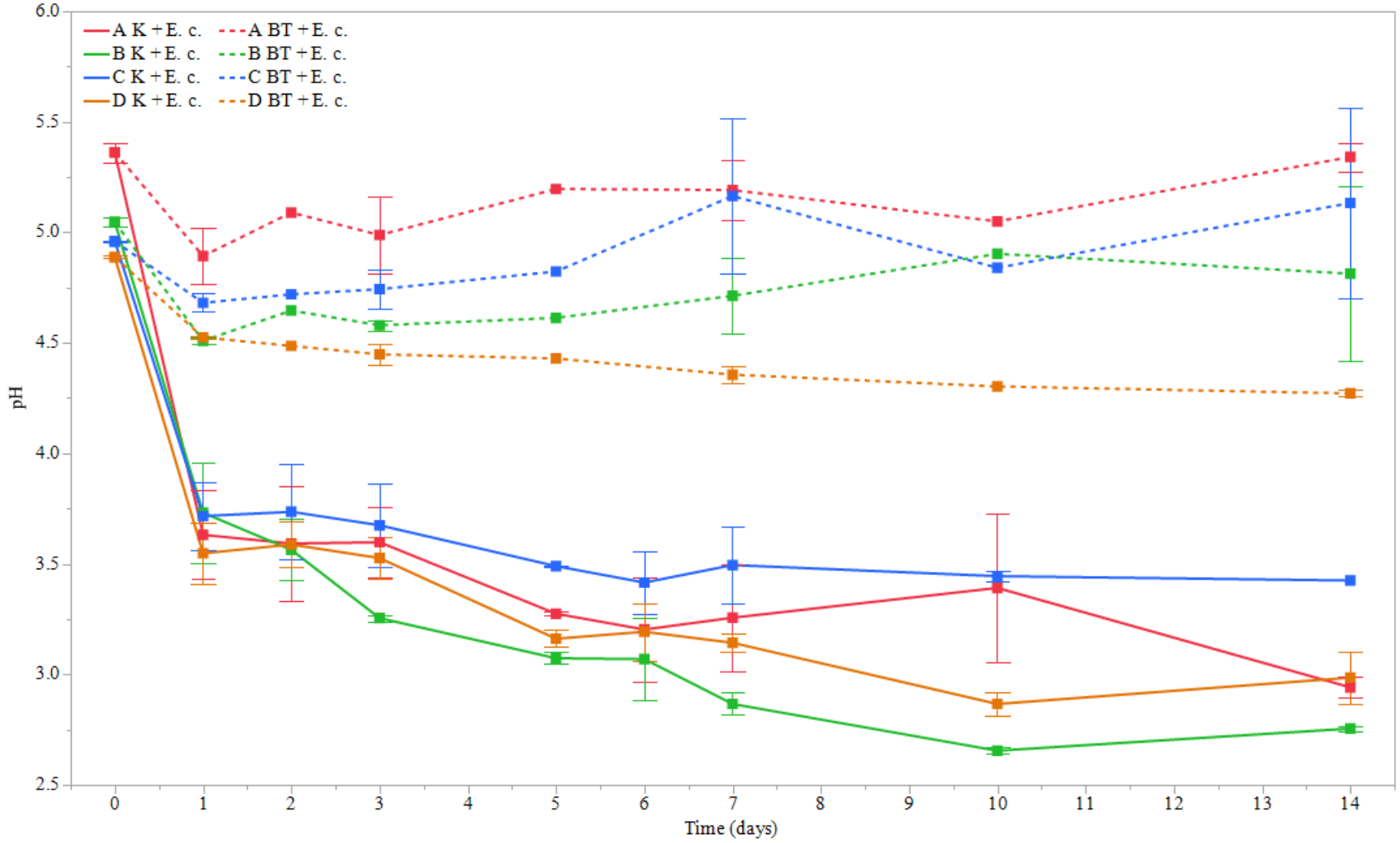

A K +E. c.
A BT +E. c.
B K +E. c.
B BT +E. c.
C K +E. c.
C BT +E. c.
D K +E. c.
D BT +E. c.
pH
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Time (days)

TABLE 13. Immediate pH decline of kombucha between brands for each pathogen trial.

Decline of pH in each kombucha brand after 6 h of adding SC to BT in the initial stages of pathogen trials[a]					
Kombucha Brand	pH Drop[b]	*Salmonella*		STEC	
		Trial 1	Trial 2	Trial 1	Trial 2
A	SC	3.23	2.69	2.81	3.21
	BT (0 h)	5.41	5.35	5.41	5.32
	6 h	4.09	3.84	3.62	3.77
B	SC	3.28	2.99	2.64	3.30
	BT (0 h)	5.27	5.24	5.02	5.07
	6 h	4.33	3.89	3.65	3.93
C	SC	3.42	2.97	3.00	3.24
	BT (0 h)	5.24	5.28	4.96	4.96
	6 h	4.10	3.95	3.73	3.82
D	SC	3.20	2.87	2.99	3.13
	BT (0 h)	5.10	5.19	4.89	4.88
	6 h	4.01	3.62	3.59	3.71

[a] The reported initial pH decline values of kombucha for each pathogen trial are taken from negative control kombucha (NK) and not those treatments inoculated with pathogen. However, any differences between the pathogen inoculation might have on pH changes in the initial 6 h would likely be very minimal.

[b] The pH values are reported for initial starter culture (SC), base tea (0 h), and 6 h (0.25 d) after SC has been added to the base tea. Each pH drop value is significantly different ($P \leq 0.05$) from each other based on Tukey mean comparisons following a two-way ANOVA.

