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Microbial Diversity of the Symbiotic Colony of Bacteria and Yeast (SCOBY) and its Impact on the Organoleptic Properties of Kombucha

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MICROBIAL DIVERSITY OF THE SYMBIOTIC COLONY OF BACTERIA AND YEAST (SCOBY) AND ITS IMPACT ON THE ORGANOLEPTIC PROPERTIES OF KOMBUCHA

By

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An Abstract of the Thesis Presented
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Kombucha is an effervescent fermented tea beverage that is gaining popularity for its probiotic nature and purported health benefits. The market for kombucha is expected to reach $1.8 billion by the year 2020. The composition of microbes that compose the symbiotic colony of bacteria and yeast (SCOBY) is highly variable with some species commonly found from the *Gluconobacter*, *Acetobacter*, *Zygosaccharomyces*, *Saccharomyces*, and *Schizosaccharomyces* genera.

It was hypothesized that different SCOBYs, obtained from different sources would vary in microbial diversity and produce different biochemical and flavor profiles in the resulting beverage over several generations. Kombucha is a fermented product and ethanol is often present in the final beverage, so it is important that a quality control method exist. The main objectives of this research were: (1a) to investigate the microbial variation between three SCOBYs of different origins and (1b) determine if there are significant differences within SCOBYs over 10 generations; (2) to determine the impact that the kombucha SCOBY has on the biochemical

profile of the beverage; and (3) to learn the vocabulary words that consumers use to characterize the flavor notes in kombucha.

Kombucha was produced in a laboratory setting with three kombucha SCOBY pellicles prescreened for fermentate heterogeneity by High-performance liquid chromatography (HPLC) analysis. Two batches from each unique SCOBY were produced every 14 days. The liquid from batches 1, 5, 10, and the corresponding mother SCOBY were saved for downstream analysis including DNA sequencing with Oxford Nanopore's MinION and HPLC analysis. A sensory evaluation study was also conducted to determine the vocabulary that consumers use to describe kombucha.

The two main microbes present in the SCOBYs tested in this research were *Komagataeibacter xylinus* and *Gluconobacter oxydans*. The diversity of the SCOBY did change slightly with time; however, over ten generations, the slight change in diversity was not significant (p-value > 0.05). By calculating beta diversity, Fisher's alpha, Shannon diversity, and Simpson diversity, a clearer picture of the diversity of the SCOBY community between SCOBYs of different origins could be determined and demonstrated consortia to be quite different.

Investigating the two main microbes in kombucha, *K. xylinus* and *G. oxydans*, the Pearson correlations between the microbe and the flavor compounds acidic acid, lactic acid, glucose, fructose and sucrose were determined. *K. xylinus* was negatively correlated to glucose, fructose, lactic acid and acetic acid, and positively correlated to sucrose (part of the formulation) concentrations suggesting this microbe dominates earlier in the fermentation. *G. oxydans* was positively correlated to the concentrations of glucose, fructose, lactic acid and acetic acid, but negatively correlated to sucrose, suggesting it dominates later in the fermentation. However, these correlation coefficients were low and not significant.

A sensory evaluation study using 66 untrained panelists revealed the top 3 favorite flavors of kombucha amongst these panelists were ginger + other flavorings, ginger, and tropical flavors. Comparing two commercial and one lab-made kombucha sample showed that consumers found a significant difference in the vinegar, sour and bubbly flavor notes.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Kombucha is an effervescent fermented tea beverage that is growing in popularity for its reported health benefits. This probiotic beverage is made by combining sweetened black tea with a cellulose matrix of bacteria and yeast, which is more commonly referred to as a SCOBY. Kombucha is known to contain healthy organic acids, such as gluconic and glucuronic acid, sugars, vitamins, probiotics, polyphenols, antioxidants, amino acids and trace amounts of alcohol (Jayabalan et al., 2014).

Some of the reported health benefits obtained through the consumption of kombucha include reduced blood pressure, arthritis relief, gut health and resistance to cancer (Jayabalan, Marimuthu, Swaminathan, 2007; Leal, Valenzuela, Jayabalan, Huerta, Escalante-Aburto, 2018). However, many of these health benefits have not been scientifically proven, especially with human models (Leal et al., 2018).

Kombucha tea originated in East Asia during the Tsin Dynasty around 220 B.C. It was later used in China by Dr. Kombu in 414 A.D. in hopes of curing the digestive issues of Emperor Inkyo. Other names for this beverage include “Mother of Vinegar”, “Tea Mould”, “Mo-Go” and the “Chinese Tea of Immortality” (Kombucha Kamp, 2007). Today, kombucha SCOBYs are sold online for home brewing of kombucha. Since a new SCOBY (“daughter”) is produced when brewing every batch, many kombucha home-brewers receive their first SCOBY from a friend. The finished kombucha tea product is also made commercially and can be purchased bottled at health food stores and supermarkets.

Since kombucha is produced through the process of alcoholic fermentation, ethanol is a product usually found in the final beverage. Kombucha is usually sold as a non-alcoholic beverage (with the exception of a few companies that label it as containing alcohol). By law, for

a product to be merchandised as a non-alcoholic beverage, it must contain less than 0.5% alcohol by volume (ABV) (Martinez-Belkin, 2015). In 2015, kombucha beverages spanning several different brands and flavors received warning letters from the Alcohol and Tobacco Tax and Trade Bureau, because alcohol levels in these bottles were above 0.5% ABV (Cole, 2018). These violations were most likely caused by the lack of adequate quality control methods during production.

The American market for kombucha is expected to reach $1.8 billion by the year 2020 (Ebersole, Liu, Schmidt, Eckert, Brown, 2017). In 2016, kombucha sales reached $600 million and were expected to continue increasing by 25% a year (Cole, 2018; Hamblin, 2016). As recently as 2017, the market for probiotic beverages (such as kombucha) grew by 37.4% (Firman, 2018). The general consensus was that the beverages were compliant when they left the facility, however, due to potential temperature abuse during retail the ethanol fermentation was accelerated. In this expanding market, quality control procedures are lacking. This is an issue the research described in this thesis helps to address.

While the composition of microorganisms that make up the kombucha SCOBY is highly variable, some common microbes are consistently found. The most common bacteria include aerobic bacteria genera *Gluconobacter*, and *Acetobacter* (Amarasinghe, Weerakkody, Waisundara, 2018). In addition to the aerobic bacteria, the SCOBY contains up to 30% *Lactobacillus* (Marsh, O'Sullivan, Hill, Ross, Cotter, 2013). *Schizosaccharomyces pombe*, *Brettanomyces bruxellensis*, *Saccharomyces cereviseae*, and *Zygosaccharomyces rouxii* are some of the most common yeast species present in the kombucha SCOBY (Villarreal-Soto et al., 2018). *Zygosaccharomyces* has been found at levels above 95% in most SCOBY samples (Marsh et al., 2013). The yeast hydrolyzes the sucrose in the initial sweet tea into glucose and fructose

and utilizes these monosaccharides to produce ethanol and a number of other metabolites. The bacteria then metabolize the ethanol into organic acids. All of the bacteria and yeast that are present in the kombucha SCOBY exist within a cellulose matrix. *Acetobacter xylinum* and *Komagataeibacter rhaeticus* strain P 1463 are partly responsible for producing the cellulose (De Roos & De Vuyst, 2018 and Semjonovs et al., 2017).

The current methods for determining the diversity of microbes present in a SCOBY are either cultural techniques or DNA sequencing. The first part of this research project used Oxford Nanopore's portable DNA and RNA sequencer, the MinION, to determine the microbial community diversity of three kombucha SCOBYs over ten subsequent batches.
It was predicted that the microbial composition would change over time, and the microbial diversity would increase.

As previously mentioned, in today's market there is a lack of suitable analytical methodology that can be used to promote kombucha quality control. Many of the current analytical methods were not developed for kombucha, but for other products such as wine, grape must or grapevine berries. These methods quantify some of the organic acids, sugars, and alcohols in kombucha, but not all. Citric acid, tartaric acid, malic acid, and succinic acid are some of the organic acids that these existing methods can quantify. Glucose, fructose, ethanol, and glycerol have been separated by these methods too, but none of these techniques simultaneously quantify all of these compounds in kombucha. (Eyeghe-Bickong et al., 2012; Lopez, Gomez, 1996). The second part of this research project focuses on the development of a method to simultaneously detect organic acids, sugars, and alcohols in kombucha for quality control purposes.

To date, there has been very little sensory research conducted with kombucha beverages. The work that has been done focused mainly on the type of tea or sweetener that is used. Kombucha produced from either white or yellow tea has been shown to have the highest consumer acceptability, and kombucha produced from black tea had the lowest consumer acceptability (Gramza-Michalowska et al., 2016). Kombucha has also been produced with different types of sweeteners, mainly molasses. Consumers described kombucha produced with molasses to have a dark brown color and a caramelized taste and smell (Malbasa et al., 2008).

Currently, the words that consumers typically use to describe kombucha, and how those portrayals relate to their purchase intent and overall acceptance of the beverage has not yet been determined. The final section of the research described in this thesis helps to provide some insight into this matter. It was predicted that consumers would select words such as vinegary, bubbly, acidic, and tea flavor since this is what previous studies have suggested consumers identify as the main sensory attributes of kombucha.

This thesis had three main objectives: (1) to investigate the variation between three SCOBYs of different origin and to determine if there are significant differences within SCOBYs over 10 generations; (2) to determine the impact that the kombucha SCOBY variability has on the biochemical profile of the beverage; and finally (3) to define the flavor impacts from SCOBY variability by learning the vocabulary words that consumers use to characterize the flavor notes in kombucha.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

The millennia-old fermented tea beverage, kombucha, has gained popularity over the last several years. The Champagne of Life, The Remedy of Immortality, Tea Mould, Kambotscha, Mo-Gu, Mother of Vinegar and The Tea Beast are just some of the names given to kombucha over the years (Kombucha Kamp, 2007). Kombucha is produced by placing a Symbiotic Colony of Bacteria and Yeast (SCOBY) into sweetened black tea acidified with either vinegar or starter tea. The microbes contained within the SCOBY cellulose pellicle ferment the sugar into ethanol; organic acids and live microorganisms are produced in the process. The pH of a finished kombucha tea product is usually between 2.8 and 3.2, normally finishing around 3.0 (Vazquez-Cabral et al., 2017). It is important to monitor the pH of kombucha, because a pH of 2.0 or lower is too acidic for human consumption as it can lead to acidosis (Klein, 2017).

In 2016, kombucha sales were $600 million in the United States and predicted to grow by 25% annually (Cole, 2018; Hamblin, 2016). The market for beverages high in probiotics, such as kombucha, grew 37.4% in 2017 (Firman, 2018) and sales of kombucha products are expected to reach about $1.8 billion by the year 2020 (Ebersole, Liu, Schmidt, Eckert, Brown, 2017).

2.1.1. Health benefits

The Chinese elixir of immortality, also known as kombucha, boasts numerous health benefits. It was often given to Chinese emperors in hopes to make them immortal (Swann, 2016; Troitino, 2017). Recently, researchers have been studying the methods of kombucha production with the goal of increasing the health benefits of the final product. For example, scientists discovered white oak leaf infusions for kombucha can help decrease oxidative stress in a

macrophage model (Vazquez-Cabral et al., 2017). Researchers have also determined antioxidant content can be increased by brewing with brown sugar, in place of sucrose (Watawana, Jayawardena, Ranasinghe, Waisundara, 2016).

While kombucha products are known for their health benefits, many of these benefits have not been proven scientifically with human models (Greenwalt, Steinkraus & Ledford, 2000; Leal et al., 2018). However, the daily consumption of kombucha has been correlated with a reduced incidence of cancer (Jayabalan, Marimuthu, Swaminathan, 2007; Leal et al., 2018). Polyphenols, gluconic acid, glucuronic acid, lactic acid, amino acids, micronutrients, and vitamins in the beverage are presumed to provide the health benefits. The antioxidants in kombucha, such as ascorbic acid and tea polyphenols, function by metal chelation and electron donation, but not through free radical scavenging. The fermentation process of kombucha helps to increase the bioactive compounds and thus, the health benefits from ingesting this beverage (Jayabalan et al., 2014; Lobo, Dias, Shenoy, 2017). Kombucha has shown antioxidant and immunomodulating properties in Sprague Dawley rats in previous studies (Dipti et al., 2003). The bioactive compounds act in a synergistic manner to provide the health benefits. Due to its probiotic nature, kombucha is on the list of functional beverages and species from the *Bifidobacterium* and/or *Lactobacillus* genera have been identified as probiotics in kombucha beverages (Watawana, Jayawardena, Guawardhana, Waisundara, 2015).

While the amount of kombucha one must consume to enjoy its health benefits is based on body mass and health (pregnant, immunocompromised, elderly etc), health professionals recommended limiting kombucha consumption to 4 oz per day, diluted with water (Underthun, Dekervich, Bauer, 2017). Overall, the maximum amount of kombucha that should be consumed is 32 oz (Ho, 2012). Normally, a commercial bottle contains between 12 and 16 oz.

Health benefits are closely related to fermentation time. If the tea is fermented for more than two months, the antioxidant level of the beverage decreases, which decreases the protection from free radicals that antioxidants can provide. The total levels of organic acids that can accumulate over longer fermentation times may be harmful for human consumption. The high levels of organic acids can accumulate intracellularly and lead to a decreased pH in the body, possibly inhibiting cellular processes (Amarasinghe, Weerakkody, Waisundara, 2018; Hazan, Levine, Abeliovich, 2004).

2.1.2. History

First consumed in Eastern Asia for its health benefits, kombucha was prized during the Tsin Dynasty, around 220 B.C. for its detoxifying and energizing properties. In 414 A.D., Dr. Kombu brought this tea fungus to Japan in hopes it would help cure the digestive problems of the ailing Emperor Inkyo. Once trade routes began to expand, kombucha, Mo-Gu, as it was called on the trade routes, quickly expanded to Eastern Europe. A high demand for hot tea during World War II led to a decrease in kombucha production since the tea required to produce kombucha was being used in the production of hot tea. During the 1950s it was re-introduced in Germany and France. Today, diverse flavors of kombucha can be found in retail stores worldwide. The SCOBY, which is needed to produce kombucha tea, can be purchased from many online retailers for home production (Jayabalan et al., 2014).

2.2. Modern Production Quality

Since kombucha is produced through fermentation, ethanol is a common metabolic product. Ethanol levels below 0.5% alcohol by volume (ABV) are considered trace amounts by law and such alcohol-containing products do not need to be labeled as “containing alcohol” (Cole, 2018; Martinez-Belkin, 2015). In 2015, government regulators found that the ethanol

levels in some non-alcoholic commercial kombucha bottles were above the 0.5% (ABV) threshold and labels were not reflective of this (Ebersole et al., 2017; Cabrera & Weisfeldt, 2015; Hamblin, 2016). Some brands of kombucha, such as Boochcraft (Chula Vista, CA) and Urban Farm Fermentory (Portland, ME), sell under the Bureau of Alcohol, Tobacco, Firearms and Explosives (ATF) guidelines for products containing alcohol.

