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Food Safety and Functionality Assessment of Kombucha Systems Through Bacillus cereus Spore and Probiotic Inoculations

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FOOD SAFETY AND FUNCTIONALITY ASSESSMENT OF KOMBUCHA SYSTEMS

THROUGH *BACILLUS CEREUS* SPORE AND PROBIOTIC INOCULATIONS

By

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FOOD SAFETY AND FUNCTIONALITY ASSESSMENT OF KOMBUCHA SYSTEMS THROUGH *BACILLUS CEREUS* SPORE AND PROBIOTIC INOCULATIONS

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An Abstract of the Thesis Presented
In Partial Fulfillment of the Requirements for the
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Kombucha is a SCOBY-fermented tea beverage known for its taste, sensorial qualities, and high endogenous microbial load. In recent years, kombucha has become a popular functional food with a compound annual growth rate of 25% from 2015 to 2020 in American kombucha sales alone, and sales are predicted to keep increasing significantly over the next decade. However, kombucha is lacking in scientific research, and there is still much to be explored regarding its safety and native probiotic content. This research looked into the various routes of contamination of several kombucha systems as well as the feasibility of the producing a kombucha beverage with health-promoting characteristics derived from the inclusion of lactic probiotics.

Although bacterial spore contamination and survival in the kombucha SCOBY have been documented, it is unknown whether spores can survive in the liquid, or whether they can be transmitted to daughter SCOBYs. The foodborne pathogen and spore-former *Bacillus cereus* was inoculated into the SCOBY, unfermented liquid, and fermented liquid of three different

kombucha systems. Data suggest that neither the route of contamination nor the kombucha system influenced the transmission or survival of *B. cereus* spores. The spread of the spores between culture and liquid across generation was shown to be sporadic but possible, so hygienic handling of kombucha cultures and raw materials throughout the entire production process is crucial to prevent uptake of pathogenic organisms. There was no survival of *B. cereus* spores after short-term storage or secondary fermentation, indicating that implementation of a holding step may mitigate potential food safety threats.

Kombucha is perceived to contain probiotics, but not all live cultures comprise probiotics. Some commercial kombucha products have validated probiotic strains added to them post-fermentation, but this can be costly. If probiotics, such as lactic acid bacteria, are inoculated into sweet tea prior to fermentation, they may be able to acidify the tea, replacing the need for utilizing previous kombucha or acetic acid, or survive and/or produce beneficial metabolites during fermentation in great enough amounts to convey a health benefit upon consumption. The survivability of six probiotic *Lactobacillus* sp. in acidified, sweetened tea at 25ºC during kombucha fermentation was established, and the medium (tea) and temperature (25ºC) were both revealed to affect the growth rates of the bacteria. Differences in pH indicated that the probiotics were unable to acidify the tea pre-fermentation. Although survival during fermentation was possible for four out of the six probiotics, it was concluded that probiotic *Lactobacillus* sp. are not well suited for a probiotic kombucha beverages, but out of the tested probiotics, *Lactobacillus brevis* and *Lactobacillus fermentum* were the most promising candidates.

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CHAPTER 1
LITERATURE REVIEW

1.1. Introduction

Kombucha is a fermented tea beverage with a mildly sweet, tangy, acidic taste known for its functional properties and claimed health benefits. Also referred to as mushroom tea, haipao, tea kvass, or teakwass, this beverage is made by using green, black, or oolong tea and a symbiotic colony of bacteria and yeasts (or SCOBY) fermented under aerobic conditions (Chen & Liu, 2000; Liu et al., 1996; Kumar & Joshi, 2016; Kim & Adhikari, 2020). Originating in China in 220 BCE, kombucha can now be found all over the globe from the United States to Australia to Europe to Russia and back to Asia (Chakravorty et al., 2019). While it was originally only made in the home, kombucha is now commercially produced and can be found on grocery store shelves in the refrigerated "health beverage" section. Popular brands today include Kevita (PepsiCo), Health-Ade (Coca-Cola, as investor), Brew Dr. Kombucha, GT's Synergy, Kombrewcha, Clearly Kombucha (Molson Coors), and more (PepsiCo, 2016; Molson Coors Beer & Beyond, 2018; Food Dive, 2019). The purported health benefits of kombucha include, but are not limited to, enhancing gut health, probiotic presence, antioxidant capabilities, antimicrobial characteristics, prevention of cardiovascular, kidney, and liver disease, and lowering high cholesterol and blood pressure have been reviewed in literature (Vīna et al., 2013). However, many of these health claims have little scientific evidence to support them. There are many models evaluating these health claims in animal trials, but there are few to no human trials providing evidence for these claims (Jayabalan et al., 2014). As a result, it cannot be determined whether kombucha's constituents will have the same effects in the human body as those that have been demonstrated in animal models.

1.1.1. History and Rise in Popularity

The exact origins and commencement of kombucha production are unknown, but experts estimate the beverage originated in northeast China, then Manchuria, during the Tsin dynasty in 220 BCE, although the process of fermentation significantly predates this time (Chakravorty et al., 2019). Kombucha was said to have been popular for its detoxifying and energizing properties. From there, the fermented beverage made its way to Japan around 414 CE when the physician Kombu used it to cure Emperor Inkyo's digestive problems. It is believed "kombucha" gets its name from the physician Kombu (Chakravorty et al., 2019; Bauer, n.d.). From Japan, kombucha expanded via trade routes to Russia, Eastern Europe, and eventually Germany. After WWII, when supplies were finally replenished, the beverage became popular throughout Europe and North Africa eventually making its way to the rest of the Western world (Chakravorty et al., 2019; Bauer, n.d.).

Kombucha first became popular in the United States during the HIV/AIDS epidemic in the late 1980s and early 1990s in hopes that its health benefits would slow the progression of the disease by increasing T-cell counts and supporting compromised immune systems (Petruzzello, n.d.). It was mostly made at home until the 1990s when GT Dave started the first U.S. commercial brand, GT's kombucha, in 1995 (Kombucha Brewers International, n.d.; Troitino, 2017). He started the company partially due to his belief that the beverage had cured his mother's cancer (Troitino, 2017). Dave wanted to educate people about the product and share its potential health benefits with others. However, in 1995 the Centers for Disease Control and Prevention (CDC) released a report on two cases of severe metabolic acidosis in Iowa that were suspected to have been caused by excessive kombucha consumption, although a direct cause for the acidosis was never established (CDC, 1995; Petruzzello, n.d.). Consequently, the beverage's

popularity dwindled until the beginning of the 21st century when it resurged as a health product because of public motivation towards a healthier lifestyle and greater awareness of probiotics and the health benefits of fermented foods (Petruzzello, n.d.). It was at this time that growing curiosity surrounding kombucha and growing popularity for health foods started expanding the beverage's recognition. Kombucha has since become the fastest-growing product in the functional beverage sector (Bauer, n.d.; Kombucha Brewers International, n.d.).

The rise in kombucha's popularity goes hand-in-hand with the rise of the functional foods movement. The functional foods movement is a push driven by the public to move towards a healthier lifestyle. This primarily consisted of a shift towards healthier eating habits or consuming foods that were known to be good for health or have health benefits. What distinguishes a product as a "functional food" can vary from person to person, but it is generally defined as a food that goes beyond basic nutrition and contains ingredients that beneficially influence specific body functions (Tur & Bibiloni, 2016; Corbo et al., 2014; Hasler, 2002). In the United States, the movement started around the late 1980s/early 1990s when the demand for more healthful foods and beverages began to rise. As life expectancy, health care costs, and the desire for higher quality of life increased, people became more concerned for their health and what they were consuming (Tur & Bibiloni, 2016). Heart disease, weight and obesity, cancer, diabetes, nutrition, and exercise remain some of the top health concerns among Americans (Kapsak et al., 2011). As a result, the functional foods and beverages global market grew from $33 billion in 2000 to $176.7 billion in 2013, accounting for 5% of the food market overall (Tripathi & Giri, 2014).

Functional foods are more of a concept than a well-defined group of foods, lacking universal standards or regulatory definition (Tur & Bibiloni, 2016; Corbo et al., 2014; Hasler,

2002; Siró et al., 2008). In the United States, functional foods are loosely defined as "foods and food components that provide a health benefit beyond basic nutrition" (*Bacillus cereus—BfR*, n.d.). There is no separate category or set of regulations for functional foods in United States, and thus they fall under the regulations for conventional foods (Corbo et al., 2014). Functional foods can be a natural food (found "as-is" in nature) or a food containing one or more ingredients that positively affect the health and well-being of the consumer. These ingredients can be added to or removed from food, naturally enhanced through special conditions, or modified to provide health benefits (Tur & Bibiloni, 2016). Examples include fortified milk or juice, yogurt, cholesterol-lowering spreads, eggs enhanced with omega-3 fatty acids, kefir, kombucha, and more (Siró et al., 2008; Jędrusek-Golińska et al., 2020; Bigliardi & Galati, 2013). The earliest functional foods were those fortified with vitamins and/or minerals such as vitamin C, vitamin D, calcium, iron, and folic acid (Siró et al., 2008). This quickly shifted to foods being fortified with more complex micronutrients such as omega-3 fatty acids, phytosterol, and soluble fiber with the intention to promote good health and disease prevention (Siró et al., 2008). Today, functional foods are being developed and produced to offer multiple health benefits within a single product (Siró et al., 2008). However, functional foods are not a panacea; they do not cure or prevent illnesses alone and are not essential to the diet (Tur & Bibiloni, 2016).

Kombucha is one of the most popular and recognized beverages to be associated with functional foods because of its claimed health benefits. Although the commercial kombucha market is very young, its size and product variety are growing very rapidly (Urala & Lähteenmäki, 2003; Back, 2018). Between 2010 and 2014, kombucha sales in the United States almost quadrupled, going from a little more than $100 million to almost $400 million (Troitino, 2017). By February 2019, yearly sales of kombucha were at $728.8 million, and kombucha

consumption increased 22% from the previous year (Bauer, n.d.). From 2015 to 2020, domestic kombucha sales had a compound annual growth rate (CAGR) of 25%, and sales are predicted to increase significantly over the next decade (Troitino, 2017).

1.2. Claimed Health Benefits

There is a wide variety of benefits that kombucha is said to possess. These claimed benefits span from improving gut health due to probiotic presence to antioxidant and radical scavenger capabilities to antimicrobial properties to hepatoprotection to the prevention of numerous health issues such as cardiovascular diseases, cancer, high cholesterol and blood pressure, etc. (Chakravorty et al., 2019; Chu & Chen, 2006; Velićanski et al., 2007; Wang et al., 2014; Deghrigue et al., 2013; Jayabalan, Baskaran, et al., 2010; Srihari & Satyanarayana, 2012; Sreeramulu et al., 2001; Watawana et al., 2018; Yang et al., 2009). These claims stem from either the probiotic effect, where consumed viable microorganisms interact with the host, or indirectly through the biogenic effect, caused by the ingestion of microbial metabolites produced during fermentation (Tripathi & Giri, 2014). The majority of kombucha's health benefits can be largely attributed to its level of catechin, derived from its tea base; it protects against the development of diseases in addition to being a powerful antioxidant (Leal et al., 2018; Cardoso et al., 2020).

While many studies have been conducted on these potential health benefits, most, if not all, of them lack the scientific evidence proving or guaranteeing them in humans because the studies use animal models instead of human models (Chakravorty et al., 2019). The animal models are helpful for understanding the mechanisms of the bioactive contents found in kombucha *in vivo*, however, they may act differently in mice or rats than they do in humans

(Gedela et al., 2016; SungHee et al., 2009; Kapp & Sumner, 2019). As a result, we cannot expect the same effects happening in these animal models once having consumed kombucha to be transferred over to humans. The evidence is promising, but further research in a human system is needed to validate these health benefits in humans. To date, there have been no kombucha consumption studies using human models to confirm these health benefits.

1.2.1. Antioxidant Activity

Kombucha exhibits good antioxidant and free radical scavenging activity, which increases over [a normal period of] fermentation (Chu & Chen, 2006; Srihari & Satyanarayana, 2012; Mizuta et al., 2020). The antioxidant activity is dependent on the type of tea used and the SCOBY culture composition; this determines the amount and type of antioxidants and metabolites produced (Chu & Chen, 2006; Jakubczyk et al., 2020). Other types of tea, such as green tea, red tea, and purple basil tea, have shown to be equal to or more rich in antioxidant than traditional black tea (Jakubczyk et al., 2020; Yıkmış & Tuğgüm, 2019). Although unfermented tea does exhibit some antioxidant activity, kombucha has proven to have a higher antioxidant activity than unfermented tea in many studies (Velićanski et al., 2007; Velićanski et al., 2014; Bhattacharya et al., 2013). This is mostly like due to the SCOBY metabolites produced during fermentation.

Phenolic compounds and SCOBY metabolites are responsible for kombucha's antioxidant activity and free radical scavenging properties (Velićanski et al., 2014). These phenolic compounds consist of polyphenols, which include catechins, theaflavins, thearubigins, and flavonoids (Jayabalan et al., 2014; Jakubczyk et al., 2020; Jayabalan, Baskaran, et al., 2010; Srihari & Satyanarayana, 2012). Phenolic compounds are high-level antioxidants with a strong

ability to scavenge free-radical and active oxygen species such as singlet oxygen, superoxide free radicals, and hydroxyl radicals (Velićanski et al., 2014). Conversely, the SCOBY metabolites contributing to kombucha's antioxidant capabilities mostly consist of vitamins and organic acids such as B and C vitamins, ascorbic acid, malic acid, tartaric acid, and acetic acid (Jayabalan et al., 2014; Srihari & Satyanarayana, 2012). Vitamin C especially is known to be an antioxidant with important immunological function (Riol et al., 2018).

Kombucha's antioxidant activity may also contribute to preventing the development and progression of chronic diseases such as cardiovascular disease, cancer, diabetes, and neurodegenerative diseases (Baschali et al., 2017). Folic acid and other B vitamins present in kombucha help promote proper central nervous system function at all ages as well as help prevent various central nervous system disorders (Baschali et al., 2017).

1.2.2. Antimicrobial Activity

Kombucha's antimicrobial activity is largely attributed to its low pH, presence of various organic acids (acetic acid, succinic acid, gluconic acid, etc.), large proteins, polyphenols (catechin and isorhamnetin), and SCOBY metabolites produced during fermentation (Jayabalan et al., 2014; Ivanišová et al., 2020; Leal et al., 2018; Greenwalt et al., 2000; Greenwalt et al., 1998; Deghrigue et al., 2013; Bhattacharya et al., 2016; Sreeramulu et al., 2001; Talawat et al., 2006; Battikh et al., 2013). The contribution of tea itself to the antimicrobial activity of Kombucha proved to be insignificant (Greenwalt et al., 1998). Phenolic compounds and flavonoids are known to be effective antimicrobial agents, which includes the polyphenols catechin and isorhamnetin. Catechin, which is also found in unfermented tea, and isorhamnetin, which is not, were determined to be great antibacterial compounds because of their ability to

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target bacterial virulence factors and permeate bacterial cell membranes by generating oxidative stress (Bhattacharya et al., 2020; Bhattacharya et al., 2016; Bhattacharya et al., 2018). Thus, bacterial pathogenicity is mitigated, and any contaminated kombucha is less likely to cause illness. Četojević-Simin et al. (2012)'s study determined acetic acid was one of the main antimicrobial agents of kombucha (Četojević-Simin et al., 2012). However, other key organic acids such as succinic acid and gluconic acid contribute to kombucha's antimicrobial activity as well, which increases with fermentation time (Talawat et al., 2006). The increase in kombucha's acid content reduces the product's pH. This along with the antimicrobial substances produced by the bacteria and alcohol may result in curative potential (Baschali et al., 2017).

Unfermented tea and neutralized kombucha (pH = 7.0) do not display the same antimicrobial activity as kombucha due to their lack of organic acids (Battikh et al., 2013; Ansari et al., 2019; Greenwalt et al., 1998; Velićanski et al., 2007). Therefore, it was determined that there is greater antimicrobial activity in kombucha than unfermented tea or neutralized tea.

Because of kombucha's antimicrobial components, it is able to inhibit the growth of a substantial number of pathogenic Gram-positive and Gram-negative bacteria (Jayabalan et al., 2014; Četojević-Simin et al., 2012; Velićanski et al., 2014; Battikh et al., 2013). This includes *Agrobacterium tumefaciens, Bacillus cereus,* Salmonella Typhimurium, *Staphylococcus aureus, Escherichia coli, Entamoeba cloacae, Pseudomonas aeruginosa, Aeromonas hydrophila, Salmonella enteritidis, Shigella sonnei, Staphylococcus epidermidis, Leuconostoc monocytogenes, Yersinia enterocolitica, Campylobacter jejuni, Helicobacter pylori, Alicyclobacillus* sp., and *Micrococcus luteus* (Greenwalt et al., 2000; Greenwalt et al., 1998; Deghrigue et al., 2013; Sreeramulu et al., 2001; Mizuta et al., 2020; Sreeramulu et al., (2000). A decent number of pathogenic yeasts were found to be sensitive to kombucha, but *Candida*

albicans and *Candida krusei* especially were not inhibited by kombucha (Greenwalt et al., 2000; Greenwalt et al., 1998; Battikh et al., 2013). No antimicrobial activity against molds in kombucha was demonstrated (Velićanski et al., 2007; Četojević-Simin et al., 2012).

1.2.3. Microbial Metabolites

D-saccharic acid-1,4-lactone, or DSL, is a microbial metabolite also known as a postbiotic. Tea does not contain DSL because it is produced by *Gluconacetobacter* sp. during fermentation as a product of the GlcUA (glucuronic acid) pathway derived from D-glucaric acid (Jayabalan et al., 2014; Leal et al., 2018; Baschali et al., 2017). It is believed that DSL has detoxifying, antioxidant, and antiproliferative properties (Baschali et al., 2017; Deghrigue et al., 2013). In addition, DSL also may be a key functional component for the hepatoprotective property in kombucha (Baschali et al., 2017; Wang et al., 2014).

1.3. Kombucha Preparation and Fermentation

Kombucha is made by using a symbiotic colony of bacteria and yeasts, or a SCOBY, which kick-starts the fermentation process in black, green, or oolong tea sweetened by a carbohydrate source and pre-acidified by previously prepared kombucha or acetic acid and goes through two fermentation periods: primary fermentation and secondary fermentation (Leal et al., 2018; Baschali et al., 2017; Greenwalt et al., 2000; Goh et al., 2012; Dufresne & Farnworth, 2000). Primary fermentation results in the production of the beverage traditionally consumed as kombucha. Secondary fermentation is for adding sensory qualities that consumers prefer, such as carbonation.

1.3.1.   SCOBY Growth

A symbiotic colony of bacteria and yeasts, or SCOBY, is a cellulose matrix that contains various species of bacteria and yeasts which allows fermentation to occur (Leal et al., 2018). Without a SCOBY, kombucha fermentation would not be possible. It acts as a catalyst, floating on top of the tea and initiating the chemical reactions. Some of the main bacterial species commonly found in kombucha SCOBYs include: *Acetobacter* spp. *Gluconobacter* spp*.,* *Komagataeibacter* spp., *Lactobacillus* spp*.,* and *Lactococcus* spp. (Blanc, 1996; Ivanišová et al., 2020; Jakubczyk et al., 2020; Marsh et al., 2014; Villarreal-Soto et al., 2018). Some of the main yeast species include: *Zygosaccharomyces* spp*., Candida* spp*., Saccharomyces* spp., *Saccharomycodes* spp., *Pichia* spp., *Schizosaccharomyces* spp., *Kloeckera* spp., *Torulospora* spp., and *Brettanomyces* spp. (Chakravorty et al., 2019; Vīna et al., 2013; Blanc, 1996; Chu & Chen, 2006; Chen & Liu, 2000; Ivanišová et al., 2020; Liu et al., 1996; St-Pierre, 2019; Jayabalan, Malini, et al., 2010; Kumar & Joshi, 2016; Kappel & Anken, 1993; Malbaša et al., 2011; Jakubczyk et al., 2020; Marsh et al., 2014; Wang et al., 2014; Mayser et al., 1995; Villarreal-Soto et al., 2018).

As fermentation occurs, the microbes within the SCOBY reproduce, resulting in the accumulation of a second cellulosic matrix at the air:water interface primarily produced by *Acetobacter xylinum* (Chen & Liu, 2000; Leal et al., 2018; De Roos & De Vuyst, 2018; Saichana et al., 2015; Jayabalan, Malini, et al., 2010; Villarreal-Soto et al., 2018). This new layer of cellulose is called the "daughter SCOBY" and is created on top of the original (or "parent") SCOBY. This layer can be separated from its parent SCOBY and used on its own to brew a new batch of kombucha. However, SCOBYs are often used for multiple brewing batches before the

freshly made daughter SCOBY needs to be separated from its parent SCOBY to be used for the next tea brew.

1.3.2. Primary Fermentation

Primary fermentation begins by brewing black, green, or oolong tea. Once the tea leaves are removed, a sucrose-containing carbohydrate source is added. The sweetened tea is then pre-acidified with previously made kombucha or acetic acid to a pH of ≤4.5 and inoculated with a SCOBY to discourage the growth of mold (Goh et al., 2012; Brew Buch, 2020). It is not recommended to use ceramic or metal containers for fermentation because the high acidity of kombucha causes them to leach metals, like lead from ceramics, into the kombucha, which can cause poisoning and even death (Gedela et al., 2016; CDC, 1995; Banerjee et al., 2010; Smolinske, 2005). The container is then covered with a sterile, porous material such as cheesecloth or a coffee filter to prevent insect incursion. Because the fermentation is an aerobic process, keeping the container unsealed allows the flow of oxygen to the microorganisms within the SCOBY that need it in order for the chemical reactions driving fermentation to occur (Huang, 2016; Blanc, 1996). Kombucha is most often fermented at ambient temperature (64-78°F or 18-26°C) for 7-10 days in a dark, dry environment (Jayabalan et al., 2014; Nummer, 2013; Greenwalt et al., 2000). Once the kombucha reaches a pH around 2.5, fermentation is often considered complete (Nummer, 2013; Greenwalt et al., 2000; Greenwalt et al., 1998). However, there is no definitive pH for "finished" kombucha, so the pH of the finished product is generally determined by preference. Prolonged fermentation time leads to extreme acidity and a vinegar-like flavor (Blanc, 1996; Chen & Liu, 2000).

During the 7-10 day fermentation period, a variety of chemical reactions are at work to transform tea into kombucha (Figure 1.1). Yeast species in the SCOBY, such as *Zygosaccharomyces rouxii, Brettanomyces bruxellensis, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Torulospora delbrueckii, Kloeckera apiculata, Zygosaccharomyces bailii, Saccharomycodes ludwigii,* and *Pichia membranaefaciens*, use the sugar in the tea as fuel and break down the sucrose into the monosaccharides glucose and fructose by yeast invertase (Chakravorty et al., 2019; Chen & Liu, 2000; St-Pierre, 2019; Leal et al., 2018; Jayabalan, Malini, et al., 2010; Kappel & Anken, 1993; Jakubczyk et al., 2020; Wang et al., 2014; Mayser et al., 1995; Villarreal-Soto et al., 2018). The glucose and fructose are then converted into ethanol via glycolysis, with a preference for consumption of fructose as a substrate (Jayabalan et al., 2014; Huang, 2016; Leal et al., 2018). Additionally, carbon dioxide is released as a byproduct of the yeast breaking down the sucrose. Acetic acid bacteria, including *A. xylinum, Acetobacter pasteurianus,* and *Acetobacter aceti*, convert the ethanol created by the yeast into acetic acid as well as converting the glucose into gluconic acid (Huang, 2016; Chen & Liu, 2000; Liu et al., 1996; Leal et al., 2018; Banerjee et al., 2010; Sievers et al., 1995). Notably, ethanol to acetic acid conversion happens only in the presence of oxygen. Lactic acid bacteria convert any remaining sugars into lactic acid.
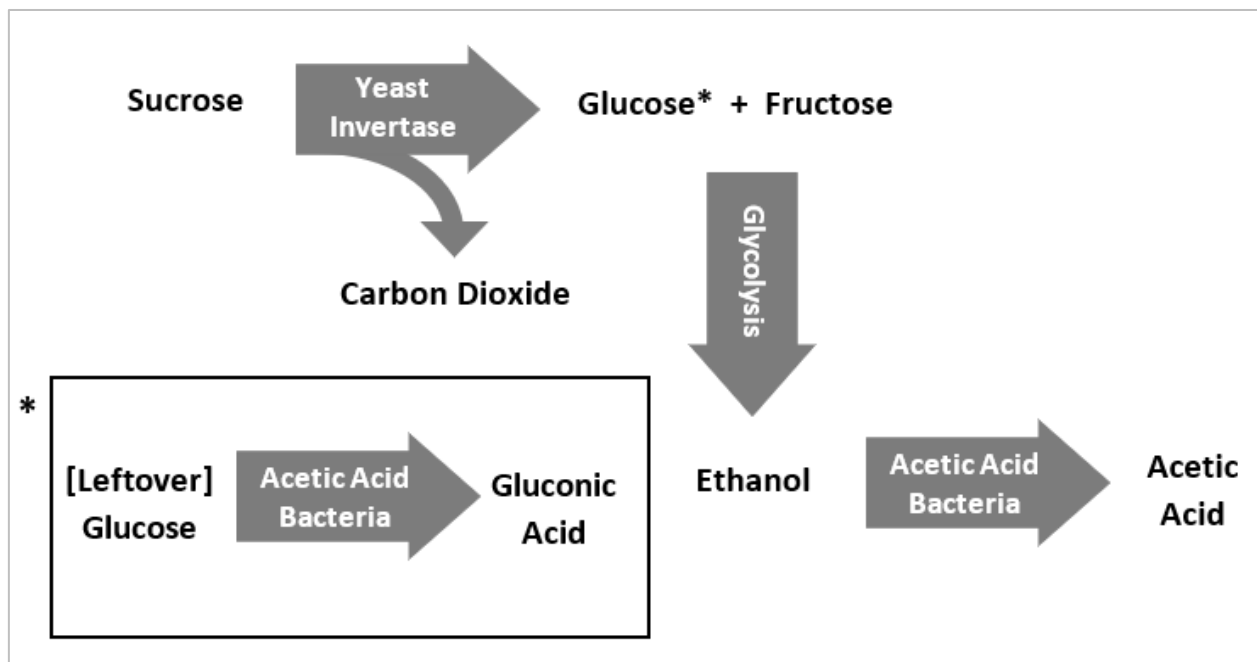
Figure 1.1. Chemical Reactions During Kombucha Fermentation

Since this is an aerobic process, none of the chemical reactions can occur in a closed

system, such as in a jar with an airtight lid, because the yeasts and acetic acid bacteria require

oxygen to produce their hydrolyzing enzymes (yeast invertase) and byproducts (Leal et al., 2018;

De Roos & De Vuyst, 2018; Saichana et al., 2015; Gullo et al., 2014). At the end of an average

fermentation period (7-10 days), studies have shown there is an overall increase in organic acids

(acetic acid, glucuronic acid, gluconic acid, etc.), DSL, amino acids, vitamins, minerals, and

ethanol while sucrose decreases (Figure 1.2) (Neffe-Skocińska et al., 2017; Chen & Liu, 2000;

Jayabalan, Malini, et al., 2010; Lončar et al., 2000; Jayabalan et al., 2007; Bauer-Petrovska &

Petrushevska-Tozi, 2000; Kaewkod et al., 2019).
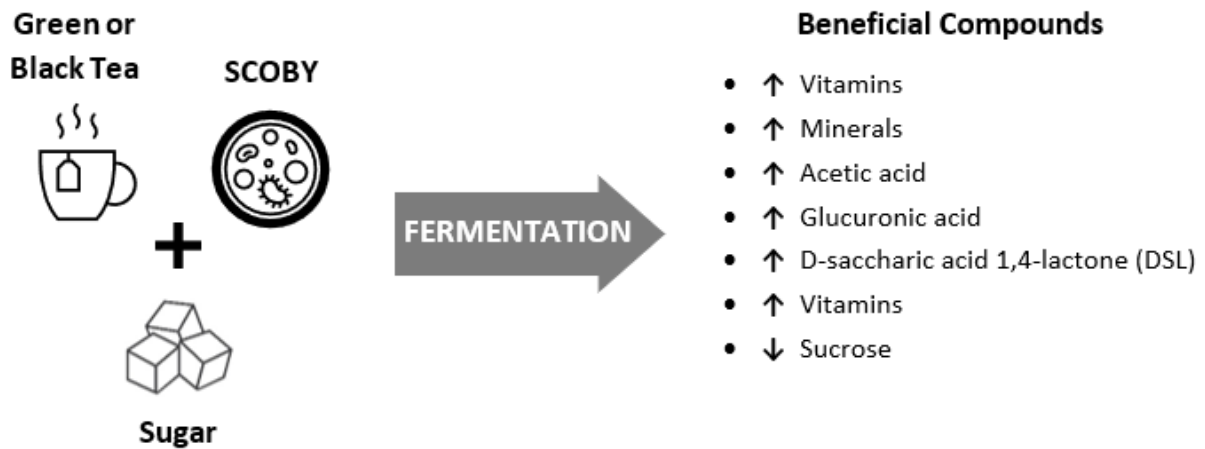
Figure 1.2. Beneficial compounds produced at the end of primary fermentation

1.3.2.1. Different Sugar and Tea Substrates

Black, green, and oolong tea are the most commonly used teas for kombucha production, but there are many studies using other varying tea types such as purple basil, Zijuan, lemon balm, white, red, and rooibos (Yıkmış & Tuğgüm, 2019; Zou et al., 2021; Gaggìa et al., 2019; Ansari et al., 2019; Velićanski et al., 2007; Velićanski et al., 2014; Četojević-Simin et al., 2012). All teas proved to be successful in kombucha fermentation and generally yield beverages well-accepted by consumers. Additionally, they were all revealed to have an equal or better phenolic, antioxidant, flavonoid, and/or antimicrobial properties than traditional kombucha, except for rooibos tea kombucha. The rooibos kombucha did not have as high an antioxidant activity as black and green tea kombucha, but it showed an important effect on the recovery of oxidative damage (Gaggìa et al., 2019).