TABLE 14. Changes in pH and Brix (°Bx) values of kombucha and base tea over 45 days of fermentation at room temperature (20.9℃ ± 1.5) when the data from the four different brands are combined.

Reported values[a] of pH and Brix of each sample type (brands combined)[b] at corresponding fermentation time										
Time	Kombucha (negative)		Kombucha + *Salmonella*		Kombucha + STEC		Base Tea + *Salmonella*		Base Tea + STEC	
(day)	pH	Brix (°Bx)	pH	Brix (°Bx)	pH	Brix (°Bx)	pH	Brix (°Bx)	pH	Brix (°Bx)
0 d	5.14 ± 0.2	6.71 ± 1.45	5.24 ± 0.17	6.69 ± 1.4	5.11 ± 0.21	7.41 ± 1.74	5.2 ± 0.18	6.88 ± 1.63	5.06 ± 0.2	6.98 ± 1.67
0.04 d	3.97 ± 0.32	7.33 ± 1.87	N/A[c]	N/A	N/A	N/A	N/A	N/A	N/A	N/A
0.25 d	3.88 ± 0.21	6.79 ± 1.45	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
1 d	3.76 ± 0.22	6.86 ± 1.59	3.84 ± 0.26	6.32 ± 1.55	3.66 ± 0.21	7 ± 1.74	4.64 ± 0.31	6.81 ± 1.57	4.65 ± 0.18	7.11 ± 1.81
2 d	3.75 ± 0.28	6.99 ± 1.64	3.67 ± 0.21	6.69 ± 1.34	3.62 ± 0.22	7.03 ± 1.76	4.7 ± 0.41	6.4 ± 2.59	4.74 ± 0.26	7.29 ± 1.76
3 d	3.67 ± 0.26	6.6 ± 1.55	3.53 ± 0.28	6.32 ± 1.53	3.51 ± 0.22	7.03 ± 1.68	4.84 ± 0.47	6.78 ± 1.61	4.69 ± 0.24	7.19 ± 1.74
5 d	3.48 ± 0.3	6.62 ± 1.58	3.47 ± 0.34	6.15 ± 1.45	3.25 ± 0.17	7.05 ± 1.72	4.84 ± 0.71	7.14 ± 1.82	4.77 ± 0.33	7.41 ± 1.85
6 d	3.27 ± 0.22	6.76 ± 1.63	N/A[d]	N/A	3.22 ± 0.23	6.87 ± 1.89	N/A	N/A	N/A	N/A
7 d	3.25 ± 0.42	6.8 ± 1.84	**3.48 ± 0.35**	**6.16 ± 1.39**	3.19 ± 0.29	7.08 ± 1.71	5.16 ± 0.76	6.59 ± 1.35	4.86 ± 0.43	7.31 ± 1.75
10 d	3.2 ± 0.35	6.38 ± 1.71	3.36 ± 0.36	6.32 ± 1.59	**3.09 ± 0.41**	**6.97 ± 1.8**	5.12 ± 0.94	6.18 ± 1.26	4.77 ± 0.33	7.41 ± 2.08
14 d	3.08 ± 0.38	6.26 ± 1.69	3.25 ± 0.44	5.99 ± 1.61	2.97 ± 0.24	6.92 ± 1.89	5.29 ± 0.88	6.68 ± 1.51	4.89 ± 0.53	7.35 ± 1.72
21 d	2.97 ± 0.35	5.9 ± 1.83	3.09 ± 0.51	5.34 ± 1.78	2.82 ± 0.19	6.41 ± 2.12	5.36 ± 0.98	6.67 ± 1.82	4.76 ± 0.49	7.38 ± 1.85
30 d	2.86 ± 0.34	5.7 ± 1.73	2.83 ± 0.28	5.57 ± 1.74	2.66 ± 0.21	6.01 ± 2.34	5.16 ± 1.3	7.25 ± 1.77	4.94 ± 0.53	7.73 ± 2.08
45 d	2.71 ± 0.3	5.51 ± 1.58	N/A[e]	N/A	N/A	N/A	N/A	N/A	N/A	N/A

[a] Reported are mean pH values and (± SD) standard deviation of the mean for at least two independent, replicate trials with single samples ($n \geq 2$) for each pathogen treatment, and at least four independent, replicate trials with single samples ($n \geq 4$) for negative control kombucha. For each pathogen treatment, the triplicate samples used for enumeration of pathogen survival were combined to create a composite sample for pH measurement, and the single sample pH values for each trial are representative of the combined triplicate test tubes.

[b] The pH and Brix were analyzed from four different kombucha brands in each trial and the reported values in this table are calculated from an increased sample size ($n \geq 8$) for pathogen treatments and ($n \geq 16$) for negative control kombucha, because data from the four brands of kombucha and base tea are combined. Values in bold correspond to the time at which a 5.0+ log reduction of pathogen was observed of the combined kombucha brand data set.

[c] Pathogen treatments were not analyzed at 0.04 d (1 h) or 0.25 d because the expected change from the negative would be expected to be minimal.

[d] Only kombucha + STEC and negative control kombucha were sampled and analyzed at 6 d.

[e] Pathogen treatments were also not analyzed at 45 d.

TABLE 15. Changes in pH and Brix (°Bx) values of negative control, non-inoculated kombucha (NK) between the four different kombucha brands (A, B, C, and D) over 45 days of fermentation at room temperature (20.9℃ ± 1.5).