During a routine audit, in 2015, of the Portland, Maine Whole Foods Market store, an inspector noticed bottles of kombucha were leaking, which got him thinking about the fermentation process and the production of ethanol. Samples were sent to the University of Maine, where analysis revealed alcohol levels as high as 2.5% ABV (Hamblin, 2016; Martinez-Belkin, 2015). Kombucha was briefly pulled from the shelves, but after some adjustments to the production process, it was quickly back on the retail market. Now, testing of the ethanol levels is required by most states. (Hamblin, 2016; Vazquez-Cabral et al., 2016). The alcohol situation seemed to be resolved, but then the Alcohol and Tobacco Tax and Trade Bureau (TTB) sent letters to several kombucha producers notifying them they were again in violation of the alcohol labeling law (Cabrera & Weisfeldt, 2015; Hamblin, 2016). Businesses found in violation of this rule were threatened with up to an $11,000 fine if they failed to change their label to reflect the true alcohol content (Cabrera & Weisfeldt, 2015).

As an example of the confusion caused by this rule, an aspiring commercial kombucha fermentory consulted the federal government to rule on their product, as it contained alcohol from the fermentation process. The government ruled that the company would need to apply for a winery license. Kombucha can contain alcohol, so the government considers the sale of kombucha to be the sale of an alcoholic beverage (Vazquex-Cabral et al., 2016). Further complicating retail sales, as a fermented beverage under the FDA's Food Code, kombucha is

categorized as a “specialized product” and requires producers to submit a food safety plan (Nummer, 2013).

In 2017, eighteen samples of kombucha were tested by gas chromatography (GC) headspace analysis and ethanol contents were determined to be between 1.12% - 2.00% (v/v); showing that the ethanol issue still has not been resolved. These samples were analyzed over a period of 60 days under either 4°C or 22°C holding temperatures. The bottles stored at 22°C had a significant increase in their alcohol content after seven days, reaching a maximum after 14 days. The alcohol content of the bottles stored at 4°C increased slowly for 14 days then remained unchanged (Talebi, Frink, Patil, Armstrong, 2017).

In January 2018, a class action lawsuit against GT’s Kombucha (Beverly Hills, CA) was settled with GT’s agreeing to pay more than $8 million dollars in fines over allegations of mislabeled sugar, antioxidant and alcohol contents. Because of this lawsuit, GT’s Kombucha changed its label to include a warning statement for the alcohol content and agreed to outside testing for alcohol compliance (CNN Wire Service, 2018).

Organoleptic properties contribute greatly to the flavor and health benefits of kombucha. For example, acetic acid is known for its acidic, astringent flavor, while gluconic acid generally has a much milder flavor (Huang, Hu, Rohrer, 2016). Besides producing a characteristic flavor, some metabolic compounds also can contribute to the health benefits that kombucha is known for (Jayabalan et al., 2007). The major organic acids contributing to health benefits are lactic, gluconic, and glucuronic acids. Glucuronic acid helps to detoxify the liver and can help to increase the bioavailability of polyphenols (Leal et al., 2018). Organic acids can also have a positive impact on sleep and have shown *in vitro* antimicrobial activity (Greenwalt, Steinkraus,

Ledford, 2000). Thus, it is important to have a quick and reliable method for the simultaneous detection of the organoleptic compounds and ethanol in kombucha beverages.

Currently, analytical methods are lacking for the simultaneous detection of organic acids, sugars, and ethanol in kombucha, but there are numerous methods for wine, beer and even vinegar (Mark, Nikfardjam, Avar, Ohmacht, 2005 & "Beverage Alcohol Methods," 2017). The published methods for kombucha have drawbacks, such as low accuracy and large RSD_r values. In 2016, a method for the determination of ethanol in kombucha products using headspace gas chromatography with flame ionization detection was validated (Ebersole et al., 2017).

The TTB tests ethanol in kombucha products using a distillation-specific gravity method from the Association of Official Analytical Chemists, International (AOAC). They use a densitometer instead of a pycnometer to complete this analysis ("Kombucha Information and Resources," 2017). The most common methods of testing include using a hydrometer, refractometer, or an Alcolyzer Beer Analyzing System. These techniques tend to be highly unreliable, especially for kombucha. Use of a High-Performance Liquid Chromatography (HPLC) system for ethanol analysis is not approved by the TTB. Only distillation, densitometry, or gas chromatography are acceptable methods for measuring alcohol content according to the TTB ("KBI Approved Alcohol Testing Methodology", 2018).

Enzytc™ Liquid Ethanol is another method of testing for the alcohol (ethanol) content in kombucha. This rapid assay kit contains both components needed for the enzymatic test, which is measured in 3 mL cuvettes at 340 nm within 20 minutes of the reaction start time. The assay is based on the catalytic activity of the enzyme alcohol dehydrogenase oxidizing the ethanol to acetaldehyde. The acetaldehyde then converts NAD^+ to NADH, which can be measured at 340 nm. Lacorn and Hektor (2019) used this AOAC Official Method (2017.07) to test four samples

of kombucha diluted 1:50, tested in duplicate by two individuals over 3 days and showed the alcohol contents ranging from 2.73 to 5.65g/L (0.346% to 0.716% ABV). A reproducibility study was conducted and showed a relative standard deviation (RSD) of 1%.

All current analytical methods for kombucha focus on a specific class of compounds; either sugars, organic acids or alcohols, requiring different assays and multiple, often expensive, analytical instruments. There is a need in the growing kombucha market for a method to simultaneously detect the organoleptic/bioactive compounds and ethanol in this fermented beverage for quality control purposes. In addition to metabolically produced organic acids, fruits added to kombucha contain additional organic acids such as malic, lactic, oxalic and citric acids and sugars (Mahmood, Anwar, Abbas, Boyce, Saari, 2012). The increased variety of organic acids increases the complexity of analysis, and the additional sugar allows for continued fermentation. Since there are yeast and bacteria still present in the finished tea, these microbes can ferment the sugar from the fruit flavoring into ethanol (Greenwalt et al., 2000 & Klein, 2017). Some of the organic acids that are produced either during the primary fermentation or from the addition of flavoring and secondary fermentation can enhance the health benefits of kombucha. One such organic acid associated with some health benefits is gluconic acid, which can help reduce the occurrence of stomach cancer (Watawana, Jayawardena, Gunawardhana, Waisundara, 2015). Thus, a quality control method is needed to ensure the organic acids that health benefits can be obtained from are present in the finished product. The sugar concentration and organic acids need to be quantified to ensure a finished product with consistent sensory attributes. The ethanol needs to be quantified for legal reasons (Cabrera & Weisfeldt, 2015; Ebersole et al., 2017; Hamblin, 2016).

2.3. Microbiology

Much of the variation in the sugar, organic acid and ethanol concentrations can be attributed to the microbial variation of the SCOBY. New species of yeast and bacteria continue to be characterized in the SCOBY (Cletus et al., 2001; Debasree et al., 2006). All kombucha is generally recognized to contain at least: *Acetobacter xylinum,* and two yeast species, *Zygosaccharomyces rouxii* and *Candida* sp. (Blanc, 1996). However, the exact ratios and concentrations of each of these microbes are highly variable (Reva et al., 2015).

2.3.1. Methods of determination

Traditionally, culture-based methods have been used to determine the microbial community composition in the kombucha SCOBY. Recently, DNA sequencing, which is the process of determining the order of the nucleotide bases in a strand of DNA, has become a rapid and useful tool ("DNA Sequencing," 2019; Marsh, O'Sullivan, Hill, Ross, Cotter, 2013). Some methods of microbial sequencing include 16S rRNA sequencing, shotgun metagenomics, microbial transcriptome analysis, and whole genome sequencing ("Microbial Sequencing Methods," 2019). Oxford Nanopore's portable DNA sequencer, the MinION, is at tool that uses nanopore sequencing technology to identify bacterial and/or fungal species in real-time. Weighing less than 100 g and needing only the USB 3.0 port of a laptop, the MinION can generate 10-20 Gb of DNA sequence data per flow cell (MinION, 2018).

Figure 2.1. MinION sequencer open and operating with a flow cell.

The MinION DNA sequencing tool has not yet been used to assess the microbial diversity of kombucha and the kombucha SCOBY, but it has been used for similar research. For example, the MinION has been used to sequence DNA from the contents of rats' stomachs to determine their diet. For this work, researchers barcoded and pooled up to 12 samples per flow cell. This study was able to obtain between 527 and 606 base pair reads, on average (Pearman, Smith, Breckell, Dale, Freed, Silander, 2018). Similar studies have shown that small DNA sequencing tasks could be completed in under three hours using the MinION as the sequencing platform, making the MinION one of the fastest of the third generation/next-generation sequencing technologies (Wei, Weiss, Williams, 2018).

A study published in 2017 using PCR-ITS RFLP technique to determine yeast biodiversity determined isolates taken from the kombucha SCOBY to be mostly *Dekkera bruxellensis*. The conserved region of the yeast was amplified using the universal primers for ITS 1 and ITS 4. Enzymatic digestion was conducted with *Hinf*I, *Hae*III, and *Hha*I. Results and ITS-RFLP patterns were compared with reported results to identify the yeast isolates (Matei, Diguta, Popa, Petruta and Matei, 2018). One of the first studies of commercial kombucha microbial communities showed the dominant bacterial populations belonged to *Acetobacter* and *Lactobacteriaceae,* and the dominant yeast genera belonged to *Dekkera, Hanseniaspora*, and *Zygosaccharomyces*. The diversity of species decreased over the course of the eight-day fermentation. This study used targeted 16S and 26S rRNA amplicon sequencing to determine the dominant microbial species following PCR amplification of the V1-V3 regions of the 16S rDNA. Indexing was completed using the Nextera XT index primers 1 and 2. The samples were pooled and sequenced using a MiSeq sequencer and V3 reagents (Coton, Pawtowski, Taminiau, Burgaud, Deniel, Coulloumme-Labarthe, Fall, Daube, and Coton, 2017).

2.3.2. Composition of the SCOBY

The SCOBY consists of bacteria and yeast in a symbiotic relationship within a cellulose pellicle (Marsh, O'Sullivan, Hill, Ross, Cotter, 2013). The ratio of bacteria to yeast is important in the flavor development and ethanol content of the final product (Eddy, 2006). The yeasts present in the SCOBY convert the sucrose into glucose and fructose, producing ethanol among other products. The bacteria then metabolize the ethanol into organic acids (Greenwalt et al., 2000; Klein, 2017). If the bacteria are present in lesser amounts or are sluggish, the ethanol content will rise quickly (Klein, 2017).

The microbial composition of the kombucha SCOBY is constantly changing, which leads to varied product composition and ethanol concerns. In a 2013 study, several sub-dominant genera not previously associated with the kombucha SCOBY were discovered (Marsh, O'Sullivan, Hill, Ross, Cotter, 2013). As the SCOBY is a microbial system, it is inherently complex. This causes the determination of the biotic and abiotic interactions of these systems to be challenging (Shade, 2011). The constant microbial flux of the SCOBY results in a lack of predictability. Quality control processes are needed to ensure that a consistent kombucha product is sent to the market, despite the variability associated with the SCOBY.

2.3.3. Impacts on Microbial diversity

Temperature has recently been discovered to play an important role in the kombucha microbial community. Samples that are fermented at 30°C have a higher microbial diversity than those fermented at 20°C. Regardless of the temperature, *Gluconacetobacter* dominated the fermentation, and *Acetobacter* was found in lower levels, in this study. A higher temperature results in a desirable increase in the fermentation rate. However, high temperatures have some drawbacks as well. One such drawback is a higher abundance of environmental contaminants (such as lactic acid bacteria). Differences are mostly observed at the sub-species level, which

accounts for the differences in the organic acid composition, specifically, with regard to the content of gluconic acid. This study shows the temperature during fermentation can be used to select for acetic acid bacteria of interest. Thus, the organic acids that are produced can be selected for as well (De Filippis, Troise, Vitaglione, Ercolini, 2018).

2.3.4. Antimicrobial activity

Kombucha has antimicrobial activity. Work conducted in 2000 showed that pathogenic microorganisms such as *Staphylococcus aureus, Salmonella Enteritidis, Bacillus cereus*, and *Listeria monocytogenes* are sensitive to kombucha (Sreeramulu, Zhu, Knol, 2001). The production process, especially the acid production, is effective in reducing the level of *Salmonella* that may be present in the raw ingredients (Rock, Unruh, Gragg, 2017). The main agents suggested to be responsible for these antimicrobial properties are acetic acid, large proteins, and other compounds present in the kombucha liquid (Greenwalt, Ledford, Steinkraus, 1998; Sreeramulu, Zhu, Knol, 2000). The polyphenolic content of this beverage has also been suggested to provide some antimicrobial function against *Vibrio cholerae* strains by way of a membrane-damage mechanism (Bhattacharya, Ghosh, Bhattacharya, Sarkar, Karmakar, Koley, Gachhui, 2018).

2.3.5. Bacterial species

The primary bacteria in the kombucha culture are aerobic and can use alcohol as their substrate to produce acetic acid. This type of bacteria requires large amounts of oxygen to grow and carry out metabolic activities. Previous research has shown that the major bacterial genus present in the bacterial SCOBY is *Gluconacetobacter*, which is present at >85% of the total bacterial populations for most samples (Marsh et al., 2013; Jayabalan et al., 2014). When looking throughout the whole fermentation medium and the broth, 86% to 99% relative abundance of

Gluconobacter was found by rRNA sequence analysis. This finding was supported by a study from 2016 by Watawana et al. (Vallarreal-Soto et al., 2018; Watawana et al., 2016).

Marsh et al. detected only trace amounts (<2% of the total bacterial load) of the genus *Acetobacter* in the kombucha pellicle (2013). Recently, the acetic acid bacteria in the microbial mat have been classified to include: *Acetobacter xylinum, A. aceti, A. pausterianus, A. xylinoides* and *Bacterium gluconicum* (Amarasinghe, Weerakkody, Waisundara, 2018). *Acetobacter xylinum* composes the majority of the acetobacter species in the pellicle (Jayabalan et al., 2014). *Acetobacter xylinum* is known for its cellulose production and fixing nitrogen (Anonymous, 2016; De Roos & De Vuyst, 2018). Some of the other predominant acetic acid bacteria found in the tea fungus include: *A. pasteurianus* and *Gluconobacter oxydans* (Jayabalan et al., 2014).

2.3.6. Yeast and other fungal species

While the composition of the yeasts present in the SCOBY is highly variable, some of the predominant species are: *Schizosaccharomyces pombe, Brettanomyces bruxellensis, Saccharomyces cereviseae* and *Zygosaccharomyces rouxii* (Villarreal-Sota et al., 2018). *Zygosaccharomyces* composes >95% of the yeast population present in the tea in most samples (Marsh et al., 2013). Most recently, the spectrum of yeast species in the kombucha culture have been reported as: *Zygosaccharomyces, Candida, Klueckera/Hanseniaspora, Torulaspora, Pichia, Brettanomyces/Dekkera, Saccharomyces, Lanchancea, Saccharomycoides, Schizosaccharomyces,* and *Kluyveromyces* (Villarreal-Sota et al., 2018). Compared to earlier studies this list identifies more yeast taxa, showing the variable nature of the SCOBY.