Kombucha may not strictly use white granulated sugar as its carbohydrate source during fermentation. Brown sugar, molasses, maple syrup, coconut palm sugar, and more can also be

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used, but white sugar is most commonly used and often preferred. The use of different sugars will result in different flavor profiles and metabolites produced because each source has a different amount of sucrose available to be broken down into subsequent metabolites via chemical reactions during fermentation (Chen & Liu, 2000). Studies have shown the use of alternative sugar sources are successful in producing a sweetened kombucha-style beverage (Muhialdin et al., 2019; Perry et al., 2020; Malbaša, Lončar, & Djurić, 2008; Malbaša, Lončar, Djurić, & Došenović, 2008). The coconut palm sugar took it a step further in producing kombucha with the highest antioxidant activity and total phenolic content when compared to kombucha made with white refined sugar and molasses sugar (Muhialdin et al., 2019).

1.3.3. Secondary Fermentation

Secondary fermentation allows flavor and carbonation to be incorporated into the beverage. The SCOBY is removed from the container, and any flavorings and/or fruits are added to the liquid broth. Residual microorganisms present in the fermentate metabolize the sugars present in the added fruit/juice to yield carbon dioxide. This step is performed in a sealed vessel in order to retain the CO_2 produced. Secondary fermentation is not mandatory to produce kombucha, but it is preferred by consumers, so many commercial brewers opt to do it. Taste is the most influential factor in consumers' food choices, and the flavorings aid in combatting the overly acidic, vinegar-like taste (Siró et al., 2008; Urala & Lähteenmäki, 2003).

1.3.4. Microorganisms Present in the Finished Beverage

Many of the organisms in the SCOBY can be found in the finished product. Yeasts and acetic acid bacteria make up a majority of these organisms. This includes *Zygosaccharomyces bailii, Brettanomyces bruxellensis, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Torulospora delbrueckii, Torulopsis* sp., *Kloeckera* sp.*, Candida* sp.*,* and *Pichia* sp. for yeast species and *A. xylinum, A. pasteurianus, A. aceti, Gluconobacter* sp., and *Komagataeibacter* sp. for acetic acid bacteria species (Liu et al., 1996; Teoh et al., 2004; Steinkraus et al., 1996; Reva et al., 2015).

1.4. Probiotic Presence in Kombucha

The FAO/WHO defines probiotics as "live microorganisms that when administered in adequate amounts are able to provide benefits to the health of the consumer" (2001). These health benefits can involve either direct or indirect interactions with the gut microbiota (Tur & Bibiloni, 2016). *Lactobacillus* and *Bifidobacterium* species are the most commonly used probiotics in commercial foods. Other probiotics include certain strains of *Lactococcus* spp.*, Enterococcus faecalis, Enterococcus faecium, Leuconostoc*, and yeast, but they are not as well-known or used as often (Diez-Gutiérrez et al., 2020; Tripathi & Giri, 2014; Marsh et al., 2014). As of 2014, *Saccharomyces boulardii* is the only recognized probiotic yeast (Marsh et al., 2014). Approved probiotics are classified as generally recognized as safe, or GRAS, because of their very low probability of causing human infection (Diez-Gutiérrez et al., 2020). Over the years, people have become more conscious of their health and know that food and eating habits have a large impact, so as a result, the demand for probiotic functional foods has increased dramatically.

At the moment, probiotic foods are the most reasonable, practical, and popular way to ingest your daily dose of probiotics (Tripathi & Giri, 2014).

Probiotics provide numerous health benefits, but these benefits can only be realized when a food contains the minimum effective dose of viable or heat-killed probiotic cells at consumption. This minimum, recommended by the Food and Drug Administration (FDA) and adopted by the food industry, is set at a minimum level of 10^6 CFU/mL at the time of consumption (Tripathi & Giri, 2014). However, this may not be enough to receive the full effects of some probiotics. It is recommended that a daily intake of probiotic foods or beverages containing $10^8 - 10^9$ viable cells should be consumed in order for the probiotic to reach the intestine, although necessary dosage varies considerably depending on strain (Tripathi & Giri, 2014; Marsh et al., 2014). Since probiotics are not retained in the gut, regular consumption is needed to maintain the claimed health benefits (Tur & Bibiloni, 2016).

First and foremost, the most important job of a probiotic is to maintain a healthy intestinal microbiota by protecting the gut against gastrointestinal pathogens. Additionally, certain probiotics have been shown to alleviate lactose intolerance, enhance the immune system, reduce high cholesterol and blood pressure levels, have anti-carcinogenic properties, improve utilization of nutrients, and improve the nutritional value of food (Tur & Bibiloni, 2016; Tripathi & Giri, 2014; Baschali et al., 2017). Regardless of all these health benefits, the FDA currently does not support probiotic health claims, yet it does agree with the claim that probiotics may aid in disease prevention. The reason the FDA does not currently support probiotic health claims may be due to the lack of international regulations for probiotic products (Diez-Gutiérrez et al., 2020).

1.4.1. Supposed Probiotics Present in Kombucha: Lactic Acid Bacteria

A majority of the microorganisms present in a kombucha SCOBY have not demonstrated probiotic activity. As a result, commercial probiotic strains, such as *Bacillus coagulans*, are often added to the beverage post-fermentation in order to claim it is a probiotic beverage. Just consuming live organisms does not necessarily confer a benefit, contrary to what many consumers tend to believe. Therefore, it is important to identify the live organisms in finished kombucha and incorporate any proven probiotics if a probiotic beverage is desired.

Lactic acid bacteria, or LAB, are known to be a part of kombucha's microbial makeup and can possibly act as probiotics. Although many probiotics are categorized under the umbrella of "lactic acid bacteria", not all LAB are probiotics. Even members of the same species may differ in their probiotic potential. LAB strains can vary from culture to culture and batch to batch in regard to which species can be found in the SCOBY, so it is unknown which strains are present in one particular batch of kombucha unless the brewer has added additional commercial probiotics of a known strain to the batch or if analysis of the microbial community is performed. LAB have GRAS status and possess special physiological activities that make them very appealing to the food industry. They have been utilized extensively as probiotics in dairy products, bread, fermented vegetables, meats, fish, etc. because of their notable properties such as immunomodulation, inhibition of pathogenic bacteria, control of intestinal homeostasis, resistance to gastric acidity, bile acid resistance, and anti-allergic activity (Li & Cao, 2010; Cui et al., 2020). Moreover, as a probiotic in foods, LAB have the capability to extend shelf life, enhance safety, improve the texture, and add to a desirable sensory profile (Li & Cao, 2010).

1.5. Storage

There are several different ways kombucha is preserved and stored. The most common method is to use a combination of refrigeration and antifungal preservatives (0.1% sodium benzoate and 0.1% potassium sorbate) (Nummer, 2013; Leal et al., 2018). This ensures foodborne hazards and spoilage are minimized by preventing spoilage organism growth and keeping yeast growth at a minimum. Many commercial cultures today will contain yeasts that do not grow well at refrigeration temperatures so that once bottled, the carbon dioxide and alcohol production will be minimized (Nummer, 2013). Otherwise, this is a major concern; yeasts unaffected by refrigeration temperatures will continue to produce carbon dioxide and alcohol, thus threatening explosion of the bottles and raising the alcohol content to a level where it needs to be labeled as a full-alcoholic beverage rather than a low-alcohol beverage. As mentioned previously, kombucha does contain traces of alcohol, but it is considered to be a low-alcohol beverage. Legally, "low-alcohol" or "reduced alcohol" beverages must contain less than 2.5% ABV and this terminology can be used only on malt beverages (27 CFR § 7.71, n.d.). However, under federal law if kombucha's alcohol content is ≥0.5% ABV at any point during production, it must be sold and regulated according to the Alcohol and Tobacco Tax and Trade Bureau (TTB) as an alcoholic beverage (TTB, 2017).

While relying on refrigeration alone is often a preferred method for homebrewers, using a combination of preservative treatments is the most effective way to ensure the required amount of protection needed for commercial kombucha to retain its sensory qualities, such as color, texture, flavor, and nutritional value (Nummer, 2013; Brul & Coote, 1999). Filtration is another method sometimes used by commercial brewers to clarify and stabilize the beverage for storage

by physically removing the majority of the yeast cells before bottling (Rektor & Vatai, 2004; Peña-Gómez et al., 2020).

Pasteurization of bottled kombucha is another common preservation method because pasteurized kombucha is shelf stable at room temperature so long as the product has a pH of 4.2 or below (Nummer, 2013; Leal et al., 2018). However, pasteurization kills any "good microorganisms" present in the liquid rendering any potential health benefits involving the consumption of these beneficial microorganisms null. As a result, it is not uncommon for commercial producers to add probiotics to the finished beverage especially after pasteurization to restore those benefits lost from killing off the "good microorganisms" (Figure 1.3). Based on Jayabalan et al. (2008)'s study, it was determined that heat treatments of ≥60°C for one minute or longer were not ideal for kombucha preservation because there was a degradation of the tea components and a steady decrease in the beverage's free radical scavenging properties during the 90-day storage period.

Figure 1.3. KeVita's Pasteurized Kombucha Bottle and Label

Label indicates live probiotics (*Bacillus coagulans* MTCC 5856) were added and this bottle has a 0.5% ABV content.

1.6. Key Characteristics of Kombucha Make It Unlikely for Harmful Microorganism Survival

Kombucha has unique characteristics unlike most other foods and beverages that allows only specific groups of microorganisms to survive and thrive. The chemical composition of kombucha, the high acidity levels, and the high microbial load providing ample competition for resources significantly contribute to this environment.

1.6.1. Chemical Composition

Kombucha's chemical composition consists of numerous components that deter potentially harmful microorganisms from surviving or thriving in the liquid. Various organic acids, including acetic acid, lactic acid, gluconic acid, glucuronic acid, citric acid, malic acid, tartaric acid, malonic acid, oxalic acid, succinic acid, pyruvic acid, and usnic acid, make up the majority of kombucha's composition which lowers the beverage to a pH where many harmful molds, yeasts, and other microorganisms cannot survive (Blanc, 1996; Neffe-Skocińska et al., 2017; Chu & Chen, 2006; Chen & Liu, 2000; Ivanišová et al., 2020; St-Pierre, 2019; Leal et al., 2018). Kombucha also contains polyphenols (catechin and isorhamnetin), D-saccharic acid-1,4-lactone (DSL), carbon dioxide, vitamins, and [low levels of] alcohol (ethanol, glycerol), which contribute to suppressing bacterial growth and/or have antimicrobial properties (Deghrigue et al., 2013; Tarver, 2016; Chatterjee et al., 2006; Daniels et al., 1985; Bhattacharya et al., 2020; Bhattacharya et al., 2016).

Many of these components are used as natural or added preservatives for numerous foods and beverages. They are necessary to extend shelf-life, deter unwanted microbial growth and spoilage, and ensure the safety of the product. Weak organic acids, such as acetic acid, lactic acid, benzoic acid, and sorbic acid, are the most common classical preservative agents. Other common food preservatives consist of phenolics and alcohol, all of which can be found in kombucha (Brul & Coote, 1999). These preservatives have an optimal inhibitory activity at a low pH and the ability to inhibit the outgrowth of bacterial and fungal cells (Brul & Coote, 1999). While bacterial spores are not killed by any of these preservatives, phenolics, organic acids, and alcohol are able to inhibit spore germination as well as the growth of vegetative bacteria (Russell, 1991). Carbon dioxide is inhibitory to many food spoilage organisms, including

psychrotrophs, thus ensuring no contamination of psychrotrophic food spoilage organisms in the kombucha during refrigeration and storage (Russell, 1991).

1.6.2. Acidity

The multitude of organic acids found in kombucha, especially acetic acid, not only act as preservative agents but they also contribute to kombucha's high acidity levels. The finished product of kombucha usually has a pH of about 2.5 (Nummer, 2013; Greenwalt et al., 2000; Greenwalt et al., 1998). Very few microorganisms are capable of surviving at such low pH levels. Acetic acid bacteria, *Acetobacter* and *Gluconobacter* spp., and lactic acid bacteria, *Lactobacillus,* and *Leuconostoc* spp., thrive at pH levels as low as 3.5 whereas other bacteria such as Lancefield Group N streptococci, *Clostridium butyricum* and *C. pasteurianum, Bacillus acidoterrestris, B. coagulans, B. macerans,* and *B. polymyxa* can only tolerate, but not increase in population, at these pH levels (Booth & Stratford, 2003; Ray, 2004). A considerable amount of other bacteria will not grow below pH 4.5. This low pH environment may not necessarily kill the bacteria, but it will prevent its growth. The combination of low pH and a high weak acid concentration in an environment often leads to the acidification of the bacteria's cytoplasm, which is usually enough to halt microbial growth (Booth & Stratford, 2003).

Most food spoilage organisms and foodborne pathogens grow within a pH range of 4.0-8.0. This does not account for spoilage yeasts and molds which are able to grow and survive at lower pH levels (Booth & Stratford, 2003). With consumable kombucha consistently at pH under 4, and oftentimes significantly lower, spoilage yeasts, molds, and spore-formers are more of a concern than harmful vegetative bacteria. However, bacterial spores are not able to germinate at a pH less than 4.5 (Booth & Stratford, 2003). The few organisms capable of growing outside

these pH ranges have never presented a significant food spoilage problem in the past because there are seldom truly acid-resistant organisms found naturally in foods (Booth & Stratford, 2003).

Acid shock syndrome is also a very common occurrence when organisms adapted to a near neutral or basic pH are suddenly thrown into an acidic environment. When placed into an environment with a pH of 3.5 or below, bacteria are killed rapidly (Booth & Stratford, 2003). Many different organisms are capable of enhancing their "acid tolerance" by previous exposure and growth at mildly acidic conditions (pH of 4.5-6). This is known as a bacterium's acid tolerance response (ATR) (Booth & Stratford, 2003; Browne & Dowds, 2002). Several organisms such as *Escherichia coli,* Salmonella Typhimurium*, Yersinia, Listeria monocytogenes,* and *Lactobacillus* have been found to demonstrate strong ATR responses (Booth & Stratford, 2003). Such responses can be concerning in long-duration fermentations if the pace of acidification is slow enough to permit adaptation.

1.6.3. Microbial Competition

Because of the abundance of bacterial and yeast species present in kombucha, a very competitive environment is created, allowing no other bacterial, fungal, or spoilage species room or resources to invade. In this sense, already-present bacterial and yeast species practice competitive exclusion. Together they exhibit metabolic cooperation, where one species metabolizes a nutrient and their byproducts are utilized by another species, which increases their reliance on one another and strengthening their community rather than if a species were alone (Elias & Banin, 2012). In several studies regarding multi-species vs. mono-species biofilms, the mixed-species biofilm in most cases was considerably more resistant to invaders (Elias & Banin,

2012). The less "good" microorganisms already present, the more chances for possible pathogenic contamination.

Acidity and the number of possible pathogenic microbial cells have an inverse relationship in kombucha. When kombucha's acidity increases due to the organic acids produced during fermentation, there is a lack of oxygen present in the liquid, resulting in a decrease of aerobic bacteria (Leal et al., 2018). The ethanol and acetic acid produced by yeasts, *Acetobacter*, and *Gluconobacter* contribute to inhibiting the growth of pathogenic bacteria and might prevent competition from other microorganisms (Liu et al., 1996; Leal et al., 2018; De Roos & De Vuyst, 2018; Saichana et al., 2015). Heat treatments that are known to reduce competitive microbiota in kombucha have been shown to support the growth of vegetative bacterial cells as well as the germination of bacterial spores (*Bacillus cereus—BfR*, n.d.).

When evaluating all these factors simultaneously, kombucha demonstrates the unlikeliness of foodborne pathogen contamination. However, because of this there is little to no research being pursued on potential contamination that could occur throughout the brewing process or with varying types and severities of foodborne pathogens.

1.7. Safety Concerns of Kombucha

Several sporadic cases have been reported where people consumed kombucha and shortly after have, allegedly, fallen ill, been hospitalized, and even died because of it. This raised great concern for kombucha drinkers, and people began to wonder if kombucha was safe to consume. However, these cases are rare and spread out over the course of 20 years with the last case being in 2017 (Holbourn & Hurdman, 2017; Murphy et al., 2018). Most of these cases involved individuals with underlying health issues who then drank an excessive amount of kombucha

within a short amount of time, leading to serious health problems like acidosis, hepatotoxicity, renal failure, etc. (Gedela et al., 2016; SungHee et al., 2009; CDC, 1995; Holbourn & Hurdman, 2017; Srinivasan et al., 1997; Phan et al., 1998; Kovacevic et al., 2014). Medical professionals deduced that it was the only logical conclusion that kombucha must have been the cause of these illnesses, yet no definitive direct link can be established. As of 2009, there have been no cases reported determining that "a clinically significant bacterial or fungal infection" of an individual was caused by kombucha directly (SungHee et al., 2009). However, the FDA has not ruled out the possibility of fungal contamination in kombucha that is known to cause disease in susceptible individuals (SungHee et al., 2009).

Because kombucha consumption has proven to be harmful in only a few documented instances, the CDC recommends an individual should not consume more than 4 oz per day at a pH no higher than 4.2 (which may allow for mold growth) or no lower than 2.5 (too acidic) (Nummer, 2013). Drinking excessive amounts of kombucha in a short period, especially at a pH of ≤2.5, could cause acidosis (SungHee et al., 2009; CDC, 1995; Baschali et al., 2017; Petruzzello, n.d.). By not over-fermenting kombucha brews and limiting servings, the CDC suggests that kombucha is a fairly safe product fit for consumption (Nummer, 2013). To date, the FDA has not found any pathogenic organisms or hygienic violations in the practices of the commercial manufacturers of this product (Nummer, 2013; Gedela et al., 2016). On the contrary, home brewing of kombucha is conducted under varying conditions, without strict sanitation controls and therefore, homebrews are more likely to be contaminated with pathogenic organisms like *Aspergillus* and cause illness (CDC, 1995; Srinivasan et al., 1997). This also could occur due to improper technique (such as not pre-acidifying the sweetened tea before fermentation with previously prepared kombucha or not taking pH measurements to know when

to end fermentation), misuse of equipment (such as mishandling of SCOBY or using the wrong container type), or being uneducated about kombucha and the fermentation process. For example, if homebrewers prepare or store their brews in ceramic or lead containers, toxic material can leach into the tea because of the high acidity (Gedela et al., 2016; SungHee et al., 2009; CDC, 1995; Srinivasan et al., 1997). Unwashed fruit used for secondary fermentation may introduce various soilborne microorganisms including fungi and bacterial spores.

In 1996, there was a documented case of twenty people contracting cutaneous anthrax after applying a kombucha SCOBY to the skin as a topical painkiller (Smolinske, 2005; Watawana et al., 2015; Sadjadi, 1998). *Bacillus anthracis* was identified as the culprit, and seeing as it is a spore-former, it is possible *Bacillus* spores are capable of becoming associated with kombucha SCOBYs. The cause of contamination was determined to be the unhygienic preparation of the tea in plastic containers in a farmyard in close proximity to cattle (Watawana et al., 2015; Sadjadi, 1998).

It is recommended by the CDC and other various sources that people with pre-existing health conditions should not consume [unpasteurized] kombucha because of the wide variety of microorganisms it contains. Potential health risks are unknown for those who have pre-existing health problems or drink excessive amounts of kombucha, and the cases in literature suggest adverse effects are more likely to occur in these circumstances (Nummer, 2013; Srinivasan et al., 1997).

Even after bottling, biochemical processes can continue to occur in kombucha, leading to carbon dioxide pressure buildup in a closed container. Because the pressure has nowhere to escape, the buildup could potentially cause leakage or explosion of the packaging, threatening projectile hazards (Nummer, 2013). The best prevention method would be to pasteurize the

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product, killing any active microorganisms, thus halting any biochemical processes (Nummer, 2013). As a result, the carbon dioxide pressure buildup is prevented, but the potential health benefits from the live organisms are no longer available.

1.7.1. Amino Acid and Biogenic Amine Presence in Kombucha

The presence of amino acids in a food product is usually a positive attribute since amino acids are essential to proper bodily function. However, these amino acids can often be transformed into biogenic amines via microbial decarboxylation during fermentation. Some biogenic amines are beneficial while others can be harmful to humans if ingested in high enough concentrations, such as histamine (Santos, 1996; Ruiz-Capillas & Herrero, 2019). The formation of biogenic amines is also influenced by the raw materials used, the microorganisms present in the starter culture, and processing and storage conditions (Doeun et al., 2017; Ruiz-Capillas & Herrero, 2019). While consuming low amounts of biogenic amines normally does not have a harmful effect on human health, their levels are often elevated to toxic amounts in fermented foods (Doeun et al., 2017). On the other hand, beneficial metabolites, such as gamma-aminobutyric acid, can also be produced during fermentation (Section 1.8).

The biogenic amines found in food that pose the biggest threats for foodborne poisoning and illness include histamine, tyramine, putrescine, cadaverine, β-phenylethylamine, agmatine, tryptamine, serotonin (SRT), spermidine, and spermine (Santos, 1996; Ruiz-Capillas & Herrero, 2019; Shalaby, 1996). Scombroid poisoning, or histamine poisoning, is caused when too much histamine is consumed and is most associated with fish, but outbreaks have also occurred with cheese, wine, and meat products (Santos, 1996). Symptoms of scombroid poisoning include dizziness, faintness, burning sensation in the mouth, inability to swallow, and itching

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(Hungerford, 2010). Putrescine and cadaverine are responsible for enhancing the toxicity of histamine (Tabanelli, 2020). Overconsumption of tyramine, phenylethylamine, and tryptamine leads to hypertension, headaches, pupil dilatation, palpebral tissue dilatation, and respiration increasing (Shalaby, 1996). Spermine and spermidine have been shown to help lower blood pressure, inhibit blood clotting, provoke respiratory symptoms, and reduce neurotoxicity which induced renal insufficiency (Pegg, 2013).

Many scholarly articles and reviews on kombucha discuss its chemical composition and what it consists of, which includes amino acids and biogenic amines, however, there is seldom any in-depth literature on specific amino acids and biogenic amines that can be found in kombucha. There is some research on amino acids and biogenic amines that have been found within the SCOBY or "tea fungus" but none on which constituents can be found in the broth, which is the portion consumed. The SCOBY was found to contain higher concentrations of lysine, isoleucine, and leucine and lower concentrations of phenylalanine, valine, methionine, threonine, tryptophan, glutamic acid, alanine, aspartic acid, and proline (Leal et al., 2018). Of these amino acids, only lysine, phenylalanine, and tryptophan are precursors for the aforementioned harmful biogenic amines (Doeun et al., 2017; Santos, 1996). However, ethylamine, choline, and adenine were the only identifiable biogenic amines in kombucha that were discussed in literature, none of which are harmful (Kappel & Anken, 1993).

1.8. GABA

Gamma-aminobutyric acid, or GABA, is a nonprotein amino acid that acts as a postbiotic, or a bioactive compound produced by probiotic metabolism, and is produced by plants, animals, and microorganisms, including fungi, yeast, and bacteria (Bown & Shelp, 1997;

Shelp et al., 1999; Diez-Gutiérrez et al., 2020; Cui et al., 2020). It is converted from glutamic

acid via glutamic acid decarboxylase (GAD) into two isoforms, GAD65 and GAD67 (Ting

Wong et al., 2003). GABA is well-known as a major inhibitory neurotransmitter in the central

nervous system and operates via the brain-gut connection (My, 2018; Diez-Gutiérrez et al., 2020;

Cui et al., 2020). Lactic acid bacteria can disable food spoilage pathogens from growing in foods

by producing GABA in the presence of glutamic acid, while also acting as probiotics in the

gastrointestinal tract once consumed (My, 2018; (Diez-Gutiérrez et al., 2020; Dhakal et al.,

2012).

GABA is said to have a wide variety of beneficial health effects. It is most known for its

roles in regulating hypertension, moderating neurological disorders, such as epilepsy,

schizophrenia, Alzheimer's disease, and aiding in alleviating psychiatric disorders, such as

anxiety, stress, sleeplessness, depression, alcoholism, and mood (Diez-Gutiérrez et al., 2020;

Sang et al., 2018; Ting Wong et al., 2003; Shimada et al., 2009; Inoue et al., 2003; Aoki et al.,

2003; Yoto et al., 2012; Nakamura et al., 2009; Abdou et al., 2006; Byun et al., 2018; Petty,

1995; Seidl et al., 2001; Opolski et al., 2000). In addition, the postbiotic helps protect against

cancer, enhances immunity and vital organ function, aids in preventing diabetes, and acts as a

hormonal regulator and potential antioxidant (Sang et al., 2018; Oh & Oh, 2003; Adeghate &

Ponery, 2002; Oh & Oh, 2004; Opolski et al., 2000; Schuller et al., 2008; Sasaki et al., 2006;

Leventhal et al., 2003; Wiens & Trudeau, 2006). In fermented foods specifically, GABA aids

with gut microbiota dysbiosis reversal, neurostimulation, and cardioprotection (Diez-Gutiérrez et

al., 2020).

Chemical synthesis of GABA was originally used to meet the demand for it as a

supplement, however it has since been replaced by the use of microorganisms. By using

microorganisms such as lactic acid bacteria to produce GABA, yields are higher, costs are lowered, and the use of corrosive reagents is excluded (Diez-Gutiérrez et al., 2020). As of 2008, the FDA granted GABA generally recognized as safe (GRAS) status (PubChem, n.d.; FDA, n.d.).

Temperature, pH, fermentation time, and different media additives are the main environmental factors that affect GABA production throughout fermentation because they modulate the *gad* gene expression, which controls GABA production (Diez-Gutiérrez et al., 2020; Dhakal et al., 2012). These circumstances are different depending on which strain is being utilized, but studies show the ideal temperature and pH for optimal GABA production is between 30-40°C and 4.5-6.0, respectively (Diez-Gutiérrez et al., 2020; Dhakal et al., 2012; Sang et al., 2018). Since glutamic acid needs to be present for the production of GABA, foods such as Korean kimchi, Chinese paocai, yogurt, cheese, and sourdough are ideal for GABA-producing LAB growth (Sang et al., 2018).

