KEY PROCESS PARAMETERS ON

KOMBUCHA'S BIOACTIVES & FLAVOR QUALITY

by

SAIKIRAN CHALUVADI

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ABSTRACT OF THE DISSERTATION

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Kombucha, a fermented tea beverage produced using yeast and acetic acid bacteria, tastes like apple-cider vinegar and contains bioactives such as tea polyphenols, acetic acid, and gluconic acid. While traditional probiotic bacteria were not observed in Kombucha, the bioactives could potentially act as postbiotics and correct dysbiosis, an imbalance in gut microbiota. Challenges in Kombucha industry include batch to batch inconsistencies in flavor quality and mislabeling the amounts of alcohol, acid and sugar. Producers frequently change tea types, inoculum types (age and solid or liquid), and fermenter characteristics without a clear understanding of their effect on bioactives and flavor quality. Our objective was to understand the relationship between key process parameters and microbial activity to help design processes that could achieve better quality control. Microbial activity was measured in terms of change in Kombucha sugars, acids and catechins using HPLC methods and correlated with total plate counts. Flavor quality was evaluated by taste and compared to a matrix developed using a 9-point hedonic scale.

Black tea Kombucha tasted harshly acidic (sugar/acid ratio (S/A) of ~10), compared to a pleasant tasting green tea Kombucha (S/A of ~14) that corresponds to an overall taste acceptance rate of 64%. The differences could be explained by the antimicrobial activity of green tea catechins. Black tea when dosed with green tea catechins (EGC and EGCG) reduced the bacteria counts in

Kombucha. While there was no difference in yeast counts, metagenomic sequence analysis showed a different microbial composition in Black and Green Tea Kombuchas'.

Compared to a 10-day inoculum, Kombucha made with a 15-day solid and liquid inoculum was overly fermented and unpalatable (lower S/A of 6.6) which corresponds to an overall taste acceptance rate of 27%. These taste characteristics were not necessarily due to higher bacteria and yeast counts in 15-day inoculum but has to do more with different microbial composition, as confirmed by sequencing data. When only liquid inoculum was used, the rate of fermentation reduced in half, resulting in an overly sweet Kombucha with S/A ratio of 28 which corresponds to an overall taste acceptance rate of 16%. There was an increase in total polyphenol content and catechins (EGC+EGCG) in Kombucha made without solid inoculum.

Kombucha made in a vessel with a specific interfacial area 0.16 (SA/V) produced glucose and fructose 5-7 times as much as the one with lowest SA/V 0.09. Specific interfacial area of the fermentation vessel was found to be directly proportional to the rate of fermentation as confirmed by S/A ratios, residual sucrose and the flavor profile.

A comprehensive analysis was performed to provide a better understanding of relationship between few key process parameters, microbial activity and quality parameters. Changing key process parameters resulted in wide range of sugars, acids and alcohol concentration. These findings will add to a list of parameters that producers will need to monitor for better Kombucha quality control and to deliver a balance between bioactive content and flavor profile.

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Table of Contents

LIST OF FIGURES

LIST OF TABLES

1 INTRODUCTION

Kombucha is a sparkling fermented beverage that is considered a healthier alternative to sugary carbonated soft drinks and widely perceived to improve digestive health. In addition to perceived gut health benefits, studies have indicated antimicrobial, antioxidant and hepato-protective properties of Kombucha which can further bolster the consumer appeal (Sreeramulu *et al.,* 2000, Hyun *et al.,* 2016, Jayabalan *et al.,* 2008). The benefits are primarily attributed to the presence of functional bioactive components such as tea polyphenols, catechins, organic short chain fatty acids (SCFAs) and in some cases lactic acid bacteria (Marsh *et al.,* 2014). Flavanoid bioactive components of black tea can be fermented and utilized for growth by bacteria in human large intestines, very much behaving like prebiotics (Lee *et al.,* 2006, Najgebauer-Lejko *et al.,* 2011, Banerjee *et al.,* 2010). Presence of L-theanine, caffeine and B vitamins along with sugars can provide mood elevation and energy benefits. While there is commercial interest to promote Kombucha as a probiotic beverage, the survival, activity and persistence of organic acid producing starter cultures from Kombucha upon ingestion is yet to be determined. Stability and robustness of these bacteria and yeast in harsh acidic and anaerobic conditions in gastrointestinal tract, along with actual health benefits needs to be well understood before qualifying it as a probiotic beverage. Assumptions made in earlier literature have not taken the consensus panel recommendations on the definition of probiotic into account (Kozyrovska et al., 2012, Fu et al., 2014, Hill et al., 2014, Reva et al., 2015, Panghal et al., 2018).

A three-step process of making Kombucha starts with preparing sweetened tea base, followed by inoculation of sweetened tea with mixed starter cultures of bacteria and yeast and ends with the incubation/fermentation process. Kombucha is traditionally prepared using black tea, *Camellia sinensis* spp., that has a characteristic astringent and puckering mouthfeel which complements the

mildly acidic fermented flavor in Kombucha. Nitrogen based phytochemicals namely caffeine, L-theanine and theobromine, ~15% (w/w), and ~6% (w/w) amino acids (Harbowy *et al.*, 1997) have been postulated as growth nutrients essential for microbial fermentation (Kallel *et al.*, 2012, Sreeramulu *et al.*, 2001, Watawana *et al.*, 2015). Other *Camellia sinsensis* based substrates like green, oolong, white and puerh teas and herbal teas such as echinacea, coffee, hibiscus, lemon balm, mint and yerba mate are also used to make Kombuchas (Jayabalan *et al.*, 2014, Malbasa *et al.*, 2008), however the role of herbal teas in Kombucha fermentation is unknown. While cane sugar is most commonly used carbon source, other complex sugar syrups like agave nectar, beet molasses, cane juice, Jerusalem artichoke extracts, sweet whey are also used in the fermentation process.

Composition of Kombucha starter culture can vary significantly with geographical location and process conditions. Typical starter culture is a mixed consortium of at least 10 different types of unstandardized bacteria and yeast species (Chakravorty *et al.*, 2016, Marsh *et. al.*, 2014). These cultures are bound to the surface of a solid cellulose matrix known as SCOBY (Symbiotic culture of bacteria and yeast) and also in a freely suspended form in the liquid fermented tea. Marsh *et. al.*, (2014) studied Kombuchas from few different origins and identified *Zygosaccharomyces* and *Gluconacetobacter* species as the dominant yeast and bacteria types responsible for alcohol and acetic acid fermentation (Marsh *et al.*, 2014). Back-slopping, a technique that uses fermented material from a prior batch to inoculate and a start fresh batch, is still a widely used method in Kombucha fermentation. The composition of these cultures is largely controlled based on ‘age of inoculum’ and pH. The inherent variability associated with all these steps is exaggerated on a commercial scale. Due to the presence of alcohol and sugar, the consequences are also much more dramatic in Kombucha when compared to other mixed culture fermented products like artisanal cheeses, kimchi and sauerkraut, etc.

The fact that Kombucha is one of the fast growing beverage categories in US is drawing more attention to the issues. In 2015, retail supermarket sales have exceeded 500 million USD, expected to grow 25% year over year, exceed 1 billion USD in 2020 (Markets and Markets, 2015). This growing demand is forcing brands and industry to scale up manufacturing processes, incorporate creative ingredients such as complex sugars, fruit juices, different types of teas, herbs and spices to differentiate from one another, explore different packaging types and explore ingredient cost saving measures to compete in the marketplace (Markets and Markets, 2015).

Fermentation characteristics change in a scaled up process, which when combined with i) new type of ingredients, ii) inherent variability in unstandardized starter cultures and iii) drawbacks of back slopping inoculation technique can only lead to quality control issues. As a result, inconsistent alcohol and bioactive content, variations in flavor profile and microbial composition has become more prevalent. Most of the Kombucha in US is sold as raw or unpasteurized but marketed as a non-alcoholic beverage, which means alcohol by volume (ABV) should be less than 0.5%. Disruption in refrigerated supply chain can cause secondary alcohol production and lead to spoilage and food safety issues. Varying amounts of residual sugar, alcohol and organic acids not only affect flavor and health benefits of Kombucha but also lead to nutrition and regulatory compliance issues (Elaine Watson 2018, Elizabeth Crawford 2017). Uncontrolled yeast fermentation has led to product recalls on accounts of presence of alcohol beyond allowable legal limits (Kim Severson 2010, James Hamblin 2016). This is prompting manufacturers to address the issue.

Other large scale fermented products like yogurt and bread use standardized starter cultures to ensure consistent quality. While this can go a long way in addressing variability in Kombucha, there are other process parameters that are equally as important and their impact on

quality parameters needs to be better understood. Compositional parameters like type of tea, carbon source, amount and type of inoculum and fermenter characteristics such as specific interfacial area have been known to affect the composition of Kombucha metabolites (Cvetkovic et al., 2008, Dufresne et al., 2010, Jayabalan et al., 2016, Loncar et al., 2006, Malbasa et al., 2011, Reiss 1994, Vitas et al., 2018). Given the nature of mixed starter cultures, it is difficult to extrapolate results from one study to another beyond a point. So, there is a need for one comprehensive study to capture the effects of multiple key process parameters on microbial activity and resulting quality attributes. Hence, the purpose of this research is to gain further insights into the mechanisms of Kombucha fermentation to help achieve better quality control.

2 LITERATURE REVIEW

2.1 Fermented foods: role in human nutrition and health

Fermentation is among the oldest food processing methods known to man. Throughout the human civilization, the techniques of traditional fermentation processes were passed down through generations. It can be argued that during the second industrial revolution, most societies have paused this tradition by moving food production from kitchens to commercial manufacturing facilities. As a result, more so in developed societies, the need to explore and teach the art and science of fermentation had become far less important. But having said that, fermented foods are still a major part of staple diets around the world. Transforming simple ingredients into complex flavors and textures by controlling microbial growth has led to creation of numerous traditional and culturally prominent food and beverage recipes like Coffee, Cocoa, Bread, Buttermilk or Chass, Cheese, Dhokla (made with fermented rice and chickpea paste), Dosa (made with fermented rice and lentils), Gochujang (made with red chili powder, rice, fermented soy beans), Kefir, Kimchi, Kombucha, Miso, Sauerkraut, Sour cream, Stinky tofu, Tabasco™ hot sauce, Vinegars, Yogurt etc. Most of these fermented foods are typically produced in small batches by either spontaneous fermentation process due to environmental microorganisms or by an inoculation using back slopping/pitching. Depending on the type of product and variations in composition of wild starter cultures, the duration of fermentation can range from few hours to weeks. This ambiguity with traditional processes could not have met the demand of growing population in the last century. So, large scale industrialized food manufacturing took over, successfully replicated some of the traditional methods but only produced a small fraction of known staple fermented foods. To replicate quality, the concept of using pure standardized starter monocultures was introduced. But in general, lack of a good understanding of fermentation science, especially in those foods involving

complex microbial communities had discouraged the commercial production of many complex staple fermented foods.

2.1.1 Gut microbiota and dysbiosis

Our recent understanding of role of gut microbiota in overall health and well-being has rekindled consumer and commercial interest in different types of fermented foods.

NIH Human Microbiome Project (HMP) was commissioned to understand the role of bacteria in healthy and disease cohorts of human population. Composition of microbiota from nasal passages, oral cavities, skin, gastrointestinal and urogenital tracts was determined and classified into various phylogenetic and taxonomical groups using traditional Sanger method and 16S ribosomal RNA gene sequencing. Along with genomic data, functional -omics such as proteomics, transcriptomics and metabolomics are being utilized to study three models of microbiome-related human conditions – pregnancy and preterm birth; inflammatory bowel disease and gut disease onset; respiratory viral infection and the onset of type 2 diabetes (Gevers *et al*., 2012, Methe 2012, Proctor, 2014). The gut microbiota composition obtained from metagenomics data of the HMP project showed staggering taxonomic, phylogenetic and species level diversity between healthy individuals of one community. However, functional data from metabolites and related –omics as mentioned above showed about 70% similarity between these healthy individuals, suggesting the need to consider bacterial communities as an assembly of functional genes but not diverse species (Burke *et al*., 2011, Turnbaugh *et al*., 2009).

Instability in the composition of gut microbiota is currently known as dysbiosis. This instability has been recently linked to health conditions like allergies, obesity, metabolic syndrome, Crohn's disease and irritable bowel syndrome. Diet, lifestyle, antibiotic use and chronic disease are some other major factors attributed to dysbiosis (Backhed *et al*., 2012, McFarland, 2014). Dietary

changes have been shown to alter the gut microbiota composition of healthy individuals. Animal based diets increased the amounts of bile-tolerant organisms from *Bacteroides* genus and decreased the level of *Firmicutes*, more specifically *Roseburia, Eubacterium rectale and Ruminococcus bromii* which are known to metabolize plant polysaccharides (Clemente *et al*., 2012, David *et al*., 2014). A significant shift in gut microbiota was found in 24 hours when diet in healthy individuals was shifted from high-fat, low fiber to low-fat, high fiber composition. Recent studies showed long term protein and animal fat diets selected for *Bacteroides* genus of *Bacteroidetes* phylum over *Prevotella* genus of *Firmicutes* phylum (Wu *et al*., 2011). However, plant based diet of African population showed dominance of *Bacteroidetes* and animal based diet of European population showed dominance of *Firmicutes* (De Filippo *et al*., 2010). Due to inter-individual variability in gut microbiota composition within healthy population, a healthy microbiota may not be defined without better understanding gene expression, metabolic profiles and other functional aspects of the microbiome. However, behavior of healthy microbiota may be defined as its ability to resist changes induced by ecological stress and/or return to its native state after the stress is released (Backhed *et al*., 2012). Less diversity in microbiota may be a direct result of less diverse diet and this was found to show least resistance to changes, especially in the elderly population (Claesson *et al*., 2012).