2.3.7. Role of the bacteria and yeast

Within a SCOBY pellicle the roles of bacteria and yeast are unique. The microbes in the SCOBY are held together by cellulose, which is produced by bacteria such as *Gluconacetobacter*

xylinus (Nguyen, Flanagan, Gidley, Dykes, 2008). Recently, *Komagataeibacter rhaeticus* strain P 1463 was isolated from kombucha and determined to be another synthesizer of bacterial cellulose (Semjonovs et al., 2017). Bacterial cellulose is unique from plant cellulose in that it has a high degree of polymerization and higher stability (Zhang, Wang, Qi, Ren, Qiang, 2018). These properties allow the bacterial cellulose to rehydrate quickly, which is essential for the survival of bacteria and yeast. The unique properties of the bacterial cellulose of the SCOBY cause it to be of interest for applications such as food packaging or wearable technology such as clothing (Zhang, Wang, Qui, Ren, Qiang, 2018).

When first placed into the sugary tea, the yeast begins the production process by metabolizing the sugar (sucrose) into simpler sugars (glucose and fructose), eventually producing ethanol. Sucrose concentrations drop linearly with time, and glucose is produced at a lower initial rate than fructose (Chen, Liu, 2000). The SCOBY bacteria then metabolize the ethanol into organic acids (Greenwalt et al., 2000 & Klein, 2017). Thus, different ratios of yeast to bacteria produce different flavor profiles of the final product, hence the variability. A study conducted in 2015, identified the optimal ratio of yeast to acetic bacteria to be 4:24, as this ratio maximized glucuronic acid production. (Nguyen et al., 2015).

Fermentation time can greatly impact the characteristics of both the microbial mat and the fermented liquid. As the fermentation time increases, the turbidity of the liquid and SCOBY size and thickness also increase. An extended fermentation time can lead to an increase in acetic acid. From a sensory perspective, this increase in acetic acid is considered an undesirable change (Amarasinghe, Weerakkody, Waisundara, 2018).

2.4. Organoleptic Properties

Kombucha is known to contain organic acids including: acetic, gluconic, glucuronic, citric, L-lactic, malic, malonic, oxalic, and succinic acids. Also present are several sugars such as sucrose, glucose, and fructose. (Huang, Hu, Rohrer, 2016 & Leal, et al., 2018). A comprehensive list of the compounds that have been identified in kombucha to date can be found in Table 2.1.

Table 2.1. Complete list of compounds commonly found in kombucha.

Compound	Type of Compound	Source	Reference
Acetic acid	Organic acid	Metabolic	Chen, Liu, 2000
Gluconic acid	Organic acid	Metabolic	Chen, Liu, 2000
Glucuronic acid	Organic acid	Metabolic	Chu, Chen, 2006
Citric acid	Organic acid	Metabolic and fruit	Neffe-Skocinska, Sioneck, Scibisz, Kolozyn-Krajewska, 2017
Lactic acid	Organic acid	Metabolic and fruit	Chu, Chen, 2006
Malic acid	Organic acid	Mostly fruit, occasionally metabolic	Neffe-Skocinska, Sioneck, Scibisz, Kolozyn-Krajewska 2017
Tartaric acid	Organic acid	Fruit	Neffe-Skocinska, Sioneck, Scibisz, Kolozyn-Krajewska, 2017
Malonic acid	Organic acid	Metabolic	Neffe-Skocinska, Sioneck, Scibisz, Kolozyn-Krajewska, 2017
Oxalic acid	Organic acid	Fruit	Neffe-Skocinska, Sioneck, Scibisz, Kolozyn-Krajewska, 2017
Succinic acid	Organic acid	Metabolic	Neffe-Skocinska, Sioneck, Scibisz, Kolozyn-Krajewska, 2017
Pyruvic acid	Organic acid	Metabolic	Neffe-Skocinska, Sioneck, Scibisz, Kolozyn-Krajewska, 2017

Table 2.1 Continued

Usnic acid	Organic acid	Metabolic	Neffe-Skocinska, Sioneck, Scibisz, Kolozyn-Krajewska, 2017
Glucose	Sugar	Metabolic	Chen, Liu, 2000
Fructose	Sugar	Metabolic	Chen, Liu, 2000
Sucrose	Sugar	Starting compound	Chen, Liu, 2000
Ethanol	Alcohol	Metabolic	Ebersole, Liu, Schmidt, Eckert, Brown, 2017
Glycerol	Alcohol	Metabolic	Vukic, Hrnjez, Milanovic, Ilicic, Torbica, Tomic, 2014
Methanol (trace amounts)	Alcohol	Metabolic	Eddy, 2006
B Vitamins (B_1, B_6, B_{12})	Vitamins	Starting tea and metabolic	Blanc, 1996
Vitamin C	Vitamins	Starting tea and metabolic	Huang, Hu, Rohrer, 2016
Biogenic amines	Amines	Unknown	Jayabalan, Malbasa, Loncar, Vitas, Sathishkumar, 2014
Purines	Aromatic organic compounds	Unknown	Jayabalan, Malbasa, Loncar, Vitas, Sathishkumar, 2014
Pigments	Pigments	Unknown	Jayabalan, Malbasa, Loncar, Vitas, Sathishkumar, 2014
Lipids	Lipids	Unknown	Jayabalan, Malini, Sathishkumar, Swaminathan, Yun, 2010
Proteins	Proteins	Unknown	Jayabalan, Malini, Sathishkumar, Swaminathan, Yun, 2010
Hydrolytic enzymes	Enzymes	Unknown	Malbaša, Lončar, Vitas, & Čanadanović-Brunet, 2011
Tea polyphenols	Polyphenols	Starting compounds (tea)	Huang, Hu, Rohrer, 2016
Phenol	Aromatic organic compounds	Unknown	Jayabalan, Malbasa, Loncar, Vitas, Sathishkumar, 2014

Table 2.1 Continued

Minerals	Minerals	Starting compounds (water)	Bauer-Petrovska, Petrushevska-Tozi, 2000
Trace elements	Ions	Starting compounds (water and tea)	Bauer-Petrovska, Petrushevska-Tozi, 2000
D-saccharic acid-1, 4-lactone (DSL)	5-membered ring acid	Unknown	Bhattacharya, Gachhui, Sil, 2013
Amino acids	Amino Acids	Unknown	Huang, Hu, Rohrer, 2016

The predominant compounds commonly found in kombucha in both the liquid and the biofilm are acetic acid, gluconic acid and ethanol. The finished fermented tea is thought to contain several carboxylic acids, 14 amino acids, some hydrolytic enzymes, and vitamins (Malbaˇsa, Loncˇar, Vitas, & Cˇanadanovic´-Brunet, 2011). Kombucha can contain a total of 19 different identified volatile compounds. Of these, seven are alcohols, four are acids, one is a phenol, six are esters, and one is a hydrocarbon (Yuan, Zhang, Sadiqu, He, 2017). The exact concentration of each of these compounds varies from kombucha to kombucha. This variation is again, thought to be due to the variability of the SCOBY and conditions used to produce the beverage.

Gluconic acid is a major organic acid of interest in kombucha, because of its detoxifying and therapeutic properties (Kumar & Joshi, 2016; Nguyen N., Nguyen P., Nguyen H., Le, 2015). D-gluconic acid is known to increase during fermentation, reaching a maximum of 2.3 g/L on or around the twelfth day of fermentation (Jayabalan et al., 2007). The presence of *Gluconacetobacter* dominates fermentations at both 20°C and 30°C, producing maximal levels of gluconic acid. However, the increased temperature allows for increased growth of the sub-dominant species, which are mainly environmental contaminants (Filippis, Troise, Vitaglione, Ercolini, 2018). Thus, despite the increased rate of production at 30°C, this is not the best

fermentation temperature, due to the proliferative growth of other less desirable microbes. The optimal balance of yeast to acetic acid bacteria to maximize gluconic acid production has been reported as an initial ratio of 4 (*Dekkera bruxellenis* KN89) to 6 (*Gluconacetobacter intermedius* KN89) and a seven-day fermentation process (Nguyen, Nguyen, Nguyen, Le, 2015).

2.4.1. Consumer Preferences

Many people describe kombucha tea as having a sour or vinegar-y taste from the acetic acid present in the tea (Sreeramulu, Zhu, Knol, 2000). Due to the relationship between the yeast and bacteria, the acetic acid content rarely rises above 2% (v/v) (Eddy, 2006). A 2007 study recorded an increasing concentration of acetic acid, up to a maximum of 9.5g/L (about 0.90% (v/v)), after a 15-day fermentation period (Jayablan et al., 2007).

Although it is an important flavor component, acetic acid is only one of the organic acids present in a kombucha product. A typical finished kombucha beverage will contain 33 g/L of organic acids. Acetic acid has the sharpest taste, while other acids such as lactic or gluconic acid, are milder. The presence of gluconic acid helps to balance the sourness of the acetic acid. Normally, gluconic acid is present at levels more than 30% greater than acetic acid, which contributes to a more balanced flavor profile of the final product (Eddy, 2006).

2.5. Sensory Evaluation

Currently, there are very few sensory evaluation studies involving consumer acceptance of kombucha and a lexicon used to characterize the beverage are lacking. Much of the sensory research involving kombucha is recent, and many sensory studies involving novel fermented beverages have focused on kefir or water kefir.

2.5.1. Types of tea used

A study conducted by Gramza-Michalowska et al. (2016) included a sensory evaluation study using kombucha made from different types of tea (black, green, white, and yellow tea). The kombucha beverages with the highest overall acceptance were made with white or yellow tea. Black tea kombucha had the lowest overall acceptance (Gramza-Michalowska et al., 2016). Part of this evaluation focused specifically on clarity scores for all samples. On a 10-point scale for clarity the consumers rated the clarity between 4 and 6 for all samples. The clarity scores were lower than expected due to a higher presence *Acetobacter xylinum*, which can produce a fibrous structure resulting in a cloudy tea (Gramza-Michalowska et al., 2016). In evaluating the taste of the kombucha samples, panelists were asked to rate the following flavors on a 10-point scale: citrus, tea, sweet, beer/fermented, sour, bitter, and unfamiliar. Some notable results from the study were that panelists noted a slightly noticeable tea, beer, and unfamiliar taste for all teas. Also, an increased fermentation time resulted in a decreased bitterness, due to the increase in amino acid production. Amino acids reduce the bitterness of tea alkaloids (Gramza-Michalowska et al., 2016). The presence of a SCOBY caused the greatest change in color and taste of the tea in the tea samples presented to the panelists compared to the control tea (without SCOBY) (Gramza-Michalowska et al., 2016).

2.5.2. Type of sweetener

The sweetener and microbial carbon source for kombucha tea is traditionally sucrose, but other sweeteners have been studied. Fermenting kombucha using molasses, instead of sucrose, produced kombucha that was dark brown in color, slightly carbonated with a sweet and caramelized taste and smell. In contrast, the kombucha produced with sucrose was light brown in color, slightly carbonated, sour, and refreshing (Malbasa et al., 2008).

2.5.3. Quantitative – Descriptive Analysis of Kombucha

The Quantitative-Descriptive Analysis (QDA) method has been used to determine the general quality of kombucha. In a study with sixteen sensory panelists, the smell discriminants: tea, lemon, acetic, yeast, sour, and other, were chosen to describe kombucha. The taste discriminants that were chosen by the panelists included tea, lemon, acetic, yeast, sour, bitter, storage – stale, and others (Neffe-Skocinska, Sioneck, Scibisz, Kolozyn-Krajewska, 2017).

2.5.4. Fermentation duration

If sensory characteristics are the determining factor for fermentation time, the optimal duration for fermentation has been identified in some studies as 10 days (Malbasa, Loncar, Djuric, 2008; Neffe-Skocinska, Sioneck, Scibisz, Kolozyn-Krajewska, 2017). This study showed that this length of fermentation resulted in a beverage with a high pH value and high content of desirable acids (ie: L-lactic acid) and low content of less desirable acids (ie: acetic acid) (Malbasa, Loncar, Djuric, 2008).

2.6. Potential Hazards

While kombucha is known for its health benefits, there are also potential hazards that consumers need to be aware of. In 1995, the Centers for Disease Control and Prevention (CDC) received two reports of unexplained severe illness (including one death) following kombucha consumption. These two individuals had been consuming kombucha daily for two months and suffered lead poisoning from homemade kombucha brewed in a ceramic pot (CDC, 1995; Phan, Estell, Duggin, Beer, Smith and Ferson, 1998). Anthrax has also been found in kombucha tea home-brewed under unhygienic conditions (Soloway, 2012). In one particularly serious case, a 59-year old woman who was on medication for anemia, hypertension and renal insufficiency was found unconscious after consuming approximately 4 oz of kombucha daily for 2 months prior to

the event. She suffered severe metabolic acidosis and died shortly after (Murphy, Walia, Farber, 2018). These examples illustrate how the lack of quality and safety measures associated with homebrewing of kombucha can be dangerous, even life-threatening.

Side effects, such as allergic reactions, jaundice, nausea, vomiting, head, and neck pain have also been correlated with consumption of kombucha. These side effects were found mainly in HIV-positive patients (Jayabalan et al., 2014). In 2009, cases of hyperthermia, lactic acidosis and acute renal failure were all reported within 15 hours of kombucha consumption. Several cases of serious and sometimes fatal, hepatic dysfunction and lactic acidosis have also been reported (SungHee, Jones, Christensen & Gladstein, 2009). Overall, the largest health concern that is raised with kombucha consumption is life-threatening metabolic acidosis.

There has also been evidence that usnic acid (an acid found in kombucha) can lead to liver toxicity or contact allergenicity (Araujo et al., 2015). After receiving reports linking dietary supplements containing usnic acid with liver toxicity, the US Food and Drug Administration (FDA) issued a warning about one of these usnic acid supplements (Guo, Shi, Fang, Mei, Ali et al., 2008). An assessment of usnic acid toxicity in rat primary hepatocytes showed augmented oxidative phosphorylation at low concentrations of usnic acid (1 or 5μM), which was suggested to be in response to diminished mitochondrial function (Sonko, Schmitt, Guo, Shi, Boros, Leakey, Beger, 2011).

The yeast, *Candida krusei*, which is present in kombucha, has been determined to account for about 2% of all yeast infections in humans. If this yeast infection occurs in the blood it can be extremely challenging to treat, because most of the isolates of *C. krusei* are fluconazole-resistant (Douglass et al., 2018). Fluconazole is the drug commonly used for the treatment of human yeast infections. As a species, the pathogenic *Candida* yeasts are known to cause over

46,000 invasive infections annually with a 30% mortality rate in the United States (Douglass et al., 2018).

2.7. Analysis by Chromatography

2.7.1. Gas Chromatography

The AOAC official method in 2016 for the detection of ethanol in kombucha products couples headspace gas chromatography with flame-ionization detection. The headspace conditions include an incubation temperature of 80°C, a syringe temperature of 85°C and a heating time of 15-20 minutes. A plain, unflavored tea was used as a control with a standard curve developed from stock ethanol solution with concentrations of 0.05, 0.10, 0.25, 1.02, 2.54 and 5.09% ABV (Ebersole, Liu, Schmidt, Eckert and Brown, 2016). This is currently the validated and accepted method according to the AOAC.