1.9. *Bacillus cereus* as a Foodborne Pathogen

1.9.1. Gram-positive bacteria and Spore-formers

Some Gram-positive bacteria, known as spore-formers, have ability to produce endospores under stressed conditions such as extreme pH, extreme temperature, drought, and lack of proper nutrients. This process is called sporulation (Karki, 2017). Examples of spore-formers include *Bacillus* spp*., Clostridium* spp*.,* and *Sporosarcina* spp*.* (Karki, 2017)*.* When sporulation occurs, the vegetative cell forms in a hardened seed-like structure containing the DNA of the bacterium and becomes metabolically dormant (Figure 1.4) (Karki, 2017). Endospores can stay in this state for years and years and are resistant to nutrition starvation,

temperature, extreme pH, antibiotics, radiation, etc. (Karki, 2017; Kotiranta et al., 2000). Some mature spores may even survive various food production procedures such as pasteurization, heating, and gamma radiation that act as "fail-safes" to rid foods of harmful pathogens (Kotiranta et al., 2000). Once nutrients are available, the spores are then activated and return to their full vegetative cell state again (Figure 1.4). This process is called spore transformation (Aissa Ouaissi Sekkouti, 2019).

Spore-formers can be found everywhere in the environment, and their wide range of diversity leads to their natural prevalence in foods. Consequently, it is not unrealistic or impossible for spores to be found on or in foods and food ingredients at any stage of food production (Stenfors Arnesen et al., 2008).
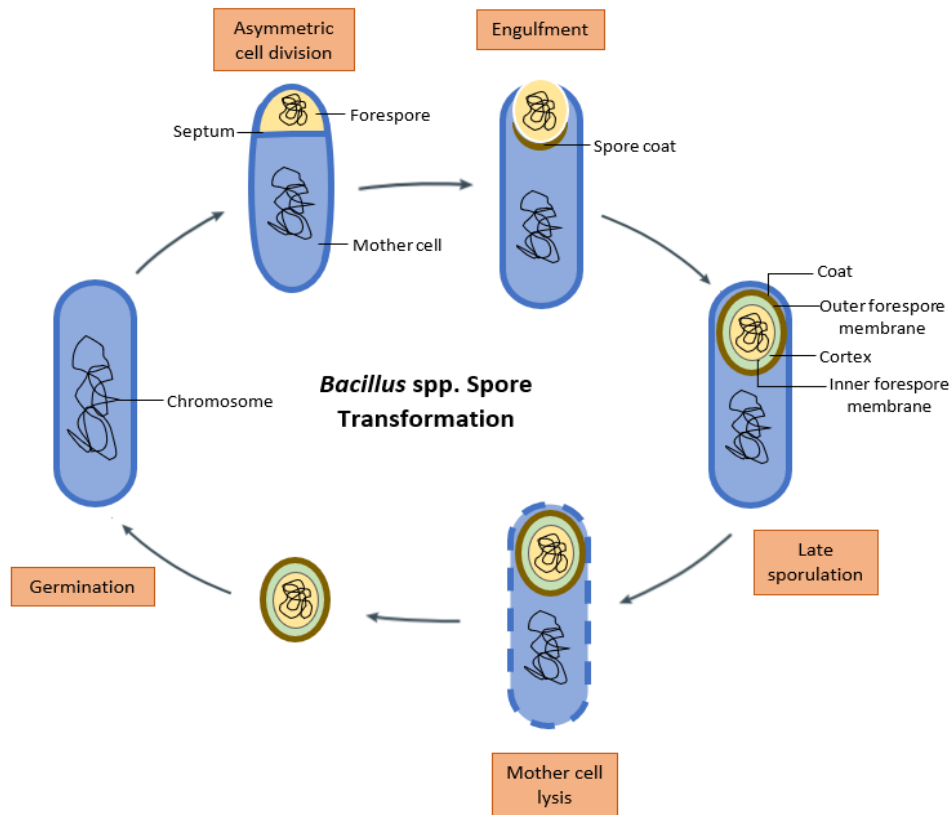
Figure 1.4. Spore Transformation Cycle of *Bacillus* spp. Including Sporulation and Germination

Adapted from McKenney et al. (2013).

1.9.2. *Bacillus cereus* Pathogenicity and Illnesses

Bacillus cereus is a Gram-positive bacillus, spore-forming foodborne pathogen that, out of the *Bacillus* genus, is the species most associated with food. As a normal soil inhabitant, *B. cereus* is frequently found in the environment, in soil samples and vegetation, and is commonly isolated from foods (Granum & Lund, 1997). *B. cereus* spores have been known to adhere to stainless steel pipes and tanks, which presents a serious challenge since stainless steel is the most commonly used material for food industry equipment (Wijman et al., 2007). *B. cereus* is also capable of forming biofilms, enabling the organism to persist in food production environments (*Bacillus cereus—BfR*, n.d.). Being a facultative anaerobe, *B. cereus* is able to survive an environment with or without oxygen, indicating that it is capable of surviving through various processes including fermentation (Examining Food, 2013). As a mesophilic organism, *B. cereus*'s optimum growth temperature is between 35-40°C, but it is capable of growing from 10°C to 50°C, which includes the temperature at which fermentation of kombucha takes place (Stenfors Arnesen et al., 2008). In the last few decades, the number of psychrotolerant *B. cereus* strains, or strains tolerant to colder temperatures, have increased, making it more of a risk to food safety than ever before (Stenfors Arnesen et al., 2008).

It was not until Hauge's experiments in the 1950s, after investigation into outbreaks in Norway hospitals from 1947-1949 caused by an unknown source, that *B. cereus* was established as an organism capable of causing foodborne diseases (Drobniewski, 1993; Stenfors Arnesen et al., 2008). Today, there are around 64,000 cases of illnesses caused by *B. cereus* reported annually, but this number is most likely underreported. This is due to the short duration of the illness, the mild symptoms, and misdiagnosis (Examining Food, 2013; Ding et al., 2013; Stenfors Arnesen et al., 2008). Symptoms generally resolve within 24 hours and are mild enough that a

hospital visit is not often required, and fatality is rare (Shinagawa, 1990; Notermans & Batt, 1998; Kotiranta et al., 2000). Cases of *B. cereus* gastroenteritis of food origin also seem to be on the rise in more industrialized countries such as Korea, Germany, Finland, and the United States (Ding et al., 2013).

There are two types of illnesses associated with *B. cereus*: emetic and diarrheal. The emetic illness is frequently confused for *Staphylococcus aureus* intoxication while the diarrheal illness is often mistaken for *Clostridium perfringens* food poisoning because they have overlapping symptoms and resemble one another (Shinagawa, 1990; Stenfors Arnesen et al., 2008). The emetic illness is an intoxication, meaning toxin(s) is produced within the food by the bacteria, and illness occurs when that toxin is consumed (*Bacillus cereus—BfR*, n.d.; Ministry of Agriculture and Forestry of Alberta, Canada, 2006). *Bacillus cereus* was identified in the early 1970's in the United Kingdom after several outbreaks were determined to be caused by consumption of contaminated cooked rice (Stenfors Arnesen et al., 2008). Emetic illness is associated with nausea, vomiting, and malaise starting 1-5 hours after ingestion and usually lasting 6-24 hours (*Bacillus cereus—BfR*, n.d.; Notermans & Batt, 1998; Kotiranta et al., 2000). Foods most associated with emetic illness are starchy foods such as pasta, fried rice, and cooked rice (Notermans & Batt, 1998; Ding et al., 2013; Stenfors Arnesen et al., 2008). The illness is caused by the emetic toxin pre-formed in food; spores not killed off by cooking processes germinate in food during instances of temperature abuse, and cereulide, a small ring-shaped dodecadepsipeptide that acts as the emetic toxin, forms in the food as the vegetative cells grow (*Bacillus cereus—BfR*, n.d.; Shinagawa, 1990; Stenfors Arnesen et al., 2008). Cereulide is extremely resistant to factors such as low pH, heat, and proteolysis. However, only a small percentage of *B. cereus* strains are capable of producing cereulide (*Bacillus cereus—BfR*, n.d.).

The infectious dose of emetic-toxin producing *B. cereus* causing illness in half of those infected (ID_{50}) has not yet been determined, although foods involved in cases of emetic disease have had levels of cells ranging from 10^3-10^{10} CFU/g with at least 10^5 CFU/g food in most cases (Stenfors Arnesen et al., 2008).

The diarrheal illness is a toxicoinfection, meaning the illness is caused by toxins produced by the bacteria after consumption (*Bacillus cereus—BfR*, n.d.; Stenfors Arnesen et al., 2008; Ministry of Agriculture and Forestry of Alberta, Canada, 2006). It is most associated with abdominal pain, cramps, and watery diarrhea occurring 8-16 hours after ingestion of contaminated food and generally resolves within 12-24 hours (*Bacillus cereus—BfR*, n.d.; Notermans & Batt, 1998; Kotiranta et al., 2000). The minimal ID_{50} for the diarrheal type illness has been established as a range between 10^5-10^8 *B. cereus* cells, however doses as low as 10^3 *B. cereus* CFU/g of food have been identified in foods causing disease (Stenfors Arnesen et al., 2008). Foods most associated with diarrheal illness are proteinaceous foods, sauces, desserts, vegetables, and dairy products (Notermans & Batt, 1998; Ding et al., 2013; Stenfors Arnesen et al., 2008). In recent years, psychrotrophic diarrheal strains have become an increasing problem for the dairy industry (Granum & Lund, 1997). Diarrheal illness is caused by an enterotoxin produced by vegetative *B. cereus* cells in the small intestine. Unlike cereulide, however, the enterotoxin is sensitive to heat, acid, and proteolysis (*Bacillus cereus—BfR*, n.d.; Ding et al., 2013).

Various mutant strains of *B. cereus* exist that are able to survive in extreme heat and/or acidic conditions (*Bacillus cereus—BfR*, n.d.). There are several strains of *B. cereus* known to flourish at both extremes of the pH scale, ranging from pH 2 to 10 (Drobniewski, 1993). However, it is a rare occurrence to have vegetative cells survive in foods that are dry and/or

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acidic, although this does not apply for spores (*Bacillus cereus—BfR*, n.d.). Spores of certain *B. cereus* strains are adept at tolerating foods at high temperatures with a high fat content as this acts as an added level of protection (Drobniewski, 1993; Johnson, 1984).

Illness due to *B. cereus* is most likely to occur from foods left out at room temperature for a prolonged time (emetic) or from improper storage of foods (diarrheal) (Examining Food, 2013). It only takes $>10^4$ CFU/g of *B. cereus* cells for foods to be considered potentially unsafe for consumption (Ding et al., 2013). As a result, it is not unreasonable or uncommon for outbreaks to occur. Inadequate cooking, inadequate cooling, preparation of food too far in advance, and infected personnel are important factors in contributing to foodborne outbreaks, with inadequate cooling deemed the most important factor causing disease outbreaks (Johnson, 1984). If storage temperature conditions are poorly controlled, the possibility of spore germination and multiplication of vegetative cells in the stored food increases dramatically. Nonetheless, this can be easily prevented by the rapid cold or hot storage of heated foods (*Bacillus cereus—BfR*, n.d.). *B. cereus* strains also demonstrate a poor ability to grow at temperatures below 10°C, resulting in a low spore germination rate (Carlin et al., 2006). By practicing good hygiene and food preparation practices, the incidence of gastrointestinal disease can be significantly reduced, especially in restaurants and other food service establishments (Drobniewski, 1993).

Outbreaks involving *B. cereus* are nothing new. A high presence of *B. cereus* does not necessarily always cause illness (Johnson, 1984). At least 230 outbreaks of the diarrheal type gastroenteritis have been reported worldwide from 1950 to 1976, and at least 170 outbreaks of the emetic type have been reported from 1971 to 1990 (Shinagawa, 1990). Between 1993 and

1998 in the Netherlands, 12% of foodborne outbreaks were caused by *B. cereus* (Stenfors Arnesen et al., 2008).

1.10. Conclusions and Experimental Objectives

Fermented foods, such as kombucha, are gaining traction among consumers in part due to the functional foods movement and the desire to start living healthier lifestyles. However, there is very little about kombucha that we know with 100% certainty, especially when it comes to potential contamination with spore-forming foodborne pathogens and probiotic presence in the finished product. Therefore, it is imperative that a better understanding of kombucha is developed. Food scientists and companies want consumers of kombucha to be informed, safe, and trusting that what is on the label is what they are getting.

This research involves two different experiments with two main experimental objectives: (i) to determine if *Bacillus cereus* spores can be incorporated into a kombucha system, survive in kombucha's unsuitable experiment, be passed on from mother SCOBY to daughter SCOBY, or persist in the final consumable product and (ii) to determine if known lactic acid probiotics can grow and survive during kombucha fermentation, guaranteeing their presence in the finished product and producing a product with the potential to provide a health benefit.

1.11. References

1) *27 CFR § 7.71—Alcoholic content.* (n.d.). LII / Legal Information Institute. Retrieved December 2, 2021, from https://www.law.cornell.edu/cfr/text/27/7.71

2) Abdou, A. M., Higashiguchi, S., Horie, K., Kim, M., Hatta, H., & Yokogoshi, H. (2006). Relaxation and immunity enhancement effects of γ-Aminobutyric acid (GABA) administration in humans. *BioFactors*, *26*(3), 201–208. https://doi.org/10.1002/biof.5520260305

3) Adeghate, E., & Ponery, A. S. (2002). GABA in the endocrine pancreas: Cellular localization and function in normal and diabetic rats. *Tissue and Cell*, *34*(1), 1–6. https://doi.org/10.1054/tice.2002.0217

4) Aissa Ouaissi Sekkouti, A. (2019). *A theoretical study and a micro-scale sensing platform for the detection of germination in Bacillus stearothermophilus spores*. https://acikbilim.yok.gov.tr/handle/20.500.12812/589656

5) Ansari, F., Pourjafar, H., Kangari, A., & Homayouni, A. (2019). Evaluation of the Glucuronic Acid Production and Antibacterial Properties of Kombucha Black Tea. *Current Pharmaceutical Biotechnology*, *20*(11), 985–990. https://doi.org/10.2174/1389201020666190717100958

6) Aoki, H., Furuya, Y., Endo, Y., Fujimoto, K. (2003). Effect of γ-Aminobutyric Acid-enriched Tempeh-like Fermented Soybean (GABA-Tempeh) on the Blood Pressure of Spontaneously Hypertensive Rats. *Bioscience, Biotechnology, and Biochemistry*, *67*(8), 1806–1808. https://doi.org/10.1271/bbb.67.1806

7) *Bacillus cereus—BfR*. (n.d.). Retrieved March 26, 2021, from https://www.bfr.bund.de/en/bacillus_cereus-54345.html

8) Back, A. (2018). The Year of Fancy Water and Kombucha. *Wall Street Journal*. https://www.wsj.com/articles/the-year-of-fancy-water-and-kombucha-11546269901

9) Banerjee, D., A. Hassarajani, S., Maity, B., Narayan, G., K. Bandyopadhyay, S., & Chattopadhyay, S. (2010). Comparative healing property of kombucha tea and black tea against indomethacin -induced gastric ulceration in mice: Possible mechanism of action. *Food & Function*, *1*(3), 284–293. https://doi.org/10.1039/C0FO00025F

10) Baschali, A., Tsakalidou, E., Kyriacou, A., Karavasiloglou, N., Matalas, A. L. (2017). Traditional low-alcoholic and non-alcoholic fermented beverages consumed in European countries: A neglected food group - ProQuest. *Nutrition Research Reviews*, *30*(1), 1–24. https://doi.org/10.1017/S0954422416000202

11) Battikh, H., Chaieb, K., Bakhrouf, A., & Ammar, E. (2013). Antibacterial and Antifungal Activities of Black and Green Kombucha Teas. *Journal of Food Biochemistry*, *37*(2), 231–236. https://doi.org/10.1111/j.1745-4514.2011.00629.x

12) Bauer, L., Dekevich, D., & Underthun, K. (n.d.). *Kombucha*. Food Source Information. Retrieved January 21, 2021, from https://fsi.colostate.edu/kombucha/.

13) Bauer-Petrovska, B., & Petrushevska-Tozi, L. (2000). Mineral and water soluble vitamin content in the Kombucha drink. *International Journal of Food Science & Technology*, *35*(2), 201–205. https://doi.org/10.1046/j.1365-2621.2000.00342.x

14) Bhattacharya, D., Bhattacharya, S., Patra, M. M., Chakravorty, S., Sarkar, S., Chakraborty, W., Koley, H., & Gachhui, R. (2016). Antibacterial Activity of Polyphenolic Fraction of Kombucha Against Enteric Bacterial Pathogens. *Current Microbiology*, *73*(6), 885–896. https://doi.org/10.1007/s00284-016-1136-3

15) Bhattacharya, D., Ghosh, D., Bhattacharya, S., Sarkar, S., Karmakar, P., Koley, H., & Gachhui, R. (2018). Antibacterial activity of polyphenolic fraction of Kombucha against Vibrio cholerae: Targeting cell membrane. *Letters in Applied Microbiology*, *66*(2), 145–152. https://doi.org/10.1111/lam.12829

16) Bhattacharya, D., Sinha, R., Mukherjee, P., Howlader, D. R., Nag, D., Sarkar, S., Koley, H., Withey, J. H., & Gachhui, R. (2020). Anti-virulence activity of polyphenolic fraction isolated from Kombucha against Vibrio cholerae. *Microbial Pathogenesis*, *140*, 103927. https://doi.org/10.1016/j.micpath.2019.103927

17) Bhattacharya, S., Gachhui, R., & Sil, P. C. (2013). Effect of Kombucha, a fermented black tea in attenuating oxidative stress mediated tissue damage in alloxan induced diabetic rats. *Food and Chemical Toxicology*, *60*, 328–340. https://doi.org/10.1016/j.fct.2013.07.051

18) Bigliardi, B., & Galati, F. (2013). Innovation trends in the food industry: The case of functional foods. *Trends in Food Science & Technology*, *31*(2), 118–129. https://doi.org/10.1016/j.tifs.2013.03.006

19) Blanc, P. J. (1996). Characterization of the tea fungus metabolites. *Biotechnology Letters*, *18*(2), 139–142. https://doi.org/10.1007/BF00128667

20) Booth, I. R., & Stratford, M. (2003). Acidulants and low pH. In N. J. Russell & G. W. Gould (Eds.), *Food Preservatives* (pp. 25–47). Springer US. https://doi.org/10.1007/978-0-387-30042-9_3

21) Bown, A. W., & Shelp, B. J. (1997). The Metabolism and Functions of [gamma]-Aminobutyric Acid. *Plant Physiology*, *115*(1), 1–5.

22) Browne, N., & Dowds, B. C. A. (2002). Acid stress in the food pathogen Bacillus cereus. *Journal of Applied Microbiology*, *92*(3), 404–414. https://doi.org/10.1046/j.1365-2672.2002.01541.x

23) Brul, S., & Coote, P. (1999). Preservative agents in foods: Mode of action and microbial resistance mechanisms. *International Journal of Food Microbiology*, *50*(1), 1–17. https://doi.org/10.1016/S0168-1605(99)00072-0

24) Byun, J.-I., Shin, Y. Y., Chung, S.-E., & Shin, W. C. (2018). Safety and Efficacy of Gamma-Aminobutyric Acid from Fermented Rice Germ in Patients with Insomnia Symptoms: A Randomized, Double-Blind Trial. *Journal of Clinical Neurology*, *14*(3), 291–295. https://doi.org/10.3988/jcn.2018.14.3.291

25) Cardoso, R. R., Neto, R. O., dos Santos D'Almeida, C. T., do Nascimento, T. P., Pressete, C. G., Azevedo, L., Martino, H. S. D., Cameron, L. C., Ferreira, M. S. L., & Barros, F. A. R. de. (2020). Kombuchas from green and black teas have different phenolic profile, which impacts their antioxidant capacities, antibacterial and antiproliferative activities. *Food Research International*, *128*, 108782. https://doi.org/10.1016/j.foodres.2019.108782

26) Carlin, F., Fricker, M., Pielaat, A., Heisterkamp, S., Shaheen, R., Salkinoja Salonen, M., Svensson, B., Nguyen-the, C., & Ehling-Schulz, M. (2006). Emetic toxin-producing strains of Bacillus cereus show distinct characteristics within the Bacillus cereus group. *International Journal of Food Microbiology*, *109*(1), 132–138. https://doi.org/10.1016/j.ijfoodmicro.2006.01.022

27) Centers for Disease Control and Prevention (CDC). (1995). Unexplained severe illness possibly associated with consumption of Kombucha tea—Iowa, 1995. *MMWR. Morbidity and Mortality Weekly Report*, *44*(48), 892–893, 899–900.

28) Četojević-Simin, D. D., Velićanski, A. S., Cvetković, D. D., Markov, S. L., Mrđanović, J. Ž., Bogdanović, V. V., & Šolajić, S. V. (2012). Bioactivity of Lemon Balm Kombucha. *Food and Bioprocess Technology*, *5*(5), 1756–1765. https://doi.org/10.1007/s11947-010-0458-6

29) Chakravorty, S., Bhattacharya, S., Bhattacharya, D., Sarkar, S., & Gachhui, R. (2019). 10 - Kombucha: A Promising Functional Beverage Prepared From Tea. In A. M. Grumezescu & A. M. Holban (Eds.), *Non-Alcoholic Beverages* (pp. 285–327). Woodhead Publishing. https://doi.org/10.1016/B978-0-12-815270-6.00010-4

30) Chatterjee, I., Somerville, G. A., Heilmann, C., Sahl, H.-G., Maurer, H. H., & Herrmann, M. (2006). Very Low Ethanol Concentrations Affect the Viability and Growth Recovery in Post-Stationary-Phase Staphylococcus aureus Populations. *Applied and Environmental Microbiology*, *72*(4), 2627–2636. https://doi.org/10.1128/AEM.72.4.2627-2636.2006

31) Chen, C., & Liu, B. Y. (2000). Changes in major components of tea fungus metabolites during prolonged fermentation. *Journal of Applied Microbiology*, *89*(5), 834–839. https://doi.org/10.1046/j.1365-2672.2000.01188.x

32) Chu, S.-C., & Chen, C. (2006). Effects of origins and fermentation time on the antioxidant activities of kombucha. *Food Chemistry*, *98*(3), 502–507. https://doi.org/10.1016/j.foodchem.2005.05.080

33) Coca-Cola invests $20M in Health-Ade Kombucha. (2019). Food Dive. Retrieved January 11, 2021, from https://www.fooddive.com/news/coca-cola-invests-20m-in-health-ade-kombucha/559171/

34) Corbo, M. R., Bevilacqua, A., Petruzzi, L., Casanova, F. P., & Sinigaglia, M. (2014). Functional Beverages: The Emerging Side of Functional Foods. *Comprehensive Reviews in Food Science and Food Safety*, *13*(6), 1192–1206. https://doi.org/10.1111/1541-4337.12109

35) Cui, Y., Miao, K., Niyaphorn, S., & Qu, X. (2020). Production of Gamma-Aminobutyric Acid from Lactic Acid Bacteria: A Systematic Review. *International Journal of Molecular Sciences*, *21*(3). https://doi.org/10.3390/ijms21030995

36) Daniels, J. A., Krishnamurthi, R., & Rizvi, S. S. H. (1985). A Review of Effects of Carbon Dioxide on Microbial Growth and Food Quality. *Journal of Food Protection*, *48*(6), 532–537. https://doi.org/10.4315/0362-028X-48.6.532

37) De Roos, J., & De Vuyst, L. (2018). Acetic acid bacteria in fermented foods and beverages. *Current Opinion in Biotechnology*, *49*, 115–119. https://doi.org/10.1016/j.copbio.2017.08.007

38) Deghrigue, M., Chriaa, J., Battikh, H., Abid, K., & Bakhrouf, A. (2013). Antiproliferative and antimicrobial activities of kombucha tea. *African Journal of Microbiology Research*, *7*(27), 3466–3470. https://doi.org/10.5897/AJMR12.1230

39) Dhakal, R., Bajpai, V. K., & Baek, K.-H. (2012). Production of gaba (γ - Aminobutyric acid) by microorganisms: A review. *Brazilian Journal of Microbiology: [Publication of the Brazilian Society for Microbiology]*, *43*(4), 1230–1241. https://doi.org/10.1590/S1517-83822012000400001

40) Diez-Gutiérrez, L., San Vicente, L., R. Barrón, L. J., Villarán, M. del C., & Chávarri, M. (2020). Gamma-aminobutyric acid and probiotics: Multiple health benefits and their future in the global functional food and nutraceuticals market. *Journal of Functional Foods*, *64*, 103669. https://doi.org/10.1016/j.jff.2019.103669

41) Ding, T., Wang, J., Park, M.-S., Hwang, C.-A., & Oh, D.-H. (2013). A probability model for enterotoxin production of Bacillus cereus as a function of pH and temperature. *Journal of Food Protection*, *76*(2), 343–347. https://doi.org/10.4315/0362-028X.JFP-12-174

42) Doeun, D., Davaatseren, M., & Chung, M.-S. (2017). Biogenic amines in foods. *Food Science and Biotechnology*, *26*(6), 1463–1474. https://doi.org/10.1007/s10068-017-0239-3

43) Drobniewski, F. A. (1993). Bacillus cereus and related species. *Clinical Microbiology Reviews*, *6*(4), 324–338. https://doi.org/10.1128/CMR.6.4.324

44) Dufresne, C., & Farnworth, E. (2000). Tea, Kombucha, and health: A review. *Food Research International*, *33*(6), 409–421. https://doi.org/10.1016/S0963-9969(00)00067-3

45) Elias, S., & Banin, E. (2012). Multi-species biofilms: Living with friendly neighbors. *FEMS Microbiology Reviews*, *36*(5), 990–1004. https://doi.org/10.1111/j.1574-6976.2012.00325.x

46) *Fact sheet on Bacillus cereus*. (2013). Examining Food. https://www.thermofisher.com/blog/food/fact-sheet-on-bacillus-cereus/

47) Gaggìa, F., Baffoni, L., Galiano, M., Nielsen, D. S., Jakobsen, R. R., Castro-Mejía, J. L., Bosi, S., Truzzi, F., Musumeci, F., Dinelli, G., & Di Gioia, D. (2019). Kombucha Beverage from Green, Black and Rooibos Teas: A Comparative Study Looking at Microbiology, Chemistry and Antioxidant Activity. *Nutrients*, *11*(1), 1. https://doi.org/10.3390/nu11010001

48) Gedela, M., Potu, K. C., Gali, V. L., Alyamany, K., & Jha, L. K. (2016). A Case of Hepatotoxicity Related to Kombucha Tea Consumption. *South Dakota Medicine: The Journal of the South Dakota State Medical Association*, *69*(1), 26–28.

49) Goh, W.N., Rosma A., Kaur, B., Fazilah, A., Karim A.A. & Bhat, R. (2012). Fermentation of black tea broth (Kombucha): I. Effects of sucrose concentration and fermentation time on the yield of microbial cellulose. *International Food Research Journal, 19*(1), 109-117.

50) Granum, P. E., & Lund, T. (1997). Bacillus cereus and its food poisoning toxins. *FEMS Microbiology Letters*, *157*(2), 223–228. https://doi.org/10.1111/j.1574-6968.1997.tb12776.x

51) Greenwalt, C. J., Ledford, R. A., & Steinkraus, K. (1998). Determination and Characterization of the Antimicrobial Activity of the Fermented TeaKombucha. *LWT - Food Science and Technology*, *31*(3), 291–296. https://doi.org/10.1006/fstl.1997.0354

52) Greenwalt, C. J., Steinkraus, K. H., & Ledford, R. A. (2000). Kombucha, the Fermented Tea: Microbiology, Composition, and Claimed Health Effects. *Journal of Food Protection*, *63*(7), 976–981. https://doi.org/10.4315/0362-028X-63.7.976

53) Gullo, M., Verzelloni, E., & Canonico, M. (2014). Aerobic submerged fermentation by acetic acid bacteria for vinegar production: Process and biotechnological aspects. *Process Biochemistry*, *49*(10), 1571–1579. https://doi.org/10.1016/j.procbio.2014.07.003

54) Hasler, C. M. (2002). Functional Foods: Benefits, Concerns and Challenges—A Position Paper from the American Council on Science and Health. *The Journal of Nutrition*, *132*(12), 3772–3781. https://doi.org/10.1093/jn/132.12.3772

55) Holbourn, A., & Hurdman, J. (2017). Kombucha: Is a cup of tea good for you? *BMJ Case Reports*, *2017*. https://doi.org/10.1136/bcr-2017-221702

56) Huang, D. B. (2016). Determination of Organic Acids in Kombucha Using a High-Pressure Ion Chromatography System. *Thermo Scientific*. 11.