2.1.2 Fermented foods are candidate Postbiotics

Postbiotics is a recently coined term used to describe molecules depleted in an abnormal or a dysbiotic gut condition which when supplemented in whole or precursor form will help restore the balance in the gut (Klemashevich *et al*., 2014). The idea behind postbiotics is to include all bioactive functional molecules that can be used or produced by microbial community for promoting health. Postbiotics produced by gut microbiota could be metabolites such as short chain fatty acids from carbohydrates, indole from amino acids, gamma-aminobutyric acid from glutamic acid

and polyphenolic acids and other functional compounds derived from the diet. It was suggested that metabolomics approach may be used to identify postbiotics from biological systems (Klemashevich *et al*., 2014). Identifying and understanding postbiotics can help develop dietary intervention methods, like fortifying or processing food to include bioactive materials that can alter/correct dysbiosis. There are several foods that have postbiotic type bioactive compounds or their precursor molecules, such as short chain fatty acids, vitamins and polyphenols.

Fermentation is one such dietary intervention method that can enhance nutritional value of food. Myriad different bioactive end products can be expected depending on the type of substrate, microorganism/s and process (aerobic/anaerobic). Peptides with an antibacterial activity (bacteriocins) or an enzymatic activity (β-glucosidase), exopolysaccharides with prebiotic activity (digested by gut microorganisms), phenolics with antioxidant/anti-inflammatory activity (conversion of anthocyanidins to proanthocyanidins), neurotransmitters like γ-amino butyric acid that can modulate behavior by reducing stress, vitamins like B2, B9 and B12 that play a role in cellular metabolism and energy production, organic short chain fatty acids like lactate that reduce pro-inflammatory cytokine secretion and reactive oxygen species in intestinal enterocytes; acetate, propionate, butyrate with inhibitory activities against enterobacteria; increasing the bioavailability of phytochemicals by releasing esterified compounds to free form were all reported (Marco *et al*., 2017, Ruijssenaars et al., 2000).

Fermented food products such as yogurt, sauerkraut, pickled vegetables and Kombucha are candidate postbiotic foods. There are more than 4000 unique flavonoids in nature that can be derived from the diet. Most of these phenolic compounds remain unabsorbed in the gut. Complex interaction between these polyphenols and intestinal microbiota result in microbial transformation products. These products have been shown to repress the growth of pathogenic strains of *Clostridium*

spp. while commensal bacteria were either less or positively affected (Lee *et al*., 2006). Long term consumption of tea polyphenols correlated with body weight reduction in obese individuals. *Bacteriodetes* produce more glycosidases, enzymes required to metabolize polyphenols, giving them a selective advantage over the *Firmicutes* (Rastmanesh, 2011). Hence, it has been hypothesized that weight loss may result from restoration of the *Firmicutes* to *Bacteriodetes* ratio to that found in healthy counterparts (Rastmanesh, 2011).

2.1.3 Food microbial cultures: their role as beneficial commensals or probiotics?

The benefits from fermented foods can extend beyond microbial transformation products and postbiotics. Microbial communities in fermented foods have the potential to colonize human gut and improve microbial diversity. As mentioned earlier, diversity offers resistance to Dysbiosis, by competing for nutrition, upregulating virulence factors, metabolites etc. This deters pathogen growth by discouraging pathogenic bacteria from infecting, colonizing, and causing disease in host. (Chaluvadi *et al*., 2015).

Most fermented foods contain live microbial cultures from well-known bacterial groups such as Lactic acid bacteria (LAB), Acetic acid bacteria (AAB) and also other bacterial groups. That is microbes that are well-established and characterized like *Saccharomyces cerevisae* to not so well known or understood yeasts like *Lachancea fermentati or Kluyveromyces spp.* can be found fermented foods (Marsh *et al.,* 2014, Chakravorty *et al*., 2016). Most staple artisanal fermented foods like cheese, kimchi, kefir, Kombucha have complex microbial ecosystems. These foods are composed of different microorganisms that may have come from the different substrates or raw materials and/or from starter cultures added by artisans and/or the surrounding environment.

A recent study (Wolfe *et al*.,2015) on microbial diversity in 100+ different cheese rinds sourced from ~10 countries showed reproducible communities of bacteria and fungi, independent of geographical location. Type of rind and moisture were the two main factors that influenced the composition of these communities. While dominant genera of identified microbes have originated from starter cultures, at least 60% of bacteria and 25% of the fungi present originated from the environment. A co-occurrence and non-co-occurrence patterns between different fungi and bacteria indicated the interdependency and co-evolution of microbial communities. Many bacteria showed strong growth responses to the presence of the fungi including poor growth in the absence of a fungal partner, indicating some type of alteration in cheese environment in the presence or absence of certain fungi types. This interdependency can apply to other microbes in other complex ecosystems.

In a study related to microbial communities in 25 different kefir milks from 8 geographically distinct regions (Marsh *et al.,* 2013), bacterial population was found to be more consistent and less diverse than in their corresponding starter cultures known as kefir grains. It is reasonable to expect a similar pattern in Kombucha broth and SCOBY. The most common fungal genus across both kefir milk and grains was *Kazachstania* followed by *Naumovozyma and Kluyveromyces*. >50% of bacteria in most samples was dominated by lactic acid bacteria, with high proportions of *Lactobacillus* followed by *Streptococcus* genera. *Lactococcus* and *Leuconostoc* were significantly higher in kefir milk. The authors have cautiously suggested that these bacteria are health beneficial as these genera are most commonly associated with probiotic bacteria. However, consistent with the recommendations for the scope of probiotics (Hill *et al*., 2014), the authors have instead advocated to alter starter culture composition to include pre-established, proven and certified probiotic strains. Kefir, like Kombucha, showed presence of low quantities of few different acetic

acid bacteria (AAB) from the genus *Acetobacter*. AABs are mostly known for production of vinegar, Belgian sour/lambic beer, Kombucha and also notorious spoilage microorganisms in beer and cider fermentation. Since they are difficult to isolate and grow using standard culture techniques, AABs are not very well studied or understood (De Roos *et al*., 2018).

While there is sufficient evidence to support beneficial relationship between consumption of fermented foods and reduced risk of certain diseases (Soedamah-Muthu *et al*., 2013, Tong *et al*., 2011, Wang *et al*., 2013) it is not possible to clearly ascertain the role of complex live microbes in a particular health benefit. In addition to this, survival and efficacy of microbes in various fermented foods during the storage shelf life is not properly understood or evaluated. Therefore, instead of describing fermented foods as probiotic, the expert panel had recommended describing them as foods containing 'live and active cultures' treating them more like beneficial commensal organisms (Hill *et al*., 2014).

2.1.4 Fermented foods: quality, safety and need for caution

Mixed starter cultures that are unstandardized are inherently risky, especially if the microbial communities have not adapted to an environment. With growing popularity for artisanal fermented food and beverage brands, for example in kefir, kimchi and kombucha categories, there is a temptation and often times a need to differentiate using different substrates. Different bacteria and yeast can be selected from a community based on different biotic and abiotic factors (Wolfe *et al*.,2015). Understanding the effect of these factors including different substrates on microbes is crucial for controlling quality. The fact that these microbes are unstandardized makes it even more challenging.

Earlier studies have shown presence and also dominance of certain organisms that are either unknown commensals or opportunistic pathogens like *Candida Kefyr* (Marsh *et al*., 2013), *Candida*

species (Jayabalan et al., 2014, Chakravorty *et al*., 2016). While horizontal gene transfers amongst prokaryotes and eukaryotes can allow them to thrive in complex microbial ecosystems, if not properly understood or studied can result in quality and safety issues. In a community setting, horizontal transfer of virulence or antimicrobial resistance genes to commensal or starter cultures has been reported numerous times in the past and can be a real concern in unstandardized mixed culture systems (Friesen *et al*., 2006, Ma *et al.*, 2000, Sanders 2006, Slot *et al*., 2011).

Toxic compounds like biogenic amines are present in fermented foods and beverages. Many species of lactic acid bacteria and gram negative bacteria have the ability to produce biogenic amines like histamine, β-phenylethylamine and polyamine (Mohedano *et al*., 2015). While amino oxidase enzyme in human gut can remove or reduce the impact of these amines, alcohol consumption through food is known to retard this enzymatic activity. Starter cultures that contain alcohol producing yeast and histamine/biogenic amine producing lactic acid bacteria starter cultures, (for example, *Oenococcus, Pediococcus,* certain *Lactobacillus),* can lead to long term accumulation of these amines in causing potential health disorders (Mohedano *et al*., 2015). Since both Kombucha and Kefir cultures use bacteria and yeast cultures, it is important to understand the composition of starter cultures and screen for biogenic amine producers.

2.2 Kombucha

2.2.1 History

To understand the history of Kombucha it is important to understand the historical significance of tea. Consumption of tea as a health beverage dates back to 5000 years ago in China. In ancient Chinese practices, tea was used as a medicine and till date part of social rituals in several countries. Various benefits like stimulation of mind, detoxification, immunity to diseases were attributed to consumption of tea (Dufresne *et al*., 2000). Tea is consumed in many forms across the

world, with classic and more familiar traditional hot tea where tea leaves are infused in hot water and liquor consumed as a refreshment; ground green tea leaves whisked into water and consumed as a ceremonial matcha green tea in Japan; fermented tea leaves known as Lahpet consumed in the form of a salad in Myanmar; tea brewed in milk and sugar known as chai is consumed as a refreshment in many parts of south Asia. Tea when fermented by bacteria and yeasts can produce a complex beverage which is now popularly known as Kombucha. A famous legend about this beverage dates back to 220 BC, calling it as "Divine Tsche or Tea of Immortality" during Tsin dynasty for its energizing and detoxification benefits. The origin of the name comes from 414 AD when Dr. Kombu used this beverage to cure ailments of a Japanese emperor. Cha relates to the tea component of the beverage. Kombucha is known by different names across the world including in Asia (haipoa, kocha kinoko, hongo, suancha), in Russia (cainiigrib, cainii kvass, japonskigrib), in eastern Europe (Heldenpilz, Kombuchaschwamm), Italy (Funkochinese) (Jayabalan et al., 2014). Traditionally, Kombucha is made at homes using tea fungus that is passed along over several generations. Around the turn of this century, Kombucha was introduced in mainstream US retail stores as a health promoting fermented beverage. Today, it has grown into a $500 million category in US and gaining traction in many other parts of the world.

Table 1: Potential health benefits of Kombucha

	Health Benefits	**References**
Kombucha	Antioxidant, antimicrobial, antiglycemic, hepatoprotective agent, mood elevation, prebiotic, weight management	
Polyphenols	1. Tea has shown to act as a prebiotic and modulated intestinal bacterial population 2. Kombucha showed enhanced antioxidant activity confirmed by DPPH (a,a-diphenyl-b-picrylhydrazyl) assay 3. Kombucha showed 40% higher ABTS scavenging (2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) 4. 50-100% increase in total polyphenol content	Chu *et al*, 2006 Jayabalan *et al*, 2008 Lee *et al*., 2006

	observed after fermentation	
Acetic acid	1. 2-5% acetic acid in vinegar when added to various foods has shown reduction in glycemic index of foods 2. Antimicrobial effects of kombucha against a range of gram positive and negative bacteria was attributed to acetic acid	Johnston *et al*., 2006 Greenwalt *et al.*,1998
Gluconic acid	Unknown	
Glucuronic acid	1. Glucuronidation process is one of the mechanisms of excretion of exogenous chemicals from human body 2. Metabolic conjugation of phenolic or alcoholic hydroxyl radicals with glucuronic acid in liver can result in removal of toxins in glucuronide form 3. Severe jaundice or liver disease is observed in case of inability of bilirubin to conjugate with glucuronic acid	Barniville *et al*., 1959 Fishman *et al*., 1951 Saltzman *et al.*, 1953 Teoh *et al*., 2004
Saccharic acid 1,4-lactone	Found to inhibit glucuronidase enzyme that deconjugates glucuronides	Dufresne *et al.*, 2000
Caffeine, L-theanine, B vitamins	Mood elevation, mental alertness and energy could be attributed to kombucha due to presence of these compounds	Owen *et al*., 2006, Institute of medicine 1998

2.2.2 Home preparation

Kombucha can be prepared by a batch or continuous brew process. Most home brewers commonly prepare kombucha using a batch process in a 1 to 5-gallon glass jar. Seasoned regular kombucha makers use a more classic continuous brew method (Jayabalan *et al*., 2014, Crum *et al*., 2016).