2.7.2. Liquid Chromatography

Currently, there are a lack of methods to simultaneously detect organic acids, sugars, and ethanol in kombucha by liquid chromatography. The main organic acids of interest are D-glucuronic acid, acetic acid, lactic acid, and citric acid (Jayabalan et al., 2007). However, the overall content of organic acids (and sugars) that are present in kombucha beverages are much greater, as can be seen in Table 2.1. Previous methods have been developed to simultaneously identify the major organic acids, sugars and ethanol in wines and grape musts. One such technique used an ion-exchange column, isocratic separation, a mobile phase of dilute H_2SO_4 and a UV detector set at 214 nm and a refractive index detector (Lopez and Gomez, 1996). This method focused on the separation of carboxylic acids, sugars, glycerol, and ethanol and produced reliable results in under 45 mins (Lopez and Gomez 1996).

A similar method has been developed to simultaneously quantify the major sugars and organic acids present in grapevine berries. In this method the samples were purified by

chloroform/polyvinylpolypyrrolidone purification. The extraction was conducted using an ion-exchange HPLC column (Aminex HPX-87H) heated to 55°C and 5 mM sulfuric acid as the mobile phase. The chromatography was visualized using a diode array detector and a refractive index detector. This method tested isocratic mobile phases that ranged in concentration from 2.5 to 22.5 mM sulfuric acid in deionized water with or without an acetonitrile modifier (6%) to determine the optimal chromatographic separation. It was determined that the 5 mM sulfuric acid mobile phase without acetonitrile worked best (Eyeghe-Bickong, Alexandersson, Gouws, Young, Vivier, 2012).

A study conducted in 2002 focused on method development for the simultaneous detection of the main carboxylic acids (citric, tartaric, malic, succinic, lactic, and acetic), sugars (glucose and fructose) and alcohols (ethanol and glycerol) in grape musts and wine. The use of a cation exchange column (Aminex HPX-87H) at 30°C was shown to improve the separation of the components that were co-eluting (fructose and malic acid, succinic and shikimic acids). The mobile phase was tetrahydrofuran (THF) with *n*-propyl alcohol as an organic modifier and 0.01N phosphoric acid as the eluent (Chinnici, Spinabelli, Amati, 2002). Detection was at 215 nm on a UV detector connected in series to a refractive index detector.

LC methods have also been developed to investigate the short-chained organic acids such as malic, oxalic, lactic, acetic, formic, citric, pyruvic, succinic, tartaric, and propionic acids in fermented foods using a diode array detector (DAD) (Mortera, Zuljan, Magni, Bortolato, Alarcon 2018). A C18 column (Zorbax SB C_{18}) at room temperature was used with a 20 mM phosphate buffer at a pH of 2.20 and a flow rate of 1.0 mL/min. All compounds of interest could be separated within ten minutes. This method had an outstanding limit of detection of 0.15 to 10.0

mM for the organic acids during the validation samples. (Mortera, Zuljan, Magni, Bortolato, Alarcon, 2018).

Chromatographic separation was used to determine the chemical composition of the organic acids and carbohydrates in naturally fermented buttermilk. In this study, 25 µL of sample was injected onto an Aminex HPX-87H column at 32°C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.4 mL/min. Detection of the organic acids was completed by using a UV detector set at 210 nm, and detection of carbohydrates was completed using a refractive index (RI) detector (Gebreselassie, Abrahamsen, Beyene, Abay & Narvhus, 2016).

2.7.3. Column Chemistry

In HPLC analysis most of the separation is done by the column, which is usually a stainless-steel tube filled with either a silica or polymer matrix. The stationary phase matrix of the column is normally silica (Sadjadi, 2017). The column is highly customizable by particle size, shape and porosity, column length and inner matrix material. The column classification (reverse phase, ion-exchange etc.) is determined by the material(s) that compose the inner matrix of the column. In general, the classes of columns are: reverse phase, ion-exchange, normal phase, and mixed mode. The suggested mobile phase for each of these columns also differ, based on the chemistry of the packing material of the column.

Column length needs to be considered when developing an HPLC analytical method. A longer column improves the efficiency and resolution (theoretical plates), but also increases the retention time (HPLC Column Dimensions, n.d.). Larger molecules such as proteins are not impacted by column length. However, for the smaller organic molecules that are commonly found in kombucha (sugars, organic acids, alcohols) the column length does have an impact ("Hydrophobic Interactions and Reversed Phase Chromatography," 2006). It is important to

select a column that provides a high enough resolution for analyte separation, while still maintaining a short elution run time.

Column diameter also has an impact on the resolution and sensitivity of the method. As internal diameter decreases, sensitivity increases, due to an increase in the amount of analyte present in the mobile phase (HPLC Column Dimensions, n.d.). The decreased internal diameter leads to a decreased volume of mobile phase and thus an increased concentration of analyte in the mobile phase.

Columns can be purchased with different particle size, shape, porosity and chemical ligands, which allows for further customization of each individual column to its application. A smaller particle size offers benefits such as efficacy and an increased speed of analysis. However, smaller particle size will lead to a higher back pressure, which causes increased pressure stress on the HPLC instrument. Short columns with small particles can provide a high resolution without exceeding the backpressure limit of 400 bar of most HPLC systems (Pereira & Milton, 2010). Particle shape, porosity and chemistry represent a range of often proprietary options available from the column manufacturers and can allow the chromatographer a wide range of alternatives for the separation of chemicals in complex matrices.

2.7.4. Ion-exchange Chromatography

Ion-exchange chromatography is a chromatographic technique which can be used to separate compounds based on their surface charge. The type of ion-exchange column used (anion or cation) should be chosen based on the charge of the compound of interest when it is in the mobile phase conditions. An ion-exchange column is developed by creating a medium (resin), which contains positively or negatively charged functional groups covalently bonded to the silica resin (Bio-rad, 2018). The ionic form of the bound counter ion should also be considered,

because this factor impacts the affinity of the counter ion to the stationary support phase. The lower the affinity of the counter ion to the support phase, the easier it is for the counter ion to be exchanged with another charged ion passing through the column, which leads to a higher selectivity in the column (Bio-rad, 2018).

Cation exchange chromatography is a type of ion-exchange chromatography which uses a negatively-charged stationary phase to separate cationic compounds (Bio-Rad, 2018). Anion-exchange chromatography uses a positively-charged stationary phase to separate anions. The charge on an organic acid changes with the pH, based on the association/dissociation constant, so pKa plays an important role in the separation of the compounds (Nielson, 2010). Therefore, choosing a mobile phase with an appropriate pH based on the pKa of the compounds of interest is important. For example, if a cation-exchange column is used, the pH of the mobile phase needs to be lower than the pKa to ensure that the compounds of interest are separated.

Compounds can either be eluted with a linear gradient or with a step isocratic gradient elution. In many cases a gradient elution is the best approach to separate the compounds of interest (Bio-rad, 2018). When using an ion-exchange column the use of a gradient mobile phase can separate a wider range of compounds as the changing pH will change the charges on the compounds (based on pKa) to increase separation. The same concept can be applied to polarity, for reverse and normal phase columns.

Gradient elution is a technique where the eluent strength is increased over the separation by modifying the concentration, polarity or pH of the mobile phase. For analytes with widely ranging polarities or for separation with wide k-ranges, gradient elution is recommended (Heinisch, 2018). However, detection by refractive index is not recommended when using a

gradient mobile phase, as baseline shifting occurs with changing mobile phase concentration (“Refractive Index Detector,”, 2019).

A column that is frequently used for fermentation monitoring and evaluation is the Aminex HPX-87H column (Eyeghe-Bickong, Alexandersson, Gouws, Young, Vivier, 2012; Lopez & Gomez, 1996). These organic acids, sugars and ethanol can all be detected by a Refractive Index (RI) detector. Some methods also use a DAD in tandem with the RI detector to improve detection of poorly separated compounds, because some of the target analytes may be undetected to one of the detectors (Coelho, Padilha, Miskinis, Barroso de Sa, Pereira, Cavalcanti de Azevedo, Lima, 2018; Eyeghe-Bickong, et al., 2012; Lopez, Gomez, 1996). The most common column temperatures for these separations is 55-75°C and mobile phases generally consist of dilute concentrations of sulfuric acid in water (Lopez, Gomez, 1996; Ma, Ouyang, Li, Lian, Cai, 2012).

2.7.5. Reverse Phase Chromatography

Reverse phase chromatography separation is based on the hydrophobicity of the molecules and the most commonly used column has a C18 stationary phase (Sadjadi, 2017). The stationary phase is more hydrophobic and non-polar, so a mobile phase consisting of polar solvent(s) is needed to elute the compound of interest from the column (“HPLC separation modes”, n.d.).

One of the most important considerations with reverse phase separation is pH. If an incorrect pH is used, a complete separation may not be possible (Dolan, 2017). Using a polystyrene medium can allow separations to be conducted over a wider pH range, because the synthetic polymer is stable over a wider pH range than traditional column packing materials (“Hydrophobic Interactions and Reversed Phase Chromatography, 2006).

2.7.6. Mixed Mode Chromatography

Reverse phase columns (such as C18) are commonly used for a range of applications. However, they often fail to retain highly hydrophilic molecules such as counter ions. In such situations the use of mixed mode chromatography is recommended, as more than one method of separation can be employed. Normally, mixed mode is a combination of reverse phase and ion-exchange chromatography (Taylor, 2014). Some benefits that mixed-mode columns offer include a simplified mobile phase and simultaneous separation of different types of analytes, including polar and non-polar compounds (Liu, Tracy, Aich, Pohl, 2014; Taylor, 2014). A column with a reversed phase/ ion-exchange bimodal phase has mixed packing for ion-exchange and reversed-phase. Such packing forms mixed ligands for improved separations (Liu, Tracy, Aich, Pohl, 2014). A drawback of mixed-mode retention is peak broadening and tailing (decreased resolution), leading to decreased sensitivity. This is due to ionic interactions between the silica groups and amino groups on the analytes of interest ("Hydrophobic Interaction and Reversed Phase Chromatography," 2006).

2.8. Conclusions

Kombucha is a sweetened effervescent fermented tea beverage with potential health benefits and is produced from sweetened tea fermented with a symbiotic colony of bacteria and yeast (SCOBY). This beverage originated out of China thousands of years ago. The market for kombucha is expected to reach $1.8 billion by the year 2020 (Ebersole, Liu, Schmidt, Eckert, Brown, 2017). There is currently a lack of methods to simultaneously determine the organic acids, sugars and ethanol content in kombucha. There is also a lack of understanding of SCOBY variability and words consumers use to classify kombucha. As the kombucha industry continues to grow and gain popularity a need for a rapid method of the simultaneous detection of ethanol,

sugars and organic acids and an understanding of SCOBY variability will be needed to ensure proper quality control. To ensure consumer acceptance of the beverage and determine the flavor profile of the beverage that consumers desire, sensory evaluation studies should be conducted.

CHAPTER 3

MATERIALS AND METHODS

3.1. Experimental Design

Kombucha was produced in a laboratory setting with three kombucha SCOBYs determined to yield heterogeneous product based on HPLC analysis of the kombucha produced from the SCOBY. SCOBYs were obtained from a lab colleague in the Food Microbiology Lab at the University of Maine. Two batches of kombucha from each unique SCOBY were produced every fourteen days (Figure 3.1). The liquid from every fifth batch of kombucha produced, as well as the mother SCOBY (bottom layer) were collected for further analysis, including HPLC, and metagenomic sequencing.

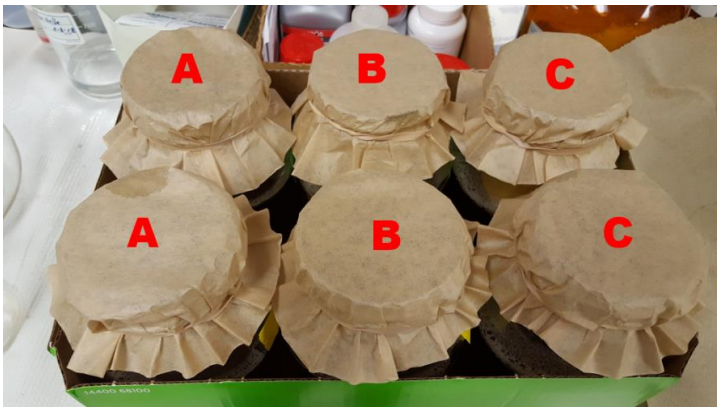

Figure 3.1. Three kombucha SCOBY[a]s fermenting in duplicate
[a] Symbiotic colony of bacteria and yeast

Genomic DNA was extracted separately from the liquid and the SCOBY of generations 1, 5 and 10 from each SCOBY and subjected to shotgun metagenomic sequencing with Oxford Nanopore's MinION, DNA and RNA sequencer (Figure 2.1).

The vocabulary that consumers use to characterize kombucha and how that relates to their overall acceptance of the beverage was assessed through a consumer sensory evaluation study. A high-performance liquid chromatography (HPLC) method was developed for the

simultaneous detection of the major organic acids, sugars, and alcohols in kombucha and was used to test both lab-made kombucha and commercial kombucha.

3.2. Kombucha Production and Storage

The three unique SCOBYs that were used for kombucha production were determined to be different based on the relative concentrations of gluconic acid and the ratio of lactic to acetic acid in the fermentate as determined in preliminary testing. The SCOBY mother from a total of 9 samples were used for the diversity sequencing. The diversity of the SCOBYs were determined by DNA sequencing. Different ratios of microbes result in a very different kombucha tea.

Each of the SCOBYs were cut into fourths using vinegar-rinsed scissors. One SCOBY was already at room temperature on the first day of inoculation. First generation kombucha production was accomplished with room temperature mother cultures. During subsequent kombucha production all sweetened teas were simultaneously inoculated with the their respective daughter SCOBY. The mother SCOBYs from batches 1, 5 and 10 were saved for sequencing. Mother SCOBYS from other batches were discarded.

Sweet tea production followed a standard recipe, based on production methods compiled from the literature. All glassware and gloves that came in direct contact with the SCOBY were rinsed (sanitized) with generic white vinegar (Hannaford, Scarborough, ME) to prevent microbial contamination. Next, 7.57 L of spring water (Great Value, West Seneca, NY) was brought to a boil in a large 19 L stainless-steel pot on a Jade commercial gas range. Once the water reached a rolling boil the heat was turned off and two cups of granulated cane sugar (Domino, Brooklyn, NY) were dissolved in the water. As soon as the sugar was added and while the water was still very hot, ten black tea bags (Salada, Little Falls, NY) were added and steeped for 15 minutes before removal of teabags. The tea was then left covered to cool overnight to

ensure it reached room temperature. Every fourteen days, two gallons of sweet tea were produced and divided equally into six 1.9 L canning jars (Mason, Salem, NJ) for SCOBY inoculation.