	Reported values[a] of pH and Brix (°Bx) of negative kombucha (NK) of each kombucha brand at fermentation time							
Time	Kombucha A		Kombucha B		Kombucha C		Kombucha D	
(days)	pH	Brix (°Bx)	pH	Brix (°Bx)	pH	Brix (°Bx)	pH	Brix (°Bx)
0 d	5.37 ± 0.04	9.36 ± 0.61	5.15 ± 0.12	6.8 ± 0.28	5.14 ± 0.23	5.94 ± 0.05	5.03 ± 0.2	5.7 ± 0.25
0.04 d	3.89 ± 0.53	9.99 ± 0.48	3.95 ± 0.42	7.08 ± 0.45	4.1 ± 0.36	6.01 ± 0.11	3.9 ± 0.26	6.12 ± 0.27
0.25 d	3.83 ± 0.19	9.31 ± 0.28	3.95 ± 0.28	6.58 ± 0.42	3.94 ± 0.17	6.05 ± 0.1	3.79 ± 0.21	5.68 ± 0.37
1 d	3.71 ± 0.21	9.52 ± 0.61	3.83 ± 0.31	6.69 ± 0.6	3.85 ± 0.19	5.92 ± 0.24	3.66 ± 0.16	5.71 ± 0.44
2 d	3.91 ± 0.51	9.49 ± 0.35	3.75 ± 0.26	6.81 ± 0.42	3.78 ± 0.13	5.89 ± 0.07	3.59 ± 0.14	5.73 ± 0.35
3 d	3.7 ± 0.25	9.3 ± 0.21	3.56 ± 0.4	6.44 ± 0.17	3.77 ± 0.24	5.67 ± 0.41	3.62 ± 0.16	5.51 ± 0.28
5 d	3.42 ± 0.2	9.33 ± 0.66	3.38 ± 0.5	6.44 ± 0.44	3.63 ± 0.28	5.7 ± 0.43	3.48 ± 0.21	5.53 ± 0.25
6 d	3.26 ± 0.25	9.3 ± 0.3	3.07 ± 0.26	6.51 ± 0.11	3.49 ± 0.1	5.73 ± 0.04	3.24 ± 0.12	5.5 ± 0.29
7 d	3.1 ± 0.27	8.94 ± 0.96	3.02 ± 0.66	7.76 ± 2.12	3.54 ± 0.23	5.6 ± 0.48	3.27 ± 0.3	5.42 ± 0.41
10 d	3.05 ± 0.16	9.3 ± 0.71	3.15 ± 0.67	6.09 ± 0.49	3.44 ± 0.21	5.49 ± 0.5	3.13 ± 0.15	5.17 ± 0.51
14 d	2.83 ± 0.14	9.04 ± 0.96	3.08 ± 0.7	6.02 ± 0.67	3.32 ± 0.25	5.48 ± 0.5	3.05 ± 0.16	5 ± 0.54
21 d	2.76 ± 0.18	8.85 ± 1.35	2.91 ± 0.58	5.65 ± 0.92	3.18 ± 0.16	5.24 ± 0.75	2.98 ± 0.27	4.46 ± 0.68
30 d	2.66 ± 0.12	8.08 ± 1.18	2.73 ± 0.3	4.72 ± 0.93	3.19 ± 0.48	5.42 ± 0.38	2.82 ± 0.12	4.34 ± 0.93
45 d	2.52 ± 0.08	7.4 ± 2.06	2.69 ± 0.4	4.96 ± 0.52	3.04 ± 0.34	4.94 ± 0.47	2.59 ± 0.08	4.57 ± 0.65

[a] Reported are mean pH values and (± SD) standard deviation of the mean of at least four independent, replicate trials with single samples ($n \geq 4$). For each pathogen challenge study trial, there was a negative control kombucha (NK) sample analyzed and because there are four pathogen trials total, this results in a higher sample number for NK than other the sample types.

TABLE 16. Changes in pH and Brix (°Bx) values of kombucha inoculated with *Salmonella* between the four different kombucha brands (A, B, C, and D) over 30 days of fermentation at room temperature (20.9°C ± 1.5).

Reported values[a] of pH and Brix (°Bx) of kombucha + *Salmonella* of each brand at fermentation time								
Time[b]	Kombucha A		Kombucha B		Kombucha C		Kombucha D	
(days)	pH	Brix (°Bx)	pH	Brix (°Bx)	pH	Brix (°Bx)	pH	Brix (°Bx)
0 d	5.38 ± 0.04	9.21 ± 0.11	5.26 ± 0.02	6.79 ± 0.29	5.25 ± 0.22	5.91 ± 0.06	5.13 ± 0.2	5.73 ± 0.26
1 d	3.68 ± 0.22	9.10 ± 0.35	4.08 ± 0.28	6.2 ± 0.16	3.92 ± 0.24	5.54 ± 0.53	3.71 ± 0.25	5.32 ± 0.58
2 d	3.58 ± 0.08	8.46 ± 1.17	3.87 ± 0.36	6.59	3.73 ± 0.10	5.98 ± 0.16	3.54 ± 0.19	5.70 ± 0.39
3 d	3.33 ± 0.03	9.13 ± 0.08	3.68 ± 0.56	6.09 ± 0.24	3.63 ± 0.11	5.57 ± 0.31	3.47 ± 0.29	5.37 ± 0.50
5 d	3.26 ± 0.18	8.80 ± 0.39	3.58 ± 0.67	5.90 ± 0.11	3.62 ± 0.26	5.54 ± 0.51	**3.37 ± 0.31**	**5.17 ± 0.32**
7 d	**3.13 ± 0.04**	**8.49 ± 1.05**	3.69 ± 0.63	6.28 ± 0.82	**3.71 ± 0.11**	**5.51 ± 0.47**	3.34 ± 0.26	5.17 ± 0.19
10 d	3.07 ± 0.01	9.12 ± 0.54	**3.60 ± 0.66**	**6.04 ± 0.70**	3.57 ± 0.31	5.35 ± 0.67	3.18 ± 0.08	5.61 ± 0.64
14 d	2.95 ± 0.13	8.74 ± 0.56	3.46 ± 0.87	5.75 ± 1.09	3.47 ± 0.46	5.37 ± 0.69	3.09 ± 0.18	4.93 ± 0.78
21 d	2.56	8.85	3.26 ± 0.86	5.68 ± 0.9	3.25 ± 0.28	4.6 ± 0.79	3.01 ± 0.58	4.01 ± 0.52
30 d	2.68 ± 0.14	7.83 ± 0.27	2.60	4.59	3 ± 0.42	4.98 ± 1.1	2.95 ± 0.29	4.37 ± 1.43