57) Hungerford, J. M. (2010). Scombroid poisoning: A review. *Toxicon*, *56*(2), 231–243. https://doi.org/10.1016/j.toxicon.2010.02.006

58) Inoue, K., Shirai, T., Ochiai, H., Kasao, M., Hayakawa, K., Kimura, M., & Sansawa, H. (2003). Blood-pressure-lowering effect of a novel fermented milk containing γ-aminobutyric acid (GABA) in mild hypertensives. *European Journal of Clinical Nutrition*, *57*(3), 490–495. https://doi.org/10.1038/sj.ejcn.1601555

59) Ivanišová, E., Meňhartová, K., Terentjeva, M., Harangozo, Ľ., Kántor, A., & Kačániová, M. (2020). The evaluation of chemical, antioxidant, antimicrobial and sensory properties of kombucha tea beverage. *Journal of Food Science and Technology*, *57*(5), 1840–1846. https://doi.org/10.1007/s13197-019-04217-3

60) Jakubczyk, K., Kałduńska, J., Kochman, J., & Janda, K. (2020). Chemical Profile and Antioxidant Activity of the Kombucha Beverage Derived from White, Green, Black and Red Tea. *Antioxidants*, *9*(5), 447. https://doi.org/10.3390/antiox9050447

61) Jayabalan, R., Baskaran, S., Marimuthu, S., Swaminathan, K., & Yun, S. E. (2010). Effect of Kombucha Tea on Aflatoxin B1 Induced Acute Hepatotoxicity in Albino Rats-prophylactic and Curative Studies. *Journal of the Korean Society for Applied Biological Chemistry*, *53*(4), 407–416. https://doi.org/10.3839/jksabc.2010.063

62) Jayabalan, R., Malbaša, R. V., Lončar, E. S., Vitas, J. S., & Sathishkumar, M. (2014). A Review on Kombucha Tea—Microbiology, Composition, Fermentation, Beneficial Effects,

Toxicity, and Tea Fungus. *Comprehensive Reviews in Food Science and Food Safety*, *13*(4), 538–550. https://doi.org/10.1111/1541-4337.12073

63) Jayabalan, R., Malini, K., Sathishkumar, M., Swaminathan, K., & Yun, S.-E. (2010). Biochemical characteristics of tea fungus produced during kombucha fermentation. *Food Science and Biotechnology*, *19*(3), 843–847. https://doi.org/10.1007/s10068-010-0119-6

64) Jayabalan, R., Marimuthu, S., & Swaminathan, K. (2007). Changes in content of organic acids and tea polyphenols during kombucha tea fermentation. *Food Chemistry*, *102*(1), 392–398. https://doi.org/10.1016/j.foodchem.2006.05.032

65) Jędrusek-Golińska, A., Górecka, D., Buchowski, M., Wieczorowska-Tobis, K., Gramza-Michałowska, A., & Szymandera-Buszka, K. (2020). Recent progress in the use of functional foods for older adults: A narrative review. *Comprehensive Reviews in Food Science and Food Safety*, *19*(2), 835–856. https://doi.org/10.1111/1541-4337.12530

66) Johnson, K. M. (1984). Bacillus cereus Foodborne lllness-An Update. *Journal of Food Protection*, *47*(2), 145–153. https://doi.org/10.4315/0362-028X-47.2.145

67) *Joint FAO/WHO Expert Consultation on*. (2001). 34.

68) Kaewkod, T., Bovonsombut, S., & Tragoolpua, Y. (2019). Efficacy of Kombucha Obtained from Green, Oolong, and Black Teas on Inhibition of Pathogenic Bacteria, Antioxidation, and Toxicity on Colorectal Cancer Cell Line. *Microorganisms*, *7*(12), 700. https://doi.org/10.3390/microorganisms7120700

69) Kapp, J. M., & Sumner, W. (2019). Kombucha: A systematic review of the empirical evidence of human health benefit. *Annals of Epidemiology*, *30*, 66–70. https://doi.org/10.1016/j.annepidem.2018.11.001

70) Kappel, T., & Anken, R. H. (1993). The tea-mushroom. *Mycologist*, *7*(1), 12–13. https://doi.org/10.1016/S0269-915X(09)80616-2

71) Kapsak, W. R., Rahavi, E. B., Childs, N. M., & White, C. (2011). Functional Foods: Consumer Attitudes, Perceptions, and Behaviors in a Growing Market. *Journal of the American Dietetic Association*, *111*(6), 804–810. https://doi.org/10.1016/j.jada.2011.04.003

72) Karki, G. (2017). Bacterial Spore: Structure, types, sporulation and germination. *Online Biology Notes*. https://www.onlinebiologynotes.com/bacterial-spore-structure-types-sporulation-germination/

73) Kim, J., & Adhikari, K. (2020). Current Trends in Kombucha: Marketing Perspectives and the Need for Improved Sensory Research. *Beverages*, *6*(1), 15. https://doi.org/10.3390/beverages6010015

74) *Kombucha Brewers International – An Association of Commercial Kombucha Brewers*. (n.d.). Retrieved January 11, 2021, from https://kombuchabrewers.org/

75) Kotiranta, A., Lounatmaa, K., Haapasalo, M. (2000). Epidemiology and pathogenesis of Bacillus cereus infections. *Microbes and Infection*, *2*(2), 189–198. https://doi.org/10.1016/S1286-4579(00)00269-0

76) Kovacevic, Z., Davidovic G., Vuckovic-Filipovic, J., Janicijevic-Petrovic, M. A., Janicijevic, K., & Popovic, A. (2014). A Toxic Hepatitis Caused the Kombucha Tea – Case Report. *Macedonian Journal of Medical Sciences*, *7*(1). https://doi.org/10.3889/MJMS.1857-5773.2013.0341

77) Kumar, V., & Joshi, V. K. (2016). Kombucha: Technology, Microbiology, Production, Composition and Therapeutic Value. *International Journal of Food and Fermentation Technology*, *6*(1), 13–24. http://dx.doi.org.wv-o-ursus-proxy02.ursus.maine.edu/10.5958/2277-9396.2016.00022.2

78) Leal, J. M., Suárez, L. V., Jayabalan, R., Oros, J. H., & Escalante-Aburto, A. (2018). A review on health benefits of kombucha nutritional compounds and metabolites. *CyTA - Journal of Food*, *16*(1), 390–399. https://doi.org/10.1080/19476337.2017.1410499

79) Leventhal, A. G., Wang, Y., Pu, M., Zhou, Y., & Ma, Y. (2003). GABA and Its Agonists Improved Visual Cortical Function in Senescent Monkeys. *Science*, *300*(5620), 812–815. https://doi.org/10.1126/science.1082874

80) Li, H., & Cao, Y. (2010). Lactic acid bacterial cell factories for gamma-aminobutyric acid. *Amino Acids*, *39*(5), 1107–1116. https://doi.org/10.1007/s00726-010-0582-7

81) Liu, C.-H., Hsu, W.-H., Lee, F.-L., & Liao, C.-C. (1996). The isolation and identification of microbes from a fermented tea beverage, Haipao, and their interactions during Haipao fermentation. *Food Microbiology*, *13*(6), 407–415. https://doi.org/10.1006/fmic.1996.0047

82) Lončar, E. S., Petrovič, S. E., Malbača, R. V., & Verac, R. M. (2000). Biosynthesis of glucuronic acid by means of tea fungus. *Food / Nahrung*, *44*(2), 138–139. https://doi.org/10.1002/(SICI)1521-3803(20000301)44:2<138::AID-FOOD138>3.0.CO;2-#

83) Malbaša, R. V., Lončar, E. S., Vitas, J. S., & Čanadanović-Brunet, J. M. (2011). Influence of starter cultures on the antioxidant activity of kombucha beverage. *Food Chemistry*, *127*(4), 1727–1731. https://doi.org/10.1016/j.foodchem.2011.02.048

84) Malbaša, R., Lončar, E., & Djurić, M. (2008). Comparison of the products of Kombucha fermentation on sucrose and molasses. *Food Chemistry*, *106*(3), 1039–1045. https://doi.org/10.1016/j.foodchem.2007.07.020

85) Malbaša, R., Lončar, E., Djurić, M., & Došenović, I. (2008). Effect of sucrose concentration on the products of Kombucha fermentation on molasses. *Food Chemistry*, *108*(3), 926–932. https://doi.org/10.1016/j.foodchem.2007.11.069

86) Marsh, A. J., Hill, C., Ross, R. P., & Cotter, P. D. (2014). Fermented beverages with health-promoting potential: Past and future perspectives. *Trends in Food Science & Technology*, *38*(2), 113–124. https://doi.org/10.1016/j.tifs.2014.05.002

87) Mayser, P., Fromme, S., Leitzmann, C., & Gründer, K. (1995). The yeast spectrum of the "tea fungus Kombucha." *Mycoses*, *38*(7–8), 289–295. https://doi.org/10.1111/j.1439-0507.1995.tb00410.x

88) McKenney, P. T., Driks, A., & Eichenberger, P. (2013). The Bacillus subtilis endospore: Assembly and functions of the multilayered coat. *Nature Reviews Microbiology*, *11*(1), 33–44. https://doi.org/10.1038/nrmicro2921

89) Ministry of Agriculture and Forestry of Alberta, Canada (2006). Chapter 3: Bad bugs. Farmers market (1st ed., pp. 1-13). https://www1.agric.gov.ab.ca/$Department/deptdocs.nsf/all/explore13314/$FILE/Chapter3.pdf

90) Mizuta, A. G., de Menezes, J. L., Dutra, T. V., Ferreira, T. V., Castro, J. C., da Silva, C. A. J., Pilau, E. J., Machinski Junior, M., & Abreu Filho, B. A. de. (2020). Evaluation of antimicrobial activity of green tea kombucha at two fermentation time points against Alicyclobacillus spp. *LWT*, *130*, 109641. https://doi.org/10.1016/j.lwt.2020.109641

91) Molson Coors acquires Clearly Kombucha. (2018). Molson Coors Beer & Beyond. https://www.molsoncoorsblog.com/news/molson-coors-acquires-clearly-kombucha

92) Muhialdin, B. J., Osman, F. A., Muhamad, R., Sapawi, C. W. C. W. N. S., Anzian, A., Voon, W. W. Y., & Hussin, M. A. S. (2019). Effects of sugar sources and fermentation time on the properties of tea fungus (kombucha) beverage. *International Food Research Journal*, *26*(2), 481–487.

93) Murphy, T. E., Walia, K., & Farber, J. M. (2018). Safety Aspects and Guidance for Consumers on the Safe Preparation, Handling and Storage of Kombucha — A Fermented Tea Beverage. *Food Protection Trends*, 38(5), 329-337.

94) My, T. K. (2018). *Screening for gamma-aminobutyric acid (GABA)—Producing lactic acid bacteria (LAB) isolated from Vietnnam sources* [Thesis, International University - HCMC]. http://125.234.238.104:8080/handle/123456789/3205

95) Nakamura, H., Takishima, T., Kometani, T., & Yokogoshi, H. (2009). Psychological stress-reducing effect of chocolate enriched with γ-aminobutyric acid (GABA) in humans: Assessment of stress using heart rate variability and salivary chromogranin A. *International Journal of Food Sciences and Nutrition*, *60*(sup5), 106–113. https://doi.org/10.1080/09637480802558508

96) Neffe-Skocińska, K., Sionek, B., Ścibisz, I., & Kołożyn-Krajewska, D. (2017). Acid contents and the effect of fermentation condition of Kombucha tea beverages on physicochemical, microbiological and sensory properties. *CyTA - Journal of Food*, *15*(4), 601–607. https://doi.org/10.1080/19476337.2017.1321588

97) Notermans, & Batt. (1998). A risk assessment approach for food-borne Bacillus cereus and its toxins. *Journal of Applied Microbiology*, *84*(S1), 51S-61S. https://doi.org/10.1046/j.1365-2672.1998.0840s151S.x

98) Nummer, B. A. (2013). Kombucha brewing under the Food and Drug Administration model Food Code: Risk analysis and processing guidance. *Journal of Environmental Health*, *76*(4), 8–11.

99) Oh, C.-H., & Oh, S.-H. (2004). Effects of Germinated Brown Rice Extracts with Enhanced Levels of GABA on Cancer Cell Proliferation and Apoptosis. *Journal of Medicinal Food*, *7*(1), 19–23. https://doi.org/10.1089/109662004322984653

100) Oh, S., & Oh, C. (2003). Brown rice extracts with enhanced level of GABA stimulate immune cells. *Food Sci. Biotechnol.*, *12*, 248–252.

101) Opolski, A., Mazurkiewicz, M., Wietrzyk, J., Kleinrok, Z., & Radzikowski, C. (2000). The role of GABA-ergic system in human mammary gland pathology and in growth of transplantable murine mammary cancer. *Journal of Experimental & Clinical Cancer Research: CR*, *19*(3), 383–390.

102) Pegg, A. E. (2013). Toxicity of Polyamines and Their Metabolic Products. *Chemical Research in Toxicology*, *26*(12), 1782–1800. https://doi.org/10.1021/tx400316s

103) Peña-Gómez, N., Ruiz-Rico, M., Pérez-Esteve, É., Fernández-Segovia, I., & Barat, J. M. (2020). Microbial stabilization of craft beer by filtration through silica supports functionalized with essential oil components. *LWT*, *117*, 108626. https://doi.org/10.1016/j.lwt.2019.108626

104) PepsiCo Announces Definitive Agreement to Acquire KeVita, a Leader in Fermented Probiotic Beverages. (2016). PepsiCo, Inc. Official Website. Retrieved January 11, 2021, from http://www.pepsico.com/news/press-release/pepsico-announces-definitive-agreement-to-acquire-kevita-a-leader-in-fermented-p11222016

105) Perry, J. J., Nile, K., Martel, A., Fiore, M., Davis-Dentici, K., Hopkins, K., & Calder, B. (2020). Pasteurized and fermented beverages for valorization of maple sap. *Journal of Food Processing and Preservation*, *44*(8), e14623. https://doi.org/10.1111/jfpp.14623

106) Petruzzello, M. (n.d.). *Kombucha | Description, History, & Nutrition | Britannica*. Retrieved September 28, 2021, from https://www.britannica.com/topic/kombucha

107) Petty, F. (1995). GABA and mood disorders: A brief review and hypothesis. *Journal of Affective Disorders*, *34*(4), 275–281. https://doi.org/10.1016/0165-0327(95)00025-I

108) Phan, T. G., Duggin, G., Estell, J., Beer, I., Smith, D., & Ferson, M. J. (1998). Lead poisoning from drinking Kombucha tea brewed in a ceramic pot. *Medical Journal of Australia*, *169*(11–12), 644–646. https://doi.org/10.5694/j.1326-5377.1998.tb123448.x

109) PubChem. (n.d.). *gamma-Aminobutyric acid FDA Generally Recognized as Safe—GRAS Notices*. Retrieved December 1, 2021, from https://pubchem.ncbi.nlm.nih.gov/compound/119

110) Ray, B. (2004). *Fundamental food microbiology* (3rd ed). CRC Press.

111) Rektor, A. & Vatai, G. (2004). Application of membrane filtration methods for must processing and preservation. *Desalination*, *162*, 271–277. https://doi.org/10.1016/S0011-9164(04)00051-7

112) Reva, O. N., Zaets, I. E., Ovcharenko, L. P., Kukharenko, O. E., Shpylova, S. P., Podolich, O. V., de Vera, J.-P., & Kozyrovska, N. O. (2015). Metabarcoding of the kombucha microbial community grown in different microenvironments. *AMB Express*, *5*(1), 35. https://doi.org/10.1186/s13568-015-0124-5

113) Riol, C. D., Dietrich, R., Märtlbauer, E., & Jessberger, N. (2018). Consumed Foodstuffs Have a Crucial Impact on the Toxic Activity of Enteropathogenic Bacillus cereus. *Frontiers in Microbiology*, *9*, 1946. https://doi.org/10.3389/fmicb.2018.01946

114) Ruiz-Capillas, C., & Herrero, A. M. (2019). Impact of Biogenic Amines on Food Quality and Safety. *Foods*, *8*(2), 62. https://doi.org/10.3390/foods8020062

115) Russell, A. D. (1991). Mechanisms of bacterial resistance to non-antibiotics: Food additives and food and pharmaceutical preservatives. *Journal of Applied Bacteriology*, *71*(3), 191–201. https://doi.org/10.1111/j.1365-2672.1991.tb04447.x

116) Sadjadi, J. (1998). Cutaneous Anthrax Associated With the Kombucha "Mushroom" in Iran. *JAMA*, *280*(18), 1567–1568.

117) Saichana, N., Matsushita, K., Adachi, O., Frébort, I., & Frebortova, J. (2015). Acetic acid bacteria: A group of bacteria with versatile biotechnological applications. *Biotechnology Advances*, *33*(6, Part 2), 1260–1271. https://doi.org/10.1016/j.biotechadv.2014.12.001

118) Sang, V. T., Uyen, L. P., Hung, N. D. (2018). The increased gamma-aminobutyric acid content by optimizing fermentation conditions of bacteria from kimchi and investigation of its biological activities. *EurAsian Journal of BioSciences*, *12*, 369–376.

119) Santos, M. H. S. (1996). Biogenic amines: Their importance in foods. *International Journal of Food Microbiology*, *29*(2), 213–231. https://doi.org/10.1016/0168-1605(95)00032-1

120) Sasaki, S., Yokozawa, T., Cho, E. J., Oowada, S., & Kim, M. (2006). Protective role of γ-aminobutyric acid against chronic renal failure in rats. *Journal of Pharmacy and Pharmacology*, *58*(11), 1515–1525. https://doi.org/10.1211/jpp.58.11.0013

121) Schuller, H. M., Al-Wadei, H. A. N., & Majidi, M. (2008). Gamma-aminobutyric acid, a potential tumor suppressor for small airway-derived lung adenocarcinoma. *Carcinogenesis*, *29*(10), 1979–1985. https://doi.org/10.1093/carcin/bgn041

122) Seidl, R., Cairns, N., Singewald, N., Kaehler, S. T., & Lubec, G. (2001). Differences between GABA levels in Alzheimer's disease and Down syndrome with Alzheimer-like neuropathology. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *363*(2), 139–145. https://doi.org/10.1007/s002100000346

123) Shalaby, A. R. (1996). Significance of biogenic amines to food safety and human health. *Food Research International*, *29*(7), 675–690. https://doi.org/10.1016/S0963-9969(96)00066-X

124) Shelp, B. J., Bown, A. W., & McLean, M. D. (1999). Metabolism and functions of gamma-aminobutyric acid. *Trends in Plant Science*, *4*(11), 446–452. https://doi.org/10.1016/S1360-1385(99)01486-7

125) Shimada, M., Hasegawa, T., Nishimura, C., Kan, H., Kanno, T., Nakamura, T., & Matsubayashi, T. (2009). Anti-hypertensive effect of gamma-aminobutyric acid (GABA)-rich Chlorella on high-normal blood pressure and borderline hypertension in placebo-controlled double blind study. *Clinical and Experimental Hypertension (New York, N.Y.: 1993)*, *31*(4), 342–354. https://doi.org/10.1080/10641960902977908

126) Shinagawa, K. (1990). Analytical methods for Bacillus cereus and other Bacillus species. *International Journal of Food Microbiology*, *10*(2), 125–141. https://doi.org/10.1016/0168-1605(90)90061-9

127) Sievers, M., Lanini, C., Weber, A., Schuler-Schmid, U., & Teuber, M. (1995). Microbiology and Fermentation Balance in a Kombucha Beverage Obtained from a Tea Fungus Fermentation. *Systematic and Applied Microbiology*, *18*(4), 590–594. https://doi.org/10.1016/S0723-2020(11)80420-0

128) Siró, I., Kápolna, E., Kápolna, B., & Lugasi, A. (2008). Functional food. Product development, marketing and consumer acceptance—A review. *Appetite*, *51*(3), 456–467. https://doi.org/10.1016/j.appet.2008.05.060

129) Smolinske, S. C. (2005). Herbal Product Contamination and Toxicity. *Journal of Pharmacy Practice*, *18*(3), 188–208. https://doi.org/10.1177/0897190005277217

130) Sreeramulu, G., Zhu, Y., & Knol, W. (2000). Kombucha Fermentation and Its Antimicrobial Activity. *Journal of Agricultural and Food Chemistry*, *48*(6), 2589–2594. https://doi.org/10.1021/jf991333m

131) Sreeramulu, G., Zhu, Y., & Knol, W. (2001). Characterization of Antimicrobial Activity in Kombucha Fermentation. *Acta Biotechnologica*, *21*(1), 49–56. https://doi.org/10.1002/1521-3846(200102)21:1<49::AID-ABIO49>3.0.CO;2-G

132) Srihari, T., & Satyanarayana, U. (2012). Changes in Free Radical Scavenging Activity of Kombucha during Fermentation. *J. Pharm. Sci.*, 4.

133) Srinivasan, R., Smolinske, S., PharmD, & Greenbaum, D. (1997). Probable Gastrointestinal Toxicity of Kombucha Tea. *Journal of General Internal Medicine*, *12*(10), 643–645. https://doi.org/10.1046/j.1525-1497.1997.07127.x

134) Steinkraus, K. H., Shapiro, K. B., Hotchkiss, J. H., & Mortlock, R. P. (1996). Investigations into the antibiotic activity of tea fungus/kombucha beverage. *Acta Biotechnologica*, *16*(2–3), 199–205. https://doi.org/10.1002/abio.370160219

135) Stenfors Arnesen, L. P., Fagerlund, A., & Granum, P. E. (2008). From soil to gut: Bacillus cereus and its food poisoning toxins. *FEMS Microbiology Reviews*, *32*(4), 579–606. https://doi.org/10.1111/j.1574-6976.2008.00112.x

136) St-Pierre, D. L. (2019). *Microbial Diversity of the Symbiotic Colony of Bacteria and Yeast (SCOBY) and its Impact on the Organoleptic Properties of Kombucha* [The University of Maine]. https://digitalcommons.library.umaine.edu/etd/3063

137) SungHee Kole, A., Jones, H. D., Christensen, R., & Gladstein, J. (2009). A case of Kombucha tea toxicity. *Journal of Intensive Care Medicine*, *24*(3), 205–207. https://doi.org/10.1177/0885066609332963

138) Tabanelli, G. (2020). Biogenic Amines and Food Quality: Emerging Challenges and Public Health Concerns. *Foods*, *9*(7), 859. https://doi.org/10.3390/foods9070859

139) Talawat, S., Ahantharik, P., Laohawiwattanakul, S., Premsuk, A., & Ratanapo, S. (2006). Efficacy of Fermented Teas in Antibacterial Activity. *Agriculture and Natural Resources*, *40*(4), 925–933.

140) Tarver, T. (2016). *The Good, the Bad, and the Ugly: A Fermentation Saga*. Retrieved November 30, 2021, from https://www.ift.org/news-and-publications/food-technology-magazine/issues/2016/december/columns/inside-academia-fermentation-science-washington-state-university-university-of-idaho

141) Teoh, A. L., Heard, G., & Cox, J. (2004). Yeast ecology of Kombucha fermentation. *International Journal of Food Microbiology*, *95*(2), 119–126. https://doi.org/10.1016/j.ijfoodmicro.2003.12.020

142) The Quick Guide to Kombucha pH. (2020). *Brew Buch*. https://brewbuch.com/kombucha-ph-acid-balance/

143) Ting Wong, C. G., Bottiglieri, T., & Snead III, O. C. (2003). GABA, γ-hydroxybutyric acid, and neurological disease. *Annals of Neurology*, *54*(S6), S3–S12. https://doi.org/10.1002/ana.10696

144) Tripathi, M. K., & Giri, S. K. (2014). Probiotic functional foods: Survival of probiotics during processing and storage. *Journal of Functional Foods*, *9*, 225–241. https://doi.org/10.1016/j.jff.2014.04.030

145) Troitino, C. (2017). *Kombucha 101: Demystifying The Past, Present And Future Of The Fermented Tea Drink*. Forbes. Retrieved January 21, 2021, from https://www.forbes.com/sites/christinatroitino/2017/02/01/kombucha-101-demystifying-the-past-present-and-future-of-the-fermented-tea-drink/

146) TTB | kombucha | kombucha information and resources. (2017). Retrieved from https://www.ttb.gov/kombucha/kombucha-general.shtml. Date accessed: January 11, 2021.

147) Tur, J. A., & Bibiloni, M. M. (2016). Functional Foods. In B. Caballero, P. M. Finglas, & F. Toldrá (Eds.), *Encyclopedia of Food and Health* (pp. 157–161). Academic Press. https://doi.org/10.1016/B978-0-12-384947-2.00340-8

148) U.S. Food and Drug Administration. (n.d.). *Substances Added to Food (formerly EAFUS)*. Retrieved December 1, 2021, from https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=FoodSubstances&id=AMINOBUTYRICACID

149) Urala, N., & Lähteenmäki, L. (2003). Reasons behind consumers' functional food choices. *Nutrition & Food Science*, *33*(4), 148–158. https://doi.org/10.1108/00346650310488499

150) Velićanski, A. S., Cvetković, D. D., Markov, S. L., Šaponjac, V. T. T., & Vulić, J. J. (2014). Antioxidant and Antibacterial Activity of the Beverage Obtained by Fermentation of Sweetened Lemon Balm (Melissa officinalis L.) Tea with Symbiotic Consortium of Bacteria and Yeasts. *Food Technology and Biotechnology*, *52*(4), 420–429. https://doi.org/10.17113/ftb.52.04.14.3611

151) Velićanski, A. S., Cvetković, D. D., Markov, S. L., Tumbas, V. T., & Savatović, S. M. (2007). Antimicrobial and antioxidant activity of lemon balm Kombucha. *Acta Periodica Technologica*, *38*, 165–172.

152) Villarreal-Soto, S. A., Beaufort, S., Bouajila, J., Souchard, J.-P., & Taillandier, P. (2018). Understanding Kombucha Tea Fermentation: A Review. *Journal of Food Science*, *83*(3), 580–588. https://doi.org/10.1111/1750-3841.14068

153) Vīna, I., Semjonovs, P., Linde, R., & Deniņa, I. (2013). Current Evidence on Physiological Activity and Expected Health Effects of Kombucha Fermented Beverage. *Journal of Medicinal Food*, *17*(2), 179–188. https://doi.org/10.1089/jmf.2013.0031

154) Wang, Y., Ji, B., Wu, W., Wang, R., Yang, Z., Zhang, D., & Tian, W. (2014). Hepatoprotective effects of kombucha tea: Identification of functional strains and quantification of functional components. *Journal of the Science of Food and Agriculture*, *94*(2), 265–272. https://doi.org/10.1002/jsfa.6245

155) Watawana, M. I., Jayawardena, N., & Waisundara, V. Y. (2018). Value-added Tea (Camellia sinesis) as a Functional Food using the Kombucha 'Tea Fungus.' *Chiang Mai J. Sci.*, *45*(1), 136–146.