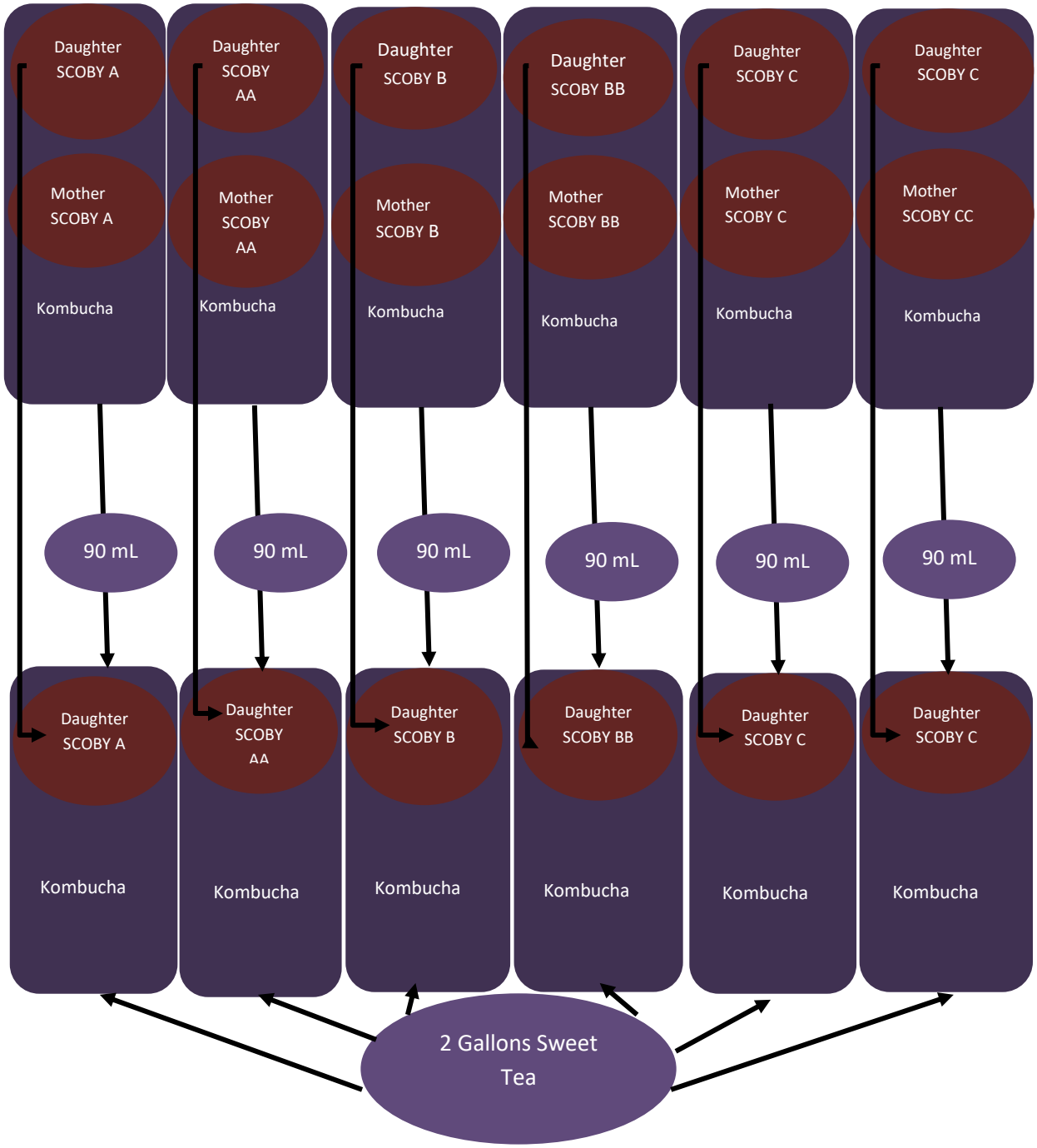

Figure 3.2. Diagram of Kombucha Production

To the 1.9 L jars of sweet tea, 90 mL of starter tea from the previous batch was added. Finally, this mixture was inoculated with the corresponding daughter SCOBY. The jar was then covered with an unbleached brown coffee filter (Hannaford, natural unbleached coffee filters, 8" basket style, Scarborough, ME) and secured with a rubber band. Each of these six jars were placed into a cardboard box (25cm x 25cm x 35cm) at ambient temperature (between 20°C and 25°C) and left for 14 days to ferment.

On day fourteen, the "mother" cellulose pellicle was removed. Every batch 5 mL of the finished tea was collected and stored at -18°C in a freezer compartment located in the Food Analysis lab at the University of Maine for later analysis. The kombucha tea and the mother pellicles were saved from batches 1, 5, and 10. The 1.9 L jars were rinsed with spring water to remove any yeast residue. The kombucha tea and the mother pellicles were saved from batches 1, 5, and 10. The 2 gallons of fresh sweet tea was evenly divided amongst the 6 containers. Then, 90 mL of kombucha from the previous batch was added and the daughter SCOBY placed on top (Figure 3.2). The jars were then covered with a coffee filter and left to ferment for 14 days.

Both the liquid and the pellicle were stored in a 473 mL glass jars covered with a coffee filters at 2°C for no more than two weeks until they could be moved to long-term storage. For long- term storage of the samples that would be used for sequencing the kombucha liquid was stored 50/50 (sample/80% glycerol) at -80°C. For the samples that were to be used for HPLC analysis 5 mL was stored at -18°C.

3.3. DNA Extraction

DNA extraction was completed using the DNeasy PowerFood Microbial Kit from Qiagen (Hilden, Germany) with the following modifications. For the extraction from the SCOBY, 0.25g of SCOBY was first sliced using scissors into pieces approximately 0.5cm^2 and mixed with 750

µL of cellulase (Millipore Sigma, Bedford, MA) in a 2 mL microcentrifuge tube and incubated at room temperature for 30 minutes. Following incubation, the mixture was spun at 7,000 x *g* using a Thermo Scientific Sorvall Legend Micro 21 centrifuge for 1 minute and the liquid was extracted. The cells were resuspended in 450 µL "Solution MBL" and the solution was boiled for 1 minute to increase cell lysis prior to transferring the mixture to PowerBead tubes. This boiling step modification was completed for both the SCOBY and the liquid.

3.4. Kombucha Diversity Sequencing

Samples of the isolated DNA from the SCOBY were stored frozen (-80°C) after determining the DNA concentration using a NanoDrop 2000 (Thermo Fisher, Waltham, MA). Extracted DNA from the duplicates were compiled prior to sequencing. For example, the DNA from SCOBY A, batch 1 was pooled with the DNA from SCOBY AA, batch 1. The kombucha SCOBY samples were prepared for sequencing by attaching a barcode to each of the samples, which allowed samples to be pooled and sequenced simultaneously. Barcoding was completed following the instructions included with the 1D Native Barcoding Kit from Oxford Nanopore (Oxford, UK). The nine SCOBY samples were sequenced together on May 7, 2019. The sequencer was run for 6 hours. At the beginning of the run the MUX scan results showed 1298 active channels and 750 inactive channels, with about 200 pores sequencing. At the end of the run there were only 1163 active channels and 934 inactive channels, with about 185 pores sequencing. Sequencing was conducted using Oxford Nanopore's MinION sequencer and the MinKNOW software was used to operate the MinION.

3.5. Diversity Sequencing Analysis

Once the data was collected and the sequencing was complete, all data was uploaded to the EPI2ME software provided by Oxford Nanopore. The "What's in my Pot" (WIMP)

Workflow was used to determine the microbial taxa present. For the analysis, any fragments with a q-score less than 7 were automatically discarded.

3.6. High Performance Liquid Chromatography Method

A method was developed for the simultaneous detection of the major organic acids, sugars, and alcohol in kombucha. With the exception of gluconic acid, this method successfully separated major organic acids, sugars, and alcohol in kombucha (acetic acid, L-lactic acid, sucrose, glucose, fructose and ethanol) within 25 minutes.

Analytical standards target analytes were obtained from Fisher Scientific (Hampton, NH) and stock solutions were prepared by dissolving 0.2 g of each compound in 100 mL of the HPLC mobile phase. Five-point standard curves, ranging from 5 to 200 mg/g were prepared for each compound by further diluting stock solutions with mobile phase.

The samples were assayed using an Agilent Technologies (Wilmington, DE) 1200 series HPLC with an Agilent 1200 series Refractive index detector set at 35°C. Samples were filtered using 0.45µL nylon syringe filters (Advanced Microdevices, India) and 20 µL of each filtrate was injected onto an Agilent Hi-Plex H, 300 x 6.5 mm (part # PL1F70-6830) column heated to 35°C. A mobile phase of 0.005N H_2SO_4 was used at a flow rate of 0.6 mL/min and the total run time was 25 minutes.

3.7. High Performance Liquid Chromatography Data Analysis

Agilent Chem Station (Wilmington, DE) was used for data collection and analysis. Target analytes were identified by comparing retention times with corresponding analytical standards. Each peak was manually integrated to determine peak area and analyte concentrations were calculated by comparing sample/standard area ratios.

3.8. Sample Collection and Preparation for HPLC Analysis

Samples of lab-brewed kombucha from all 10 batches of fermentation were removed from the -18°C freezer five hours prior to the sample preparation and allowed to thaw. Samples of original flavor, commercially- produced kombucha were purchased from Amazon (B-tea, Original Flavor (Mahwah, NJ) and Captain's Kombucha, original flavor (Sintra, Portugal)) and stored at 4°C until testing.

3.9. Sensory Evaluation

Sensory evaluation of one lab-made and two commercial kombucha samples was conducted. The lab-made sample was produced using a SCOBY from a colleague in the Food Microbiology Lab at the University of Maine. The sweetened black tea was made following the procedure discussed in Section 3.2 of this thesis and fermented for 14 days at room temperature (between 20°C and 25°C). SIMS 2000 Sensory Software (Version 6, Berkeley Heights, NJ) was used to design the questionnaire, establish test design and execute the test. SIMS generated random, three-digit codes used to blind samples and provided a randomized and balanced design of the samples in order to prevent positional bias from the panelists.

Approval for testing was provided by the University of Maine Institutional Review Board for the Protection of Human Subjects (2018-09-14). The test panel consisted of 66 participants, all over the age of 18. The panelists received no training prior to participating in this study. Panelists that enjoyed consuming kombucha beverages were recruited through the University of Maine Sensory Testing Center email mailing list, Facebook page and through flyers hung around buildings on campus (Appendix A).

Each panelist evaluated the samples in their own private booth in an ambient temperature room. There was a positive pressure to prevent any aroma bias from the kitchen. The samples

were served at 2.9 ± 1.2°C. Before beginning testing, participants were asked to read an informed consent statement (Appendix B). Their participation in the study assumed their consent. Panelists began by answering a series of demographic questions that asked about their gender, age, how often they purchased kombucha, their knowledge of the health benefits, how the health benefits impact their decision to purchase the beverage and their favorite flavors of kombucha. Once the demographic questions were answered, panelists were provided with three samples in 162.65 mL tasting glasses in an order randomized by SIMS software. Each sample was labeled with a random 3-digit blinding code determined by SIMS. The samples were delivered to the panelists on a beige colored tray with a napkin (Vanity Fair, Every day collection, Atlanta, GA) and 265 mL plastic cup (Great Value, West Seneca, NY) of spring water (Poland Springs, Poland Spring, ME) (Figure 3.3). Panelists were instructed to take a sip of water between each sample to prevent flavor carryover and to cleanse their palates between samples.

Figure 3.3. Kombucha samples as presented to panelists for the Sensory Evaluation study

Panelists were first asked a check-all-that-apply question for the flavor notes they detected in kombucha. The options provided were: vinegar, fermented, sweet, sour, acidic, bubbly, refreshing, tea flavor, or none of the above. A comment box was provided for panelists to write

in other flavor notes, if they chose to do so. The questions regarding purchase likelihood, cloudiness acceptability and overall liking were asked using a standard 9-point hedonic scales (Peryam and Pilgrim, 1957). These scales were anchored at 1 – "Dislike extremely" and 9 – "Like extremely". Panelists selected the option on the scale they thought most appropriate. Upon completion of the test participants were compensated with two dollars.

3.10. Statistical Analysis

3.10.1. Microbiology

Data from metagenomic sequencing was filtered such that identified microbes were only kept if the cumulative reads were greater than 50 reads. A series of 1-way ANOVA tests were conducted using R Studio, running R (Version 3.6.0, Vienna, Austria). All statistics were run comparing the number of microbes and thus diversity in each SCOBY. To determine if the generation had a significant impact on the diversity of the SCOBY, a Multiway ANOVA was run comparing generations 1, 5, and 10. To determine if there was a significant difference between the individual SCOBYs the number of microbes present in each SCOBY over generations were averaged and a 1-way ANOVA was run. Finally, to better understand the diversity that was present in each of the pellicles, the Fisher's alpha, Simpson diversity, Shannon diversity, beta diversity and beta diversity indices were calculated. Beta diversity was calculated by the summation of the number of species minus the species in common and the beta diversity index was calculated by taking the number of species in common and dividing that by the sum of all the species. Fisher's alpha was calculated with the formula $\frac{S}{n} = \left(\frac{1-x}{x}\right) * (-\log(1-x))$, where S is the number of species and n is the number of individuals. Once x was known the equation $\tau = \frac{1}{z}$ was used, where z is the number of species. Finally, Fishers alpha (α) was determined from the equation $\tau = \frac{1}{\alpha+1}$. Shannon's index (H) was calculated from the formula $H = -\sum_{i=1}^{S} p_i ln p_i$

where p_i = n/N and n is the number of individuals in one species and N is the total individuals. S is the number of species. To calculate the Simpson index (D) the following formula was used $D = \frac{1}{\sum_{i=1}^{S} p_i^2}$, where S is again the number of species, and $p = \frac{n}{N}$ with n being the number of individuals and N being the total individuals.

3.10.2. HPLC Analysis

To determine the impact that the SCOBY had on the biochemical profile of the resulting beverage the sensory evaluation results (flavor notes consumers selected) were compared to the concentrations of acetic acid, lactic acid and total sugars (shown in Figure 4.3). Mean values for each of the major analytes (sucrose, glucose, fructose, lactic acid, acetic acid and ethanol) were calculated from replicates of each of the three SCOBYs. Pearson correlations were used to determine the correlation of each major metabolite to the two main bacterial species in the pellicles.

3.10.3. Sensory

To analyze the data obtained from the sensory evaluation, XLStat (Addinsoft, Long Island City, NY) was used to conduct the nonparametric Cochran's Q test for all the check-all-that-apply data. The hedonic data was analyzed by Analysis of Variance followed by Tukey's HSD test using SAS (Version 9.4, SAS Institute Inc., Cary, North Carolina). Overall means for the demographic data were determined using Microsoft Excel (Microsoft Office 16, Redmond, WA).

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Microbiological Results

The data from the results of sequencing were filtered such that only microbes classified with greater than 50 reads were kept (Appendix C). For further analysis, only microbes that composed more than 5% of the SCOBY were kept.