<u>Home Brewer's Batch process</u>

1. 4 to 6 tea bags are steeped in ½ gallon hot to boiling reverse osmosis (RO) water for 5 to 10 minutes
2. ¼ to ½ cup of granulated sugar is dissolved in hot tea

3. ½ gallon cold RO water is added and mixture is brought to room temperature
4. ~1 cup of Kombucha from a previous batch (liquid inoculum) to 1 gallon of sweet tea is added
5. ~½ cup or 4 oz. of SCOBY also known as solid inoculum is then added. During the fermentation, a new SCOBY is formed on top of this inoculum
6. The setup is covered with a clean medium to tight weave breathable cloth and left to ferment in ambient temperature 20 to 28 ºC
7. Depending on type of tea, amount of sugar and inoculum, desired flavor profile the fermentation could take 5 to 30 days

Home Brewer's Continuous process

1. A glass jar with a spigot is used for this process
2. Amount of tea, sugar, water and inoculum are same as batch process
3. The process of setting up the ferment is same as batch process
4. After desired level of fermentation, 30-50% of fermented Kombucha is decanted through the spigot and the jar is replenished with sweetened tea to continue the process
5. Regular maintenance of the jar is required which involves removing excess SCOBYs, cleaning spigot and yeast debris from the bottom of the jar.

2.2.3 Microbial composition

2.2.3.1 Starter cultures

Two types of starter cultures are used to produce a traditional Kombucha. The liquid cultures or inoculum is composed of both bacteria and yeasts but their relative distribution can vary significantly between different Kombuchas. Solid inoculum is more commonly known as SCOBY, *Medusomyces gisevii*, is a symbiotic culture of bacteria and yeast bound to the surface of a cellulose

matrix. Few metagenomics studies (Table 2, Table 3) have characterized the composition of inoculum around the world.

Table 2: Dominant bacteria (by relative abundance) among various kombucha inocula

	References
Komagataeibacter xylinus, Komagataeibacter saccharivorans, Komagataeibacter intermedius	Reva *et al*., 2015, Marsh *et al*., 2014, Chakravorty *et al*., 2016
Gluconobacter oxydans	Reva *et al.*, 2015
Lactobacillus sp.	Reva *et al.*, 2015, Marsh *et al*., 2014
Acetobacter xylinum (same as *Komagataeibacter xylinus*)	Jayabalan *et al.*, 2014

Table 3: Dominant yeast species (by relative abundance) among various kombucha inocula

	References
Dekkera anamola (Bretannomyces)	Reva *et al.*, 2015
Zygosaccharomyces kombuchaensis	Marsh *et al*., 2014
Pichia occidentalis.	Reva *et al.*, 2015
Candida stellimalicola	Chakravorty *et al*., 2016
Lachancea fermentati	Chakravorty *et al*., 2016

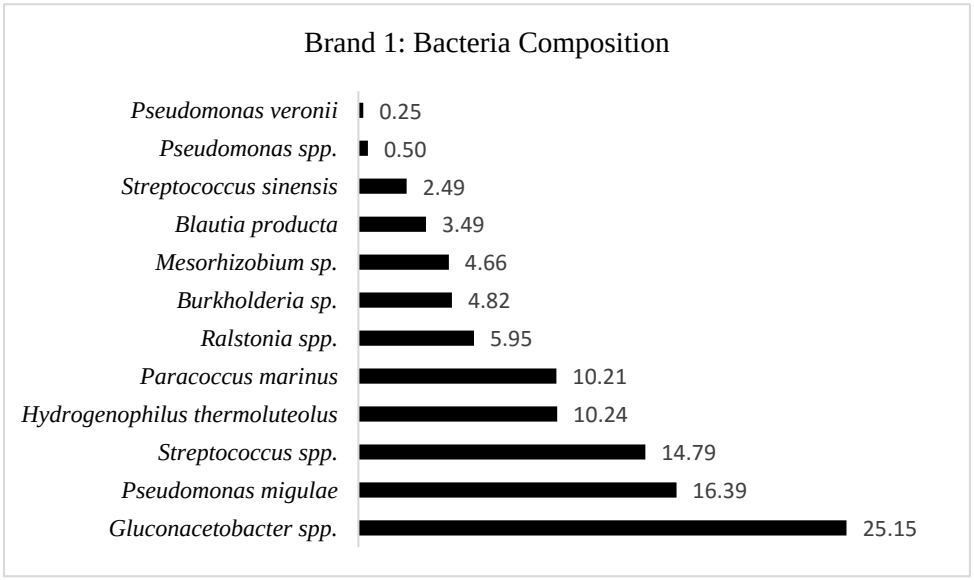
Komagataeibacter sp. (previously *Gluconacetobacter* sp. and *Acetobacter sp.*) can produce cellulose, gluconic and acetic acids and vitamins (Jayabalan *et al.,* 2014, Nguyen *et al.,* 2015). Along with these dominant bacteria, Marsh *et al*., (2014) showed presence of *Lactococcus, Leuconostoc, Propionibacterium, Enterococcus* genera and Chakravorty et al., reported small percentages of *Bifidobacterium, Enterobacter, Lyngbya* genera.

Zygosaccharomyces kombuchaensis is one of the more popular yeast species found in Kombucha and different from other members of this genus. Members of *Zygosaccharomyces* genus, are generally known to be spoilage organisms found in high sugar environment like fruit and vegetable juices. This particular species is acetic acid and low pH tolerant, prefers to grow in static conditions and sensitive to high sugar content (Steels *et al.,* 2002). *Bretannomyces (Dekkera)* another dominant yeast in Kombucha, is a high acid tolerant yeast which can produce both acetic acid and alcohol. It is also a contaminant and a spoilage organism in Belgian beers (Teoh *et al*., 2004).

Along with these dominant yeasts, other genera like *Eremothecium, Debaryomyces, Saccharomyces, Hanseniaspora, Pichia, Candida, Lachancea* can be present in small quantities.

2.2.3.2 Composition of store bought samples

Metagenomic sequencing of few US store bought Kombucha samples showed remarkable diversity in microbial composition (Figures 1-4). Predominant bacteria phylum in all 4 brands marketed as 'Raw Kombuchas' is Proteobacteria: *Gluconacetobacter spp., Acetobacter spp., Paracoccus spp., Pseudomonas spp.,* followed by Firmicutes: *Streptococcus spp., Bacillus spp.* The only known/proven probiotic organism detected was *Bacillus coagulans*. Predominant bacteria in Kombucha seems to vary by brand and by flavor within the same brand. *Oenococcus oeni* (Figure 3), a lactic acid bacterium used in malolactic fermentation, was found predominantly in one of the brands. Depending on the type of flavor/substrate *Oenococcus* spp. was preferentially selected over *Gluconacetobacter* spp (Figure 3). *Acinetobacter johnsonii*, a commensal organism part of human skin microbiota, was a predominant organism in one of the brands (Figure 4). Other members of genus Acinetobacter have been implicated in burn injuries related hospital infections and gained wider public attention during military operations in Iraq and Afghanistan. Of importance, most strains were found to be multi-drug antibiotic resistant (Davis *et al*., 2005).



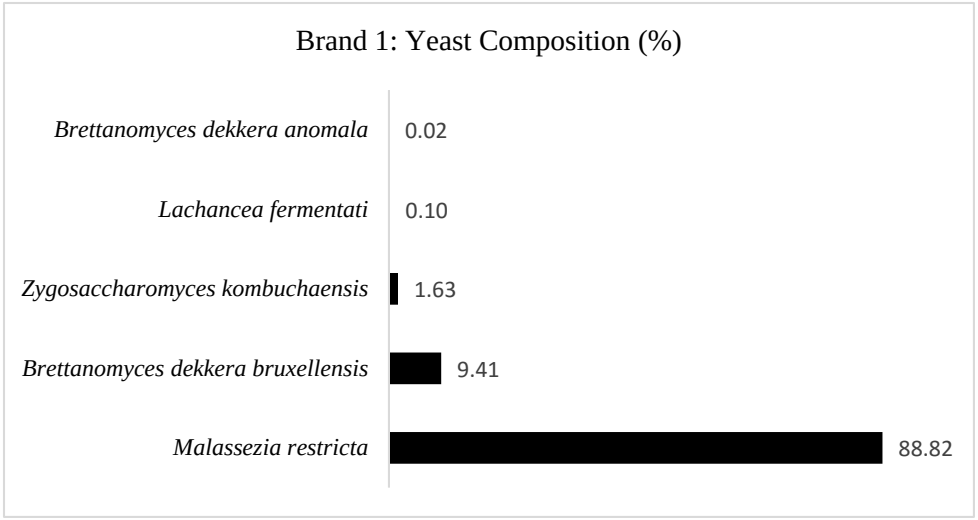


Figure 1: Relative abundances (%) of 16S bacterial and ITS fungal species in a commercial Kombucha product (Brand 1)

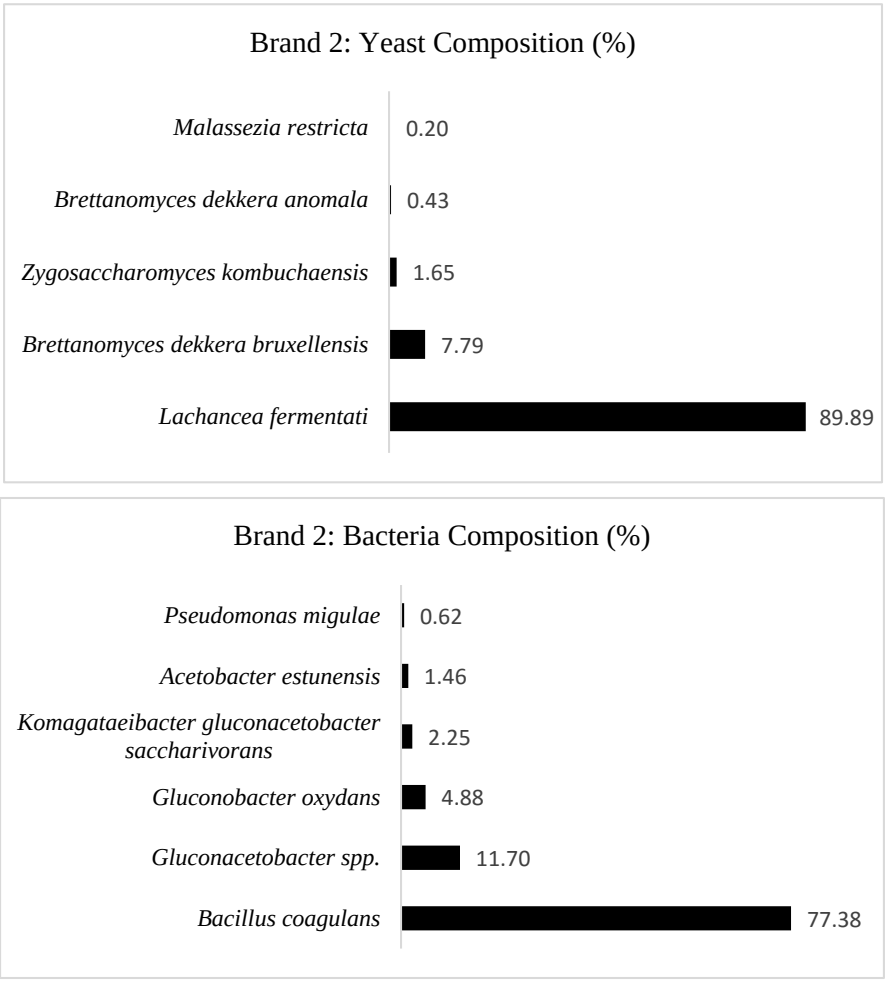
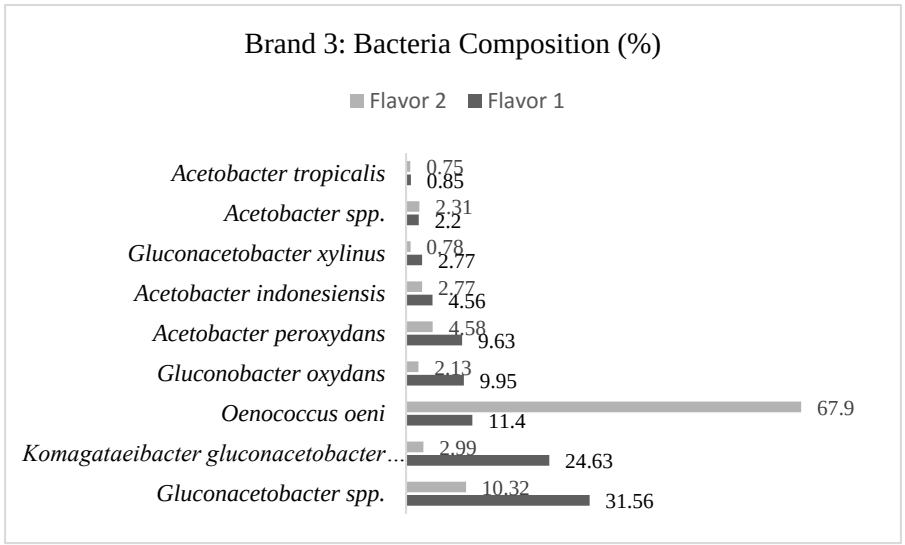


Figure 2: Relative abundances (%) of 16S bacterial and ITS fungal species in a commercial Kombucha product (Brand 2)