156) Watawana, M. I., Jayawardena, N., Gunawardhana, C. B., & Waisundara, V. Y. (2015). Health, wellness, and safety aspects of the consumption of Kombucha. *Journal of Chemistry*, *2015*. https://doi.org/10.1155/2015/591869

157) Wiens, S. C., & Trudeau, V. L. (2006). Thyroid hormone and γ-aminobutyric acid (GABA) interactions in neuroendocrine systems. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *144*(3), 332–344. https://doi.org/10.1016/j.cbpa.2006.01.033

158) Wijman, J. G. E., Leeuw, P. P. L. A. de, Moezelaar, R., Zwietering, M. H., & Abee, T. (2007). Air-Liquid Interface Biofilms of Bacillus cereus: Formation, Sporulation, and Dispersion. *Applied and Environmental Microbiology*, *73*(5), 1481–1488. https://doi.org/10.1128/AEM.01781-06

159) Yang, Z.-W., Ji, B.-P., Zhou, F., Li, B., Luo, Y., Yang, L., & Li, T. (2009). Hypocholesterolaemic and antioxidant effects of kombucha tea in high-cholesterol fed mice. *Journal of the Science of Food and Agriculture*, *89*(1), 150–156. https://doi.org/10.1002/jsfa.3422

160) Yıkmış, S., & Tuğgüm, S. (2019). Evaluation of Microbiological, Physicochemical and Sensorial Properties of Purple Basil Kombucha Beverage. *Turkish Journal of Agriculture - Food Science and Technology*, *7*(9), 1321–1327. https://doi.org/10.24925/turjaf.v7i9.1321-1327.2550

161) Yoto, A., Murao, S., Motoki, M., Yokoyama, Y., Horie, N., Takeshima, K., Masuda, K., Kim, M., & Yokogoshi, H. (2012). Oral intake of γ-aminobutyric acid affects mood and activities of central nervous system during stressed condition induced by mental tasks. *Amino Acids*, *43*(3), 1331–1337. https://doi.org/10.1007/s00726-011-1206-6

162) Zou, C., Li, R.-Y., Chen, J.-X., Wang, F., Gao, Y., Fu, Y.-Q., Xu, Y.-Q., & Yin, J.-F. (2021). Zijuan tea- based kombucha: Physicochemical, sensorial, and antioxidant profile. *Food Chemistry*, *363*, 130322. https://doi.org/10.1016/j.foodchem.2021.130322

CHAPTER 2

BACILLUS CEREUS SPORE CONTAMINATION OF KOMBUCHA SYSTEMS BEFORE AND AFTER FERMENTATION VIA THE SCOBY, UNFERMENTED LIQUID, OR FERMENTED LIQUID

2.1 Abstract

Kombucha is known for its high endogenous microbial load and highly acidic nature that inhibits most extraneous microorganisms from surviving in the beverage. Although bacterial spore contamination and survival in the culture has been documented, it is unknown whether spores can survive in the liquid, or whether they are transmitted to daughter SCOBYs. In this study, *Bacillus cereus* spores were inoculated onto SCOBY, in unfermented tea, or in fermented kombucha, in triplicate. Survival and redistribution (to daughter SCOBY, finished, or stored fermentate) during and after fermentation were assessed by cultural selective enrichment. The data collected from the presence or absence of *B. cereus* in each sample were analyzed using best fit logistic models (glm function) in R studio to determine any significant differences ($p < 0.05$) for each inoculation treatment. Retention of spores was observed in the mother SCOBY after direct SCOBY inoculation, and *B. cereus* was inconsistently transferred to the daughter SCOBY or liquid. Only one replicate showed survival of spores in the finished kombucha after direct liquid (post-fermentation) inoculation, and *B. cereus* was not detected in kombucha after secondary fermentation or refrigerated storage. While no apparent pattern in survival was observed, results suggest that contamination from bacterial spores may occur at any stage of fermentation. Additional investigation should explore whether this risk is mitigated by subsequent product storage.

2.2 Introduction

Kombucha, a sweetened tea beverage fermented by a symbiotic colony of bacteria and yeast (SCOBY), has seen a significant recent increase in popularity. The fermented beverage has been produced and consumed since 220 BCE (Greenwalt et al., 2000; Jayabalan et al., 2014). It did not enter the US commercial market until the 1990s and was only homebrewed before that time with the starter culture (SCOBY) being passed on from friends and family members (Greenwalt et al., 2000). Kombucha's market entry as a packaged good coincided with the building momentum of the functional foods movement in the late 1990s, and as a result, kombucha and other fermented foods have become emblems of the movement due to claims such as improved gut health, disease prevention, and high antioxidant content (Jayabalan et al., 2014; Chakravorty, 2019). These health benefits have not been substantiated due to a lack of research in human test subjects. Only animal models have been used, and they may not necessarily simulate the same effects in humans (Greenwalt et al., 2000). By the 2010s, kombucha was increasingly familiar to American consumers. However, today there is still much uncertainty surrounding kombucha.

It is widely assumed that kombucha poses a low risk from contamination with foodborne pathogens because it is highly acidic, has high microbial competition from yeast and bacteria species from the SCOBY, and contains ethanol and other chemical components, deterring harmful organism growth (Greenwalt et al., 1998; Jayabalan et al., 2014; Bhattacharya, 2018; Battikh et al., 2013; Deghrigne et al., 2013; Četojević-Simin et al., 2012; Sreeramulu et al., 2001; Bauer et al., n.d.) Considered a high acid food by the food industry, kombucha's final pH of ~2.5 often limits the ability of many spoilage organisms to grow because most of them cannot grow below a pH of 4.0 (Greenwalt et al., 2000). However, there are no studies ensuring kombucha is

100% safe from contaminants in its final product form. The available research that has been done

on kombucha mostly focuses on its composition and/or potential health benefits. This includes

the antimicrobial activity of kombucha, regardless of green or black tea origin, but these studies

only focus on Gram-positive and Gram-negative vegetative cells in the final product after

fermentation (Greenwalt et al., 1998; Bhattacharya, 2016). Bacterial spores, in contrast, are

hardier than vegetative microorganisms and are able to survive in harsh conditions. There has

been a documented case of twenty people contracting cutaneous anthrax after applying a

kombucha SCOBY to the skin as a painkiller (Sadjadi, 1998; Smolinske, 2005). *Bacillus*

anthracis was identified as the culprit, and seeing as it is a spore-former, it was concluded that

Bacillus spores are capable of surviving in kombucha SCOBYs. Knowing this, it is possible that

spore-contaminated SCOBYs could potentially disseminate the spores into the kombucha liquid,

presenting a food safety hazard for kombucha consumers.

Bacillus cereus is a Gram-positive mesophilic spore-former capable of causing diarrheal

and emetic foodborne illnesses in those that consume it or its toxins (Granum & Lindbäck, 2012;

Batt, 2014). Although these illnesses do not usually warrant a hospital visit or cause fatality, *B.*

cereus is considered one of the two most important food spoilage and food poisoning spore-

formers (Russell, 1991). Its spores are heat- and acid-stable, making it conceivable that they may

survive in kombucha's harsh environment and end up in the final product (Notermans & Batt,

1998). One report describes the minimum pH for the growth of *B. cereus* as being 4.35, yet this

only describes vegetative cells and not endospores (Notermans & Batt, 1998). However, the

growth of *B. cereus* is not the only concern. The presence and survival of endospores in a

product, which is inevitably at a pH lower than 4.35, is also of great concern. *B. cereus* spores

could germination in the human GI tract and thus threaten a foodborne outbreak, putting public

health at risk. *B. cereus* spores are also known to be more hydrophobic than any other *Bacillus* spp. spores, making them capable of adhering to various surface types. This makes them less susceptible to cleaning practices and disinfection, and as a result, there is a greater chance of spores remaining on equipment surfaces, potentially contaminating foods like kombucha (Granum & Lindbäck, 2012).

There are few published studies that assess survival of foodborne pathogens introduced to the substrate (sweetened tea) before kombucha fermentation. Research on kombucha's antimicrobial abilities against pathogenic Gram-positive and Gram-negative microorganisms has been conducted by inoculating the fully fermented liquid (Jayabalan et al., 2014). Realistically, kombucha could be contaminated at any stage of production, and contaminants could be introduced, either via the SCOBY, the broth, etc. A better understanding of contamination dynamics could aid in increasing the safety of this product, such as producing more effective HACCP plans and proactive safety measures in brewing facilities, and providing guidance for home brewers who do not follow established food safety and sanitation plans.

This research involved examining the different routes of contamination in a kombucha system (i.e., the SCOBY, the substrate, and the finished product) before, during, and after fermentation. *B. cereus* spores were inoculated in three different kombucha systems three different ways before and after fermentation. There were four main objectives throughout the study, which were to determine: (i) if *B. cereus* spores could be more readily incorporated into kombucha systems through the SCOBY, unfermented liquid, or fermented liquid, (ii) if *B. cereus* spores could survive in kombucha's hostile environment during and/or after fermentation, (iii) if *B. cereus* spores be passed on from mother SCOBY/liquid to daughter SCOBY, and (iv) if *B. cereus* spores could survive and be found in the final "consumable" product.

2.3 Methods and Materials

2.3.1 SCOBY Preparation

Kombucha SCOBYs from three different sources (IBIV Kombucha, Chesapeake, VA), designated throughout this chapter as A, B, and C, were grown until a biomass of 225+ grams was reached. This was accomplished by transferring the SCOBYs from a container of finished kombucha to a container of freshly brewed, acidified sweetened black tea every 14 days to increase the biomass. For each container, (inoculated with one SCOBY), 1000 mL of deionized water was boiled, and four bags of Lipton (Hoboken, NJ) black tea were brewed for 10 minutes. The tea was cooled in an ice bath until approximately ambient temperature (~20°C) was reached. The tea bags were removed, and the tea was combined with 100 grams of Domino's (Yonkers, NY) pure white cane sugar and 5% (vol/vol) of the liquid from the previous kombucha batch (produced with the same SCOBY) was added for acidification. Once thoroughly mixed until the sugar dissolved, the SCOBY was added to the container which was then covered with a clean (sterile) cheesecloth and placed in a bacteriostatic incubator set to 25°C. pH was taken using the Thermo Scientific (Waltham, MA) Orion Star A111 pH meter at the start of the fermentation cycle, every three days thereafter, and at the end of the fermentation cycle (pH of ≤ 3.0). The pH meter was calibrated before every grouping of pH reading samples with an accuracy of $\geq 95\%$ every time. The approximate starting pH of each fermentation cycle was 3.4, and the approximate ending pH was 2.5. Once a SCOBY accumulated the appropriate amount of biomass, it was covered with the container lid and placed in the refrigerator until later use.

2.3.2 Spore Suspension

Bacillus cereus ATCC 14579 was obtained from the American Type Culture Collection (Manassas, VA). Spore suspension preparation procedure was adapted from Sawai et al. (1995). A 0.5 mL portion of tryptone soy broth (TSB) (Difco, Spark, MD) culture inoculated with *B. cereus* (incubated at 32°C for 24 hours) was pipetted into 100 mL of a sporulation medium, which consisted of 5 g beef extract (Oxoid, Basingstoke, England), 1 g bacto peptone (Difco, Spark, MD), 0.5 g NaCl (Fisher Scientific, Waltham, MA), 0.05 g MnSO$_4$ (MP Biomedicals, Solon, OH), and 100 mL of neutral deionized water. The sporulation medium was autoclaved before *B. cereus* inoculation. Inoculated sporulation medium was incubated in 100 mL aliquots at 37°C for seven days. The sporulation medium was centrifuged in an Eppendorf (Hauppauge, NY) Centrifuge 5810 R. The spores were washed three times with a sterile 0.9% saline solution by centrifugation at 12,857 x *g* at 20°C then resuspended in saline. The final spore suspensions were stored at 4°C until use. Spore suspension concentrations were confirmed using a Neubauer improved hemocytometer (Hausser Scientific, Horsham, PA) and compound light microscope, according to established methods (*Counting Cells in a Hemocytometer*, n.d.). A working stock was created by diluting the original spore solution in sterile 0.9% saline as appropriate to achieve ~7.0 log spores/mL.

2.3.3 SCOBY Inoculation

A schematic representation of inoculation and analysis workflow is shown in Figure 2.2. Pieces of SCOBY, weighing 25 g each, were cut from each SCOBY system using a kitchen knife in replicates of three. Each 25 g SCOBY piece was placed in a clean, labeled 24 oz mason jar (Ball, Broomfield, CO) and inoculated with 1,000 *B. cereus* spores. Jars were sealed and

incubated overnight (18 hours) at ambient temperature. The following day black tea was prepared by brewing four Lipton black tea bags per jar in 600 mL of boiling water for 10 minutes. Once cooled to room temperature in an ice bath, the tea was acidified to a pH of 4.0 with kombucha produced by the appropriate SCOBY. About 50 grams of white sugar was added to the 600 mL of tea as a carbohydrate source for the SCOBY. The acidified sweet tea was then added to each jar containing inoculated SCOBY, covered with cheesecloth, and put in a 25°C incubator to ferment. pH was taken every two days until a pH of ≤ 3.0 was reached, indicating fermentation was complete.

Once fermentation was completed, the mother and daughter SCOBYs were removed, separated from each other, and weighed. The mother SCOBY was enriched in tryptone soy polymyxin (TSP) broth (Difco, Sparks, MD and HiMedia, Lincoln University, PA) at a 1:9 ratio of SCOBY:enrichment broth, incubated under conditions detailed in Table 2.1, and blended in a Waring (Stamford, CT) 700S blender for one minute (Post, n.d.). The daughter SCOBY was cut approximately in half using a sterile scalpel (Cynamed, Lorton, VA). One half was enriched in TSP broth at a 1:9 ratio of SCOBY:enrichment broth and blended as previously described. The other was kept under refrigeration for further analyses as needed. Aliquots of fermented kombucha liquid were enriched as well. Remaining kombucha liquid was split into two. Half of the fermented liquid was refrigerated at 4°C to simulate storage; the other half was subjected to secondary fermentation by the addition of 20% (vol/vol) of Hannaford (Scarborough, ME) 100% apple juice and incubated according to Table 2.1. Lastly, samples of fermented kombucha liquid were directly spread plated in duplicate onto mannitol yolk polymyxin (MYP) agar (HiMedia, Lincoln University, PA). The plates were incubated according to Table 2.1 to allow for any bacterial colony formation.

The following day all enrichments (mother SCOBY, daughter SCOBY, and fermented liquid) were spread plated onto MYP agar in duplicate and incubated consistent with conditions detailed in Table 2.1. After 18 hours of incubation, all plates were checked for growth of colonies characteristic of *B. cereus* and results recorded (*MYP Agar Base Technical Data sheet,* 2015). If the enriched daughter SCOBY was positive for *B. cereus* (Figure 2.1), the reserved daughter SCOBY half was used in the next brewing batch with all fractions analyzed as described here to check for the presence or absence of *B. cereus*.

vs.

Negative   Positive

Figure 2.1. Mannitol Yolk Polymyxin (MYP) agar plates comparing negative and positive growth for *B. cereus*.

The image on the left shows confluent growth, but the media color has changed to from pale pink to a bright yellow, indicating this bacterial growth is not *B. cereus*. The image on the right shows confluent growth, opaque zones around the colonies, and a media color change from pale pink to hot pink, indicating the presence of *B. cereus*.

2.3.4 Liquid Inoculation

Black tea was prepared and acidified as previously described. The acidified sweet tea was then divided among clean and labeled jars and inoculated with 24,000 *B. cereus* spores. At this point, inoculated tea was plated onto MYP in duplicate and incubated according to Table 2.1. Pieces of SCOBY, weighing 25 grams each, were cut from each SCOBY system in replicates of three, totaling 9 pieces of SCOBY across all systems. The SCOBYs were added to the inoculated

tea, covered with cheesecloth, and put in a 25°C incubator to ferment. pH was taken every two

days until a pH of ≤ 3.0 was reached, indicating fermentation was complete.

Table 2.1. Treatments performed after fermentation and their respective temperature and duration throughout all *B. cereus* inoculations

Treatment	Temperature (°C)	Duration (hours)	Inoculations Performed In
Enrichments	37	24	SCOBY, Liquid, Finished Product
MYP Plating	32	18	SCOBY, Liquid, Finished Product
Secondary Fermentation	25	48	SCOBY, Liquid, Finished Product
Refrigeration	4	1 week	Finished Product

After fermentation, the mother and daughter SCOBYs were removed from the jars. The

mother SCOBY was disposed of while the daughter SCOBY was weighed and cut in half. Half

of the daughter SCOBY was stored for additional analyses, and the other half was enriched in

TSP broth as previously described. Simultaneously, a portion (25 mL) of the fermented liquid

was enriched in TSP broth. The remaining liquid was split into two. Half of the fermented liquid

was refrigerated at 4°C to simulate storage; the other half was subjected to secondary

fermentation by the addition of 20% (vol/vol) of Hannaford (Scarborough, ME) 100% apple

juice and incubated according to Table 2.1. Lastly, samples of fermented kombucha liquid were

directly spread plated in duplicate onto MYP agar. The plates were incubated according to Table

2.1 to allow for any bacterial colony formation.

The following day all enrichments (daughter SCOBY and fermented liquid) were plated

onto MYP agar in duplicate and incubated consistent with conditions detailed in Table 2.1. If the

enriched fermented liquid plates were positive for *B. cereus*, the secondary fermentate was plated

onto MYP agar in duplicate and subsequently assessed for the presence of *B. cereus* after 18

hours. Additionally, if the enriched fermented liquid plates were positive for *B. cereus*, the

fermented liquid stored under refrigeration was enriched with TSP broth after one week and

plated onto MYP agar in duplicate to see if viable *B. cereus* spores were still present in the liquid

after storage. If the enriched daughter SCOBY plates were positive for *B. cereus*, the saved

daughter SCOBY half was used in the next brewing batch and went through the process again to

check for the presence or absence of *B. cereus*.

2.3.5 Finished Product Inoculation

Black tea was prepared and acidified as previously described. The acidified sweet tea and

pieces of SCOBY, weighing 25 g each, were simultaneously added to clean and labeled jars,

covered with cheesecloth, and put in a 25°C incubator to ferment. pH was taken every two days

until a pH of 3.0 was reached, indicating fermentation was complete.

Once fermentation was complete, the mother and daughter SCOBYs were removed from

the jars. The mother SCOBY was disposed of while the daughter SCOBY was weighed and

stored at 4ºC for additional analyses. The fermented liquid was then inoculated with 24,000 *B.*

cereus spores per jar. Samples of inoculated tea were plated onto MYP in duplicate and placed in

a 32°C incubator for 18 hours. The remaining liquid was split into two. Half of the fermented

liquid was refrigerated at 4°C to simulate storage; the other half was subjected to secondary

fermentation by the addition of 20% (vol/vol) of Hannaford (Scarborough, ME) 100% apple

juice and incubated in conditions consistent with Table 2.1. After 48 hours, a portion (25 mL) of

the secondary fermentate was enriched in TSP broth and incubated according to Table 2.1. The remaining secondary fermentate was stored at 4ºC for additional analyses.

The following day the secondary fermentate enrichments were plated onto MYP agar in duplicate and incubated according to Table 2.1. If the enriched secondary fermentate plates were positive for *B. cereus*, the secondary fermentate stored under refrigeration was enriched with TSP broth after one week of storage week and plated onto MYP agar in duplicate to see if viable *B. cereus* spores were still present in the liquid after storage. The half of the fermented liquid that did not go through secondary fermentation was refrigerated consistent with conditions detailed in Table 2.1. A portion (25 mL) of the fermented liquid was then enriched in TSP broth and incubated according to Table 2.1. The next day samples of this enrichment were plated onto MYP in duplicate and incubated according to Table 2.1. The plates were checked and recorded for *B. cereus* growth, determining whether the spores could survive storage without having gone through secondary fermentation.

2.3.6 Statistical Analysis

Data were analyzed in R and R studio Version 1.4.1103 (Boston, MA) using multiway ANOVA and the best fit logistic regression models (glm function) to generate a worldwide model to recognize the interactions defined by the inoculation methods and by the portion of the kombucha system inoculated. Tukey's post hoc test was then used as a pairwise comparison to identify any significant differences ($p < 0.05$) among the interactions.

Figure 2.2. Overall inoculation methods of B. cereus spores into the SCOBY, unfermented tea (liquid), or fermented tea (finished product)

2.4 Results and Discussion

Table 2.2 displays the presence and absence results of the SCOBY inoculations among the three tested kombucha systems. All enrichments had a detection limit of 1 CFU/25 mL or 1 CFU/25 grams. For the direct liquid samples, which were not enriched, a negative result indicated <10 CFU/mL. All but one mother SCOBYs were positive for presence of *B. cereus*, as expected. Kombucha system A was negative for the presence of *B. cereus* in the enriched daughter SCOBY, but two of three enriched liquid samples were positive for presence of *B. cereus*. Even though the replicates were positive, there was a decrease of *B. cereus* from the amount initially inoculated. Consequently, those two replicate jars went through secondary fermentation and were plated onto MYP agar. As indicated by Table 2.2, liquid from secondary fermentation tested negative, suggesting that subsequent fermentation was sufficient to inactivate the spores.

Table 2.2. Presence or absence of *B. cereus* in inoculated kombucha cultures and fermentate at various stages of preparation [SCOBY inoculation of *B. cereus*]

Kombucha System	Enriched Mother SCOBY[3]	Enriched Daughter SCOBY[3]	Direct Liquid [after fermentation][4]	Enriched Liquid[3]	Secondary Fermentation[3]	Total[2]
A	2/3	0/3	0/3	2/3	0/2	4/12
B	3/3	1/3	0/3	1/3	0/1	5/12
C	3/3	0/3	0/3	0/3	N/A[1]	3/12

[1]: No enriched liquid samples were positive, so there was no need to check secondary fermentation samples because it is already known there is no *B. cereus* present.
[2]: Excludes secondary fermentation column from total count
[3]: Positive enrichment means at least 1 CFU/25 mL
[4]: Negative liquid means <10 CFU/mL

Kombucha system B had one replicate out of three return positive for both the enriched liquid samples and the enriched daughter SCOBY samples. The secondary fermentation of the one positive enriched liquid replicate was hence plated and returned negative for the presence of *B. cereus*. The saved daughter SCOBY half of the one positive enriched daughter SCOBY replicate was used to rebrew a new batch of kombucha under the same conditions as the mother SCOBY was brewed in (Section 2.3.3). The enriched daughter SCOBY (now acting as the "mother SCOBY" in this scenario) was positive, as expected, but the enriched [grand]daughter SCOBY, direct liquid, and enriched liquid samples were all negative, meaning the transfer and presence of *B. cereus* ended here. This kombucha system showed a 33% transfer rate from mother to daughter SCOBY and a 33% transfer rate from SCOBY to liquid, however the liquid was only positive for *B. cereus* when enriched and not when directly plated onto MYP without enrichment, indicating a low level of survival. Presence of *B. cereus* was no longer detected once secondary fermentation had occurred.

All samples taken for kombucha system C, besides the enriched mother SCOBY, were negative for the presence of *B. cereus*. No enriched liquid samples were positive, indicating the absence of *B. cereus*. Because there was no *B. cereus* detected in the enriched liquid samples, secondary fermentation was not performed.

Among all three kombucha systems, the inoculated mother SCOBY samples were almost always positive, as expected, but the respective daughter SCOBY and enriched liquid samples were not always positive. This implies that the *B. cereus* spores were able to be incorporated into the SCOBY, and the transfer of spores from mother SCOBY to daughter SCOBY or from mother SCOBY to the liquid was sporadic. Upon contamination, this tells us pathogenic spore-formers

are capable of being taken up by the SCOBY but have a low chance of being passed on either to the daughter SCOBY or liquid.

Table 2.3 displays the presence and absence results of *B. cereus* resulting from inoculation of tea before fermentation. There were no positives for any of the replicates of all three kombucha systems for the direct liquid samples taken at inoculation and samples taken immediately after fermentation. These samples were not enriched with the TSP enrichment broth. Theoretically, there should be about 4 spores per plating of direct liquid at inoculation and after fermentation based on the amount of spores inoculated into the broth (24,000 spores/600 mL), but samples were taken from the top inch of the broth which may have been void of spores, regardless of stirring beforehand. However, two of the three systems displayed positive replicates later on in testing. This is because there was not enough detectable *B. cereus* to warrant presence without enrichment when taken at inoculation and right after fermentation.

Table 2.3. Presence or absence of *B. cereus* in inoculated kombucha fermentate and associated cultures at various stages of preparation [Liquid inoculation of *B. cereus*]

Kombucha System	Liquid at Inoculation	Direct Liquid [after fermentation]	Enriched Daughter SCOBY	Enriched Liquid	Secondary Fermentation
A[2]	0/3	0/3	0/3	**1/3**	0/1
B[3]	0/3	0/3	**1/3**	0/3	N/A[1]
C[4]	0/3	0/3	0/3	0/3	N/A[1]

[1]: No enriched liquid samples were positive, so there was no need to check secondary fermentation samples because it is already known there is no *B. cereus* present.
[2]: Average pH at the end of fermentation for kombucha system A was 3.01
[3]: Average pH at the end of fermentation for kombucha system B was 2.99
[4]: Average pH at the end of fermentation for kombucha system C was 3.00

Kombucha system A revealed only one positive replicate for enriched liquid among the entirety of the samples tested. Consequently, the secondary fermentation of this positive replicate was plated onto MYP agar and returned negative for the presence of *B. cereus*. Kombucha system B also exhibited only one positive replicate for the enriched daughter SCOBY among the entirety of the samples tested. This positive result warranted the second brewing of daughter SCOBY, using the same conditions as the mother SCOBY was brewed in. However, because no enriched liquid plates were positive, secondary fermentation was not necessary to carry out because it was already determined there was no *B. cereus* present in the liquid. The second brewing results of the daughter SCOBY indicated the absence of *B. cereus* everywhere except in the original daughter SCOBY which remained positive for *B. cereus*, as established from before. Much like the SCOBY inoculation, all samples taken from kombucha system C were negative for the presence of *B. cereus*. No enriched liquid samples were positive, indicating the absence of *B. cereus* in the liquid, so plating the secondary fermentation samples to check for the presence of *B. cereus* was unnecessary.

Survival of spores inoculated into the liquid was observed in only one out of nine replicates. Transfer of spores from the liquid to the culture was not evident except for one daughter SCOBY replicate in the Kombucha system B and therefore displays the transfer of spores from contaminated tea (pre-fermentation) is less likely than transfer from contaminated SCOBY.

Table 2.4 displays the presence and absence results of *B. cereus* inoculation directly into finished product, or fermented liquid, among the three tested kombucha systems. No positives were detected for any of the replicates of all three systems for the liquid samples taken after fermentation when inoculated with *B. cereus* spores. These samples were not enriched with the

TSP enrichment broth. Theoretically, there should be about 4 spores per plating of direct liquid at inoculation and after fermentation based on the amount of spores inoculated into the broth (24,000 spores/600 mL), but samples were taken from the top inch of the broth which may have been void of spores, regardless of stirring beforehand. Additionally, there was mostly likely not enough detectable *B. cereus* to warrant presence without enrichment. As a result, enrichment of the one week wait [primary fermentation] samples and the secondary fermentation samples were necessary to lower the detection limit and confirm the presence or absence of *B. cereus*. As it turned out, two of the nine samples analyzed displayed positive results after one week of storage at 4°C.

Table 2.4. Presence or absence of *B. cereus* in inoculated kombucha after fermentation and subsequent refrigerated storage [Finished product inoculation of *B. cereus*]

Kombucha System	Direct Liquid [after fermentation]	One week wait [primary fermentation]	Secondary Fermentation
A	0/3	**1/3**	0/3
B	0/3	**1/3**	0/3
C	0/3	0/3	0/3

In Kombucha systems A and B, one out of the three replicates was positive after primary fermentation and stored in the refrigerator at 4°C for one week. However, there was no *B. cereus* present in the samples taken from the post-fermentation inoculated liquid or secondary fermentation in both systems. The secondary fermented liquid was not tested after one week storage in the refrigerator because the absence of *B. cereus* right after the conclusion of secondary fermentation implies it would still be absent after storage. Kombucha system C was

negative for all samples tested for *B. cereus* presence including post-fermentation inoculation, one week storage, and secondary fermentation.

While rare, survival of post-fermentation spore contaminants during storage is possible. Table 2.4 illustrates that contamination during any part of the brewing process could pose a risk to consumers, as even extended storage does not completely inactivate spores.

Overall, samples positive for the presence of *B. cereus* were sporadic. Positive replicates were only confirmed after the respective samples were enriched for 24 hours. This indicates there is only a small amount of *B. cereus* present ($\geq$1 CFU/25 mL or $\geq$1 CFU/25 g), if any at all, and it needs enrichment in order to produce growth. If ingested, the number of spores may be too little to cause illness since the estimated infectious dose is at least $>10^5$ spores or vegetative cells (Batt, 2014). Although, doses as low as 10^3 *B. cereus* CFU/g of food have been found in foods causing disease (Stenfors Arnesen, Fagerlund, & Granum, 2008). In addition, all secondary fermentation samples were negative regardless of inoculation method or interaction. This signifies that if the spores can survive the initial fermentation, they are most likely not able to survive the secondary fermentation. No significant differences ($p \leq 0.05$) were found in likelihood of spore recovery between the inoculation methods or in the presence of *B. cereus* in a specific portion (daughter SCOBY or liquid) of the product. However, data collected in this study reveals some kombucha systems (kombucha system C) may be more robust and less likely to become contaminated than others (kombucha systems A and B). Therefore, antibacterial differences between kombucha systems should be further explored to determine which systems are more likely to prevent contamination of spore-formers and should be used for brewing.