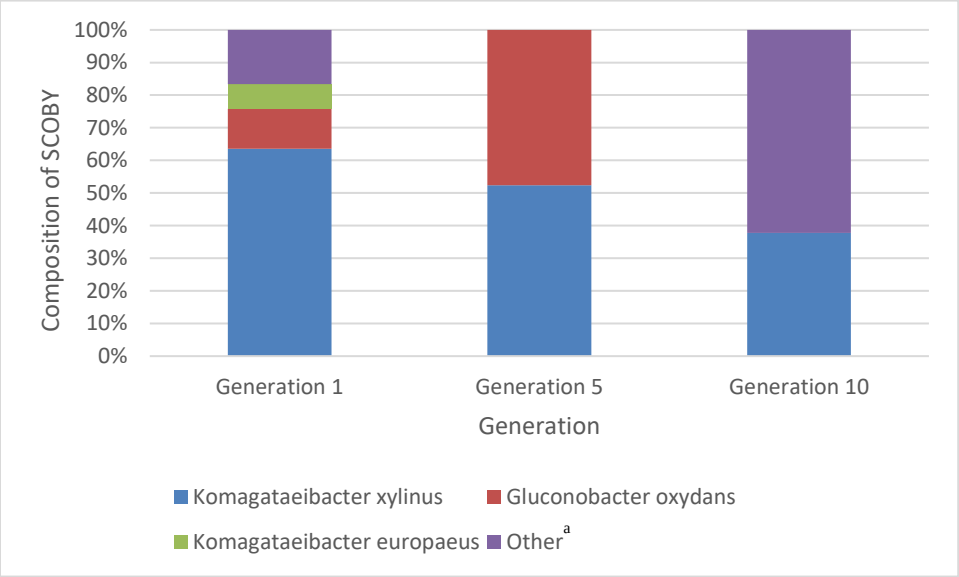

Figure 4.1. Microbial composition of SCOBY[b] A by percentage over generations
[a] Microbes with fewer than 5% of the cumulative reads from sequencing
[b] Symbiotic colony of bacteria and yeast
n= 9

As can be seen in Figure 4.1, the greatest diversity of microbes was present after the first generation of SCOBY A and decreased over subsequent generations. In generation 5 and 10 the only microbes that were present above the 5% threshold were *K. xylinus*, *Gluconobacter oxydans*. *Komagataeibacter xylinus* was initially present and made up more than 60% of the kombucha SCOBY community, but then decreased by generation 5 and then increased again to compose over 70% of the SCOBY in generation 10.

K. xylinus is known for being one of the major cellulose producers in the kombucha SCOBY ("*Komagateibacter xylinus*," n.d.). The other main microbe present was *G. oxydans* which is a member of the *Acetobacteraceeae* family. It incompletely oxidizes alcohols and carbohydrates. This microbe is known to produce ascorbic acid, and tartaric acid. ("*Gluconobacter Oxydans*," n.d.).

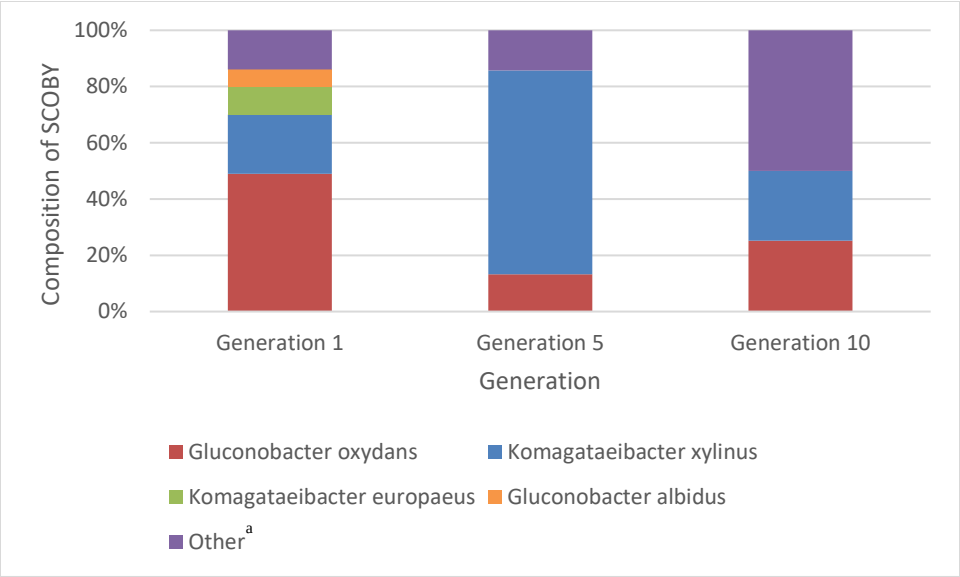

Figure 4.2. Microbial composition of SCOBY[b] B by percentage over generations
[a] Microbes with fewer than 5% of the cumulative reads from sequencing
[b] Symbiotic colony of bacteria and yeast
n= 9

SCOBY B also exhibited reduced diversity over time, and the two major species changed over time. For *G. oxydans* over the ten generations starts at over 40% of the SCOBY, decreases to less than 20% by generation 5 and finally at generation 10 is back to over 40% microflora. A similar trend can be observed with *K. xylinus*; however, it composes the greatest proportion of the SCOBY at generation 5 (Figure 4.2).

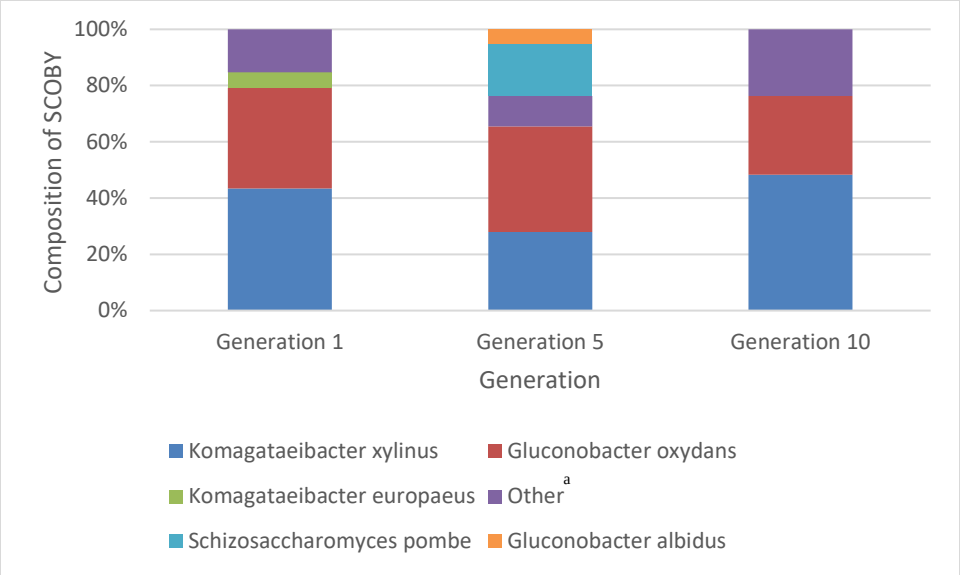

Figure 4.3. Microbial composition of SCOBY[b] C by percentage over generations
[a] Microbes with fewer than 5% of the cumulative reads from sequencing
[b] Symbiotic colony of bacteria and yeast
n= 9

Sequencing reads from SCOBY C contained the greatest percentage of yeast species of those cultures tested (Figure 4.1a-c). More specifically, generation 5 had the greatest percentage of yeast species, mainly consisting of *Schizosaccharomyces pombe*. In SCOBY C, *K. xylinus* initially compromises around 40% of the kombucha SCOBY, then decreases to less than 30% by generation 5, and back up to almost 50% of the pellicle in generation 10. The proportion of the SCOBY represented by *G. oxydans* remained consistently around 35% (Figure 4.1c).

In general, the community that begins in generation one tended to shift in favor of a larger percentage of minor contributors (other) as each generation progressed. This suggests an increase in richness over the ten generations. This trend can be observed for all three different SCOBYs.

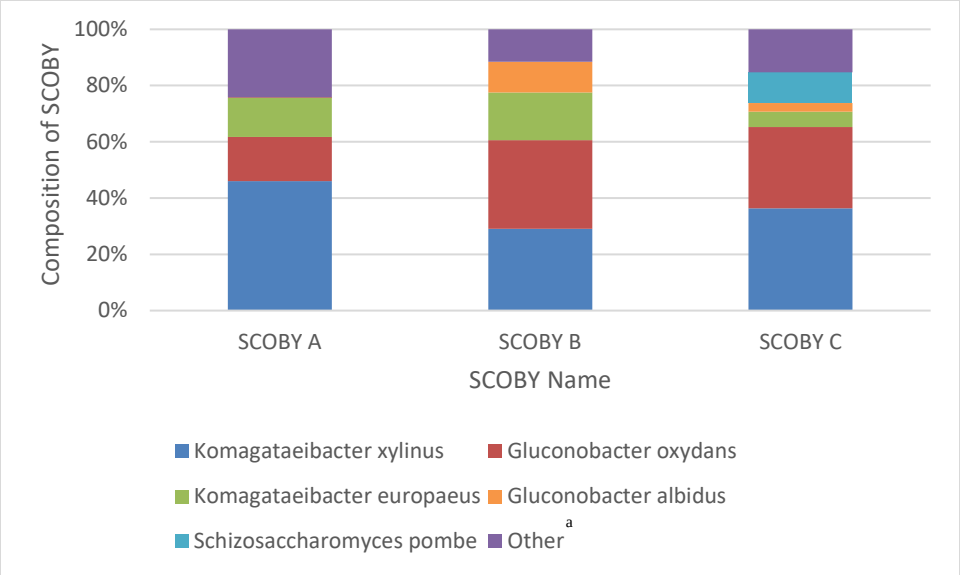

Figure 4.4. Microbial composition of different SCOBY[b] by percentage

[a] Microbes with fewer than 5% of the cumulative reads from sequencing

[b] Average of three duplicate SCOBYs (symbiotic colony of bacteria and yeast), average of 10 generations

n= 9

On average, 94% of the kombucha SCOBY reads consisted of bacteria with the remaining 6% corresponding to yeast species. The two most prevalent bacterial species identified in all three kombucha SCOBYs were *K. xylinus* and *G. oxydans*, but the two most prevent yeast species were *Z. rouxii* and *S. pombe* (Figure 4.2). However, it was not found in this study that all kombucha SCOBYs over all generations contained yeast. In fact, the diversity between the SCOBYs was determined to not be significant (p-value 0.383).

The data in Figure 4.3 show that there was ethanol production from all the pellicles. Yeast are required for ethanol production, so there must have been yeast species present in each of the pellicles. Experimental error may be responsible for the discrepancy. For example, the difference in the cell structure of yeast makes yeast DNA often more challenging to extract. The DNA extraction procedure that was used for this research was modified to include a step that boiled the cells for one minute in hopes of improving the lysis of the yeast cells. While the extraction procedure was modified to account for the cellulose matrix the cells were in, the yeast

cells may still have been bound in the cellulose and thus discarded with the pellet before the DNA was fully extracted.

Table 4.1. Beta Diversity & Beta Diversity Index for SCOBYs comparing generations within SCOBY[a]s

SCOBY	Beta Diversity	Beta Diversity Index
A	8	0.286
B	31	0.108
C	5	0.571

n= 9

[a] Symbiotic Colony of Bacteria and yeast

Beta diversity is a measure of the change in diversity of species from one environment to another. In other words, it is the number of species that are not the same in the different environments. A high beta diversity index (close to 1) is indicative of low species diversity and a low beta diversity index (close to 0) indicates a high level of diversity. Comparing each of the SCOBYs, SCOBY B has the highest level of diversity with a Beta diversity index of 0.108 in comparison to the other SCOBYs that were used for this study. SCOBY C has the lowest level of diversity with a beta diversity of 5 and beta diversity index of 0.571 (Table 4.1).

Fisher's Alpha diversity is a measure of "richness" and is a way to determine if the community is mostly composed of one species or a more equal mixture of multiple species. Similar, to Fisher's alpha, Shannon Diversity index (H) is another measure of species diversity, and it accounts for both abundance (overall contribution of taxa) and evenness (across taxa) of the species (Beals, Gross, Harrell, 1999). The Simpson's Diversity index (D) is another way of measuring the species "diversity" in a community. This index is often used to compare the rarity or commonness of species within a community (Beals, Gross, Harrell, 2000).

To get the best understanding of the community diversity among the three pellicles investigated in this study, all three measures of diversity should be considered. Fisher's alpha,

Shannon Diversity and Simpson Diversity it is very clear that SCOBY C is the most diverse. The large Shannon Index of 1.14 indicates a high diversity and a high Simpson Index of 2.50 indicates a higher species richness (Table 4.2). SCOBY A had the lowest species diversity and richness as it had the lowest values for all three measures of diversity.

Table 4.2. Diversity Measures for SCOBY[a]'s comparing generations within SCOBY

SCOBY	Fisher's Alpha Diversity	Shannon Diversity Index	Simpson Diversity Index
A	2.76	0.712 *	1.62
B	7.16	1.00 *	2.35
C	15.6	1.14 *	2.50

n= 9
[a] Symbiotic colony of bacteria and yeast
* Significant to 0.05

Table 4.3. Diversity Measures for Generation 1, 5, 10 of SCOBY C

Generation	Fisher's Alpha Diversity	Shannon Diversity Index	Simpson Diversity Index
1	3.98	0.712	1.62
5	14.5	1.01	2.35
10	8.85	1.41	2.50

n= 9
[a] Symbiotic colony of bacteria and yeast

Using SCOBY C, as an example, the impact that generation has on the diversity of the pellicle could be determined. Since SCOBY C was the only SCOBY for which the diversity was great enough at every generation that the diversity measures had a solution, it was chosen as an example. Again, three measures of diversity were used to get a better picture of the diversity across generations (Table 4.3.). Only the Shannon Diversity Indices showed a significant difference between the generations (p-value<0.05). The rest of the diversity indices were not significantly different between generations (p-value >0.05).

Thus, based on the lack of significant differences in diversity between the generations it can be concluded that the microbial diversity of a kombucha pellicle does not significantly change over 10 generations of brewing. However, even though the diversity does not change it is still likely that the composition of the kombucha pellicle does change. To better understand if the

composition of the pellicle changes with time factors such as the sensory characteristics and/or biochemical profile need to be considered. These factors are considered in the next sections of this thesis.

4.2. Biochemical Profile of Kombucha by HPLC Analysis and Sensory Evaluation

The main factor responsible for determining the biochemical profile of the kombucha beverage is the microbiological makeup of the SCOBY. The ratio of the bacteria to yeast and the levels of each will determine the consumption of sucrose and production of monosaccharides, organic acids and ethanol, all of which impact the flavor profile of the kombucha.

Table 4.4. Pearson correlation of percentage of *K. xylinus* in SCOBY to biochemical profile

	Sucrose	Glucose	Fructose	Lactic Acid	Acetic Acid
Correlation	0.358	-0.639*	-0.522	-0.017	-0.481

n= 12
[a] Symbiotic colony of bacteria and yeast
* Significant to 0.05

Table 4.5. Pearson correlation of percentage of *G. oxydans* in SCOBY[a] to biochemical profile

	Sucrose	Glucose	Fructose	Lactic Acid	Acetic Acid
Correlation	-0.114	0.227	0.116	0.159	0.176

n= 12
[a] Symbiotic colony of bacteria and yeast

K. xylinus and *G. oxydans* are the two dominant bacterial species identified in the pellicles used for this study. The amount of *K. xylinus* (as a percentage of the SCOBY) is inversely related to the concentration of sucrose, glucose, fructose, lactic acid and acetic acid (in mg/mL) in the resulting fermentate. Thus, during fermentation with a SCOBY containing higher levels of *K. xylinus*, the concentration of these compounds in the finished product should be low. However, since *K. xylinus* is positively correlated to the concentration of sucrose the higher the

amount of the microbe in the SCOBY the higher the concentration of sucrose should be in the finished tea (Table 4.4).

A different trend is observed of the compounds present int the finished product with a high percentage of *G. oxydans*. Based on the correlation between this microbe and sucrose, *G. oxydans* probably results in a lower concentration of sucrose in the resultant fermentate, because of the negative (but not significant) correlation (Table 4.5). A kombucha beverage with a high concentration of glucose, fructose, acetic acid and lactic acid in the finished beverage would probably contain a high percentage of *G. oxydans,* as shown by the positive correlations between these microbes and the concentrations of these biochemical compounds (Table 4.5).