[a] Reported are mean pH values and (± SD) standard deviation of the mean for at least two independent, replicate trials with single samples ($n \geq 2$) for each kombucha + *Salmonella* treatment at each sampling time. The triplicate samples used for enumeration of pathogen survival were combined to create a composite sample for pH measurement, and the single sample pH values for each trial are representative of the combined triplicate test tubes.

[b] Values in bold correspond to the time at which a 5.0+ log reduction of *Salmonella* surviving populations was observed for each kombucha brand of the combined, replicate trials.

TABLE 17. Changes in pH and Brix (°Bx) values of kombucha inoculated with STEC between the four different kombucha brands (A, B, C, and D) over 30 days of fermentation at room temperature (20.9°C ± 1.5).

Reported values[a] of pH and Brix (°Bx) of kombucha + STEC of each brand at fermentation time								
Time[b]	Kombucha A		Kombucha B		Kombucha C		Kombucha D	
(days)	pH	Brix (°Bx)	pH	Brix (°Bx)	pH	Brix (°Bx)	pH	Brix (°Bx)
0 d	5.36 ± 0.06	9.50 ± 1.01	5.05 ± 0.03	6.81 ± 0.39	4.97 ± 0.02	6.27 ± 0.42	4.89 ± 0.02	5.65 ± 0.31
1 d	3.63 ± 0.29	9.70 ± 0.33	3.73 ± 0.32	6.78 ± 0.19	3.72 ± 0.22	5.90 ± 0.11	3.55 ± 0.20	5.61 ± 0.35
2 d	3.59 ± 0.37	9.73 ± 0.69	3.56 ± 0.19	6.86 ± 0.46	3.74 ± 0.31	5.84 ± 0.04	3.59 ± 0.15	5.70 ± 0.39
3 d	3.60 ± 0.22	9.56 ± 0.93	3.26 ± 0.02	6.78 ± 0.82	3.68 ± 0.27	5.95 ± 0.35	3.53 ± 0.13	5.84 ± 0.35
5 d	3.28 ± 0.01	9.53 ± 1.28	3.07 ± 0.04	6.95 ± 0.74	3.49 ± 0.00	5.95 ± 0.04	3.16 ± 0.05	5.76 ± 0.63
6 d	3.20 ± 0.33	9.74 ± 0.92	3.07 ± 0.26	6.72 ± 0.42	3.42 ± 0.20	5.67 ± 0.11	3.19 ± 0.18	5.36 ± 0.10
7 d	3.26 ± 0.34	9.75 ± 0.28	2.87 ± 0.07	6.81 ± 0.32	3.50 ± 0.24	5.95 ± 0.04	**3.14 ± 0.06**	**5.81 ± 0.39**
10 d	**3.39 ± 0.48**	**9.75 ± 0.28**	**2.66 ± 0.02**	**6.75 ± 0.40**	3.45 ± 0.03	5.70 ± 0.23	2.87 ± 0.08	5.68 ± 0.51
14 d	2.94 ± 0.07	9.81 ± 0.58	2.76 ± 0.02	6.42 ± 1.09	**3.32 ± 0.14**	**5.90 ± 0.11**	2.87 ± 0.01	5.56 ± 0.67
21 d	2.83 ± 0.34	9.62 ± 0.54	2.69 ± 0.12	5.93 ± 0.70	2.99 ± 0.15	5.54 ± 0.32	2.76 ± 0.07	4.56 ± 1.06
30 d	2.59 ± 0.10	9.56 ± 0.77	2.50 ± 0.02	5.28 ± 0.98	2.93 ± 0.28	5.23 ± 0.59	2.61 ± 0.01	3.98 ± 0.87

[a] Reported are mean pH values and (± SD) standard deviation of the mean for at least two independent, replicate trials with single samples ($n \geq 2$) for each kombucha + STEC treatment at each sampling time. The triplicate samples used for enumeration of pathogen survival were combined to create a composite sample for pH measurement, and the single sample pH values for each trial are representative of the combined triplicate test tubes.

[b] Values in bold correspond to the time at which a 5.0+ log reduction of STEC surviving populations was observed for each kombucha brand of the combined, replicate trials.

TABLE 18. Changes in pH and Brix (°Bx) values of base tea inoculated with *Salmonella* (positive control) between the four different kombucha brands (A, B, C, and D) over 21 days at room temperature (20.9℃ ± 1.5).