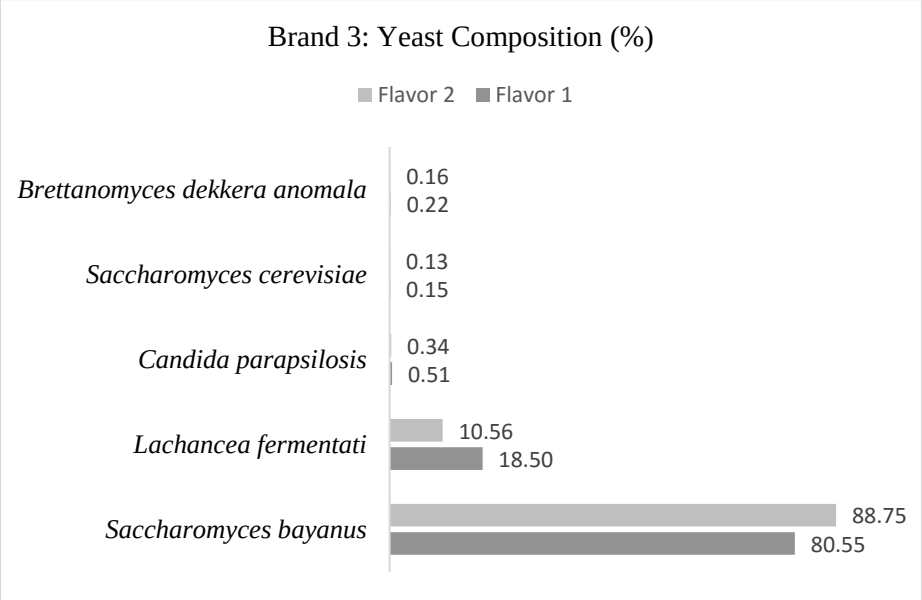
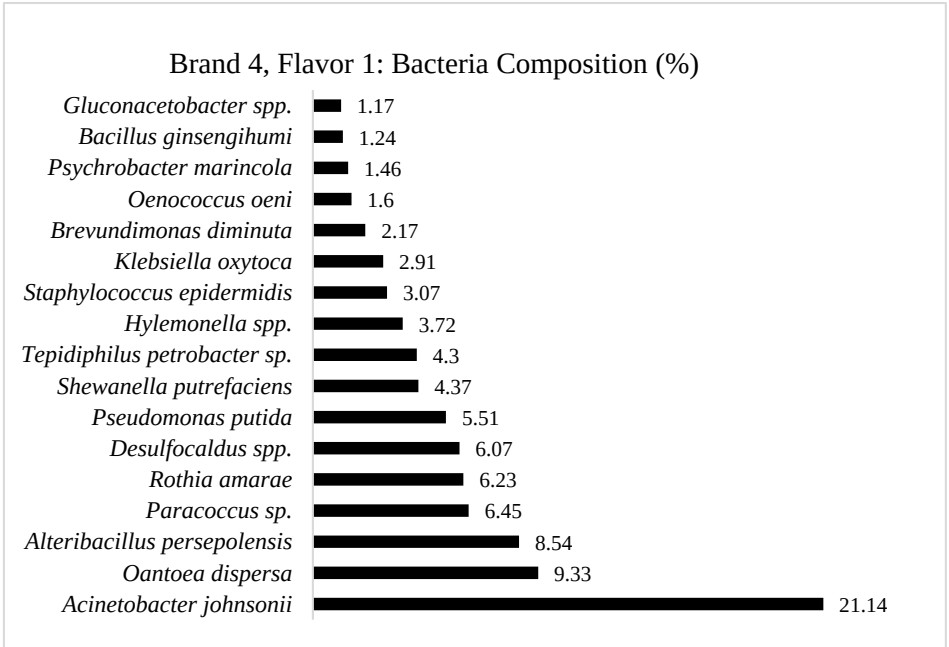


Figure 3: Relative abundances (%) of 16S bacterial and ITS fungal species in a commercial Kombucha product (Brand 3)



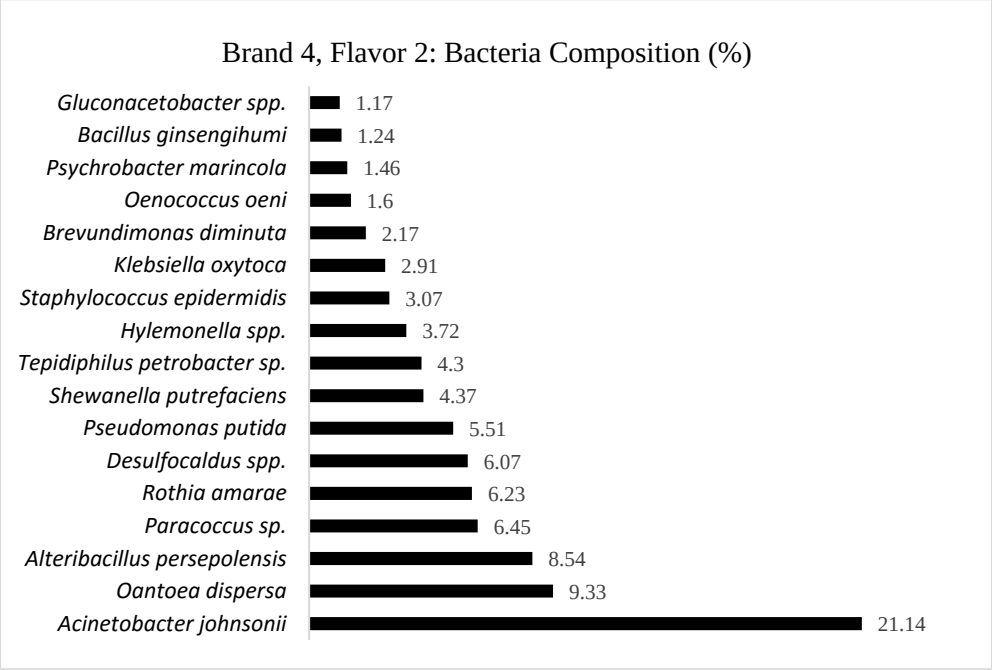


Figure 4: Relative abundances (%) of 16S bacterial species in commercial Kombucha product (2 different flavors)

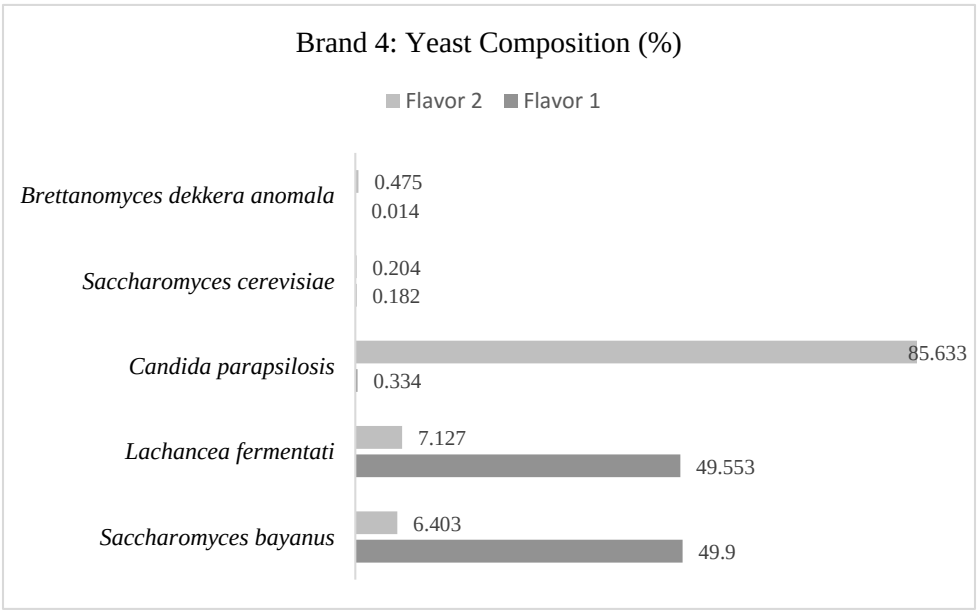


Figure 5: Relative abundances (%) of ITS fungal species in commercial Kombucha product

Lachancea fermentati was the most common yeast followed by *Saccharomyces spp., Candida spp., and Brettanomyces spp.* Predominant fungus in one of the brands was *Malassezia restricta* (~90%) (Figure 1), a commensal yeast that is an opportunistic pathogen and was previously implicated in variety of atopic dermatitis/skin infections including hypo/hyperpigmentation and dandruff in humans (Gupta *et al.*, 2004). *Candida parapsilosis*, another human commensal and an emerging pathogen, implicated in several sepsis, wound infections and a leading cause of invasive candidal disease (Trofa *et al*., 2008) was found in high abundance (~85%) in one of the Kombuchas (Figure 5). Fermentation using mixed culture ecosystems is not new to mankind. However, since every ecosystem is different, it is important to understand all key variables that affect the composition and balance of bacteria and yeasts. Most times, metagenomics sequencing generates more questions than answers. The variety of unknown bacteria and yeast types is remarkably high in few samples. While there is a clear dominant bacteria and yeast type in most samples, the role and safety of few organisms discussed in this section is unknown. Malbasa et al., (2011) successfully demonstrated use of standardized Kombucha bacteria and yeast cultures. This standardized approach can offer immense control on quality and safety.

2.2.4 Chemical composition

Kombucha is a complex functional beverage with varied composition based on type of ingredients. Classic kombucha is made with black or green tea, sugar and cultures. Tea compounds namely catechins, theaflavins, thearubigins, caffeine, L-theanine; sugar and yeast metabolites namely fructose, glucose, ethanol and bacterial metabolites like acetic, gluconic, glucuronic, citric, L-lactic, malic, tartaric, oxalic, succinic, pyruvic and usnic acids, vitamins B1, B2, B6, B12 and C were reported to be present in Kombucha. Several proteins, amino acids and minerals are also present in kombucha (Jayabalan *et al*, 2016, Greenwalt *et al*, 2000).

2.2.4.1 Composition of store bought samples

Composition of starter cultures can affect the type of metabolites produced in Kombuchas (Table 4). Gluconic, glucuronic acids and residual sucrose were not found in Kombuchas made using cultures with *Saccharomyces bayanus* as a dominant yeast. Also, the concentration of acetic acid was thrice as much in Brand 2. The concentration of metabolites was very similar between individual flavors under each brand. However, the flavor with higher % of *Oenococcus oeni* (Leuconstoc spp.,) produced higher concentration of lactic and succinic acids (Table 4).

Table 4: Sugar, Acid, Alcohol concentration in store bought samples

Concentration (mg/mL)									
	Sucrose	Glucose	Fructose	Acetic acid	Gluconic acid	Glucuronic acid	Lactic acid	Succinic acid	Ethanol % (ABV)
Brand 2/Flavor 1	3.24 ± 0.08	17.16 ± 0.29	21.87 ± 0.49	3 ± 0.09	1.1 ± 0.12	0.52 ± 0.03	0.88 ± 0.03	0.27 ± 0.005	2.26 ± 0.06
Brand 2/Flavor 2	2.33 ± 0.05	12.92 ± 0.27	18.12 ± 0.35	3.04 ± 0.04	1.1 ± 0.01	1.07 ± 0.35	0.8 ± 0.02	0.35 ± 0.005	3.1 ± 0.08
Brand 3/Flavor 1	-ND-	16.1 ± 0.01	19.7 ± 0.02	0.9 ± 0.06	-ND-	-ND-	0.66 ± 0.04	0.31± 0.03	3.19
Brand 3/Flavor 2	-ND-	12.6 ± 0.09	18.4 ± 0.06	0.7 ± 0.05	-ND-	-ND-	1.26 ± 0.09	0.81 ± 0.04	3.46

Alcohol in all Kombuchas was over 0.5% ABV, a legal limit in US for non-alcoholic beverage.

2.2.5 Fermentation: key process parameters

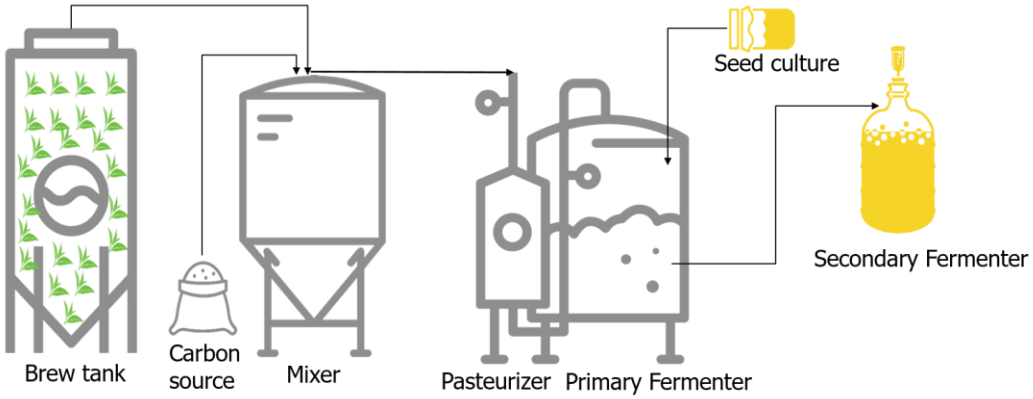


Figure 6: Process Flow Sheet

2.2.5.1 Sweetened tea base

The process of preparing Kombucha begins with selection and preparation of a tea base. Green, oolong, black are popular *Camellia sinensis* based teas that are commonly used in a tea base. Degree of chemical oxidation amongst these teas affects amounts of catechins, caffeine and polyphenols. Taste, color, aroma, antimicrobial and antioxidant properties of teas also depend on extent of oxidation. Black tea is the most oxidized tea with reddish brown color, bitter and astringent flavor, heavier mouthfeel and has least amount of catechins. Green tea is the least oxidized with lighter green color, fresh sweet and grassy flavor notes and has most amount of catechins. Oolong is semi-oxidized tea having properties of both black and green teas. Origin, growth and harvest seasons are also other important variables that affect the quality of tea and hence their selection (Liu *et al*., 2015). Factors such as total dissolved solids in water, brew time and temperature affect the extraction of tea leaves in water. Typically, Green tea, 1-2% (w/v), is brewed in reverse osmosis water between 75-90 ºC for 2-3 minutes where as black tea,1-2% (w/v), is brewed between 85-95 ºC for 3-5 minutes. Jayabalan *et al*., reported ~8 mg/mL acetic acid production with green tea but only half as much with black tea by the end of fermentation.