The flavor compounds that are produced by the microbial species directly impact the sensory properties of the beverage. For example, a high concentration of acetic acid will result in a beverage that is perceived as vinegary. Samples used for HPLC analysis were stored at 4°C for 7 months prior to the analysis. This extensive storage period likely impacted the results as the microbes present in the kombucha could have continued to ferment the beverage at 4°C, but at a slower rate, as this is not the ideal fermentation temperature for the microbes commonly present in the kombucha SCOBY.

The lab-made sample was high in acetic acid (Figure 4.5), and 66.7% of panelists said that the sample was vinegary. There was a positive correlation of 0.902 between the number of panelists that selected vinegar as a flavor attribute and the level of acetic acid in the beverage. Acetic acid is the organic acid primarily responsible for providing kombucha with its vinegar flavor note. A similar, but stronger positive correlation of 0.986 was observed between the combined acetic and lactic acid concentration and the number of panelists that described the

beverage as having an acidic flavor. Finally, the total sugar concentration and the number of panelists that selected “sweet” as a flavor attribute were positively correlated (r=0.784).

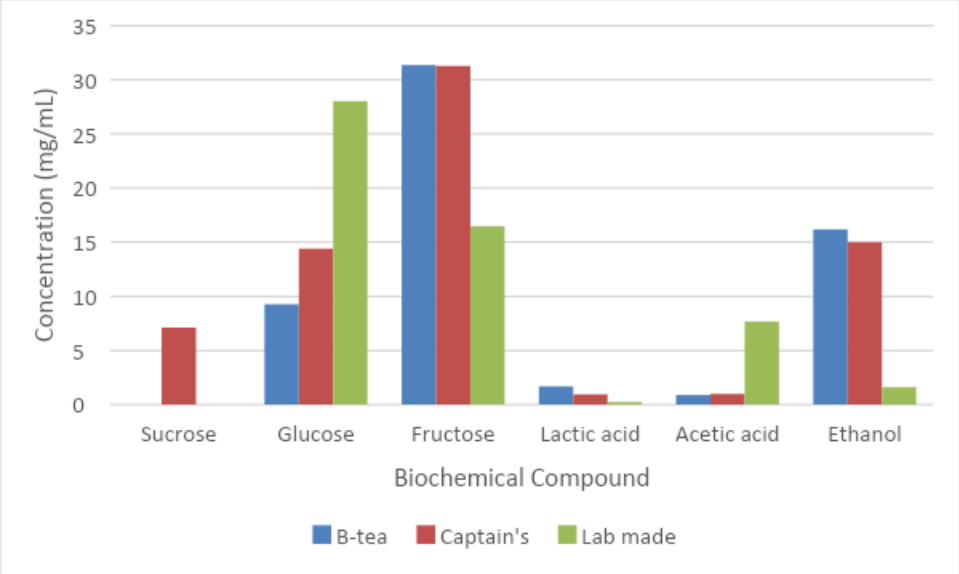

Figure 4.5. Biochemical profile of three kombucha samples used for Sensory Evaluation n=6

Comparing the results in Table 4.8 to Figure 4.5, it can be observed how the composition of the beverage directly impacts the sweet, sour, acidic and vinegar flavor notes that consumers rated in the samples. The combined concentrations of sucrose, glucose and fructose will impact the sweetness of the flavor. As can be seen in Figure 4.5., Captain’s kombucha has the highest combined concentration of sucrose, glucose and fructose, thus it is expected that consumers rate this as the sweetest sample. Table 4.8 shows the most panelists described Captain’s kombucha as sweet.

Lactic acid is known for contributing to kombucha’s sour and acidic flavor. Most panelists selected acidic and sour for the lab-made kombucha in comparison to the other samples. (Table 4.8) The data in Figure 4.5, however, does not support this conclusion. Instead, B-tea had the highest concentration of lactic acid. This discrepancy can be explained, since lactic acid is not the only compound that can provide an acidic or sour flavor to the kombucha

beverage. Organic acids, as a chemical class, are known to provide the sour and acidic flavor. Thus, the sour and acidic flavor that was perceived by consumers could have been due to high levels of organic acids, besides lactic acid, that were not tested for in this study. However, it is interesting to note that pH is not directly related to the perceived sourness of an organic acid. In fact, organic acids are known for having a poor correlation between their pH and the sourness perceived (Neta, Johanningsmeier, McFeeters, 2007).

Finally, acetic acid is known for almost exclusively providing kombucha with its vinegar flavor (Sreeramulu, Zhu, Knol, 2000). The vinegar flavor was perceived by more consumers for the lab-made kombucha than for any of the other samples. This finding was supported the HPLC analysis data. The acetic acid concentration was 7.65 mg/mL in the lab-made sample compared to only 0.880 or 0.990mg/mL in the two commercial samples.

Changes in microbial diversity can result in changes in the biochemical profile of kombucha (Eddy, 2006). The kombucha SCOBY does slightly change over time and this is reflected in the biochemical profile (Table 4.6).

Table 4.6. Biochemical profile (by HPLC Analysis) of three kombucha SCOBY[a]'s fermentate over time

SCOBY	Generation	Sucrose (mg/mL)	Glucose (mg/mL)	Fructose (mg/mL)	Lactic acid (mg/mL)	Acetic acid (mg/mL)	Ethanol (% V/V)
A	1	4.14	15.81	13.45	0.57	2.20	0.32
	2	2.97	22.71	23.60	0.80	4.05	0.29
	3	13.03	18.73	13.16	0.75	2.43	0.37
	4	9.96	21.48	17.60	0.76	3.08	0.27
	5	8.64	15.48	10.70	0.66	1.78	0.34
	6	9.98	21.86	15.34	0.91	2.21	0.42
	7	20.78	17.92	9.61	0.80	2.56	0.37
	8	14.83	21.03	15.26	0.94	2.17	0.35
	9	27.95	15.17	9.86	0.69	2.30	0.23
	10	22.28	17.89	11.27	0.67	3.06	0.27
	Average	**13.45**	**18.81**	**13.98**	**0.75**	**2.59**	**0.32**

Table 4.6 Continued

B	1	0.00	23.05	18.29	0.67	3.89	0.43
	2	0.00	24.68	20.29	0.74	5.12	0.29
	3	4.65	22.81	14.71	0.80	3.26	0.48
	4	7.09	22.75	16.17	0.74	3.41	0.37
	5	4.15	17.01	10.47	0.83	2.47	0.43
	6	9.19	21.34	15.35	1.01	2.15	0.51
	7	10.69	20.28	10.78	1.18	4.04	0.55
	8	22.94	16.30	7.42	0.96	2.47	0.52
	9	12.92	20.86	15.14	1.11	3.32	0.43
	10	24.20	15.66	7.68	0.88	2.61	0.48
	Average	**9.58**	**20.48**	**13.63**	**0.89**	**3.27**	**0.45**
C	1	0.74	24.25	21.13	0.40	4.87	0.30
	2	6.78	21.81	13.97	0.76	3.12	0.45
	3	12.13	21.06	15.96	0.68	2.11	0.34
	4	2.27	18.01	11.98	0.75	2.36	0.41
	5	9.73	21.96	15.33	0.87	3.01	0.44
	6	10.06	21.46	13.86	0.84	4.32	0.43
	7	13.97	19.60	13.55	0.80	2.85	0.43
	8	18.86	18.31	11.95	0.74	3.65	0.34
	9	13.63	21.63	15.07	0.69	2.58	0.40
	10	0.00	23.76	20.34	0.66	3.44	0.42
	Average	**8.82**	**21.19**	**15.31**	**0.72**	**3.23**	**0.40**

[a] Symbiotic colony of bacteria and yeast

4.3. Kombucha Sensory Evaluation

It is important to note that this study was conducted at the Sensory Evaluation Center at the University of Maine as this could have been a cause for a potential source of bias due to the lack of population diversity. Also, 31.8% of panelists reported never purchasing kombucha. The panel consisted of 66 panelists with an average age of 30.1 ± 13.2 years (Ages ranged from 19-68 years old). The panel consisted of 25 males and 41 females. Panelists (18.2%) indicated they had a moderate knowledge of the health benefits associated with kombucha by selecting 5 as their response on a 10-point scale (refer to Appendix D with the ballot). More panelists selected 6 or higher on the scale than did those who selected 4 or lower (Table 4.7). When asked if the health benefits associated with kombucha impacted their decision to purchase the beverage, the panels' responses did not indicate a clear trend. Thirteen persons reported that the health benefits

had no impact on their purchase of kombucha, while 42 persons said that the benefits had a slight or major impact on their purchase habits (Table 4.7).

Table 4.7. Kombucha Sensory Panel Demographic Data

Demographic Characteristic	Response	Panel (n=66)
Gender	Male	25
	Female	41
	Prefer not to answer	0
Age	Average Age	30.1 ± 13.2
Frequency of Purchasing	Once a day	0
	2-3 times a week	2
	Once a month	11
	2-3 times a month	10
	A few times a year	13
	Once a year	9
	Never	21
Knowledge of Health Benefits	0 (Low)	13
	1	1
	2	3
	3	6
	4	5
	5	12
	6	7
	7	9
	8	6
	9	3
	10 (High)	1
Impact of health benefits on intent to purchase	No impact	13
	Slight impact	22
	Neutral	10
	Major impact	20
	Not applicable	1

Participants (33.3%) most commonly selected a mixture of ginger and other flavorings (ie: ginger lemon) as their favorite flavor (Figure 4.6), followed by other (where participants had the option to write in their favorite flavor). Flavors that participants provided for "other" were: apple/spiced apple/cider, oak, cherry, lavender, herbal blends, cranberry, grapefruit/sage or earthy. The least commonly selected flavor by participants was raspberry lime (Figure 4.6). Participants could select more than one answer for this question.

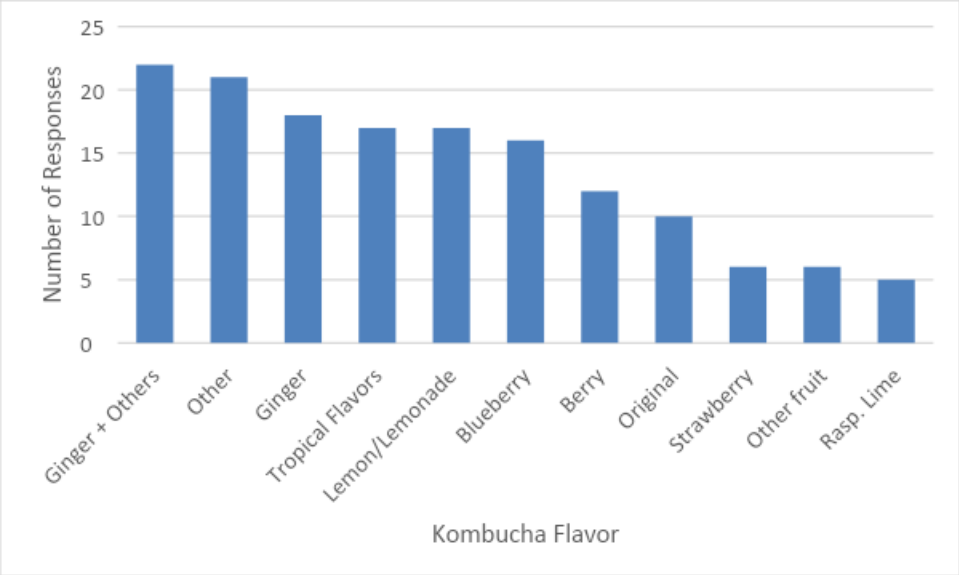

Figure 4.6. Favorite Kombucha flavors as selected by participants (n=66).

One of the main flavor components in ginger is gingerol, a very strong flavor compound capable of overpowering other less desirable flavor compounds. Ginger provides a pungent character, which humans characterize as a positive experience (Scott, Burgess, Tepper, 2019). Since ginger contains the strong flavor compound gingerol, this compound could help to explain why consumers most commonly selected ginger or ginger + others as their favorite kombucha flavor.

The purchase likelihood for Captain's kombucha was significantly different from the lab-made kombucha and B-Tea kombucha. Captain's kombucha was also significantly different from the other two samples in the attributes: refreshing, fermented and sweet (p-value < 0.05). However, the lab-made kombucha was significantly different from the other two samples for the acidic flavor note (p-value < 0.05).

Table 4.8. Kombucha descriptors selected as Check-All-That-Apply data (n =66)

Kombucha Sample	Vinegar	Fermented	Sweet	Sour	Acidic	Bubbly	Refreshing	Tea
Lab-made	44 a	35 a	22 b	45 a	44 a	6 c	21 b	20 b
B-Tea	22 b	43 a	34 b	26 b	19 b	19 b	26 b	15 b
Captain's	6 c	20 b	56 a	12 c	21 b	62 a	43 a	31 a
Probability [a]	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.007

[a] Cochran's Q test for differences in each attributes for the three samples. Counts within columns followed by different letters are significantly different.

Consumers reported that all three samples were significantly different for the vinegar, sour and bubbly flavor notes. The lab-made kombucha and the B-tea kombucha were not perceived as having significantly different flavor notes for fermented, but Captain's was significantly different. Captain's kombucha was perceived to be significantly different from the other two samples for several flavor notes. The lab-made kombucha tea was perceived as significantly different only for the acidic flavor note compared to the other samples (Table 4.8).

Table 4.9. Hedonic evaluation of kombucha samples (n = 66)[a]

Kombucha sample	Purchase likelihood	Cloudiness	Overall acceptability
Lab-made	4.7 b	5.7 ab	5.2 ab
B-Tea	4.4 b	5.4 b	4.9 b
Captain's	5.9 a	6.2 a	6.0 a

[a] Analysis of variance followed by Tukey's HSD test ($p \leq 0.05$). Means in the same column that are followed by different letters are significantly different. The hedonic scale was: 1 = dislike extremely, 2 = dislike very much, 3 = dislike moderately, 4 = dislike slightly, 5 = neither like nor dislike, 6 = like slightly, 7 = like moderately, 8 = like very much, and 9 = like extremely.

Based on the results of the hedonic questions, Captain's kombucha was the only kombucha of the three samples that had a significantly different purchase likelihood. The lab-made sample was not significantly different in cloudiness and overall acceptability from either of the other two samples. However, Captain's kombucha was significantly different from the B-tea sample in cloudiness and overall acceptability (Table 4.9).

CHAPTER 5

SUMMARY AND CONCLUSIONS

Based on the DNA sequencing conducted for this thesis research it was determined that *Komagataeibacter xylinus* and *Gluconobacter oxydans* were the two main bacterial species present in the kombucha SCOBYs. These species did not have a significant impact on the concentration of acetic acid, lactic acid, glucose, sucrose or fructose in kombucha (p-value > 0.05). The diversity of the kombucha pellicle did change over time, however, over 10 generations, this change in diversity was not significant (p-value > 0.05).

The numbers of yeast species identified in the kombucha SCOBYs were lower than expected, based on previous research. This finding could have been due to the yeast DNA not being properly extracted. Yeast cells are a challenge to lyse, which is why a boiling step was added as a procedural modification during the DNA extraction. However, despite the addition of the cellulase step, it is possible that the yeast DNA was still encased in the cellulose matrix and was discarded with the pellet.