Temperature abuse may play a role in the germination and growth of *B. cereus* endospores in a kombucha system. *B. cereus*'s optimum temperature range overlaps with the

microorganisms responsible for kombucha fermentation (mainly acetic acid bacteria) because they are both mesophilic organisms. However, *B. cereus*'s overall growth temperature range is more extensive (10ºC-50ºC) than acetic acid bacteria (20ºC-37ºC) and thus has the ability to grow at higher and lower temperatures when the working bacteria has slowed or died off also minimizing microbial competition (Lu, Lee, & Chen, 1999; *Bacillus cereus—BfR*, n.d.; "How Temperature Affects Kombucha", 2019). The reduction of competitive microbiota and poorly temperature-controlled conditions support the germination of the spores and the growth of vegetative cells (*Bacillus cereus—BfR*, n.d.).

2.5 Conclusions

The portion of the kombucha system inoculated did not significantly influence the likelihood of survival of *B. cereus* within the kombucha system. Equal survival was observed in the daughter SCOBY and the liquid regardless of inoculation method or kombucha system used. Results show the spread of *B. cereus* is sporadic but possible, so hygienic handling of kombucha cultures and raw materials throughout the entire production process is crucial to prevent uptake of pathogenic organisms. Additionally, there was no survival after 1-week storage or secondary fermentation, indicating that implementation of a holding step may mitigate potential food safety threats.

2.6 References

1) *Bacillus cereus—BfR*. (n.d.). Retrieved March 26, 2021, from https://www.bfr.bund.de/en/bacillus_cereus-54345.html

2) Batt, C. A. (2014). BACILLUS | Bacillus cereus. In C. A. Batt & M. L. Tortorello (Eds.), *Encyclopedia of Food Microbiology (Second Edition)* (pp. 124–128). Academic Press. https://doi.org/10.1016/B978-0-12-384730-0.00019-7

3) Battikh, H., Chaieb, K., Bakhrouf, A., & Ammar, E. (2013). Antibacterial and Antifungal Activities of Black and Green Kombucha Teas. *Journal of Food Biochemistry*, *37*(2), 231–236. https://doi.org/10.1111/j.1745-4514.2011.00629.x

4) Bauer, L., Dekevich, D., & Underthun, K. (n.d.). *Kombucha*. Food Source Information. Retrieved January 21, 2021, from https://fsi.colostate.edu/kombucha/.

5) Bhattacharya, D., Bhattacharya, S., Patra, M. M., Chakravorty, S., Sarkar, S., Chakraborty, W., Koley, H., & Gachhui, R. (2016). Antibacterial Activity of Polyphenolic Fraction of Kombucha Against Enteric Bacterial Pathogens. *Current Microbiology*, *73*(6), 885–896. https://doi.org/10.1007/s00284-016-1136-3

6) Bhattacharya, D., Ghosh, D., Bhattacharya, S., Sarkar, S., Karmakar, P., Koley, H., & Gachhui, R. (2018). Antibacterial activity of polyphenolic fraction of Kombucha against Vibrio cholerae: Targeting cell membrane. *Letters in Applied Microbiology*, *66*(2), 145–152. https://doi.org/10.1111/lam.12829

7) Četojević-Simin, D. D., Velićanski, A. S., Cvetković, D. D., Markov, S. L., Mrđanović, J. Ž., Bogdanović, V. V., & Šolajić, S. V. (2012). Bioactivity of Lemon Balm Kombucha. *Food and Bioprocess Technology*, *5*(5), 1756–1765. https://doi.org/10.1007/s11947-010-0458-6

8) Chakravorty, S., Bhattacharya, S., Bhattacharya, D., Sarkar, S., & Gachhui, R. (2019). 10 - Kombucha: A Promising Functional Beverage Prepared From Tea. In A. M. Grumezescu & A. M. Holban (Eds.), *Non-Alcoholic Beverages* (pp. 285–327). Woodhead Publishing. https://doi.org/10.1016/B978-0-12-815270-6.00010-4

9) *Counting Cells in a Hemocytometer | Thermo Fisher Scientific—US*. (n.d.). Retrieved October 30, 2020, from https://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/counting-cells-in-a-hemacytometer.html?gclid=CjwKCAjw8-78BRA0EiwAFUw8LF_wjERCOT-3hx0k1Tp9yF3PjV1KfjOP7f0QUeygQJulTMvFkQoqkBoCUQwQAvD_BwE&ef_id=CjwKCAjw8-78BRA0EiwAFUw8LF_wjERCOT-3hx0k1Tp9yF3PjV1KfjOP7f0QUeygQJulTMvFkQoqkBoCUQwQAvD_BwE:G:s&s_kwci

d=AL!3652!3!305473461780!b!!g!!&cid=bid_clb_cce_r01_co_cp0000_pjt0000_bid00000
_0se_gaw_dy_pur_con&s_kwcid=AL!3652!3!305473461780!b!!g!!

10) Deghrigue, M., Chriaa, J., Battikh, H., Abid, K., & Bakhrouf, A. (2013). Antiproliferative and antimicrobial activities of kombucha tea. *African Journal of Microbiology Research*, *7*(27), 3466–3470. https://doi.org/10.5897/AJMR12.1230

11) Granum, P. E., & Lindbäck, T. (2012). Bacillus cereus. In *Food Microbiology* (pp. 491–502). John Wiley & Sons, Ltd. https://doi.org/10.1128/9781555818463.ch19

12) Greenwalt, C. J., Ledford, R. A., & Steinkraus, K. (1998). Determination and Characterization of the Antimicrobial Activity of the Fermented TeaKombucha. *LWT - Food Science and Technology*, *31*(3), 291–296. https://doi.org/10.1006/fstl.1997.0354

13) Greenwalt, C. J., Steinkraus, K. H., & Ledford, R. A. (2000). Kombucha, the Fermented Tea: Microbiology, Composition, and Claimed Health Effects. *Journal of Food Protection*, *63*(7), 976–981. https://doi.org/10.4315/0362-028X-63.7.976

14) How Temperature Affects Kombucha. (2019). *Brew Buch*. https://brewbuch.com/temperature-affects-kombucha/

15) Jayabalan, R., Malbaša, R. V., Lončar, E. S., Vitas, J. S., & Sathishkumar, M. (2014). A Review on Kombucha Tea—Microbiology, Composition, Fermentation, Beneficial Effects, Toxicity, and Tea Fungus. *Comprehensive Reviews in Food Science and Food Safety*, *13*(4), 538–550. https://doi.org/10.1111/1541-4337.12073

16) Lu, S.-F., Lee, F.-L., & Chen, H.-K. (1999). A thermotolerant and high acetic acid-producing bacterium Acetobacter sp. I14–2. *Journal of Applied Microbiology*, *86*(1), 55–62. https://doi.org/10.1046/j.1365-2672.1999.00633.x

17) *MYP Agar Base (Phenol Red Egg Yolk Polymyxin Agar Base) Technical Data sheet*. (2015). HiMedia Laboratories.

18) Notermans, & Batt. (1998). A risk assessment approach for food-borne Bacillus cereus and its toxins. *Journal of Applied Microbiology*, *84*(S1), 51S-61S. https://doi.org/10.1046/j.1365-2672.1998.0840s151S.x

19) Post, D. E. (n.d.). *Food-borne Pathogens Monograph Number 4 Clostridium perfringens Bacillus cereus*. Oxoid. Retrieved September 30, 2021, from https://drive.google.com/file/d/1EBuWWvNff6PNsERdxrMSojyNqwjsrPqV/view?usp=drive_web&usp=embed_facebook

20) Russell, A. D. (1991). Mechanisms of bacterial resistance to non-antibiotics: Food additives and food and pharmaceutical preservatives. *Journal of Applied Bacteriology*, *71*(3), 191–201. https://doi.org/10.1111/j.1365-2672.1991.tb04447.x

21) Sadjadi, J. (1998). Cutaneous Anthrax Associated With the Kombucha "Mushroom" in Iran. *JAMA*, *280*(18), 1567–1568.

22) Sawai, J., Igarashi, H., Hashimoto, A., Kokugan, T., and Shimizu, M. (1995). Effect of Ceramic Powder Slurry on Spores of *Bacillus subtilis*. *Journal of Chemical Engineering of Japan*, 28, 556-561. https://www-jstage-jst-go-jp.wv-o-ursus-proxy02.ursus.maine.edu/article/jcej/28/5/28_5_556/_pdf/-char/ja

23) Smolinske, S. C. (2005). Herbal Product Contamination and Toxicity. *Journal of Pharmacy Practice*, *18*(3), 188–208. https://doi.org/10.1177/0897190005277217

24) Sreeramulu, G., Zhu, Y., & Knol, W. (2001). Characterization of Antimicrobial Activity in Kombucha Fermentation. *Acta Biotechnologica*, *21*(1), 49–56. https://doi.org/10.1002/1521-3846(200102)21:1<49::AID-ABIO49>3.0.CO;2-G

25) Stenfors Arnesen, L. P., Fagerlund, A., & Granum, P. E. (2008). From soil to gut: Bacillus cereus and its food poisoning toxins. *FEMS Microbiology Reviews*, *32*(4), 579–606. https://doi.org/10.1111/j.1574-6976.2008.00112.x

CHAPTER 3

SURVIVAL OF PROBIOTIC *LACTOBACILLUS SP*. DURING FERMENTATION TO PRODUCE A PROBIOTIC KOMBUCHA BEVERAGE

3.1 Abstract

Kombucha is noted for its taste, sensorial qualities, and supposed health benefits. The beverage is perceived to automatically contain probiotics, but not all live cultures contain probiotics as many consumers believe. In this study, the survivability of six probiotic *Lactobacillus* sp. was established in MRS or sweetened tea at recommended temperature (30ºC or 37ºC) or 25ºC to create bacterial growth curve models for predicting probiotic populations under certain conditions. Once it was determined the probiotics could grow in sweetened tea at 25ºC, kombucha fermentation was carried out with each probiotic until a pH of 3.0 was reached, and the probiotic populations were documented throughout fermentation and storage. Model comparison was performed using multiway ANOVA in R studio to decipher significant differences ($p < 0.05$) between treatments and between probiotics. Temperature and medium had an effect on the probiotic growth rates, and results showed the probiotics survived better in sweetened tea at 25ºC than in sweetened tea at recommended temperature. Differences in pH indicated the probiotics were unable to acidify the tea pre-fermentation. Fermentation lasted on average 8.3 days, and probiotic populations revealed an acidic threshold around day 6. GABA and other biogenic amines were not detected in the lactic acid bacteria inoculated kombucha samples at a detection limit of 1 μmol. It was concluded that probiotic *Lactobacillus* sp. are not well suited for a probiotic kombucha beverage but out of the tested probiotics, *L. brevis* and *L. fermentum* are the best candidates of the species tested in this study.

3.2 Introduction

Tea is the most consumed beverage in the world after water, with green and black tea being the most popular tea varieties, so it is no surprise that kombucha has become increasingly popular over the past few decades for its taste, sensory profile, and potential health benefits (Chakravorty et al., 2019). Kombucha is a tea beverage that goes through a double fermentation process using a symbiotic colony of bacteria and yeasts (SCOBY) to create a slightly carbonated drink with a tangy vinegar flavor (*Kombucha | Description, History, & Nutrition,* n.d.). It is usually consumed as a health beverage because of its functional features and is often an alternative to traditional soft drinks. As a result, kombucha has become one of the most popular low-alcoholic fermented beverages in the world (Baschali, 2017). In the US, it was solely brewed at home using a tea fungus passed from home to home until the commercialization of the product in 1995 when GT Dave started his brand "GT's Kombucha" (Dufresne & Farnworth, 2000; Bauer et al., n.d.; *Kombucha Brewers International*, n.d.). It then surfaced as a health product because of the public push towards a healthier lifestyle at the time and greater awareness of probiotics and the health benefits of fermented foods (*Kombucha | Description, History, & Nutrition,* n.d.). Kombucha has since become the fastest-growing product in the functional beverage sector (Bauer et al., n.d.; *Kombucha Brewers International*, n.d.). The fermented beverage can now be found commercially around the globe in larger chain stores and not just in health or natural foods stores although many consumers still continue to practice kombucha fermentation at home.

Most of kombucha's rise in popularity is not only due to its taste and sensorial qualities but also its supposed health benefits, such as having antioxidant, antidiabetic, hepatoprotective, antimicrobial, anti-inflammatory, and anticancer capabilities as well as being able to lower

stress, blood pressure, and cholesterol (Chakravorty et al., 2019). These health effects may be due to the known benefits of tea itself in addition to the products formed in the beverage during fermentation, which include glucuronic acid, acetic acid, polyphenols, and B-complex vitamins (Baschali, 2017). To date, here have been no kombucha consumption studies using human models to confirm these health benefits.

Kombucha is likewise promoted for its supposed probiotic presence. Many consumers think that all live cultures offer probiotic benefits. This is not true. Although unpasteurized kombucha typically contains high levels of live organisms, these do not comprise proven probiotic species, or organisms closely related to probiotics. Probiotics are generally defined as "live microbial food ingredients that, when ingested in sufficient quantities, have health benefits on the consumers by acting either directly or indirectly via interactions with the gut microbiota" (Tur & Bibiloni, 2016; Asaithambi, Singh, & Singha, 2021; Bauer et al., n.d.; Siró, Kápolna, Kápolna, & Lugasi, 2008). Upon consumption, probiotics have the ability to restore, balance, or maintain the gut microbial population, function, and composition (Asaithambi, Singh, & Singha, 2021). Their major health benefits include lactose intolerance alleviation, the reduction of digestive infections via immune stimulation, and the reduction of precancerous gastrointestinal lesions (Tur & Bibiloni, 2016).

Probiotic microorganisms mainly consist of *Lactobacillus* and *Bifidobacterium* species, which are also normal components of the human intestinal microbiota. They are the most studied and widely employed bacteria within the probiotic classification and have a long tradition of safe application within the food industry (Siró, Kápolna, Kápolna, & Lugasi, 2008). Even though these species can sometimes be found in the intestinal microbiota, added probiotics may change the microbiota, but they do not become a permanent part of the microbiota when consumed,

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passing through the intestinal tract (Tur & Bibiloni, 2016). Thus, regular consumption of 100 grams or more of probiotics are recommended to produce palpable health effects (Tur & Bibiloni, 2016; Asaithambi, Singh, & Singha, 2021). However, these organisms are only minor parts, if present at all, in the kombucha biome.

Kombucha microbiota varies from batch to batch depending on the SCOBY's microbial community, but there are a few species of bacteria and yeasts that one can find in almost every batch of kombucha. The most predominant species most often found in kombucha are *Acetobacter* and *Gluconobacter* bacteria and *Brettanomyces, Zygosaccharomyces, Saccharomyces,* and *Candida* yeasts (May, 2019; Greenwalt, Steinkraus, & Ledford, 2000). Smaller traces of *Lactobacillus, Lactococcus, Pichia, Schizosaccharomyces*, and *Kloeckera* can be found in kombucha, but they are not always present (Chakravorty et al., 2019; Dufresne & Farnworth, 2000; Reva, 2015; Ivanišová, 2020). Since probiotics are largely *Lactobacillus* and *Bifidobacterium* species, they may be naturally-occurring in the kombucha as part of the lactic acid bacteria population, but usually comprise a significant minority of the composition. Thus, it cannot be assumed these "naturally occurring" probiotics are present, or if they are present, it cannot be assumed they are present in high enough amounts to be effective. Therefore, it is recommended that exogenous probiotics be added to the beverage in order to proclaim it a probiotic beverage. The question then becomes: When should the probiotic be added to the beverage? Before or after fermentation? Additionally, which probiotic is most likely to survive in kombucha's environment and deliver benefits to the consumer? More research guaranteeing probiotic efficacy in kombucha is needed, questioning which probiotic should be added, when said probiotic should be added, and whether kombucha can deliver the said probiotic's effects.

In order to attain the full effects of probiotics, they must be maintained in the food product at sufficient levels from processing until consumption. The minimal level of probiotics required to be in the final processed product in order to assert the health benefits at the time of consumption is at least 10^6 CFU/g (Asaithambi, Singh, & Singha, 2021; Corbo et al., 2014). Successful probiotics in foods must be able to survive and be adsorbed by the gastrointestinal tract. The food matrix in which the probiotics are incorporated also plays a significant role in cell survival in the food (Asaithambi, Singh, & Singha, 2021). Temperature and the probiotic strain's resistance to withstand various food processing procedures are the two most important parameters when selecting the appropriate probiotic strains and processing conditions for a probiotic food (Asaithambi, Singh, & Singha, 2021).

Although kombucha may not be a suitable environment for these [*Lactobacillus*] probiotics, there are ways that probiotics can be beneficial even if they're not viable such as management of gastrointestinal disorders, protection against enteropathogens, maintenance of intestinal barrier integrity, and the presence of their metabolites, like gamma-aminobutyric acid (GABA), if they are produced in high enough quantities before the cells die (Piqué, Berlanga, & Miñana-Galbis, 2019). Gamma-aminobutyric acid (GABA) is a non-protein amino acid produced by various plants, animals, and microorganism through glutamate decarboxylase (GAD) activity (Diez-Gutiérrez, 2020; Corbo et al., 2014). It is known to have a substantial role in behavior, cognition, disease prevention, gut microbiota function, and the body's response to stress (Diez-Gutiérrez, 2020; Cui, Miao, Niyaphorn, & Qu, 2020). When present in adequate amounts in fermented foods, the health benefits of consuming this amino acid include gut modulation, neurostimulation, and cardioprotection (Diez-Gutiérrez, 2020). A large number of LAB species

are capable of producing GABA, but *Lactobacillus brevis* and *Lactobacillus plantarum* are noted as two of the best producers of GABA (Cui, Miao, Niyaphorn, & Qu, 2020).

This research sought to assess the feasibility of the production of a kombucha beverage with health-promoting characteristics derived from the inclusion of LAB probiotics. Specific objectives included: (i) determining the suitability of sweetened tea as a growth medium for selected probiotic LAB species (ii) determining the survivability of LAB during active kombucha fermentation and storage (iii) determining the differences in GABA production by LAB probiotics under ideal conditions compared in a kombucha system.

3.3 Methods and Materials

3.3.1 SCOBY Preparation

Only SCOBY from one system (IBIV Kombucha, Chesapeake, VA) was used throughout this study. The system was grown until a biomass of 900+ grams was reached. This was accomplished by transferring the SCOBY from a container of finished kombucha to a container of freshly brewed, acidified sweetened black tea every 14 days to increase the biomass. For each rebrewing cycle, 1000 mL of deionized water was boiled, and four bags of Lipton (Hoboken, NJ) black tea were brewed for 10 minutes. The tea was cooled in an ice bath until approximately ambient temperature (~20°C) was reached. The tea bags were removed, and the tea was combined with 100 grams of Domino's (Yonkers, NY) pure white cane sugar and 5% (vol/vol) of the liquid from the previous kombucha batch (produced with the same SCOBY) was added for acidification. Once thoroughly mixed until the sugar dissolved, the SCOBY was added to the container which was then covered with a clean (sterile) cheesecloth and placed on a clean,

isolated windowsill at approximately ambient temperature (~25°C). pH was taken using the

Thermo Scientific (Waltham, MA) Orion Star A111 pH meter at the start of the fermentation

cycle and at the end of the fermentation cycle (pH of ≤3.0). The pH meter was calibrated before

every grouping of pH reading samples with an accuracy of ≥95%. The approximate starting pH

of each fermentation cycle was 3.1, and the approximate end pH was 2.5. Once the SCOBY

accumulated the appropriate amount of biomass, it was covered with the container lid and placed

in the refrigerator until later use.

3.3.2 LAB Preparation

Six different *Lactobacillus* species, categorized as probiotic lactic acid bacteria (LAB),

all sourced from the American Type Culture Collection (Manassas, VA) were used:

Lactobacillus plantarum ATCC 8014 , *Lactobacillus bulgaricus* ATCC 11842, *Lactobacillus*

brevis ATCC 14869 strain Bb14, *Lactobacillus fermentum* ATCC 9338, *Lactobacillus casei*

ATCC 393, and *Lactobacillus sakei* ATCC 15521. A loopful of *L. plantarum*, *L. bulgaricus*, and

L. brevis -80°C freezer stock was each inoculated into separate deMan, Rogosa, and Sharpe

(MRS) broth tubes (Difco, Sparks, MD). The *L. plantarum*, *L. bulgaricus*, *L. brevis* tubes were

incubated at recommended temperature (Table 3.1) overnight. Once bacterial growth in the tubes

was visible, the tubes were vortexed for 10 seconds using the Fisher Scientific Vortex Mixer

(Waltham, MA), and each organism was streak plated onto MRS agar in duplicate for colony

isolation. The MRS plates were then placed in their respective incubators for 24-48 hours to

allow for bacterial colony formation. *L. fermentum*, *L. casei*, *and L. sakei* were regenerated using

Microbiologics's KwikStik™ (Saint Cloud, MN). Following the directions in the KwikStik kit,

the bacteria were hydrated in the stick containing the bacteria and streak plated onto MRS agar in

duplicate for colony isolation (*KWIK-STIK Plus Instructions For Use*, n.d.). The MRS plates were then incubated at recommended temperature (Table 3.1) for *L. fermentum*, *L. casei*, and *L. sakei* for 24-48 hours to allow for bacterial colony formation.

Table 3.1. Recommended Incubation Temperatures[1] Used for Each Species of Lactic Acid Bacteria

Probiotic	Recommended Incubation Temperature (^{o}C)[1]
L. plantarum	37
L. bulgaricus	37
L. brevis	30
L. fermentum	37
L. casei	37
L. sakei	37

[1]Temperatures obtained from references: *Lactobacillus plantarum (Orla-Jensen) Bergey et al. | ATCC*, n.d.; *Lactobacillus delbrueckii subsp. Bulgaricus (Orla-Jensen) Weiss et al. | ATCC*, n.d.; *Lactobacillus brevis (Orla-Jensen) Bergey et al. | ATCC*, n.d.; *Lactobacillus fermentum Beijerinck | ATCC*, n.d.; *Lactobacillus casei (Orla-Jensen) Hansen and Lessel | ATCC*, n.d.; *Lactobacillus sakei subsp. Sakei Katagiri et al. | ATCC*, n.d.

3.3.3 Sweetened Tea Preparation

Black tea was prepared by brewing four Lipton black tea bags in 600 mL of boiling deionized water for 10 minutes. The tea was cooled in an ice bath until approximately ambient temperature (~20°C) was reached. The tea bags were removed, and the tea was combined with 100 grams of Domino's (Yonkers, NY) pure white cane sugar. The tea was thoroughly mixed until the sugar was completely dissolved.

3.3.4 LAB Growth Curves

Growth curves were created in triplicate for each LAB species in the following medium/temperature treatments: MRS broth incubated at recommended temperature (Table 3.1), MRS broth incubated at 25ºC, sweetened tea (prepared as described in section 3.3.3) incubated at recommended temperature (Table 3.1), and sweetened tea incubated at 25ºC. Within each replicate, duplicate platings were performed for each time point (n=6 tubes per species/treatment/time). At each sampling time (every 4 hours over 24 hours), a unique tube (no repeated sampling) of inoculated broth was diluted as appropriate in sterilized peptone water and spread-plated in duplicate onto MRS agar, which was subsequently incubated for 48 hours at species specific recommended temperature (Table 3.1).

The bacterial colonies were counted using plate counting techniques, and the CFU/mL were calculated and log transformed (Perry, 2019). Remaining sample left in the 24-hour tubes was filter sterilized using Fisher Scientific (Waltham, MA) 0.22μm sterile 13mm syringe filter with a PVDF membrane and placed in the -80°C freezer for future chromatographic analysis. The pH of 20-hour samples was measured and compared to the non-inoculated MRS broth to assess LAB acidification of the medium.

3.3.5 LAB Culture Preparation for Kombucha Fermentation

Each LAB was inoculated into sterile MRS broth in triplicate. Within each replicate, duplicate MRS broth tubes were inoculated per species (n=6 tubes per species). The culture tubes were incubated overnight (24 hours) at the organism's recommended optimal growth temperature (Table 3.1). To confirm the amount of probiotic being put into each jar of tea,

samples from the culture tubes were diluted as appropriate in peptone water and plated onto

MRS agar in duplicate and incubated at recommended temperature (Table 3.1). Each overnight

culture had a population of ~9 log CFU/mL, confirmed by spread plating.

3.3.6 Probiotic Inoculation into Tea and Survival Through Kombucha Fermentation

The probiotic LAB cultures were prepared (as described in section 3.3.5) for pre-

fermentation inoculation into tea. Sweetened tea was prepared as previously described in section

3.3.3 and acidified to a pH of 4.0 with previously brewed kombucha produced with the same

SCOBY system. Tea, SCOBY, and LAB cultures were added to clean mason jars (Ball,

Broomfield, CO) in triplicate. Within each replicate, duplicate jars were produced to attain six

jars per species. The LAB cultures added to each jar inoculated the pre-fermented tea with a

single designated probiotic at a level of 1% of the tea by volume, representing a starting

inoculum level of approximately 9.0 log CFU/mL. The jars were then covered with cheesecloth

and incubated at 25°C.

One hour after the probiotic inoculation, samples were taken aseptically from each jar,

diluted in sterilized peptone water, plated onto MRS agar in duplicate, and incubated at

recommended temperature (Table 3.1) to assess immediate loss of viability due to inoculation

into acidified sweet tea (when compared to culture plating as described in section 3.3.5). These

bacterial counts were designated as the "Day 0" population. Kombucha sample pH and surviving

LAB population were measured daily until a pH of ≤3.0 was reached. The kombucha jars were

stirred 10 times with a sterile glass rod, and samples were taken from the top inch of liquid. The

samples were diluted in sterilized peptone water and plated onto MRS agar in duplicate. The

MRS plates were incubated for 48 hours at recommended temperature (Table 3.1) then counted

and recorded. If there was no more growth on the MRS plates because the probiotic population had decreased below detection limit (0 CFU/mL), plating ceased for that replicate, but pH was still measured every day until each kombucha jar reached a pH of 3.0, at which point fermentation was considered complete, and the SCOBY was removed. Samples of fermentate were saved in falcon tubes in duplicate in the -80°C freezer for subsequent GC-MS analysis. If probiotic population was still above detection limit in the fully fermented kombucha, samples of the liquid (sans SCOBY) were stored at 4°C and plated onto MRS agar after one week.

3.3.7 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of Biogenic Amines in Probiotic-Inoculated Kombucha

GC-MS was performed using EZ:faast^{tm} amino acid analysis kit (PN: KG0-7166) by Phenomenex (Torrance, CA). Sample and analytical standard preparation and analysis was accomplished by following the directions accompanying the kit. Preparation included a solid-phase extraction step where amine compounds in the sample matrix or standards were bound to a sorbent-packed tip. Bound amino acids within samples were evaluated while allowing interfering compounds to flow through and discarded. The amino acids on sorbent were eluted into sample vials and derivatized with reagent at room temperature in aqueous solution. The derivatized amino acids simultaneously migrated to the organic layer for additional partitioning from interfering compounds. The organic layer was removed, evaporated, suspended in redissolution solvent and injected into the GC-MS system.

Kombucha, unfermented sweetened tea, and MRS broth samples inoculated with the six tested *Lactobacillus* sp. (*L. plantarum, L. bulgaricus, L. brevis, L. fermentum, L. casei, L. sakei*) were tested. Analytical standards, purchased from Sigma-Aldrich Co. (St. Louis, MO) included

gamma aminobutyric acid (GABA), putrescine, cadaverine, histamine, tyramine, spermine, spermidine and tryptamine. Stock solutions for individual standards were prepared by dissolving 50 mg of each standard in 50 mL of deionized water.

An Agilent model 6890 gas chromatograph coupled with a 5973 mass spectrometer (GC/MS) was used to identify and quantify individual amines in the kombucha. The GC column was provided with the EZfaast[tm] kit with the dimensions of 10 M x 0.025 ID. The column contained a proprietary separation phase of unknown thickness. A split injection mode, maintained at 250°C, was chosen with a 2:1 split ratio to maximize sensitivity for targeted analytes. The mass spectrometer was operated in the scan mode with a low mass of 45 and a high mass of 450 M/z (mass/charge). The MS source was maintained at 240ºC, and the MS quad was maintained at 180°C. The GC oven was programed and held for three minutes at an initial temperature of 110°C. The temperature was raised isocratically at 30ºC/minute to 320ºC where it was held for the last five minutes of each run, for a total run time of 15 minutes. Target analytes were identified by retention time and ion fragmentation (M/z). Biogenic amines concentrations in kombucha samples were quantified by comparing peak areas of samples with peak areas of analytical standards of known concentrations. An internal standard, norvaline (included in the EZ:faast[tm] kit), was used to correct for differences in final volumes and derivatization efficiencies.