A carbon source for microbial fermentation could range from a simple cane sugar to much more complex sources like cane juice, maple syrup, agave nectar, honey, chicory root extract and Jerusalem artichoke extracts. Typically cane sugar at 3.5-10% (w/v) is dissolved in freshly brewed hot tea and the resulting tea base is pasteurized. Reiss 1994, reported production of different levels of ethanol and lactic acid in the presence of different sugars i.e., sucrose, glucose and fructose. Most ethanol was produced in presence of fructose and most lactic acid in presence of sucrose (Reiss 1994). Also, when Jerusalem artichoke extract rich in inulin was used as a carbon source, intense metabolism was observed along with production of a hydrolysis product fructo-oligosaccharide (another prebiotic fiber).

It is important to pasteurize sweetened tea base (> 90 °C for 5 minutes) to remove any natural occurring microflora, reduce competition for growth of starter cultures and prevent any spoilage issues. This will also help protect the composition of starter cultures.

Type and amount of tea compounds, complexity of sugars will affect the rate of microbial fermentation, amount and type of metabolite production, bioactive content and overall flavor profile. Key process parameters: Type of tea and carbohydrate, % tea, % carbohydrate, brewing and pasteurization time and temperature

2.2.5.2 Inoculation

Second step in the process is inoculating the tea base with Kombucha starter cultures. As discussed in an earlier section, unstandardized mixed cultures of bacteria and yeast are current used to ferment the tea base. Typically, liquid inoculum at 7-15%(v/v) and solid inoculum at 3-6%(w/v) is added to the tea base below 30 ºC.

Malbasa *et al*. (2011) reported higher amounts of total acids, antioxidant activity and vitamin c content with black tea when standardized mixture of acetic acid bacteria and *Zygosaccharomyces*

was used as inocula. When unstandardized mixed cultures were used, green tea produced higher acids and had a positive correlation with other metabolite production. Loncar *et al*. (2006) compared the difference in inoculum levels (10, 15%) on production of ethanol, change in pH and acid levels at 7 different time points. Higher inoculum yielded higher metabolites at all-time points but the differences in sensory properties was not reported. Due to the nature of unstandardized mixed culture fermentation, age of inoculum along with titratable acidity is used in manufacturing facilities.

Chun and Liu (2000), had reported significantly higher bacteria and yeast content in liquid over solid inoculum. Due to handling and storage issues with SCOBY, some manufacturers are avoiding use of SCOBY during fermentation. No studies have compared the differences in kombucha quality with and without the use of SCOBY.

Key Process Parameters: The amount, age, presence or absence of liquid and solid inoculum will affect the rate of fermentation, amount of metabolic products and flavor profile.

2.2.5.3 Fermentation

Last step of the process is fermentation. A temperature range of 22-28 ºC and 7-30 days of fermentation is typically used based on desired characteristics. The time and temperature of fermentation are important parameters that affect the flavor profile and amount of metabolites. In a study by Loncar *et al*, higher fermentation temperatures yielded highest amount of organic acids and vitamin C.

The dimensions on fermentation vessel especially the amount of headspace, surface area to volume ratio, also known as specific interfacial area (SIA), affects the amount of dissolved oxygen in Kombucha. This in turn influences the growth rates of yeast and bacteria (Crum et al., 2016). Cvetkovic *et al*., (2008) used 6 different fermentation vessels with varying SIAs. The Kombucha

in vessel with SIA value 0.036 reached pH 3.0 after 14 days of fermentation compared to 6-7 days for vessel with SIA value 0.06. These variations indicate SIA to be a key process parameter that needs to be controlled to maintain consistent quality.

Key Process Parameters: Incubation – time and temperature, specific interfacial area and head-space, secondary (2') fermentation in final package.

2.2.5.4 Secondary fermentation

After the completion of primary fermentation process, the Kombucha can be allowed to age in bottles, kegs or chilled brite tanks for 12-48 hours to develop natural carbonation. The anaerobic process preferentially allows growth of yeast over bacteria resulting in formation of CO_2 and more alcohol.

2.2.5.5 Mechanism

The science of Kombucha fermentation is unclear. Given the complex microbial ecosystem in the starter culture, different types of substrates and fermentation conditions, there are a number of obstacles in understanding the interactions between different bacteria and yeast in Kombucha.

Figure 7 depicts an oversimplified three step mechanism of fermentation:

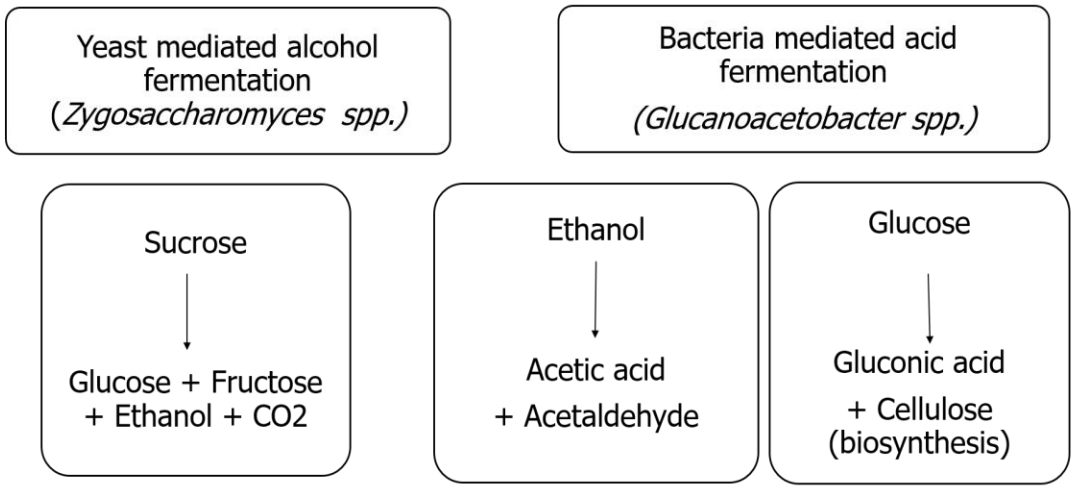
i) Anaerobic conversion of sucrose to glucose, fructose and ethanol by yeast fermentation. Sucrose in sweetened tea is metabolized and converted to ethanol by a variety of yeasts. Several studies reported different dominant yeast genera in Kombucha namely *Bretannomyces, Dekkera, Candida, Pichia, Saccharomyces* and *Zygosaccharomyces* species (Steels *et al.*, 2002).

ii) Aerobic conversion of ethanol to acetaldehyde and subsequent dehydrogenation to acetic acid by acetic acid bacteria gives this beverage a characteristic apple cider vinegar like sweet and tart flavor profile,

iii) Aerobic conversion of glucose to gluconic acid and cellulose. Last two steps are mediated by *Gluconacetobacter* species. Carbonation makes this beverage light and refreshing giving it an advantage over other fermented beverages like yogurt or kefir that have a heavier mouthfeel.

iv) The resulting Kombucha tastes mildly sweet and acidic, like an apple cider vinegar, with a titratable acidity typically not less than 0.1% (v/v) acetic acid equivalence, and alcohol can be greater than 0.5% (v/v).

Figure 7: Simplified mechanism of fermentation



v) Process

of fermentation also produce health promoting bioactive compounds known as postbiotics. Few examples include polyphenols, organic acids and vitamins Depending on the microbial composition, variety of acids such as gluconic, 2-keto-gluconic acid (sometimes misidentified as glucuronic acid), glucuronic, lactic, succinic and malic are produced, imparting new flavor subtleties (Jayabalan *et al.*, 2014).

2.2.6 In-Process Quality Control

It is important to standardize analytical parameters and measurement techniques to control and monitor quality of Kombucha.

Fermentation end point is typically determined using pH and taste. However, due to the buffering capacity of Kombucha, pH does not change significantly with increase in concentration of acids, making it an unreliable method to determine the extent of fermentation. Hence, titratable acidity is more preferred over pH measurements (Cvetkovic *et al*., 2008).

Residual sugars and alcohol are often measured using indirect methods such as refractometer or hydrometer. Not only are these methods indirect, they are also prone to matrix interference, making them highly unreliable and inaccurate. To accurately measure different types of sugars, acids and alcohol, it is important to use an HPLC type chromatographic technique. The method highlighted in Section 4.1.1 is validated and sensitive enough to accurately measure and determine any meaningful changes in metabolite concentration in Kombucha.

Kombucha taste as a release criterion is not only qualitative but it can be very subjective and unreliable. It is important to at least create or adopt a semi quantitative method to reliably measure taste.

3 RESEARCH PLAN

3.1 Rationale

Kombucha industry is facing regulatory compliance issues, class action law suits and product recalls due to incorrectly labeled sugar and alcohol content (Elaine Watson 2018, Elizabeth Crawford 2017, Kim Severson 2010, James Hamblin 2016). This could be a combination of lack of proper in-process quality control processes and/or understanding of factors that affect the mechanism of Kombucha fermentation.

Preliminary data and previous studies, as indicated in earlier sections, have shown inconsistencies in composition of inocula and metabolites. Store bought samples showed certain bacteria and yeast that could be categorized as opportunistic pathogens. Postbiotic type bioactive compounds, alcohol content and the flavor profile seem to vary significantly between flavors and batches.

Complex microbial ecosystem in Kombucha makes it difficult to detail a clear mechanism of fermentation, requiring a need to confirm key process parameters and study their effect on microbial activity.

3.2 Hypothesis

Understanding the cause and effect relationship between key process parameters and microbial activity is necessary to design processes that achieve better quality control.

Quantitative analysis of metabolites and bioactives along with a semi-quantitative analysis of flavor profile will offer insights into quality and fermentation mechanisms.

3.3 Objective

To understand the relationship between key process parameters and microbial activity to help design processes that could achieve better quality control

i. Tea Type
ii. Age of inoculum
iii. Solid inoculum - presence or absence
iv. Specific interfacial area

3.4 Materials and methods

3.4.1 Kombucha fermentation

Several teas (*Camellia sinensis* spp.) were screened for use in Kombucha. Premium quality Japanese Sencha green tea from Sugimoto, Japan and a black tea blend of different origins from Harris Tea company, NJ, USA were used. A 50:50 blend of these black and green teas was also made. 1% (w/v) infusions of loose tea leaves was made using 90 °C water, steeping green tea for 2 minutes and black tea for 5 minutes, tea leaves were removed and 7.5% (w/v) store bought granulated cane sugar was dissolved in the infusion. 0.72 L of this mixture was transferred to clean, sanitized 0.946 L mason jars in replicates and these jars were pasteurized at 90 °C for 5 minutes in a hot water bath.

Several commercial Kombucha starter cultures were screened for quality and cultures produced by Kombucha Brooklyn (KB) was selected. The KB cultures have produced a Kombucha with a better flavor profile. Sequencing analysis showed that *Lachancea fermentati* and *Brettanomyces dekkera bruxellenis* were dominant yeast species and *Gluconacetobacter* sp., were dominant bac-

teria species similar to previous findings (Marsh *et al*., 2014, Jayabalan *et al*., 2014). Fresh inoculum was produced, pH and titratable acidity were confirmed prior to inoculation. Sweetened tea base was inoculated with 11.11% (w/v) of liquid Kombucha and 4.5% (w/v) solid SCOBY inoculum from a 10- or 15-day old ferment in same type of tea. Mason jars were covered with disposable filter papers and allowed to ferment in a temperature controlled water bath at 26 ± 1°C.

All Kombuchas were sampled into sterile falcon tubes. For each experiment, multiple samples were retained at time 0 as control and end of fermentation and frozen at -20 ºC until analysis. Depending on the type of analysis, some of the samples were centrifuged at 10,000 rpm, supernatant filtered through Whatman filter paper #1, 0.45 or 0.22 µm.

3.4.2 Experimental design

1. Effect of type of tea on quality of Kombucha

   Green and black tea Kombuchas were prepared in quadruplicates and 50:50 green and black tea Kombucha was prepared in replicates per directions in Section 3.4.1. Another set of black tea Kombucha samples spiked with green tea catechins (0.05-0.15 mg/mL EGC and EGCG) were prepared in replicates. All these samples were fermented for 9 days.