Reported values[a] of pH and Brix (°Bx) of base tea + *Salmonella* of each brand at fermentation time								
Time	Base Tea A		Base Tea B[b]		Base Tea C		Base Tea D	
(days)	pH	Brix (°Bx)	pH	Brix (°Bx)	pH	Brix (°Bx)	pH	Brix (°Bx)
0 d	5.38 ± 0.04	9.21 ± 0.11	5.24	6.59	5.15 ± 0.19	5.92 ± 0.08	5.05 ± 0.20	5.65 ± 0.31
1 d	4.90 ± 0.21	9.04 ± 0.04	4.66	6.47	4.77 ± 0.19	5.95 ± 0.19	4.25 ± 0.12	5.59 ± 0.39
2 d	4.98	9.07	N/A	N/A	4.88	6.2	4.23	3.91
3 d	5.13 ± 0.03	9.07 ± 0.09	4.92	6.47	5.18 ± 0.03	5.78 ± 0.27	4.17 ± 0.16	5.65 ± 0.47
5 d	5.11	9.23	N/A	N/A	5.37	6.21	4.03	5.98
7 d	5.70 ± 0.7	8.47 ± 0.77	5.47	6.09	5.50 ± 0.06	5.95 ± 0.19	4.14 ± 0.14	5.59 ± 0.39
10 d	5.81	7.64	N/A	N/A	5.51	5.37	4.06	5.54
14 d	5.97 ± 0.35	8.85 ± 0.08	5.83	6.31	5.58 ± 0.06	5.90 ± 0.11	4.04 ± 0.12	5.48 ± 0.24
21 d	6.2	9.28	5.78	6.47	5.5	5.7	3.95	5.2

[a] Reported are mean pH values and (± SD) standard deviation of the mean for at least two independent, replicate trials with single samples (n = 2) for each base tea + *Salmonella* treatment at each sampling time. The triplicate samples used for enumeration of pathogen survival were combined to create a composite sample for pH measurement, and the single sample pH values for each trial are representative of the combined triplicate test tubes.

[b] Values of pH and Brix for Base Tea B + *Salmonella* were only collected for one trial (Trial 2). Therefore these values are reported from a single measurement (n = 1) with no standard deviation.

[c] Values of pH and Brix reported for Base Tea + *Salmonella* (all brands) for 2 d, 5 d, 10 d, and 21 d sampling times were only collected for one trial (Trial 1). Therefore, these values are reported from a single measurement (n = 1) with no standard deviation. This also results in these values not being available ("N/A") for Base Tea B at these sampling times.

TABLE 19. Changes in pH and Brix (°Bx) values of base tea inoculated with STEC (positive control) between the four different kombucha brands (A, B, C, and D) over 21 days at room temperature (20.9℃ ± 1.5).

	Reported values[a] of pH and Brix (°Bx) of base tea + STEC of each brand at fermentation time							
Time	Base Tea A		Base Tea B		Base Tea C		Base Tea D	
(days)	pH	Brix (°Bx)	pH	Brix (°Bx)	pH	Brix (°Bx)	pH	Brix (°Bx)
0 d	5.36 ± 0.06	9.50 ± 1.01	5.05 ± 0.03	6.81 ± 0.39	4.96 ± 0	5.98 ± 0	4.89 ± 0.01	5.65 ± 0.31
1 d	4.89 ± 0.18	9.84 ± 0.54	4.51 ± 0.02	7.08 ± 0.32	4.68 ± 0.06	5.81 ± 0.32	4.53 ± 0.01	5.70 ± 0.39
2 d	5.09	9.84	4.65	7.08	4.72	5.93	4.49	6.31
3 d	4.99 ± 0.25	9.78 ± 0.46	4.58 ± 0.03	7.19 ± 0.16	4.74 ± 0.12	6.14 ± 0.24	4.45 ± 0.07	5.62 ± 0.66
5 d	5.2	10.05	4.61	7.31	4.82	5.98	4.43	6.31
7 d	5.19 ± 0.20	10 ± 0.46	4.71 ± 0.25	7.17 ± 0.12	5.17 ± 0.50	6.20 ± 0.16	4.36 ± 0.05	5.86 ± 0.16
10 d	5.05	10.44	4.90	7.14	4.84	5.98	4.30	6.09
14 d	5.34 ± 0.09	10.02 ± 0.49	4.81 ± 0.56	7.11 ± 0.11	5.13 ± 0.61	6.26 ± 0.08	4.27 ± 0.02	6.01 ± 0.27
21 d	5.09 ± 0.17	10.22 ± 0.54	4.65 ± 0.29	7.23 ± 0.20	5.05 ± 0.80	6.14 ± 0.24	4.26 ± 0.11	5.93 ± 0.16

[a] Reported are mean pH values and (± SD) standard deviation of the mean for at least two independent, replicate trials with single samples (n = 2) for each base tea + STEC treatment at each sampling time. The triplicate samples used for enumeration of pathogen survival were combined to create a composite sample for pH measurement, and the single sample pH values for each trial are representative of the combined triplicate test tubes.

[b] Values of pH and Brix reported for Base Tea + STEC (all brands) for 2 d, 5 d, and 10 d, sampling times were only collected for one trial (Trial 1). Therefore, these values are reported from a single measurement (n = 1) with no standard deviation.