The detection limit (5:1, signal:noise) for each of the eight biogenic amines tested was 1 μmol and was determined using standard dilutions of each analytical standard.

3.3.8 Statistical Analysis

Data were analyzed in R and R studio Version 1.4.1103 (Boston, MA) using polynomial regression models (lm function) to represent best fit of the data. Each model was chosen based on R^2 value and likeness to data points when graphed. Four models were created per probiotic to represent each treatment (MRS broth at recommended probiotic temperature, MRS broth at 25ºC, sweet tea at recommended probiotic temperature, and sweet tea at 25ºC) for a total of 24 models. Model comparison was performed using multiway ANOVA to decipher significant differences ($p<0.05$) between treatments and between probiotics.

Multiway ANOVA was performed on the pH values taken at the 20-hour interval to identify any significant differences ($p<0.05$) among final pH values for each probiotic. Tukey's post hoc test was then used as a pairwise comparison to determine any specific significant ($p<0.05$) interactions.

3.4 Results and Discussion

The aim of this study was to determine if selected probiotics (*L. plantarum, L. bulgaricus, L. brevis, L. fermentum, L. casei, L. sakei*) could grow in sweet tea before or during kombucha fermentation and/or produce gamma-aminobutyric acid (GABA) or other biogenic amines. Because kombucha is fermented at a maximum of 25ºC, we first had to assess if the decrease in temperature from species "optimal" values would have an effect on probiotic growth, if medium (sweetened tea) would have an effect on probiotic growth, and if the combination of temperature and medium would have an effect on probiotic growth. This would help determine if the probiotics would be able to survive and grow under fermentation conditions (sweetened,

acidified tea at 25ºC). Growth in sweetened tea at recommended temperature could provide the option of acidification of the kombucha substrate with probiotic LAB (as opposed to standard practice of using previously fermented kombucha or acetic acid). Assuming the cultures could grow in tea at 25ºC, indicating that viability may be possible in the finished beverage, kombucha fermentation was carried out with the six *Lactobacillus* present, and kombucha's contents were analyzed to see if probiotics survived and if any GABA was produced.

3.4.1 Bacterial Growth Curves as Predictor Models for Probiotic Kombucha Fermentation

The bacterial growth curves generated in MRS broth at the probiotic's recommended temperature (visualized in Figure 3.1) acted as the control and theoretically represents that probiotic's optimal growth. The control was compared against MRS broth at 25ºC (temperature difference), tea at recommended temperature (medium difference), and tea at 25ºC (fermentation conditions) to identify any significant differences. Table 3.2 lists all model equations used for comparison as well as suitability of equation fit. Table 3.3 illustrates the model comparisons of each probiotic among the three different treatments and their respective p-values. In every case except *L. bulgaricus* in MRS broth at 25ºC, decreasing the temperature resulted in significantly reduced growth of the probiotic. Switching the medium from MRS broth to sweetened tea led to a significant reduction in survival. However, comparing the models of tea at recommended temperature and tea at 25ºC revealed there was better cell survival at 25ºC than at the recommended temperature.

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Table 3.2. Model Equations for *Lactobacillus* species grown under ideal conditions and in kombucha-relevant conditions

Probiotic	Medium[1]	Temperature (°C)	Model Equation	R^2
L. bulgaricus	MRS	37	$y = -0.0003x^4 + 0.0158x^3 - 0.2876x^2 + 2.1626x + 3.6383$	49%
L. bulgaricus	MRS	25	$y = -0.0088x^2 + 0.3307x + 6.5963$	94%
L. bulgaricus	Tea	37	$y = 0.0001x^4 - 0.0054x^3 + 0.0917x^2 - 0.6817x + 8.4194$	93%
L. bulgaricus	Tea	25	$y = 0.0017x^2 + 0.0949x + 7.3217$	44%
L. plantarum	MRS	37	$y = 0.0005x^3 - 0.0310x^2 + 0.5817x + 5.9533$	97%
L. plantarum	MRS	25	$y = -0.0071x^2 + 0.3320x + 5.5583$	93%
L. plantarum	Tea	37	$y = 0.0077x^2 - 0.2821x + 7.5797$	88%
L. plantarum	Tea	25	$y = 0.0023x^2 + 0.1287x + 7.1947$	79%
L. brevis	MRS	30	$y = 0.0001x^5 - 0.0069x^4 + 0.1873x^3 - 2.3181x^2 + 12.9102x + 18.4867$	68%
L. brevis	MRS	25	$y = 0.0007x^3 - 0.0374x^2 + 0.6603x + 5.7267$	98%
L. brevis	Tea	30	$y = 0.00003x^5 - 0.0020x^4 + 0.0491x^3 - 0.5706x^2 + 2.9986x + 1.0267$	62%
L. brevis	Tea	25	$y = -0.0264x + 7.2356$	37%
L. fermentum	MRS	37	$y = -0.0084x^2 + 0.3358x + 5.7133$	84%

Table 3.2. Continued

|				
L. fermentum	MRS	25	$y = -0.0021x^2 + 0.1145x + 6.796$	85%
L. fermentum	Tea	37	$y = -0.0004x^4 + 0.0201x^3 - 0.3703x^2 + 2.6344x + 0.1067$	44%
L. fermentum	Tea	25	$y = (-1.1583 * 10^{-5})x^5 + 0.0008x^4 - 0.0192x^3 + 0.2245x^2 - 1.1744x + 8.56$	37%
L. casei	MRS	37	$y = -0.0008x^3 + 0.0296x^2 - 0.2215x + 7.7767$	92%
L. casei	MRS	25	$y = -0.0009x^2 + 0.1120x + 6.3447$	87%
L. casei	Tea	37	$y = -0.0010x^2 - 0.1479x + 8.225$	81%
L. casei	Tea	25	$y = -0.0043x^2 + 0.0453x + 6.7817$	48%
L. sakei	MRS	30	$y = -0.0005x^3 + 0.0191x^2 - 0.1526x + 6.4189$	12%
L. sakei	MRS	25	$y = -0.0005x^3 + 0.0215x^2 - 0.2060x + 7.713$	91%
L. sakei	Tea	30	$y = 0.0086x^2 - 0.3182x + 6.798$	81%
L. sakei	Tea	25	$y = 0.0015x^3 - 0.0672x^2 + 0.6808x + 5.1567$	96%

[1]: Medium consisted of either MRS broth or sweetened tea

Figure 3.1 illustrates the growth treatments for each probiotic and denote these significant differences along with the final pH values for each treatment. The bacterial curves demonstrate that medium and temperature did have an effect on probiotic growth, but the effects are not necessarily negative when combined. The combination of temperature and medium differences lessens the expected negative effect. In tea medium at 25°C, although population did not increase, there was still a good amount of probiotic alive at the end of the 24 hours, notably *L.*
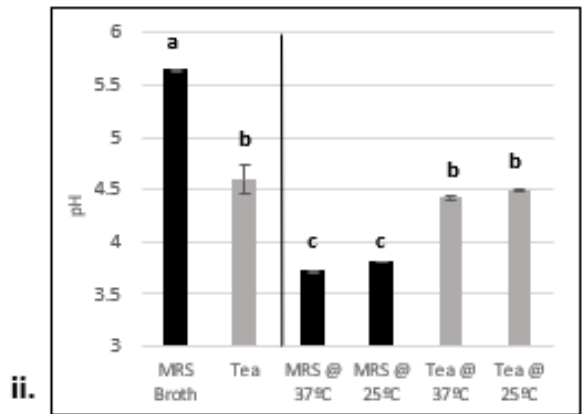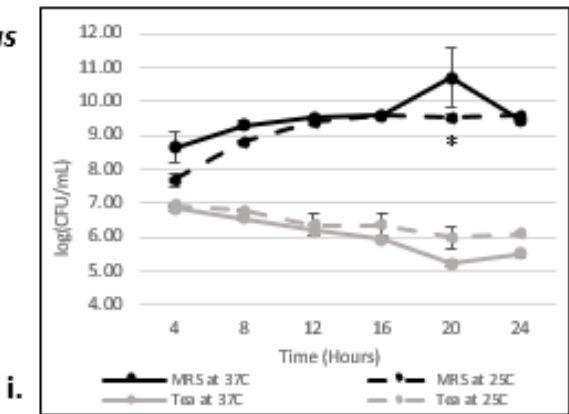
bulgaricus, L. brevis, and *L. fermentum* which all had a final population of >6.0 log. The

remaining three LAB, *L. plantarum, L. casei,* and *L. sakei*, had final populations of 5.4 log, 5.4

log, and 4.0 log, respectively. At the end of the 24 hours, every probiotic had an equal or higher

population level in sweet tea at 25ºC than sweet tea at the recommended temperature (refer to

Figure 3.1).

These observations are reinforced by the pH values associated with each growth curve. A

lower pH indicates there is more acid in the sample, and thus, there are more cells present

because they produce the acid. The difference in pH of the MRS control (no probiotic present)

and the probiotics in MRS broth is much greater than the difference in pH of the tea control (no

probiotics present) and the probiotics in the tea. This is because the cells in the tea medium did

not grow whereas the cells in the MRS broth did grow.
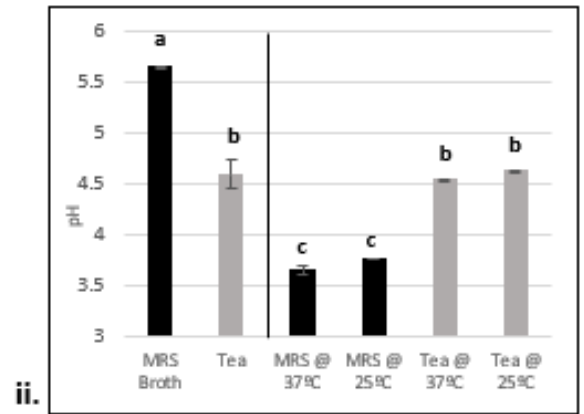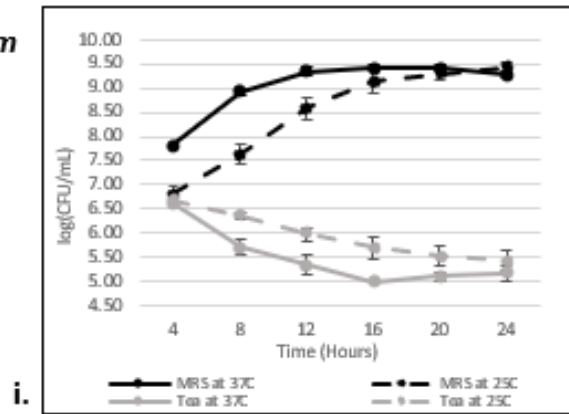
Figure 3.1. Bacterial Growth Curves (i) and pH values (ii) for A. *L. plantarum,* **B.** *L. bulgaricus,* **C.** *L. brevis,* **D.** *L. fermentum,* **E.** *L. casei,* **F.** *L. sakei*

Figure represents survival of probiotic populations within each treatment (n= 6). All growth curves within pane are significantly different unless marked with (*). Letters on pH panes indicate significant differences (p<0.05) between pH values, including controls MRS broth and sweet tea.

D. *L. fermentum*
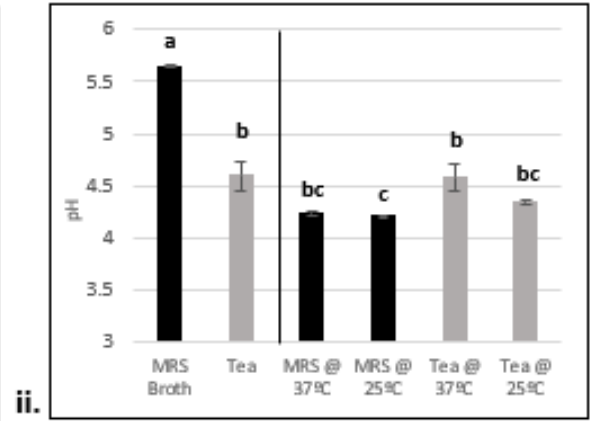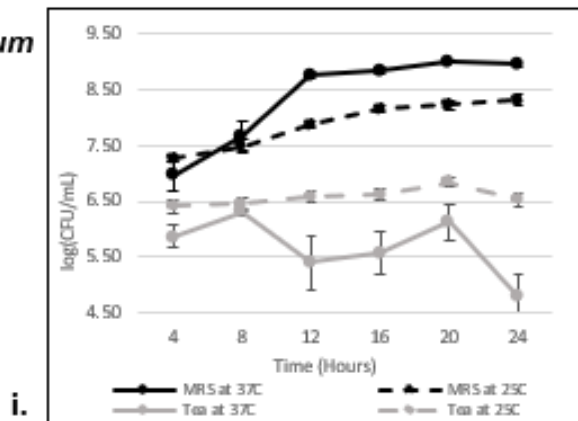
E. *L. casei*
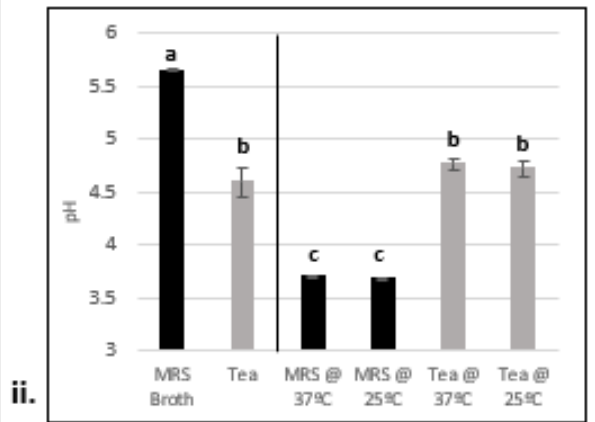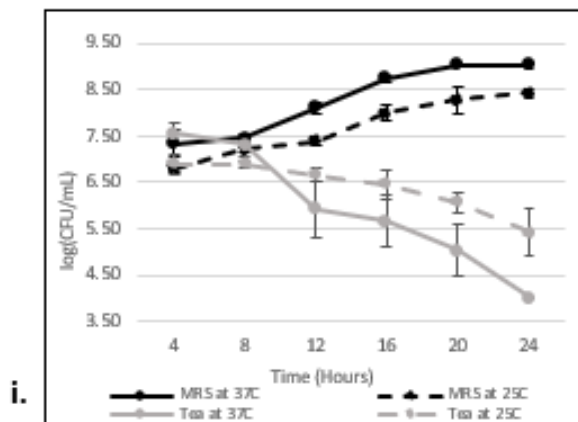
F. *L. sakei*
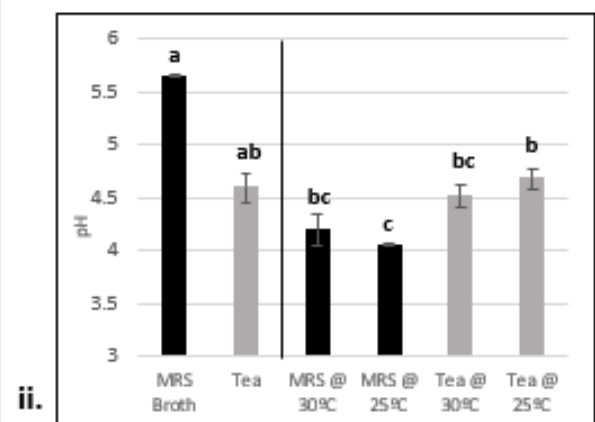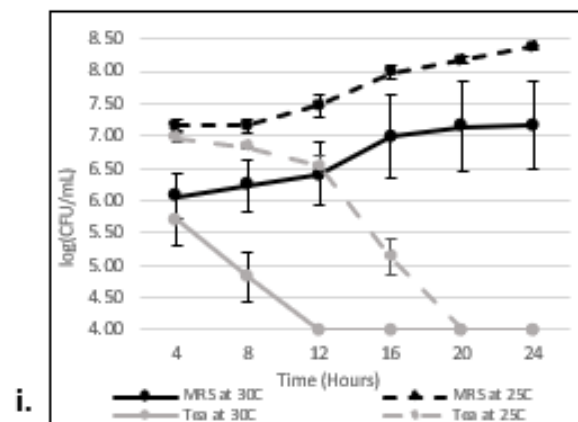
Figure 3.1. Continued

Table 3.3. Model Comparisons of Individual Probiotics

Each treatment of a probiotic compared to its control.

L. plantarum	
Comparison	**p-value[1]**
MRS at 37ºC vs. MRS at 25ºC	6.069e-9
MRS at 37ºC vs. Tea at 37ºC	2.2e-16
MRS at 37ºC vs. Tea at 25ºC	2.2e-16
Tea at 37ºC vs. Tea at 25ºC	1.126e-5
L. bulgaricus	
Comparison	**p-value[1]**
MRS at 37ºC vs. MRS at 25ºC	0.1076
MRS at 37ºC vs. Tea at 37ºC	2.2e-16
MRS at 37ºC vs. Tea at 25ºC	9.519e-15
Tea at 37ºC vs. Tea at 25ºC	0.005204
L. brevis	
Comparison	**p-value[1]**
MRS at 30ºC vs. MRS at 25ºC	1.349e-10
MRS at 30ºC vs. Tea at 37ºC	5.349e-7
MRS at 30ºC vs. Tea at 25ºC	1.283e-4
Tea at 30ºC vs. Tea at 25ºC	1.627e-5
L. fermentum	
Comparison	**p-value[1]**
MRS at 37ºC vs. MRS at 25ºC	4.559e-6
MRS at 37ºC vs. Tea at 37ºC	6.377e-14

Table 3.3. Continued

MRS at 37ºC vs. Tea at 25ºC	2.2e-16
Tea at 37ºC vs. Tea at 25ºC	0.0001564

L. casei	
Comparison	**p-value[1]**
MRS at 37ºC vs. MRS at 25ºC	3.479e-6
MRS at 37ºC vs. Tea at 37ºC	2.845e-16
MRS at 37ºC vs. Tea at 25ºC	2.446e-13
Tea at 37ºC vs. Tea at 25ºC	0.003595

L. sakei	
Comparison	**p-value[1]**
MRS at 30ºC vs. MRS at 25ºC	0.02378
MRS at 30ºC vs. Tea at 37ºC	4.228e-7
MRS at 30ºC vs. Tea at 25ºC	3.541e-5
Tea at 30ºC vs. Tea at 25ºC	3.841e-10

[1]Significance identified when obtain a p-value of <0.05

As seen in Figure 3.1, the probiotic populations do not increase in the sweetened tea, and the pH did not decrease enough from the tea control to the end of the growth period to indicate tea is able to be acidified by the acid produced from probiotics alone. Therefore, it can be concluded that this amount of cultures cannot be used alone to acidify the tea. Previous kombucha or acetic acid needs to be added to acidify the tea pre-fermentation. The question becomes if we can get the probiotics to survive by adding a higher concentration.

Having the probiotics grow in sweet tea at 25ºC mimics the conditions at which kombucha fermentation would take place. Results previously discussed allow us to have a full

picture of the response of each individual probiotic to the conditions that would be necessary to include it in a probiotic kombucha product. It is also valuable, however, to understand whether certain probiotics show more promise for this purpose than others. To this end, each probiotic was compared to every other strain in tea at 25°C to identify any significant differences. Table 3.4 illustrates the comparisons of each probiotic to one another and their respective p-values. The plethora of statistical differences (with the exception of *L. bulgaricus* compared to *L. casei*) reveals that each probiotic is going to act differently during the fermentation process. *L. brevis* (0.50 logCFU/ml reduction) and *L. fermentum* (0.53 logCFU/ml increase) survived significantly better than the remaining probiotics; *L. casei* (1.48 logCFU/ml reduction) and *L. sakei* (2.98 logCFU/ml reduction) performed significantly worse than the other four probiotics. If a certain amount of *L. brevis* and *L. fermentum* are inoculated into the kombucha beverage, the two probiotics are most likely to survive and retain most, if not all, of their populations based on their models. Therefore, it can be predicted from these comparisons that *L. brevis* and *L. fermentum* are best suited for use in a traditionally fermented kombucha product, based on their superior survival during fermentation, while *L. casei* and *L. sakei* are the least likely to be used in a fermented beverage.

L. brevis and *L. fermentum*'s acid tolerances further confirm their abilities to survive and grow in kombucha as well as a human's stomach and GI tract. Stomach acidity can range from a pH of 1.5-3.0, and consumption of food usually raises it to 3.0 or higher (Kandola et al., 2016; Zeng, Pan, & Zhou, 2011). *L. brevis* starts to decrease in population at a pH below 3.0, but its cell death rate was found to decrease at approximately <1.0 log CFU/mL per every 0.5 unit decrease in pH, meaning its survival rate is still fairly robust (Wu et al., 2018). *L. fermentum* has been found to be resistant to a pH of 2.0, and viable counts have been shown to stay constant at a

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pH of 3.0 (Zeng, Pan, & Zhou, 2011; Melia et al., 2018). Comparatively, *L. plantarum, L. casei, L. sakei,* and *L. bulgaricus* prefer a slightly higher pH, usually >3.0, or else viability and populations start to become severely reduced by several log CFU/mL (Zhang, Wu, Du, & Chen, 2012; Boke, Aslim, & Alp, 2010; Guo et al., 2017; Hong, Kim, & Pyun, 1999; Shah & Jelen, 1990). This strain of *L. bulgaricus* especially is known to be low-acid tolerant and thrives best in a less acidic pH (>4.0) (Shah & Jelen, 1990). In order to guarantee probiotic presence in the kombucha beverage and the GI tract, we should select a probiotic with a better acid tolerance.

Table 3.4. Model Comparisons Between Probiotics in Tea at 25ºC

Comparison (Tea at 25ºC)	p-value[1]	Which species performed better?
L. bulgaricus vs. *L. casei*	0.370	N/A
L. plantarum vs. *L. casei*	0.024	*L. casei*
L. brevis vs. *L. casei*	0.006	*L. brevis*
L. brevis vs. *L. fermentum*	3.165e-4	*L. brevis*
L. fermentum vs. *L. casei*	0.003	*L. fermentum*
L. bulgaricus vs. *L. fermentum*	0.002	*L. fermentum*
L. bulgaricus vs. *L. plantarum*	0.002	*L. bulgaricus*
L. bulgaricus vs. *L. brevis*	0.002	*L. brevis*
L. casei vs. *L. sakei*	2.796e-5	*L. casei*
L. plantarum vs. *L. sakei*	1.752e-6	*L. plantarum*
L. bulgaricus vs. *L. sakei*	3.123e-8	*L. bulgaricus*
L. plantarum vs. *L. fermentum*	4.833e-10	*L. fermentum*

Table 3.4. Continued

L. plantarum **vs.** *L. brevis*	9.439e-11	*L. brevis*
L. brevis **vs.** *L. sakei*	1.272e-12	*L. brevis*
L. fermentum **vs.** *L. sakei*	6.95e-13	*L. fermentum*

[1]Significance identified when obtain a p-value of <0.05

3.4.2   Probiotic Population Curves and Health Claims

Literature indicates on average that the pH of finished kombucha is around 2.5, but there is no pH defined for "finished" kombucha (Greenwalt, Steinkraus, & Ledford, 2000; Greenwalt, Ledford, & Steinkraus, 1998; Nummer, 2013). The pH of kombucha may also fluctuate during storage (shelf-life) unpredictably, and pH is often not tested after leaving the production facility (Yıkmış & Tuğgüm, 2019; La Torre, 2021). The Centers for Disease Control and Prevention (CDC) recommends kombucha under a pH of 2.5 or above a pH of 4.0 not be consumed due to safety concerns, but it seems anywhere in between this range may be acceptable. The pH chosen for this study to determine when fermentation was "complete" was 3.0. The kombucha jars completed fermentation from a range of 7 to 11 days with an average of 8.3 days, as shown in Table 3.5.

Table 3.5. Longevity of Fermentation for Each Replicate

Probiotic	**Days of Fermentation**
L. plantarum	8.2 ± 0.4
L. bulgaricus	9.0 ± 0.5
L. brevis	8.8 ± 0.5

Table 3.5. Continued

L. fermentum	8.0 ± 0.3
L. casei	8.0 ± 0.4
L. sakei	7.8 ± 0.4
Avg.	8.3 ± 0.2

Figure 3.2 illustrates the probiotic populations throughout sweet tea fermentation into kombucha. For all six probiotics, there was an initial drop in population of ~2 log CFU/mL on average when the probiotic culture is added into the acidified tea (from Culture to Day 0). The populations plateau for several days for four out of the six probiotics (*L. plantarum, L. bulgaricus, L. brevis, L. fermentum*) until decreasing markedly in the later stages of fermentation (day 6/7). At the end of the kombucha jars' fermentation periods, *L. brevis* and *L. bulgaricus* maintained the highest populations whereas *L. sakei, L. casei, L. fermentum,* and *L. plantarum* were all undetectable (0 CFU/mL). *L. sakei* was the quickest to reach undetectable levels (4 days) followed by *L. casei* (6 days). Looking at previous data (Figure 3.1), this was expected because *L. sakei* and *L. casei* had the largest decreases in population as well as the smallest populations at the end of the 24-hour sample period among the six probiotics. *L. fermentum* and *L. plantarum*, however, did not reach under the detection limit until ≥9 days, which roughly corresponded with the end of active fermentation. *L. brevis* consistently had highest population counts among all the probiotics throughout fermentation, moreover, it also retained viability during storage, despite a steep decrease in population level during that time. For this reason, *L. brevis* and *L. bulgaricus* have demonstrated the most promise for kombucha fermentation and creating a probiotic beverage.
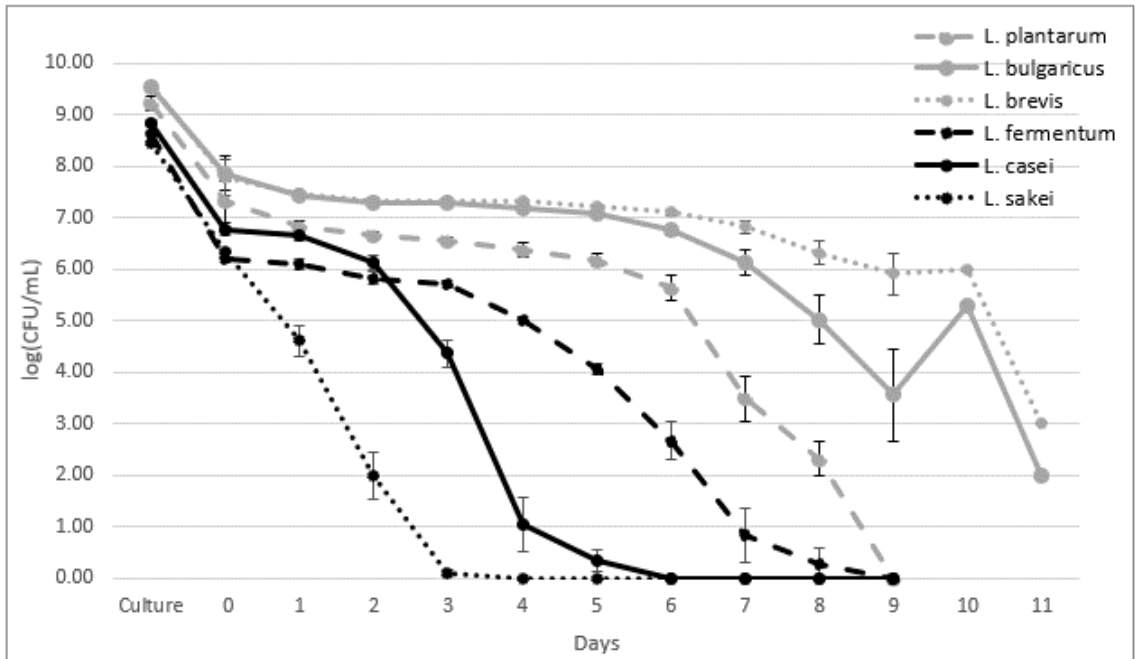
Figure 3.2. Averaged *Lactobacillus* sp. Population during Kombucha Fermentation and Storage

n=6. Error bars represent ± standard error for each data point.
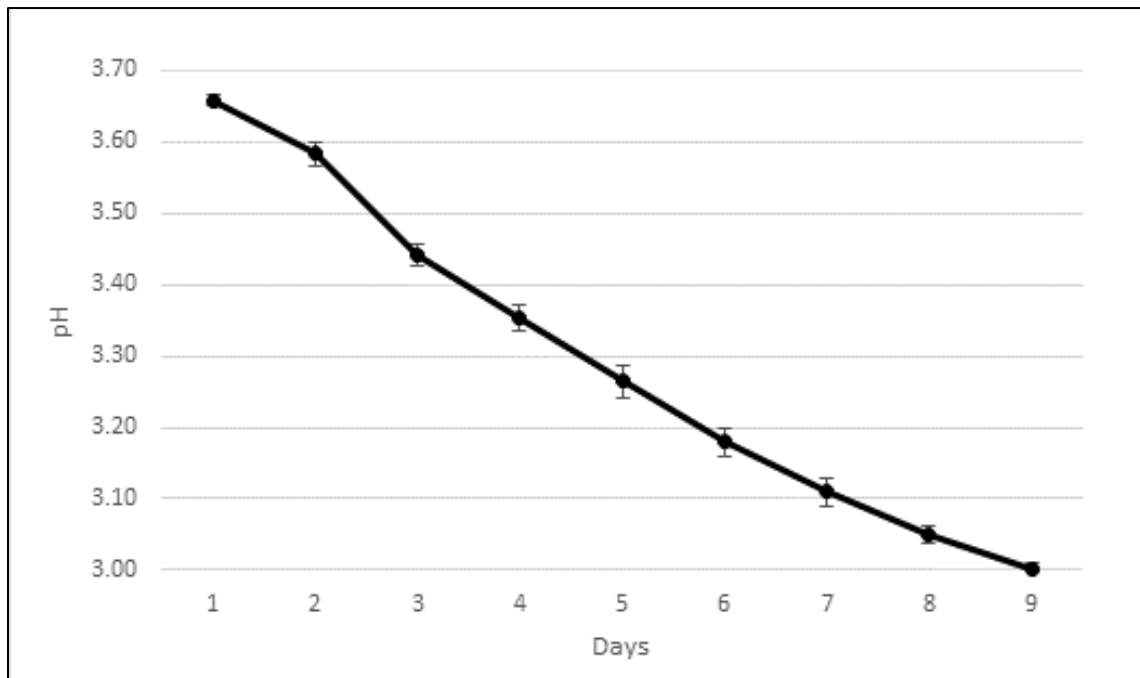

Figure 3.3. Mean *Lactobacillus* sp. pH values during Kombucha Fermentation

pH was taken every 24 hours until a pH of ≤3.≥ was reached (n=a million).