2. Effect of age of inoculum

   Green tea Kombucha and 50:50 black and green tea Kombucha were prepared in replicates per directions in Section 3.4.1. Blended tea was also used to address any bias. SCOBY/solid and liquid broth from Kombuchas fermented for 10 and 15 days were used as inocula in this experiment. All these samples were fermented for 9 days.

3. Presence or absence of solid inoculum

50:50 black and green tea Kombucha was prepared in replicates per directions in Section 3.4.1. Blended tea was used to avoid any bias. Along with liquid inoculum, one set used 4.5% (w/v) SCOBY/solid inoculum and the other set only used liquid inoculum.

4. Specific interfacial area

   50:50 black and green tea Kombucha was prepared in replicates per directions in Section 3.4.1. Blended tea was used to avoid any bias. Kombucha batches were setup in fermentation vessels of different sizes to obtain a 0.09-0.16 range of surface area to volume of liquid ratios.

3.4.3 Physico-chemical analysis

3.4.3.1 pH and Titratable Acidity

pH of the samples was measured using an Orion 420A pH meter. Titratable acidity was measured by titrating a 20ml aliquot of kombucha against a 0.1M solution of sodium hydroxide (NaOH) to an end point of pH 7 determined using the Orion pH meter. These readings were taken for Day 0 and at end of fermentation.

Titratable acid as acetic acid equivalent (g/100ml) is calculated according to the formula= $(V_{titr} \times C_{titr} \times 60.05 \times 100)/(V_{smp} \times 1000)$

Where: V_{titr} = Volume of titrant used (ml), C_{titr} =Concentration (N) of titrant used, 60.05 = Molecular weight of acetic acid, V_{smp} = Volume of sample used (ml)

3.4.3.2 Organic Acids, Sugars and Ethanol

Modified Chaluvadi *et al. 2012*, was used to separate and quantify these analytes from Kombucha. Sucrose, glucose, fructose, gluconic acid, glucuronic acid, acetic acid, lactic acid and

ethanol were measured using an HPLC-UV-RI system. 1 mg/mL mixed stock solutions of the analytes were prepared in HPLC grade water using analytical grade chemicals from Sigma Aldrich (St. Louis, MO), standards in the concentration range of 0.01-0.8 mg/mL were prepared and standard curves were plotted. Aliquots of Kombucha samples were filtered through 0.22 µm PVDF Durapore Millex-GV Millipore (Burlington, MA) membranes into autosampler vials. Isocratic elution of analytes was performed using 5 mM H_2SO_4 mobile phase with a flow rate of 0.6 ml/min in a Hewlett Packard 1100 HPLC system equipped with Aminex HPX 87H column at 35 ^oC. Detection was performed using both Thermo Scientific Dionex UltiMate 3000 Series VWD-3400RS variable wavelength (UV) and Erma Optical Works ERC-7510 refractive index (RI) detectors arranged in series. Dionex 'Chromeleon' software (version 6.8) was used to quantify the compounds present in samples with reference to standard curves. Due to the co-elution of gluconic acid and glucose, the use of the 2 detectors in series was necessary. Glucose shows a very low to no response on UV detector. Hence, the following formula was used to quantify glucose:

Concentration of glucose (mg/ml) =

$$\frac{\text{Area mixed peak (RI)}}{\text{Response Factor of (glucose + gluconic acid) (RI)}} - \frac{\text{Area gluconic acid (UV)}}{\text{Response Factor gluconic acid (UV)}}$$

Where 'Response Factor' is the slope of the relevant calibration curve or peak area/concentration.

3.4.3.3 Catechin and Caffeine Analysis

Modified Li *et al*. 2012, was used to analyze these compounds. Catechin derivatives Epicatechin (EC), Epigallocatechin (EGC), Epicatechin Gallate (ECG), Epigallocatechin Gallate (ECGC) and caffeine were measured using an HPLC-UV method. 0.3 mg/mL working mixed stock solutions of the analytes were prepared in HPLC grade 20% methanol solution using analytical grade chemicals from Sigma Aldrich (St. Louis, MO), standards in the concentration range of 0.01-0.15

mg/mL were prepared and standard curves were plotted. Aliquots of Kombucha samples were filtered through 0.22 µm PVDF Durapore Millex-GV Millipore (Burlington, MA) membranes into auto-sampler vials. Isocratic elution of analytes was performed using the same Hewlett Packard 1100 system used for organic acids, sugars and ethanol except for the YMC PackPro 250 mm x 4.6 mm ID, 5 µm particle size column operated at ambient temperature, 0.8 mL/min mixed mobile phase of 75% trifluoro acetic acid (TFA) (0.1%) and 25% methanol and detected with UV at 210, 265 and 280 nm as well as RI. The system was equipped with ERC-7510 refractive index detector, Dionex UltiMate 3000 variable wavelength detector and data analysis performed using Chromeleon 6.8 software.

Dihydroxylated/trihydroxylated catechins [(EC+ECG)/(EGC+EGCG+GCG)], a bitterness indicator, was also calculated to understand the quality of catechins in tea/kombucha (Liu *et al*., 2015). Higher the ratio better is the quality of green tea. This ratio may not be used to compare qualities of two different teas.

3.4.3.4 Total polyphenols

Total polyphenolic content was measured according to the method described by Singleton and Rossi (1965) as modified by ISO 14502 whereby 1ml of sample was reacted with 5ml of 10% v/v Folin-Ciocalteu reagent for 3-8 minutes, after which 4ml of 7.5% w/v sodium carbonate solution was added. The mixture was then incubated at room temperature for 1 hour, after which time the absorbance was measured at 765nm using a Shimadzu UV-1601 spectrophotometer. Total polyphenolic compounds were quantified as gallic acid equivalents using a calibration curve created with standard solutions of gallic acid.

The total polyphenolic content was also measured according to the method proposed by Medina (2011) based on the direct binding of gallic acid with Fast Blue BB diazonium salt which is

thought to be a more sensitive method than those using Folin-Ciocalteu reagent and subject to less interference from other compounds.

3.4.3.5 Theaflavin, Thearubigin and Highly polymerized substances

Theaflavins (TF), thearubigins (TR) and highly polymerized substances (HPS) were measured according to the method proposed by Takeo and Oosawa (1976) as modified by UPASI Tea Research Foundation. The flow diagram in Figure 8 depicts the extraction process used to produce fractions A, B, C, D and E the absorbance of which were measured at 380nm. TF was quantified using the equation proposed by Roberts and Smith (1963) which was modified according to the suggestions of Spiro and Siddique (1981) which makes use of the molar extinction coefficient of the theaflavins rather than the gram concentration extinction coefficient:

$$\text{TF (mol.dm}^{-3}) = \frac{6.25 \times Abs_C}{l \times \varepsilon}$$

Where 6.25 is a dilution factor, Abs_C is the absorbance of fraction C at 380nm, l is the path length in cm (1 cm) and ε is the mean molar extinction coefficient for theaflavins in ethanol (9180 $L.mol^{-1}.cm^{-1}$ at 378nm).

TR was quantified using the gram concentration extinction coefficient as used by Roberts and Smith (1963) as no molar extinction coefficient is available for the thearubigins which are a complex mixture of some 10,000 compounds (Kuhnert 2010). The equation was modified to give TR% (m/v) of kombucha tea rather than a percentage m/v of the dry tea leaves used in the original infusion:

TR% (m/v in kombucha tea) = $10(Abs_B + Abs_D - Abs_C) \times \frac{0.02}{0.733}$

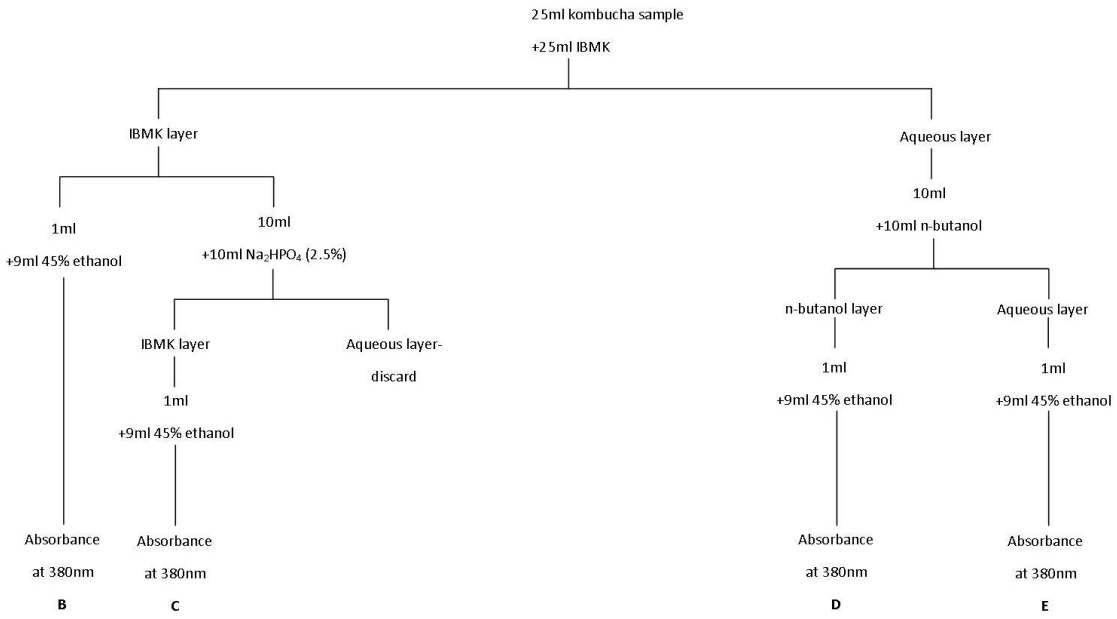


Figure 8: Flow diagram for tea fraction separation

Where 10 is a dilution factor, Abs_X is the absorbance of fraction X at 380nm, 0.02 is the percentage at which the gram extinction coefficient was measured and 0.733 is the gram extinction coefficient.

Similarly, HPS was quantified as TR using the gram concentration extinction coefficient as used by Roberts and Smith (1963) modified to give HPS% (m/v) of kombucha tea:

HPS% (m/v in kombucha tea) = $10 \times Abs_E \times \frac{0.02}{0.733}$

3.4.3.6 Vitamins

Vitamins B1, B2, B6, B12 and C were analyzed using a Hewlett Packard 1100 HPLC system with an Alltech Econosphere C18 column, length 150mm, internal diameter 4.6mm and particle size 3µm. The elution method was a modification of that reported by Heudi *et al*. (2005) using a

gradient elution with two solvents; solvent A being 0.025% TFA in HPLC grade water and solvent B methanol. Detection was performed using a Dionex UltiMate 3000 ultra violet (UV) variable wavelength detector. Dionex 'Chromeleon' software (version 6.8) was used to quantify the compounds present in samples with reference to standard curves prepared using analytical grade standard compounds obtained from Sigma Aldrich (St Louis, MO).

3.4.3.7 Total Protein

Protein content of the kombucha was measured using the method of Bradford (1976) using bovine serum albumin (BSA) as a standard. The standard protocol was used whereby 100µl of sample was transferred to a test tube then 5ml of 20% (v/v) Bradford's reagent was added and the mixture incubated at room temperature for a minimum of 5 minutes. The absorbance was then measured at 595nm using a Shimadzu UV-1601 spectrophotometer.

3.4.4 Microbial activity and composition

The total sugar to acid ratio, i.e., the sum of sucrose, glucose and fructose to the sum of acetic acid and gluconic acid concentration, was calculated to understand the rate of fermentation. The higher the ratio, the lower is the rate of fermentation. Also, higher the amount of residual sucrose in Kombucha at the end of fermentation, lower is the rate of fermentation.

Changes in total aerobic bacterial plate counts (Maturin *et al*., 2001) and yeast counts (Ryu *et al.*, 2013) were determined (Certified Laboratories, NY). A composite of replicate Kombucha samples was made for diversity sequencing assays of yeast and bacteria based on 16s rRNA V4-V5 and ITS-1 rDNA ribosomal amplicons respectively (Illumina platform, Mr DNA Lab, Shallowater, TX).

3.4.5 Sensory Analysis

The sensory analysis of the obtained Kombucha samples was performed by a panel of tasters. The level of each sensory attribute (sweet, sour, yeasty) was described in a manner similar to a modified 3-point ‘just about right’ scale from a previous lemonade study (Vickers 1988). Along with a descriptive analysis, a semi-quantitative overall acceptance score was determined by comparing sugar/acid (S/A) ratios from an experiment to a pre-calibrated chart composed of S/As and scores based on a 9-point hedonic scale. Twenty panelists, 5 trained and 15 untrained, were used to create this calibration chart (See Appendix section).

4 RESULTS AND DISCUSSION

4.1 Metabolite profile

4.1.1 Sugars, Acids, Alcohol

While the concentrations were different, the profile of sugars, acids in Kombuchas' produced under various process conditions were the same. Acetic and gluconic acids were the only organic acids identified across various conditions. This was consistent with at least one other previous study (Chen *et al.,* 2000). Other studies have indicated presence of few other organic acids like citric, glucuronic, lactic, malic, malonic, oxalic, pyruvic, and tartaric acids. The differences in acid profile was attributed to differences in starter culture composition and freshness of tea (Jayabalan *et al.*, 2014, 2016). An HPLC method using CarboPac PA10 column did not detect other organic acids, more specifically glucuronic acid (Wang *et al.*, 2012). As expected, the amount of metabolites i.e., glucose, fructose, acetic acid, gluconic acid and ethanol increased with fermentation time. A dramatic variation in the amount of each of these metabolites was observed by varying key process parameters such as tea type, age of inoculum, presence or absence of solid inoculum (SCOBY) and specific interfacial areas (Table 4).