There is quite a range in microbial diversity between kombucha SCOBYs. Comparing the beta diversity indices, the microbial diversity ranged from 0.108 to 0.571, depending on the SCOBY. To get a more holistic understanding of the microbial diversity, Fisher's alpha, Shannon diversity index and Simpson diversity index were calculated. Again, the different measures of diversity showed that the range in diversity between kombucha pellicles of different origins differed quite significantly. The Fisher's alpha diversity ranged from 2.76 to 15.6, the Shannon diversity index ranged from 0.712 to 1.14 and finally the Simpson Diversity index ranged from 1.62 to 2.50. The range in the diversity indices shows how the microbial composition between individual SCOBYs can differ greatly. This is what allows different

kombucha production companies to produce products with a different biochemical and flavor profiles. Since only the Shannon diversity index was significantly different over generations it can be concluded that a consistent product would be produced over 10 generations.

Since kombucha is a product of fermentation, ethanol is a product commonly found in the resulting beverage. In most cases, kombucha is labeled as a non-alcoholic beverage (with a few exceptions). For a product to be considered a non-alcoholic beverage in the United States, it must contain less than 0.5% ABV (Cole, 2018; Martinez-Belkin, 2015). In this growing market there is a lack of quality control methods for both the ethanol levels and flavor compounds in the beverage.

Of the lab-made kombucha samples, 10% had ethanol concentrations above 0.5%. Both commercial samples used for consumer sensory testing had ethanol levels above the legal limit. However, the fact that both these samples were past their expiration date should be considered as continued fermentation could have resulted in increased ethanol concentrations. Since both lab-made and commercial samples were measuring above the legal limit this shows the need for a quality control method, such as the one suggested in this thesis, to ensure ethanol compliance. Currently, the methods that consumers are using simply follow the guidelines presented by the TTB ("Kombucha Information and Resources," 2017).

The most significant factor in determining the biochemical profile of a kombucha beverage is the composition of the SCOBY. The two main bacterial species, *K. xylinus* and *G. oxydans*, impact the flavor profile of the final beverage by metabolizing and producing chemicals that impact the biochemical profile of the beverage. These two bacterial species were the two most prevalent species in the kombucha pellicles used for this research, however, other bacterial and yeast species also impact the biochemical profile. *K. xylinus* is negatively correlated to

glucose, fructose, lactic acid, acetic acid and positively correlated with sucrose. Thus, a sweet tasting kombucha would have a higher amount of *K. xylinus* earlier in the fermentation. However, an acidic kombucha beverage would have *G. oxydans* in higher levels later in the fermentation based on the correlations. Sucrose is one of the starting materials required to produce kombucha.

If a sweeter tasting kombucha is desired then the SCOBY should be in a tea solution with a low concentration of lactic acid (or pyruvic acid) as *K. xylinus* grows optimally at conditions with a less acidic pH (Liu, Zhong, Zhang, Xu, Qiao, Jia, 2016). This could also be accomplished by using a shorter fermentation time as residual sugar would lead to a sweeter, less acidic product. If these growth conditions are maintained over time the SCOBY will adapt to contain a higher concentration of *K. xylinus*. However, if a more acidic kombucha is desired then mannitol, sorbitol, glycerol or ethanol should be present as substrates for *G. oxydans* to metabolize into organic acids. In general, acetic acid bacteria (which include *G. oxydans*) produce high levels of acetic acid. These microbes grow best in sugary, acidic and alcoholic environments (Lynch, Zannini, Wilkinson, Daenen, Arendt, 2019).

Of the 66 participants that completed the sensory evaluation study, 31.8% of panelists never purchase kombucha, which could have impacted the results. Consumers selected ginger, ginger + other flavorings and tropical flavors as their favorite flavors of kombucha. Ginger provides a pungent character which humans characterize as a positive experience (Scott, Burgess, Tepper, 2019). A similar explanation is true for tropical flavors, because many tropical flavors have terpenes dominating their flavor profile. Terpenes as a flavor family are volatile unsaturated hydrocarbons, this allows them to effectively mask the less desirable flavors in kombucha (Jackson, 2008).

Comparing two commercial kombucha samples (B-tea, original flavor and Captain's kombucha, original flavor) with a lab-made sample, consumers determined these beverages to be significantly different in three attributes: vinegar, sour and bubbly. Other vocabulary words that were used to describe kombucha included fermented, sweet, sour, acidic, refreshing and tea flavor.

Cloudiness refers to the transparency of the beverage and includes "floaters" (floating chunks of SCOBY). Only the lab-made kombucha was significantly different from the other two samples in cloudiness and overall liking. Captain's kombucha had a significantly different purchase likelihood compared to the other samples.

Based on the research conducted as part of this thesis the two main microbes present in kombucha SCOBYs were determined to be *K. xylinus* and *G. oxydans*. By calculating beta diversity, Fisher's alpha, Shannon diversity index and Simpson diversity index it was determined that the diversity of a kombucha SCOBY does change slightly with time. However, over ten generations this change in microbial diversity is not significant (p-value > 0.05). To gain a better understanding of the microbial diversity present in the kombucha fermentation environment, studying both the liquid and the pellicle would be necessary. Future research on kombucha fermentation could provide further insight into the microbial community by sequencing both the liquid and the pellicle. In addition, this study was conducted over a relatively short time period, so a longer-term study would be recommended. It is important to further determine if the microbiological makeup of the SCOBY changes significantly over many generations and if this change impacts the biochemical profile of the beverage.

The HPLC method was unable to detect gluconic acid which is an important metabolite for its health benefits. This was a result of a method short-coming. Gluconic acid was probably

lost in the solvent front of the chromatogram. An area of future research could focus on improving this method to include determination of the gluconic acid concentration.

The results of this sensory evaluation help to provide insight into which characteristics of a kombucha beverage consumers find desirable. By understanding the characteristics that consumers find desirable in their beverages such as cloudiness and preferred flavors, kombucha producers can condition their SCOBYs to produce these characteristics in the beverage. However, the small sample size of 66 participants with 31.8% of panelists never purchasing kombucha does not give the best representation of the population of kombucha drinkers. Further research should be done with larger sample sizes across a greater geographic region to better understand consumer preferences. Also, a study could be conducted that investigates purchase intent and consumer acceptability of differently flavored (or unflavored) kombuchas.

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APPENDIX A: SENSORY EVALUATION RECRUITMENT FLYER

Consumers needed for a brief sensory evaluation of kombucha beverages

Hello-

You are invited to participate in a research project being conducted by graduate student Danielle St-Pierre and faculty sponsor Dr. Mary Ellen Camire of the University of Maine School of Food and Agriculture. If you are at least 18 years old and like the fermented tea beverage kombucha, please help researchers learn about the characteristics associated with kombucha.

If you do not like kombucha or are avoiding the consumption of trace amounts of alcohol for any reason (up to 0.5% alcohol by volume), please do not participate in this study.

This study should take no more than 20 minutes to complete and participants who complete the study will be compensated with $2.00.

Please refrain from eating or drinking anything besides plain water for at least one hour prior to testing.

If you are able and willing to participate, you can reserve a time on our Doodle poll. Walk-ins are also welcome.

https://doodle.com/poll/fifngn99r4mx4tms

When? October 11, 2018 from 10am – 3pm

Where? The Sensory Evaluation Center located in Rooms 158A and 158B in Hitchner Hall at the University of Maine.

If you have any questions, please contact Danielle St-Pierre at danielle.l.st@maine.edu or (207) 571-1733.

APPENDIX B: SENSORY EVALUATION INFORMED CONSENT STATEMENT

INFORMED CONSENT FORM

You are invited to take part in a research project. The goal is to learn how people describe kombucha. This project will be done by graduate student Danielle St-Pierre and faculty sponsor, Dr. Mary Ellen Camire from the School of Food and Agriculture. If you do not like kombucha or are avoiding the consumption of trace amounts of alcohol for any reason (up to 0.5% alcohol by volume), please do not participate in this study. You must be at least 18 years old to participate.

What Will You Be Asked to Do?
If you choose to take part, you will be asked to try three different kombucha drinks. Please drink at least two mouthfuls of each sample. Kombucha may contain up to 0.5% alcohol by volume. This is below the legal limit for alcoholic beverages. For each kombucha drink you will see a list of words. Please click the box next to all the words that you would use to describe the sample you just tasted. You will do this for all three samples. It may take up to 20 minutes of your time.

Risks
Time and inconvenience are the risks to you from participating in this study.

Benefits
There are no direct benefits to you. Results of this study will allow researchers to better understand the flavor characteristics of kombucha.

Compensation:
Persons who complete all questions will be compensated with $2.00.

Confidentiality
All data will be collected anonymously and stored indefinitely on a secure password protected computer. All data will be archived to Digital Commons for other researchers to access.

Voluntary
Participation is voluntary. If you choose to take part in this study, you may stop at any time. If you do not answer every question you will not get the $2.00.

Contact Information
If you have any questions about this study, please contact:

- Danielle St-Pierre, email: danielle.l.st@maine.edu.
- Dr. Mary Ellen Camire (Faculty sponsor), email: camire@maine.edu.

If you have any questions about your rights as a research participant, please contact the Office of Research Compliance, University of Maine, 207/581-1498 or 207/581-2657 (or e-mail umric@maine.edu).

Your participation in the study indicates consent.

APPENDIX C: DNA SEQUENCING RESULTS

		Number of reads			
		Generation 1	**Generation 5**	**Generation 10**	**Average**
SCOBY A	*Komagataeibacter xylinus*	2406	184	216	2806
	Gluconobacter oxydans	467	167	70	704
	Komagataeibacter europaeus	283	167	70	283
	Komagataeibacter nataicola	139	<50	<50	139
	Acetobacter pasteurianus	123	<50	<50	123
	Komagataeibacter medellinensis	98	<50	<50	98
	Acetobacter senegalensis	81	<50	<50	81
	Gluconobacter albidus	67	<50	<50	67
	Homo sapiens	67	<50	<50	67
	Gluconacetobacter diazotrophicus	56	<50	<50	56
SCOBY B	*Gluconobacter oxydans*	31165	3359	99	34623
	Komagataeibacter xylinus	13443	18427	97	31967
	Komagataeibacter europaeus	6188	596	<50	6784
	Gluconobacter albidus	3992	417	<50	4409
	Komagataeibacter nataicola	2024	<50	<50	2024
	Acetobacter pasteurianus	1514	693	<50	2207
	Komagataeibacter medellinensis	1264	433	<50	1697
	Acetobacter senegalensis	1124	300	<50	1424
	Gluconacetobacter diazotrophicus	898	207	<50	1105
	Kozakia baliensis	523	187	<50	710
	Acetobacter aceti	433	57	<50	490

	Neoasaia chiangmaiensis	314	<50	<50	314
	Acetobacter tropicalis	267	59	<50	326
	Acetobacter pomorum	177	<50	<50	177
	Acetobacter persici	131	<50	<50	131
	Acetobacter sp. SLV-7	76	<50	<50	76
	Homo sapiens	72	102	<50	174
	Asaia bogorensis	51	<50	<50	51
	Komagataeibacter nataicola	<50	365	<50	365
	Penicillium expansum	<50	85	<50	85
	Zygosaccharomyces rouxii	<50	80	<50	80
	Penicillium rubens	<50	75	<50	75
	Neoasaia chiangmaiensis	<50	62	<50	62
SCOBY C	*Komagataeibacter xylinus*	3823	1421	4141	9385
	Gluconobacter oxydans	3150	1916	2398	7464
	Komagataeibacter europaeus	473	95	258	826
	Gluconobacter albidus	372	265	336	973
	Acetobacter pasteurianus	236	106	267	609
	Komagataeibacter nataicola	188	<50	131	319
	Komagataeibacter medellinensis	164	<50	127	291
	Acetobacter senegalensis	149	58	129	336
	Homo sapiens	91	173	383	647
	Gluconacetobacter diazotrophicus	83	55	85	223
	Kozakia baliensis	65	<50	69	134
	Schizosaccharomyces pombe	<50	943	<50	943
	Sugiyamaella	<50	62	61	123

	lignohabitans				
	Pichia kudriavzevii	<50	<50	134	134
	Rhodotorula graminis	<50	<50	57	57

APPENDIX D: SENSORY EVALUATION BALLOT

Welcome to the Sensory Evaluation Center at the University of Maine! Thank you for taking the time to participate in our research. Please evaluate the samples in the order they are displayed to you on the computer screen. Please make sure the 3-digit code on your sample matches the code on your computer screen. For each sample please drink at least two mouthfuls. Please take a sip of water before tasting and evaluating each sample.

What is your gender?

- Male
- Female
- Prefer not to answer

Please select your age on the scale below:

[Scale ranging from 18 to 95]

How often do you purchase kombucha?

- Once a day
- 2-3 times a week
- Once a month
- 2-3 times a month
- Once a year
- A few times a year
- Never
- Prefer not to answer

How much do you know about the health benefits of kombucha?

[Scale from "nothing" to "a great deal"]

How does your knowledge of these health benefits influence your decision to consume this beverage?

- No impact
- Slight impact
- Neutral
- Major impact
- Not applicable
- Prefer not to answer

What flavors of kombucha do you normally purchase?

- Original
- Ginger
- Ginger with other flavors
- Blueberry
- Lemon/lemonade

- Berry
- Strawberry
- Tropical flavors (mango, pineapple, etc)
- Raspberry lime
- Other fruit flavors
- Other: _[textbox for participants to write in their answer]__
- None of the above

[The following questions will be displayed for all samples]

Please evaluate this sample and check all the boxes that you associate with this sample.

- ☐ Vinegar
- ☐ Fermented
- ☐ Sweet
- ☐ Sour
- ☐ Acidic
- ☐ Bubbly
- ☐ Refreshing
- ☐ Tea flavor
- ☐ Other: [box for panelists to write in answer]
- ☐ None of the above

How likely would you be to purchase this sample?

1 – Extremely unlikely
2 – Very much unlikely
3 – Moderately unlikely
4 – Slightly unlikely
5 - Neither likely nor unlikely
6 – Slightly likely
7 – Moderately likely
8 – Very much likely
9 - Extremely likely

How much do you like the cloudiness of this sample?

1 – Dislike Extremely
2 – Dislike very much
3 – Dislike moderately
4 – Dislike slightly
5 - Neither like nor dislike
6 – Like slightly
7 – Like moderately
8 – Like very much

9 - Like extremely

How much do you like this sample OVERALL?
1 – Dislike Extremely
2 – Dislike very much
3 – Dislike moderately
4 – Dislike slightly
5 - Neither like nor dislike
6 – Like slightly
7 – Like moderately
8 – Like very much
9 - Like extremely

Please leave any additional comments about this sample in the space provided below. If you wish to refer to another sample from this test, please refer to it by the three-digit code.

[Comment box]

BIOGRAPHY OF THE AUTHOR

Danielle St-Pierre was born in Markham Ontario, Canada on October 9, 1995. She was raised in Essex Junction, Vermont and graduated from Essex High School in 2014. She attended the University of Maine and graduated in 2018 with a Bachelor's degree in Food Science and Human Nutrition. She stayed in Maine and continued her accelerated graduate degree at The University of Maine in the summer of 2018. Danielle is a candidate for the Master of Science degree in Food Science and Human Nutrition from the University of Maine in August 2019.