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Based on the growth curves and pH values of the probiotics during kombucha fermentation, we could say if we want to have a probiotic kombucha, then the kombucha should not be fermented below a pH of ~3.2. At that point, as mentioned previously, the probiotics seem to hit an acidic threshold where they tend to die off if that threshold is surpassed (Zhang, Wu, Du, & Chen, 2012; G-Alegría et al., 2004; Boke, Aslim, & Alp, 2010). Based on Figure 3.2, the time it takes to reach that threshold was generally around Day 6 or Day 7. Assuming the storage period will decrease the pH of the kombucha (no SCOBY present) by 0.3-0.5 on average after six weeks (Dankwa, 2021), it can be recommended the fermentation should be stopped at 3.5 to obtain a final pH of 3.2 if the goal is to maintain viability of naturally present or inoculated LAB.

In order to have an effective probiotic drink, a certain viable concentration of specified probiotic per serving of kombucha is necessary. According to most nutrition labels, an average serving size of kombucha is 16 fluid oz. A manufacturer would need to ensure enough probiotic is being consumed per serving in order for a health effect to occur and a health claim to be made. However, it is not possible to state a minimum dose for all probiotics in all food vehicles. Each species and strain require different dosages to be effective for a specific health effect (McFarland, 2015; Sanders, n.d.). These efficacy dosages can range from 50 million CFU/day to 1 trillion CFU/day depending on the product (Sanders, n.d.). In food products, microorganisms are usually found in lower ranges from 10^6-10^7 CFU/mL or CFU/g because you consume more of it compared to a supplement capsule (Bertazzoni et al., 2013). In a 16 fluid oz serving of kombucha, this level would require approximately $4.73 \times 10^{8-9}$ CFU/mL of live probiotic.

Table 3.6 describes some recommended dosages for the six *Lactobacillus* sp. studied in this research experiment in order to make specific health claims. Nonetheless, making a health claim on a product is extremely complicated, especially when it involves probiotics. Health

claims describe the relationship between a food or food component and reducing the risk of a

disease or health-related condition, and these health claims must have supporting evidence in

human trials (Donovan et al., 2012). On top of that, as of right now there is no legal definition or

standard identity for the term 'probiotic' in the United States or Europe (Donovan et al., 2012;

Sanders, n.d.).

Table 3.6. Minimum Dosages of L. plantarum, *L. bulgaricus, L. brevis, L. fermentum, L. casei,* and *L. sakei* Needed to Make Specific Health Claims

Probiotic	Recommended Dosage	Health Effect	Reference(s)
L. plantarum	10^7 minimum dose	Alleviation of symptoms of intestinal gas in IBS patients	Jew et al., 2008; Behera, Ray, & Zdolec, 2018
	1.2×10^9 CFU/day	Lowers cholesterol	Fuentes et al., 2013
	10^{10} CFU per day	Reduction of the incidence of gastrointestinal symptoms associated with antibiotic treatment	Seddik et al., 2017
	10 billion CFU/day	Decrease of antibiotic-associated diarrhea in children	Chakravorty et al., 2019
	5×10^7 CFU/day	Decrease in IBS symptoms	Bertazzoni et al., 2013
	10×10^9 CFU/day	Increased prevention of CDI in those currently treated with antibiotics [in hospital setting]	Kujawa-Szewieczek et al., 2015
L. bulgaricus	10^7 CFU/100g	Gut homeostasis	Savard et al., 2011
	9×10^7 CFU/day	Increased innate immune system stimulation	Moro-García et al., 2013
	$\geq 10^8$ CFU/g	Increased lactose digestion in those with lactose maldigestion	EFSA Panel on Dietetic Products, Nutrition and

Table 3.6. Continued

			Allergies (NDA), 2010
L. brevis	$4x10^9$ CFU/day	Increased oral health in school children	Campus et al., 2014
	$1.8x10^{11}$ CFU/day	Decreased *H. pylori* colonization	Linsalata et al., 2004
L. fermentum	$2x10^{11}$ CFU/day	Reduction of traveler's diarrhea when taken 3 weeks before traveling	McFarland, 2007
	$3x10^9$ CFU/mL	Exhibition of anti-atherogenic effects	Kullisaar et al., 2003
	$1.6x10^9$ CFU/day	Exhibition of antioxidative effects	Songisepp et al., 2005
L. casei	10^8-10^9 dose	Improvement of bowel movements and support of a balanced gut microbiota	Jew et al., 2008
	10 billion CFU/day	Decrease of antibiotic-associated diarrhea	Guo et al., 2019
	$6.5x10^9$ CFU/day	Reduction of chronic constipation	Chmielewska & Szajewska, 2010; Koebnick et al., 2003
	$3x10^{10}$ CFU/day	Prevention of the reoccurrence of various cancers	Shida & Nomoto, 2013
	$4x10^{10}$ CFU/day	Enhancement of natural killer (NK) cell activity	Shida & Nomoto, 2013
	10^8 CFU/day minimum	Decrease in rheumatoid arthritis symptoms	Vaghef-Mehrabany et al., 2014
L. sakei	$1x10^{10}$ CFU/day	Help with body fat mass reduction in those with obesity	Lim et al., 2020

3.4.3 GC-MS Analysis of Biogenic Amines

The MRS broth and unfermented sweetened tea samples inoculated with the six LAB used to identify the bacterial growth curves were analyzed by GC-MS to identify the presence of amines. By analyzing the MRS broth inoculated samples, it possible to distinguish the amino acids and biogenic amines these probiotics can produce in an ideal medium. Various amino acids and biogenic amines were detected, but they were in too small of amounts to make a significant impact. By analyzing the unfermented sweetened tea samples, it was possible to determine if the small amounts of various amino acids and biogenic amines could be produced in a less ideal medium. Analysis determined there were no biogenic amines present in these samples. However, as would be expected in tea, the samples did contain caffeine, theanine, and traces of aspartic acid.

Although the unfermented inoculated tea samples did not contain any biogenic amines, this may not be the case after completing fermentation in conjunction with a SCOBY. The species within the SCOBY produce metabolites during fermentation that are found in kombucha but are not found in unfermented tea, hence the need to analyze the LAB inoculated kombucha samples. Several samples were screened, but all samples were negative for GABA and other biogenic amines at the detection limit of 1 μmol. This is because there were little to no free amino acids to decarboxylate into biogenic amines. For example, if there is no glutamic acid present in a sample, there will not be any GABA present in that sample either. Caffeine was detected in all samples, and lysine was identified in three out of the nine samples. Because there were no detectable biogenic amines, including GABA, it can be determined that there is not a large enough presence of biogenic amines to warrant a health benefit upon consuming a serving of kombucha.

3.5 Conclusions

A change in medium (tea), temperature, and the combination of medium and temperature all had an effect on the growth rate of the probiotics tested. Together, the change in medium and temperature ameliorated those expected negative effects and resulted in better growth rates than the medium change alone. The decrease in pH for every probiotic from the tea control to the tea treatment at 25ºC was not great enough to indicate that the lactic acid bacteria tested could acidify the sweetened tea pre-fermentation, replacing the need to use previous kombucha or acetic acid. The kombucha fermentation showed an initial 2 log CFU/mL drop in probiotic population when the cultures were added to the sweetened acidified tea. They then saw a plateau in population for a few days until they began to significantly decrease around day 6. *L. fermentum* and *L. brevis* were the only to two probiotics to survive with populations above the detection limit at the end of fermentation. GABA and other biogenic amines were not detected in the inoculated kombucha samples at a detection limit of 1 μmol. Overall, looking at the survival rates it seems *Lactobacillus* sp. are not well suited to act as probiotics in a kombucha, but of those tested, *L. brevis* and *L. fermentum* would be the best options of those tested in this work. However, other probiotics that seem more promising in kombucha, such as *Bacillus coagulans*, should be tested and further researched.

3.6 References

1) Asaithambi, N., Singh, S. K., & Singha, P. (2021). Current status of non-thermal processing of probiotic foods: A review. *Journal of Food Engineering*, *303*, 110567. https://doi.org/10.1016/j.jfoodeng.2021.110567

2) Baschali, A., Tsakalidou, E., Kyriacou, A., Karavasiloglou, N., & Matalas, A. (2017). Traditional low-alcoholic and non-alcoholic fermented beverages consumed in European countries: a neglected food group. *Nutrition Research Reviews, 30*(1), 1-24. http://dx.doi.org/10.1017/S0954422416000202

3) Bauer, L., Dekevich, D., & Underthun, K. (n.d.). *Kombucha*. Food Source Information. Retrieved January 21, 2021, from https://fsi.colostate.edu/kombucha/.

4) Behera, S. S., Ray, R. C., & Zdolec, N. (2018). Lactobacillus plantarum with Functional Properties: An Approach to Increase Safety and Shelf-Life of Fermented Foods. *BioMed Research International*, *2018*. https://doi.org/10.1155/2018/9361614

5) Bertazzoni, E., Donelli, G., Midtvedt, T., Nicoli, J., & Sanz, Y. (2013). Probiotics and clinical effects: Is the number what counts? *Journal of Chemotherapy*, *25*(4), 193–212. https://doi.org/10.1179/1973947813Y.0000000078

6) Boke, H., Aslim, B., & Alp, G. (2010). The role of resistance to bile salts and acid tolerance of exopolysaccharides (EPSS) produced by yogurt starter bacteria. *Archives of Biological Sciences*, *62*(2), 323–328. https://doi.org/10.2298/ABS1002323B

7) Campus, G., Cocco, F., Carta, G., Cagetti, M. G., Simark-Mattson, C., Strohmenger, L., & Lingström, P. (2014). Effect of a daily dose of Lactobacillus brevis CD2 lozenges in high caries risk schoolchildren. *Clinical Oral Investigations*, *18*(2), 555–561. https://doi.org/10.1007/s00784-013-0980-9

8) Chakravorty, S., Bhattacharya, S., Bhattacharya, D., Sarkar, S., & Gachhui, R. (2019). 10 - Kombucha: A Promising Functional Beverage Prepared From Tea. In A. M. Grumezescu & A. M. Holban (Eds.), *Non-Alcoholic Beverages* (pp. 285–327). Woodhead Publishing. https://doi.org/10.1016/B978-0-12-815270-6.00010-4

9) Chmielewska, A., & Szajewska, H. (2010). Systematic review of randomised controlled trials: Probiotics for functional constipation. *World Journal of Gastroenterology : WJG*, *16*(1), 69–75. https://doi.org/10.3748/wjg.v16.i1.69

10) Corbo, M. R., Bevilacqua, A., Petruzzi, L., Casanova, F. P., & Sinigaglia, M. (2014). Functional Beverages: The Emerging Side of Functional Foods. *Comprehensive Reviews in*

Food Science and Food Safety, *13*(6), 1192–1206. https://doi.org/10.1111/1541-4337.12109

11) Cui, Y., Miao, K., Niyaphorn, S., & Qu, X. (2020). Production of Gamma-Aminobutyric Acid from Lactic Acid Bacteria: A Systematic Review. *International Journal of Molecular Sciences*, *21*(3). https://doi.org/10.3390/ijms21030995

12) Dankwa, A. (2021, July 18-21). *Assessing the Microbial Variability and Chemical Composition in Kombucha during Repeated Brewing Cycles and Refrigerated Storage* [Poster presentation]. IAFP 2021 Annual Meeting, Phoenix, AZ. https://www.foodprotection.org/about/news-releases/iafp-2021-2/

13) Diez-Gutiérrez, L., San Vicente, L., R. Barrón, L. J., Villarán, M. del C., & Chávarri, M. (2020). Gamma-aminobutyric acid and probiotics: Multiple health benefits and their future in the global functional food and nutraceuticals market. *Journal of Functional Foods*, *64*, 103669. https://doi.org/10.1016/j.jff.2019.103669

14) Donovan, S. M., Schneeman, B., Gibson, G. R., & Sanders, M. E. (2012). Establishing and Evaluating Health Claims for Probiotics. *Advances in Nutrition*, *3*(5), 723–725. https://doi.org/10.3945/an.112.002592

15) Dufresne, C., & Farnworth, E. (2000). Tea, Kombucha, and health: A review. *Food Research International*, *33*(6), 409–421. https://doi.org/10.1016/S0963-9969(00)00067-3

16) EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). (2010). Scientific Opinion on the substantiation of health claims related to live yoghurt cultures and improved lactose digestion (ID 1143, 2976) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA Journal*, *8*(10), 1763. https://doi.org/10.2903/j.efsa.2010.1763

17) *English LYFO DISK, KWIK-STIK, KWIK-STIK Plus Instructions For Use*. (n.d.). Retrieved October 11, 2021, from https://www.microbiologics.com/English-LYFO-DISK-KWIK-STIK-KWIK-STIK-Plus-Instructions-For-Use

18) Fuentes, M. C., Lajo, T., Carrión, J. M., & Cuñé, J. (2013). Cholesterol-lowering efficacy of Lactobacillus plantarum CECT 7527, 7528 and 7529 in hypercholesterolaemic adults. *British Journal of Nutrition*, *109*(10), 1866–1872. https://doi.org/10.1017/S000711451200373X

19) G-Alegría, E., López, I., Ruiz, J. I., Sáenz, J., Fernández, E., Zarazaga, M., Dizy, M., Torres, C., & Ruiz-Larrea, F. (2004). High tolerance of wild Lactobacillus plantarum and Oenococcus oeni strains to lyophilisation and stress environmental conditions of acid pH and ethanol. *FEMS Microbiology Letters*, *230*(1), 53–61. https://doi.org/10.1016/S0378-1097(03)00854-1

20) Greenwalt, C. J., Ledford, R. A., & Steinkraus, K. (1998). Determination and Characterization of the Antimicrobial Activity of the Fermented TeaKombucha. *LWT - Food Science and Technology*, *31*(3), 291–296. https://doi.org/10.1006/fstl.1997.0354

21) Greenwalt, C. J., Steinkraus, K. H., & Ledford, R. A. (2000). Kombucha, the Fermented Tea: Microbiology, Composition, and Claimed Health Effects. *Journal of Food Protection*, *63*(7), 976–981. https://doi.org/10.4315/0362-028X-63.7.976

22) Guo, Q., Goldenberg, J. Z., Humphrey, C., El Dib, R., & Johnston, B. C. (2019). Probiotics for the prevention of pediatric antibiotic-associated diarrhea. *The Cochrane Database of Systematic Reviews*, *2019*(4), CD004827. https://doi.org/10.1002/14651858.CD004827.pub5

23) Guo, Y., Tian, X., Huang, R., Tao, X., Shah, N. P., Wei, H., & Wan, C. (2017). A physiological comparative study of acid tolerance of Lactobacillus plantarum ZDY 2013 and L. plantarum ATCC 8014 at membrane and cytoplasm levels. *Annals of Microbiology*, *67*(10), 669–677. https://doi.org/10.1007/s13213-017-1295-x

24) Hong, S.-I., Kim, Y.-J., & Pyun, Y.-R. (1999). Acid Tolerance of Lactobacillus plantarum from Kimchi. *LWT - Food Science and Technology*, *32*(3), 142–148. https://doi.org/10.1006/fstl.1998.0517

25) Ivanišová, E., Meňhartová, K., Terentjeva, M., Harangozo, Ľ., Kántor, A., & Kačániová, M. (2020). The evaluation of chemical, antioxidant, antimicrobial and sensory properties of kombucha tea beverage. *Journal of Food Science and Technology*, *57*(5), 1840–1846. https://doi.org/10.1007/s13197-019-04217-3

26) Jew, S., Vanstone, C. A., Antoine, J.-M., & Jones, P. J. H. (2008). Generic and product-specific health claim processes for functional foods across global jurisdictions. *The Journal of Nutrition*, *138*(6), 1228S-36S. https://doi.org/10.1093/jn/138.6.1228S

27) Kandola, S., Teotia, U. V. S., Kumar, R., Mishra, A. K., & Singh, A. (2016). Investigation of acid tolerance attribute of various Lactobacillus casei group strains. *Indian Journal of Animal Research*, *50*(2). https://doi.org/10.18805/ijar.9539

28) Koebnick, C., Wagner, I., Leitzmann, P., Stern, U., & Zunft, H. F. (2003). Probiotic Beverage Containing *Lactobacillus casei* Shirota Improves Gastrointestinal Symptoms in Patients with Chronic Constipation. *Canadian Journal of Gastroenterology*, *17*(11), 655–659. https://doi.org/10.1155/2003/654907

29) *Kombucha | Description, History, & Nutrition | Britannica*. (n.d.). Retrieved September 28, 2021, from https://www.britannica.com/topic/kombucha

30) *Kombucha Brewers International – An Association of Commercial Kombucha Brewers*. (n.d.). Retrieved January 11, 2021, from https://kombuchabrewers.org/

31) Kujawa-Szewieczek, A., Adamczak, M., Kwiecień, K., Dudzicz, S., Gazda, M., & Więcek, A. (2015). The Effect of Lactobacillus plantarum 299v on the Incidence of Clostridium difficile Infection in High Risk Patients Treated with Antibiotics. *Nutrients*, *7*(12), 10179–10188. https://doi.org/10.3390/nu7125526

32) Kullisaar, T., Songisepp, E., Mikelsaar, M., Zilmer, K., Vihalemm, T., & Zilmer, M. (2003). Antioxidative probiotic fermented goats' milk decreases oxidative stress-mediated atherogenicity in human subjects. *British Journal of Nutrition*, *90*(2), 449–456. https://doi.org/10.1079/BJN2003896

33) La Torre, C., Fazio, A., Caputo, P., Plastina, P., Caroleo, M. C., Cannataro, R., & Cione, E. (2021). Effects of Long-Term Storage on Radical Scavenging Properties and Phenolic Content of Kombucha from Black Tea. *Molecules*, *26*(18), 5474. https://doi.org/10.3390/molecules26185474

34) *Lactobacillus brevis (Orla-Jensen) Bergey et al. | ATCC*. (n.d.). Retrieved October 11, 2021, from https://www.atcc.org/products/14869

35) *Lactobacillus casei (Orla-Jensen) Hansen and Lessel | ATCC*. (n.d.). Retrieved October 11, 2021, from https://www.atcc.org/products/393

36) *Lactobacillus delbrueckii subsp. Bulgaricus (Orla-Jensen) Weiss et al. | ATCC*. (n.d.). Retrieved October 11, 2021, from https://www.atcc.org/products/11842

37) *Lactobacillus fermentum Beijerinck | ATCC*. (n.d.). Retrieved October 11, 2021, from https://www.atcc.org/products/9338

38) *Lactobacillus plantarum (Orla-Jensen) Bergey et al. | ATCC*. (n.d.). Retrieved October 11, 2021, from https://www.atcc.org/products/8014

39) *Lactobacillus sakei subsp. Sakei Katagiri et al. | ATCC*. (n.d.). Retrieved October 11, 2021, from https://www.atcc.org/products/15521

40) Lim, S., Moon, J. H., Shin, C. M., Jeong, D., & Kim, B. (2020). Effect of Lactobacillus sakei, a Probiotic Derived from Kimchi, on Body Fat in Koreans with Obesity: A Randomized Controlled Study. *Endocrinology and Metabolism*, *35*(2), 425–434. https://doi.org/10.3803/EnM.2020.35.2.425

41) Linsalata, M., Russo, F., Berloco, P., Caruso, M. L., Matteo, G. D. I., Cifone, M. G., Simone, C. D. E., Ierardi, E., & Leo, A. D. (2004). The Influence of Lactobacillus brevis

on Ornithine Decarboxylase Activity and Polyamine Profiles in Helicobacter pylori-Infected Gastric Mucosa. *Helicobacter*, *9*(2), 165–172. https://doi.org/10.1111/j.1083-4389.2004.00214.x

42) May, A., Narayanan, S., Alcock, J., Varsani, A., Maley, C., & Aktipis, A. (2019). Kombucha: A novel model system for cooperation and conflict in a complex multi-species microbial ecosystem. *PeerJ*, *7*, e7565. https://doi.org/10.7717/peerj.7565

43) McFarland, L. V. (2007). Meta-analysis of probiotics for the prevention of traveler's diarrhea—ScienceDirect. *Travel Medicine and Infectious Disease*, *5*(2), 97–105. https://doi.org/10.1016/j.tmaid.2005.10.003

44) McFarland, L. V. (2015). From Yaks to Yogurt: The History, Development, and Current Use of Probiotics | Clinical Infectious Diseases | Oxford Academic. *Clinical Infectious Diseases*, *60*(suppl_2, May 2015), S85–S90. https://doi.org/10.1093/cid/civ054

45) Melia, S., Yuherman, Y., Jaswandi, J., & Purwati, E. (2018). SELECTION OF BUFFALO MILK LACTIC ACID BACTERIA WITH PROBIOTIC POTENTIAL. *Asian Journal of Pharmaceutical and Clinical Research*, *11*(6), 186. https://doi.org/10.22159/ajpcr.2018.v11i6.24809

46) Moro-García, M. A., Alonso-Arias, R., Baltadjieva, M., Benítez, C. F., Barrial, M. A. F., Ruisánchez, E. D., Santos, R. A., Sánchez, M. A., Miján, J. S., and López-Larrea, C. (2013). Oral supplementation with Lactobacillus delbrueckii subsp. Bulgaricus 8481 enhances systemic immunity in elderly subjects. *Springer*, *35*(4), 1311–1326. https://doi.org/10.1007/s11357-012-9434-6

47) Nummer, B. A. (2013). Kombucha brewing under the Food and Drug Administration model Food Code: Risk analysis and processing guidance. *Journal of Environmental Health*, *76*(4), 8–11.

48) Perry, J. (2019). *FSN 439: Food Microbiology Lab: Enumeration Methods* [Class handout]. School of Food and Agriculture, University of Maine.

49) Piqué, N., Berlanga, M., & Miñana-Galbis, D. (2019). Health Benefits of Heat-Killed (Tyndallized) Probiotics: An Overview. *International Journal of Molecular Sciences*, *20*(10), 2534. https://doi.org/10.3390/ijms20102534

50) Reva, O. N., Zaets, I. E., Ovcharenko, L. P., Kukharenko, O. E., Shpylova, S. P., Podolich, O. V., de Vera, J.-P., & Kozyrovska, N. O. (2015). Metabarcoding of the kombucha microbial community grown in different microenvironments. *AMB Express*, *5*(1), 35. https://doi.org/10.1186/s13568-015-0124-5

51) Sanders, M. E. (n.d.). *How Do We Know When Something Called "Probiotic" Is Really a Probiotic? A Guideline for Consumers and Health Care Professionals*.

52) Savard, P., Lamarche, B., Paradis, M.-E., Thiboutot, H., Laurin, É., & Roy, D. (2011). Impact of Bifidobacterium animalis subsp. Lactis BB-12 and, Lactobacillus acidophilus LA-5-containing yoghurt, on fecal bacterial counts of healthy adults. *International Journal of Food Microbiology*, *149*(1), 50–57. https://doi.org/10.1016/j.ijfoodmicro.2010.12.026

53) Seddik, H. A., Bendali, F., Gancel, F., Fliss, I., Spano, G., & Drider, D. (2017). Lactobacillus plantarum and Its Probiotic and Food Potentialities. *Probiotics and Antimicrobial Proteins*, *9*(2), 111–122. https://doi.org/10.1007/s12602-017-9264-z

54) Shah, N. & Jelen, P. (1990). Survival of Lactic Acid Bacteria and Their Lactases under Acidic Conditions. *Journal of Food Science*, *55*(2), 506–509. https://doi-org.wv-o-ursus-proxy02.ursus.maine.edu/10.1111/j.1365-2621.1990.tb06797.x

55) Shida, K., & Nomoto, K. (2013). Probiotics as efficient immunopotentiators: Translational role in cancer prevention. *The Indian Journal of Medical Research*, *138*(5), 808–814.

56) Siró, I., Kápolna, E., Kápolna, B., & Lugasi, A. (2008). Functional food. Product development, marketing and consumer acceptance—A review. *Appetite*, *51*(3), 456–467. https://doi.org/10.1016/j.appet.2008.05.060

57) Songisepp, E., Kals, J., Kullisaar, T., Mändar, R., Hütt, P., Zilmer, M., & Mikelsaar, M. (2005). Evaluation of the functional efficacy of an antioxidative probiotic in healthy volunteers. *Nutrition Journal*, *4*(1), Article 1. https://cyberleninka.org/article/n/921704

58) Tur, J. A., & Bibiloni, M. M. (2016). Functional Foods. In B. Caballero, P. M. Finglas, & F. Toldrá (Eds.), *Encyclopedia of Food and Health* (pp. 157–161). Academic Press. https://doi.org/10.1016/B978-0-12-384947-2.00340-8

59) Vaghef-Mehrabany, E., Alipour, B., Homayouni-Rad, A., Sharif, S., Asghari-Jafarabadi, M., Zavvari, S. (2014). Probiotic supplementation improves inflammatory status in patients with rheumatoid arthritis—ScienceDirect. *Nutrition*, *30*(4), 430–435. https://doi.org/10.1016/j.nut.2013.09.007

60) Wu, C.-H., Hsueh, Y.-H., Kuo, J.-M., & Liu, S.-J. (2018). Characterization of a Potential Probiotic Lactobacillus brevis RK03 and Efficient Production of γ-Aminobutyric Acid in Batch Fermentation. *International Journal of Molecular Sciences*, *19*(1). https://doi.org/10.3390/ijms19010143

61) Yıkmış, S., & Tuğğüm, S. (2019). Evaluation of Microbiological, Physicochemical and Sensorial Properties of Purple Basil Kombucha Beverage. *Turkish Journal of Agriculture -*

Food Science and Technology, *7*(9), 1321–1327. https://doi.org/10.24925/turjaf.v7i9.1321-1327.2550

62) Zeng, X. Q., Pan, D. D., & Zhou, P. D. (2011). Functional Characteristics of Lactobacillus fermentum F1. *Current Microbiology*, *62*(1), 27–31. https://doi.org/10.1007/s00284-010-9669-3

63) Zhang, J., Wu, C., Du, G., & Chen, J. (2012). Enhanced acid tolerance in Lactobacillus casei by adaptive evolution and compared stress response during acid stress. *Biotechnology and Bioprocess Engineering*, *17*(2), 283–289. https://doi.org/10.1007/s12257-011-0346-6

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