Table 5: Key metabolite final concentration range by varying different key process parameters

Residual Sugars, Acids and Alcohol (mg/mL)					
Sucrose	Glucose	Fructose	Acetic acid	Gluconic acid	Ethanol
25.62 ± 0.83 to 73.95 ± 1.4	2.35 ± 0.07 to 22.17 ± 0.64	0.79 ± 0.17 to 20.5 ± 0.34	1.8 ± 0.1 to 3.62 ± 0.35	0.03 ± 0.003 to 3.46 ± 0.17	2.2 ± 0.17 to 5.6 ± 0.1
Total sugars/acid concentration range: 6.63 ± 0.7 to 28.15 ± 0.8					

4.1.2 Vitamins

No vitamins were detected in any of the Kombuchas. Earlier studies have shown increasing concentrations of Vitamin C and antioxidant activity in Kombucha with fermentation time (Chu and Chen *et al.*, 2006, Loncar *et al*., 2006). Loncar et al., used Boehringer Mannheim enzymatic method for L-ascorbic acid/Vitamin C detection and reported very low (2.5 µg/mL) to no vitamin C at 20 °C incubation temperature and 10-27.5 µg/mL between 30-35°C. Possible explanations for no Vitamin C detection in our study: i) Vitamin C concentration corresponding to incubation temperature and time (26 ± 1 °C, 9-15 days) from this study, falls on the lower end of the HPLC calibration range (6.25-500 µg/mL). ii) Incubation temperature range did not select for the same organisms as in Loncar *et al* study, so no vitamins were produced. Although, temperature range of 30-35°C could yield a harsh tasting, acidic Kombucha.

Bauer-Petrovska et al. (2000) used thin layer chromatography for detection of Vitamin B1, B6, B12 and C and reported a concentration range of 0.5-1.5 mg/mL. The fermentation time and temperature fall within normal ranges used in most studies. It is however, very surprising to see such high concentrations of vitamins. Based on these results, a serving (8.4 fl oz) of Kombucha would have ~350 mg of vitamin C, which is 6-7 times as much in 1 orange fruit.

4.2 Effect of tea type on chemical, microbial and sensory profile

Irrespective of the tea type, the initial/Day 0 pH and titratable acidity of Kombucha was 3.5 ± 0.06 and 0.19 % ± 0.02, respectively. Towards the end of fermentation, i.e. 9 days at 26 ºC, Kombuchas' made with black tea (BTK) and 50:50 green and black blended tea (BGTK) reduced to same pH of 2.95 and titratable acidity of 0.42%. In the same duration, Kombucha made with green tea (GTK) dropped only to pH 3.11 and 0.28% titratable acidity (Table 6).

Table 6: Tea Type - pH and Titratable Acidity

	GTK Day 9	BTK Day 9	BGTK Day 9
pH	3.11 ± 0.06	2.98 ± 0.03	2.95 ± 0.04
Titratable Acidity (acetic acid equivalent) g/100mL	0.28 ± 0.04	0.42 ± 0.02	0.42 ± 0.03

Since the type of acids produced between all three substrates remain unchanged, the increased titratable acidity in presence of black tea was primarily attributed to a higher microbial activity. After 9 days, GTK showed glucose and fructose concentration ~10 mg/mL, which is a 25-100% increase from day 0 (Figure 9). With at least 50% higher residual sugar than in BTK, green tea fermentation was at a much slower rate as also indicated by a higher S/A ratio (Table 7).

Table 7: Tea type - Flavor profile

	GTK	BTK	BGTK
Description	Fizzy, sweet, sour, green apple like flavor, no bitterness, some astringency, very complex	Not nearly sweet enough, much too sour, harsher acid, much too yeasty, slightly bitter after taste	Sweet, much too sour, slight bitter after taste, green apple like flavor
Overall acceptance % (9-point hedonic scale)	63.8 ± 13	42.8 ± 3.98	55.5 ± 1.82
Sugars/Acid ratio	14.42 ± 3.23	9.96 ± 1.02	12.61 ± 0.38
Sucrose (mg/mL)	54.70 ± 4.53	29.66 ± 3.08	25.63 ± 0.82

Both BTK, BGTK produced twice as much glucose and fructose and significantly higher gluconic acid than GTK. As expected from this data, BTK tasted not as sweet, and also had a sour and yeasty flavor whereas BGTK had a well-balanced sweet and sour taste with a slight bitter aftertaste compared to GTK. Due to a higher S/A ratio, GTK also had a higher overall sensory acceptance score range of 50-77%, followed by BGTK with 53-57% versus a low 39-47% with BTK (Table 7).

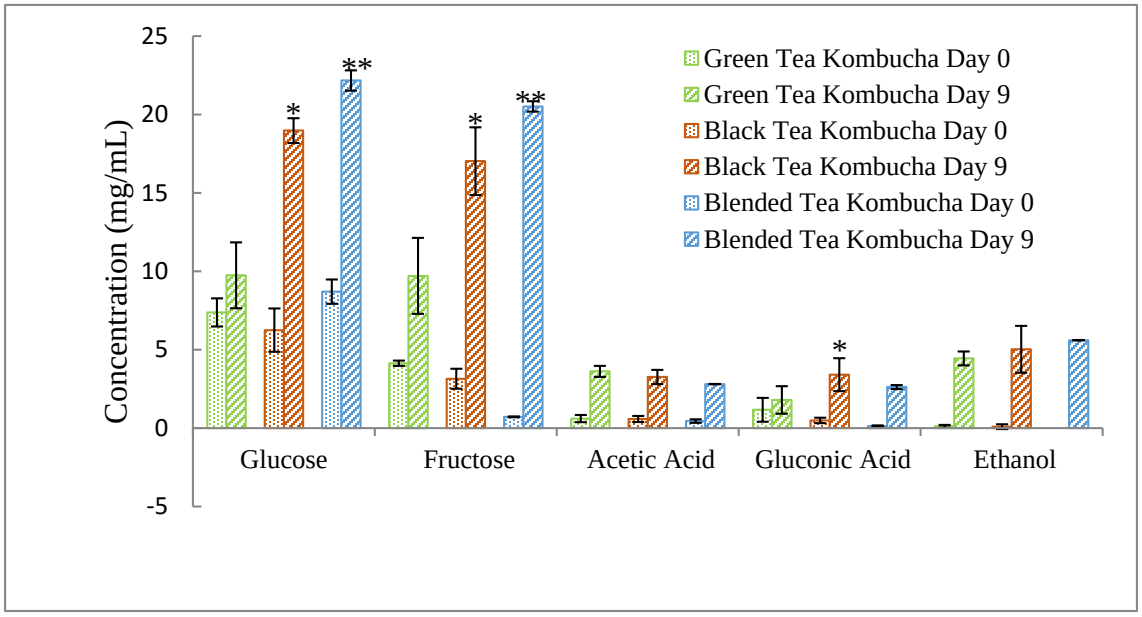


Figure 9: Tea Type - Sugars, Acids and Alcohol Concentration (mg/mL)

'*' $p \leq 0.05$ between day 9 green and black tea Kombuchas, '**' $p \leq 0.05$ between day 9 black and blend tea Kombuchas

Battikh *et al* (2013) reported higher antimicrobial activity of GTK over BTK against a range of pathogenic bacteria and yeast including *Candida* species. They predicted this activity was a function of higher organic acids and tea derived phenolic compounds in GTK. In our study, BTK had same amount of acetic acid and slightly higher gluconic acid compared to GTK. Therefore, the reduced rate of fermentation in GTK is most likely influenced by the tea compounds.

Almajano *et al* (2008), reported antimicrobial activity of green tea catechins, EGC and EGCG, against select gram positive and negative bacteria, and few yeast species when their combined concentration exceeded 14 mg/g of green tea leaf. Based on 1% tea usage level, EGC and EGCG levels in GTK is normalized to 40 mg/g of tea leaf. BTK had lowest levels of these catechins and

highest rate of fermentation and by adding green to black tea we increased the antimicrobial Catechin concentration in BGTK to 15 mg/g tea leaf. BGTK showed a significantly better rate of fermentation than green tea.

When black tea was supplemented with purified EGC and EGCG compounds, we observed a concentration dependent inhibitory effect of these catechins on bacteria in BTK broth (Figure 10). However, there was no significant adverse effect on yeast counts. Also, while we observed an antibacterial effect in the liquid broth there was no clear trend on microbes in solid SCOBY (Figure 11). It is likely that SCOBY is more robust and can resist change.

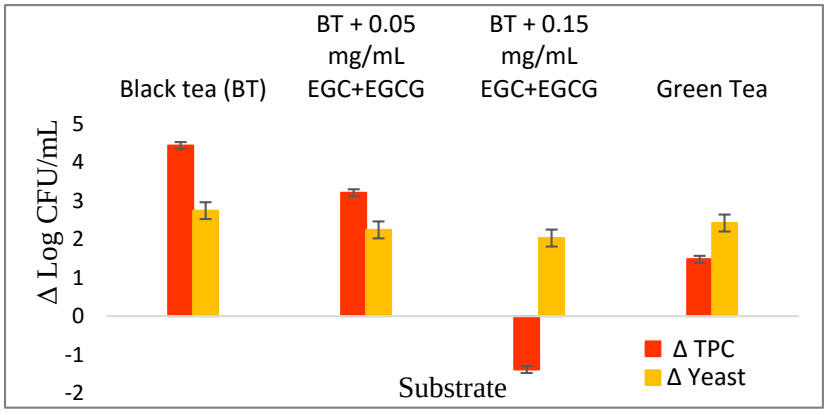


Figure 10: Effect of tea type on bacteria and yeast changes in Kombucha after 9 days of fermentation

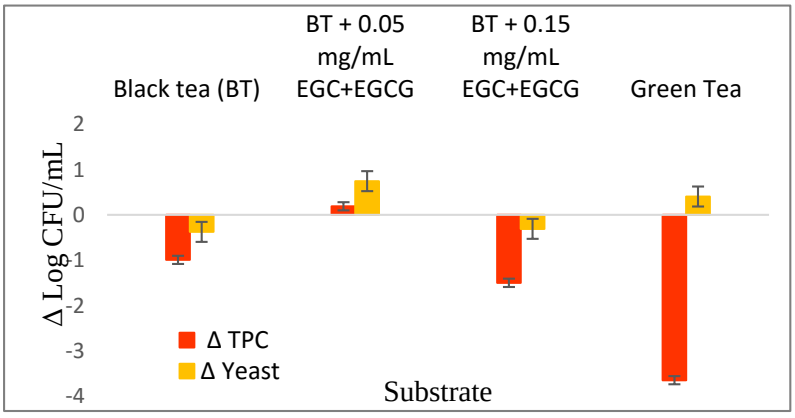


Figure 11: Effect of tea type on bacteria and yeast changes in SCOBY after 9 days of fermentation

Metagenomic sequencing data showed a different microbial composition between GTK and BTK (Table 8). This is another possible explanation for the observed differences in organic acid content and sensory profiles. Both BTK and GTK started with same SCOBY starter culture (Table 8) but after 9 days of fermentation, green tea predominantly selected for Komagataeibacter gluconacetobacter and Brettanomyces spp. while black tea selected for Gluconacetobacter spp. and Zygosaccharomyces spp. Marsh et al., 2014 also reported Gluconacetobacter spp. and Zygosaccharomyces spp.as dominant bacteria and yeast in 5 different black tea Kombucha broths fermented for 10 days using SCOBYs from different countries. Mayser et al., 1995 reported presence of Brettanomyces spp., in 22 of 34 different Kombucha samples, most of them made using black tea. One possible explanation for effect of tea type on microbial composition is that some microbes are more resistant to green tea antimicrobial polyphenols than others (Almajano *et al*., 2008). Another explanation is the prebiotic-like behavior of certain black tea polyphenols that may promote preferential growth of certain microorganisms (Banerjee *et al*., 2010). Hence, it is most likely possible that the antimicrobial and growth promoting effects are strain dependent.

GTK had a green apple like fruity flavor, which can be explained by the presence of Brettanomyces spp., a yeast known to impart a sour and fruity flavor in Belgian beers (Tonsmeire 2014). Certain Zygosaccharomyces spp., some of them currently reclassified under the genus Lachancea, like Z. bailii are known to impart a very sour and acidic taste (Gobbi *et al*., 2013, Kuanyshev *et al*., 2017). However, when controlled, Z. bailii has produced a high level of pleasant fruity aromatic esters (Garavaglia *et al*., 2015).