TABLE 20. Changes in specific gravity (SG) and theoretical ABV (Ethanol %) values when calculated from changes in specific gravity using two different formulas (ABV_1 & ABV_2) for all kombucha samples types

Changes in observed specific gravity and theoretical ABV[a] (calculated from SG)[b] in kombucha samples at fermentation time												
Time	Kombucha (negative)			Kombucha + *Salmonella*			Kombucha + STEC			Kombucha Treatments Combined		
(days)	SG	[c]ABV_1	[d]ABV_2	SG	ABV_1	ABV_2	SG	ABV_1	ABV_2	SG	ABV_1	ABV_2
0 d	1.027	0.000	0.000	1.026	0.000	0.000	1.028	0.000	0.000	1.027	0.000	0.000
1 d	1.027	0.022	0.017	1.025	0.171	0.118	1.028	-0.010	-0.006	1.027	0.053	0.038
2 d	1.028	-0.023	-0.014	1.026	0.234	0.160	1.028	-0.018	-0.011	1.027	0.039	0.028
3 d	1.026	0.084	0.057	1.025	0.164	0.110	1.028	-0.025	-0.015	1.026	0.082	0.056
5 d	1.026	0.080	0.050	1.024	0.256	0.162	1.028	-0.033	-0.020	1.026	0.104	0.066
6 d	1.027	0.113	0.068	N/A	N/A	N/A	1.027	0.048	0.028	1.027	0.080	0.048
7 d	1.025	0.328	0.200	1.025	0.243	0.142	1.028	-0.049	-0.029	1.025	0.231	0.139
10 d	1.025	0.241	0.142	1.025	0.235	0.173	1.028	0.000	0.000	1.026	0.186	0.119
14 d	1.025	0.277	0.173	1.024	0.348	0.233	1.028	0.025	0.015	1.025	0.241	0.155
21 d	1.024	0.497	0.300	1.021	0.609	0.367	1.026	0.287	0.173	1.024	0.471	0.284
30 d	1.023	0.639	0.391	1.022	0.684	0.424	1.024	0.509	0.306	1.023	0.615	0.376
45 d	1.022	0.770	0.465	1.021	0.875	0.530	N/A	N/A	N/A	1.022	0.792	0.479

[a] ABV = Alcohol by volume, ethanol percentage

[b] ABV is calculated from changes in reported values of specific gravity (SG) of kombucha samples. There are two different equations are used to theoretically calculate or estimate approximate ethanol percentage in kombucha based on these changes in specific gravity alone. Because these are only theoretical calculated values of miniscule amount of ethanol, some resulting reportable values are negative (negligible).

[c] ABV_1 is calculated by the following equation: $(SG_{initial} - SG_{final}) \times 131.25$ = approximate ABV

[d] ABV_2 is calculated by the following equation: $(76.08 * (SG_{initial} - SG_{final})) \div ((1.775 - SG_{initial}) \times (SG_{final} / 0.794))$ = approximate ABV

Parameters for a 5.0+ log reduction of pathogens through kombucha fermentation. U.S. regulations (21 CFR 120.1) dictate that all juice sold in interstate commerce must have a HACCP plan in place with a verification of a 5.0 log reduction (21 CFR 120.24) of pertinent microorganisms of public health significance. Kombucha production is technically exempt from that statute as a "acid food" (21 CFR 114.3). Acid foods are those products that have a natural pH of 4.6 or below (21 CFR 114.3). All kombucha samples in this study fell below that pH range as soon as starter culture is added to base tea (Table 13). However, as stated previously, kombucha has some comparable attributes to unpasteurized apple cider. Because of the re-occurring outbreaks in unpasteurized apple cider, the FDA has issued a guidance document (Docket No: FDA-2013-S-0610) for producers of unpasteurized apple cider which strongly encouraged producers to modify operations and include treatments and additives to achieve that 5.0 log reduction of pathogens when the cider is not pasteurized. Given that basis, it could reasonably be inferred that the same standard should be applied to kombucha producers because the product is not subject to a thermal treatment pathogen kill step.

Table 21 summaries the fermentation time, pH, and pH decline values at the time a 5.0+ log reduction is observed of inoculated pathogen populations (Table 5 – 7) across brands and trials. When data from the two replicate trials of kombucha brands are combined, 5 log reduction of *Salmonella* occurs after 7 days of fermentation with a with an endpoint pH of 3.48 ± 0.35 and a total of -1.76 log reduction for pH. The lowest observed pH for corresponding to a 5.0+ log reduction of *Salmonella* across brands was 3.13 ± 0.04, and conversely, the highest observed pH was 3.71 ± 0.11. When data from the two replicate trials of kombucha brands are combined, 5 log reduction of STEC occurs after 10 days of fermentation with a with an endpoint pH of 3.09 ± 0.41 and a total of -1.97 log reduction for pH. The lowest observed pH for corresponding to a 5.0+ log reduction of STEC across brands was 2.66 ± 0.02, and conversely, the highest observed pH was 3.39 ± 0.48.

Overall, STEC demonstrated a greater acid resistance than *Salmonella* in terms of pH values alone, which is corroborated by other studies of acid resistance of pathogens *(63, 90)*. These reported pH

values for 5.0+ log inhibition are considerably lower than some of the pH ranges reported for survival of *Salmonella* and STEC. The fermentation times are also more extended that what previous studies have reported (Table 2 & 3). Considering the survey of fermented foods, only traditional Greek olives *(4)* and tempoyak *(25)* reported pathogen populations viable in the same fermentation time frames reported here but the end point pH values of those products are higher than the pH values reported here for the viability of *Salmonella* and STEC.

The Food Science Extension of Utah State published a risk analysis guide and HACCP plan for kombucha brewing based on the FDA Model *Food Code (78)* in which the critical step for food safety was to ensure an end point of ≤ 4.2 and ≥ 2.5 pH with a recommended fermentation of 7 – 10 days with a starter culture inoculum of 10% (v/v) addition *(78)*. Our data is at least partially conflicting with these parameters. A 5-log reduction of *Salmonella* and STEC was recorded, on average, at 7 and 10 days, respectively, which supports Nummer's recommended fermentation time frame to some degree. However, the pH values at the time for which a 5-log reduction was recorded were much lower with 3.13 – 3.70 pH for *Salmonella* and 2.66 – 3.39 pH for STEC (Table 21). As reported in Table 13, all but one kombucha sample reached a pH below 4.2 within 6 h after the starter culture was added. While Nummer's recommended fermentation time frame may fall with our pathogen reduction parameters, an endpoint pH of ≤ 4.2 is not a sufficient standard to ensure pathogen reduction. Our reported data would support the notion that the endpoint pH value should be lowered in accordance with a 7 – 10 day fermentation and a 10 % (v/v) starter culture addition.

As reported in Table 21, when data from all kombucha brands and trials of both *Salmonella* and STEC are combined (n = 48), a 5.0+ log reduction of both pathogens was observed after 10 days of fermentation with a mean value pH of 3.24 ± 0.40 and -1.92 pH log reduction. Given this is the strongest data point of the pooled data set, it would be reasonable to recommend that fermentation of 10 days with a pH end point of 3.2 and decline of -1.92 pH to ensure a 5.0+ log reduction of pathogens in kombucha. Furthermore, it is worth considering that the single kombucha trial that did not have an initial pH drop

below 4.2 pH, (Brand B Trial 1) (Table 13) is the same trial at which a 5.0+ log reduction was not observed even by the end of trial at 14 days. It could be hypothesized that a pH $\leq$ 4.2 may be required for the initial pH drop when starter culture is added to base tea to prevent pathogen survival beyond a typical 10 day fermentation.

TABLE 21. Characteristics of kombucha fermentation at which point a 5.0+ log reduction of *Salmonella* and STEC is recorded for each brand of kombucha in each trial for a 14 d incubation at room temperature (20.9℃ ± 1.5)

Fermentation time[a] (day), pH[b], and pH reduction[c] values corresponding to a 5.0+ log reduction of each pathogen trial								
		Salmonella			STEC			*Salmonella* and *E.coli*
Kombucha Brand		Trial 1 (n = 3)	Trial 2 (n = 3)	Trials Combined[d] (n = 6)	Trial 1 (n = 3)	Trial 2 (n = 3)	Trials Combined[d] (n = 6)	Trials Combined[e] (n = 12)
A	day	7	5	7	7	14	10	7
	pH	3.16	3.39	3.13	3.01	2.99	3.39	3.20
	pH reduction	-2.25	-1.96	-2.25	-2.39	-2.34	-1.97	-2.18
B	day	(14)[f]	3	10	10	7	10	10
	pH	(4.07)	3.29	3.60	2.67	2.92	2.66	3.13
	pH reduction	(-1.20)	-1.95	-1.66	-2.35	-2.15	-2.39	-2.02
C	day	7	10	7	10	14	14	10
	pH	3.68	3.47	3.71	3.47	3.22	3.32	3.52
	pH reduction	-1.56	-1.82	-1.55	-1.49	-1.74	-1.64	-1.61
D	day	5	5	5	10	7	7	7
	pH	3.41	3.29	3.37	2.81	3.18	3.14	3.26
	pH reduction	-1.69	-1.90	-1.76	-2.08	-1.70	-1.75	-1.77
Brands Combined (n × 4)	day	7	5	7	10	10	10	10
	pH	3.56	3.40	3.48	3.17	3.01	3.09	3.24
	pH reduction	-1.66	-1.86	-1.76	-1.90	-2.05	-1.97	-1.92

[a] Sampling time (day) at which the mean pathogen population recovered from kombucha is at least a 5.0+ log reduction lower than initial populations. Initial populations of *Salmonella* ranged from 6.10 (SD ± 0.18) to 7.84 (SD ± 0.30) log CFU/mL. Initial populations of STEC ranged from 6.77 (SD ± 0.32) to 7.20 (SD ± 0.15) log CFU/mL.

[b] The reported pH value is analyzed from a composite sample of triplicate kombucha + pathogen samples pooled together. The pH value corresponds to the time for which a 5.0+ log reduction of pathogen is observed in each trial.

[c] The reported pH reduction values are the pH log decrease observed from the initial pH (0 h, base tea) to the pH of the corresponding time at which a 5.0+ log reduction of pathogen is observed in each trial ($pH_{inital} - pH_{5\ log\ reduction}$ = pH reduction). This value may be particularly relevant because the initial pH of the base tea varies for each brand and therefore end point pH may vary because of this.

[d] The fermentation time, pH, and pH reduction reported for "Trials Combined" of each pathogen refer values obtained when surviving pathogen populations and pH values of the two replicate trials are combined to form a single data set. Reported values correspond to the time at which a 5.0+ log reduction of pathogen is observed from the combined data set of the two replicate trials of each pathogen.
[e] The fermentation time, pH, and pH reduction reported for "Trials Combined" of both *Salmonella* and STEC refer values obtained when surviving pathogen populations and pH values of the two replicate trials of *Salmonella* and STEC are combined to form a single data set of four trials. Reported values correspond to the time at which a 5.0+ log reduction of pathogen is observed from the combined data set.
[f] The first kombucha + *Salmonella* trial of kombucha brand B did not reach a complete 5.0 log reduction even by the end of the trial (14 d). This data point should be taken with caution because only a 4.23 log reduction is observed by 14 d for that treatment in that trial.

CHAPTER 4

CONCLUSIONS

Conclusions. On average, pathogen populations reached a 5.0+ log reduction at 7 days and 10 days, for *Salmonella* and STEC, respectively. However, this rule cannot be applied to all kombucha fermentation conditions because a 5.0+ log reduction was not observed until 14 days for both pathogens in some kombucha brands. Differences in indigenous microbiota and type of base tea used for kombucha fermentation play a role in the capability of kombucha fermentation to eliminate pathogen contaminants. All microbiota populations surmounted a significant increase in population in the initial stages of fermentation by 2 – 3 days and did not significantly change after that. On average, LAB populations peaked at 5 days of fermentation with a population of 5.69 – 6.41 log (CFU/mL), and AAB populations peaked at 7 days of fermentation with a population of 5.94 – 6.63 log (CFU/mL). However, our data on microbiota populations was insufficient to establish any clear links between specific microbial groups and pathogen viability. There was not a significant decrease in sugar content (°Bx) over the course of kombucha fermentation. A 5.0+ log reduction of *Salmonella* was recorded in kombucha fermentation with pH endpoint of 3.13 – 3.71. A 5.0+ log reduction of STEC was recorded in kombucha fermentation with pH endpoint of 2.66 – 3.39.

These reported pH values are considerably lower and fermentation time frames are considerably longer than previously reported regarding viability of *Salmonella* and STEC in fermented products at room temperature. Because positive control treatments of *Salmonella* and STEC in base tea also had an initial pH decline in 24 h, it could be suggested that acid-adaptation of pathogens occurred by fermenting available carbohydrates *(13, 90)* from the excess sucrose dissolved in kombucha and tea. Numerous studies report that the gradual decline of pH that occurs with fermentation conditions result in acid-adaptation of pathogens *(61, 62, 87, 88, 90)*. Therefore, it can be concluded that the initial acid-shock

treatment pathogen cells were exposed to with addition of starter culture and immediate pH decline to ≤ 4.2, combined with the fermentation of excess carbohydrates in the tea by pathogens resulted in a natural and extreme acid-adaptation of *Salmonella* and STEC that permitted survival to fermentation time lengths and reduced pH levels that have not previously been reported. This is of particular concern because if these acid-adapted pathogens were to survive the course of fermentation the infectious dose will likely be lower, and virulence will be increased.

Criticisms. The Buchanan and Edelson study that reports the use of 1% glucose + TSB to induce acid-adaptation of pathogens also notes that non-selective media should be used to enumerate acid-adapted pathogens to recover injured cells. Acid-adapted STEC populations reported 0.5 – 1 log (CFU/mL) greater population numbers when enumerated on non-selective BHIA as opposed to SMAC *(13)*. For our study, pathogen populations were enumerated on the selective media XLT4 and SMAC to prevent the undefined indigenous microbiota of kombucha possibly confounding pathogen population enumerations. Therefore, it is possible that the values of recovered pathogen populations are considerably lower than would be observed if a non-selective medium such as BHIA or TSA had been used.

Food engineering studies on the scale up of industrial kombucha fermentation reports that the diameter of the fermentation vessel and volume of liquid kombucha have a major impact on the pH, organic acid content, sugar content, ethanol, and vitamin C of the product *(28, 66)*. Because our study was conducted in sterile 16 x 125 mm-screw cap glass test tubes (Pyrex No. 9825) instead of large glass brewing vessels that may typical be used for kombucha fermentation, this could contribute to be a confounding factor in our study. Malbasa et al. reports that the role of volume of the brewing vessel on pH is most pronounced after 5 days *(66)*.

The SCOBY (bio-cellulose pellicle) is not necessary to induce kombucha fermentation and is merely a by-product of growth of the unique AAB in kombucha, but a SCOBY is typically re-used for each new fermentation cycle by kombucha home brewers *(27, 42, 50)*. While SCOBYs did form inside

the test tubes over the course of a pathogen trial in our study, not adding a SCOBY to kombucha in a larger vessel may have also played a role in the pH of samples. Because the SCOBY also has an active microbiota *(27, 50, 102)*, it is possible that the SCOBY acts as an additional starter culture and may lower the pH considerably more so than is reported in our samples.

Future work. The indigenous microbiota population enumerations in this study came only from negative control kombucha samples and did not have duplicate or triplicate samples. Because microbiota populations were not enumerated from those samples with pathogen inoculation, it is impossible to draw firm conclusions about the role of these microbiota on the survival of pathogen populations. For these reasons, additional trials should be conducted to enumerate microbiota from negative control kombucha and kombucha inoculated with pathogens, to assess if pathogen populations had any reverse effects on kombucha microbiota populations

This same style of pathogen challenge study of kombucha should be conducted again in larger volume glass vessels to address the confounding variables raised by Malbasa et al. for a scaled-up kombucha process. This would also be more representative of what a home brewer of kombucha would use.

Kombucha fermentation is induced by using a starter culture of previously brewed kombucha to start the next fermentation cycle. This back-slopping style fermentation process of an undefined starter culture raises further concerns for acid-adapted pathogens. If acid-adapted pathogens are present and survive a fermentation cycle, that same kombucha could be re-used as a starter culture for the next batch. This could create a situation of constant recycling of increasing acid-adapted foodborne pathogens. If an outbreak were to occur in this manner for an industrial scale kombucha supplier, it could extend for unpredictable amounts of time. Given these issues, it would pertinent to conduct a similar style pathogen challenge study in kombucha with bacterial isolates from a previous fermentation cycle. In other words, colonies of *Salmonella* and STEC surviving from a kombucha challenge study could be cultured to create

kombucha-style, extreme acid-adapted *Salmonella* and STEC inoculums. It is likely that these kombucha-style, acid-adapted pathogens would persist for much longer than is reported in this study.

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