Table 8: Effect of tea type on relative abundances (%) of major 16S bacterial and ITS fungal species in Kombucha

Bacteria	**Inoculum (%)**	**GTK Day 9 (%)**	**BTK Day 9 (%)**
Gluconacetobacter xylinus	7.89	3.58	15.69
Gluconacetobacter spp.	71.96	3.07	49.76

Acetobacter pomorum	0.43	7.50	5.28
Komagataeibacter gluconacetobacter saccharivorans	12.28	66.53	5.65
Acetobacter tropicalis	0.92	9.62	8.58
Fungi	**Inoculum (%)**	**GTK Day 9 (%)**	**BTK Day 9 (%)**
Lachancea fermentati	90.43	1.91	7.75
Zygosaccharomyces bailii	0.97	0.47	53.66
Brettanomyces dekkera bruxellensis	7.44	58.02	27.91
Brettanomyces dekkera anomala	0.79	39.07	8.99

In both BTK and GTK, the change in catechins and total polyphenols over the fermentation period was not statistically significant (Figures 12 and 13). Jayabalan *et al*., 2007 reported dramatic shifts in Kombucha polyphenol profile, with a significant degradation of catechins in 9 days followed by an increase on 12th day. They speculated this increase in polyphenol content as a result of enzymatic degradation of polyphenols and conversion of one catechin to another. We could not confirm this in our study as the polyphenol content was quite stable during nine days of fermentation. Which means, the type of starter cultures may not only have a bearing on the acid profile but they also can affect the tea polyphenol composition.

Figure 12: Tea Type - Caffeine and Catechin Concentration

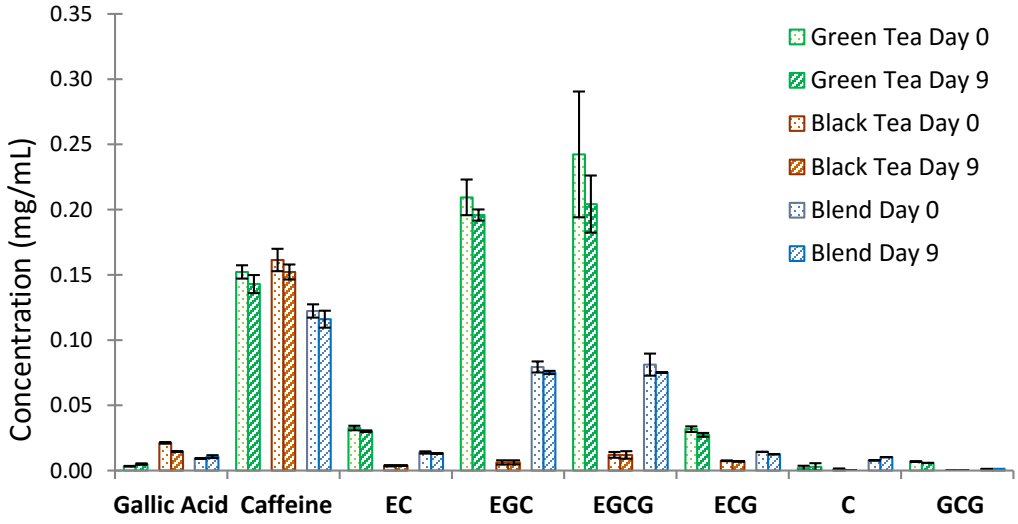


Table 9: Tea Type - Catechin Ratios

Category	GTK	BTK	BGTK
Di/Tri hydroxylated catechins ratio	0.14 ± 0.01	0.59 ± 0.048	0.17 ± 0.009
EGC+EGCG (mg/mL)	0.40 ± 0.02	0.018 ± 0.0005	0.15 ± 0.006

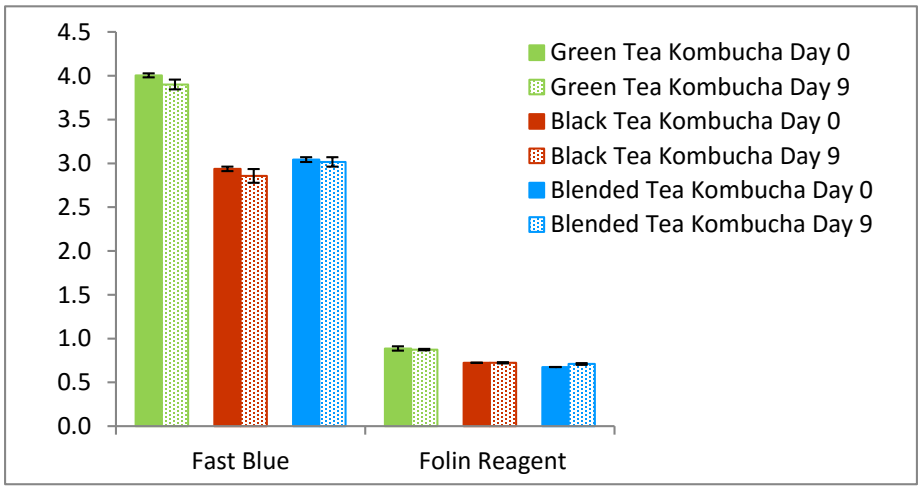


Figure 13: Tea Type - Total Polyphenols (mg/mL)

As previously reported, Fast blue BB assay captured more polyphenols (Figure 13) than indirect test using Folin reagent (Medina et al., 2011).

Table 10: Tea Type - Tea Fractions

	GTK	BTK	BGTK
Thearubigins (mg/mL) Day 0	0.01	0.19	0.17
Thearubigins (mg/mL) Day 9	0	0.26	0.06
Theaflavins (mM/mL) Day 0	0	0.005	0.021
Theaflavins (mM/mL) Day 9	0	0.029	0.04
Highly polymerized substances (mg/mL) Day 0	9.82	9	12.28
Highly polymerized substances HPS (mg/mL) Day 9	5.73	15	11.19

4.3 Effect of age of inoculum on chemical, microbial and sensory profile

Green tea Kombucha (GTK), 50:50 blended Black and Green Tea Kombucha (BGTK) made with older 15-day inoculum have shown lower pH and higher titratable acidity (Table 11). Higher concentrations of metabolites glucose, fructose, acetic and gluconic acid in Kombuchas made with

older inoculum suggests a higher microbial activity (Figure 14, Figure 15). The only exception to this trend was, likely an anomaly, a higher concentration of acetic acid in BGTK made with 10-day inoculum.

Table 11: Age of inoculum - pH and Titratable Acidity

	GTK (10 day inoculum)	**GTK (15 day inoculum)**	**BGTK (10 day inoculum)**	**BGTK (15 day inoculum)**
pH	3.11 ± 0.04	3.04 ± 0.05	3.24 ± 0.06	3.14 ± 0.03
Titratable Acidity (acetic acid equivalent) g/100mL	0.28 ± 0.04	0.64 ± 0.11	0.416 ± 0.03	0.72 ± 0.09

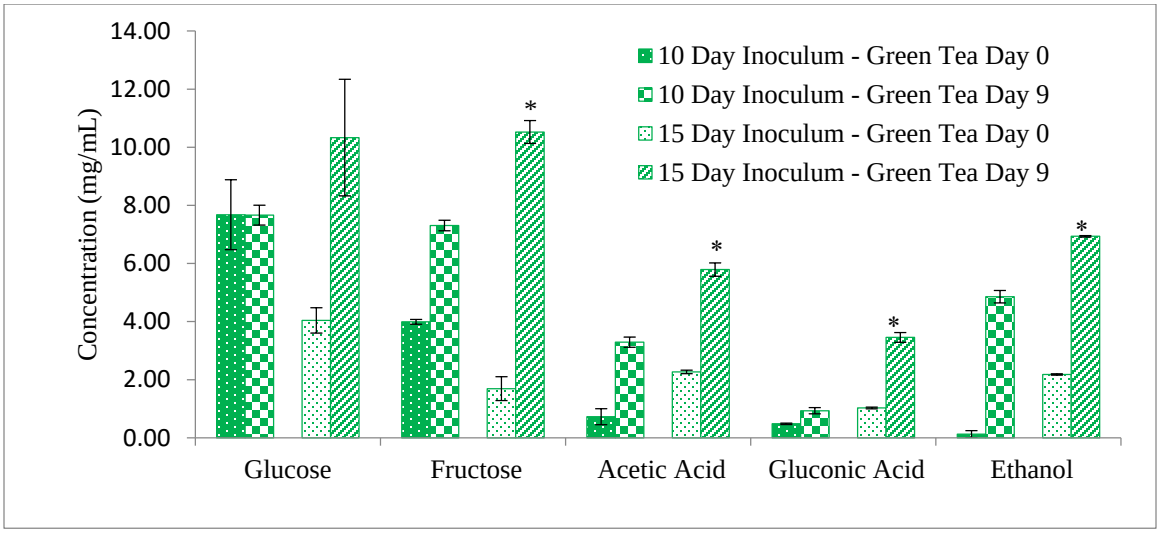


Figure 14: Age of inoculum – GTK - Sugars, Acids and Alcohol Concentration (mg/mL)

'*' $p \leq 0.05$ between 15 and 10-day inoculum fermented kombucha after 9 days

Higher microbial activity with 15-day inoculum was also confirmed by a significantly higher S/A ratio and lower residual sugar concentration. As expected, BGTK with 15-day inoculum had a lower residual sugar concentration of ~25 mg/mL compared to ~40 mg/mL in GTK (Table 12, Table 13). This can be attributed to growth promoting or prebiotic-like properties of black tea polyphenols counteracting the antimicrobial effect of green tea catechins. It is also likely for the

same reason, several commercial Kombucha producers use a blend of green and black tea in the fermentation process.

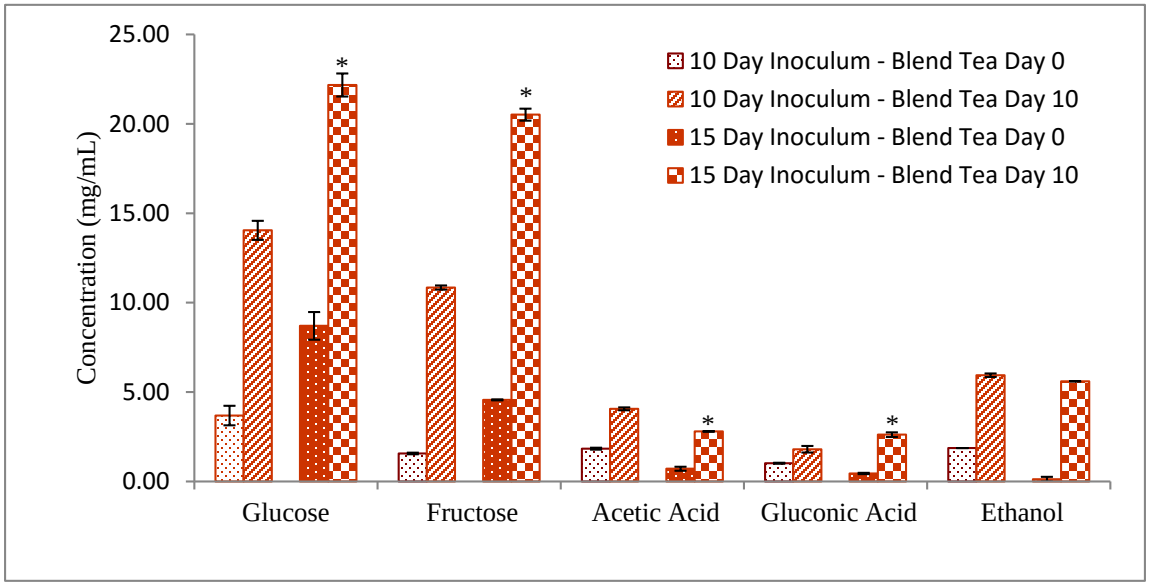


Figure 15: Age of inoculum – BGTK - Sugars, Acids, Alcohol Concentration (mg/mL)

'*' $p \leq 0.05$ between 15 and 10-day inoculum fermented kombucha after 10 days

Table 12: Age of inoculum - Flavor Profile

	GTK (10 day inoculum)	**GTK (15 day inoculum)**
Description	Sweet, Sour	Not nearly sweet enough, much too sour, much too yeasty
Overall acceptance % (9-point hedonic scale)	78.8 ± 4.13	26.8 ± 2.73
Sugar/Acid Ratio	17.5 ± 1.06	6.6 ± 0.7
Sucrose (mg/mL)	58.91 ± 0.32	40.15 ± 1.46*

	BGTK (10 day inoculum)	**BGTK (15 day inoculum)**
Description	Sweet, much too sour, slight bitter after taste, fizzy, green apple like flavor	Not sweet enough, sour, much too yeasty, no complexity
Overall acceptance % (9-point hedonic scale)	55.5 ± 1.82	50.8 ± 3.2
Sugars/Acid ratio	12.61 ± 0.38	11.64 ± 0.67
Sucrose (mg/mL)	43.17 ± 1.57	25.62 ± 0.83*

Table 13: Age of inoculum - Flavor Profile

Sreeramulu *et al* (2000) studied the growth patterns of yeasts and acetic acid bacteria in Kombucha. A rapid growth in yeast and bacteria population till 4 days followed by a rapid decline from 6 days to rest of the fermentation period with short intermittent growth periods was observed. The decline in population was attributed to acid shock and intermittent growth to acid resistance by some bacteria and yeasts. However, in our study, bacteria and yeast counts in 10 and 15-day inoculum were almost identical (Figure 16). This doesn't explain why the residual sugar in GTK made with 15-day inoculum is significantly lower at ~40 mg/mL compared to ~59 mg/mL in GTK made with 10-day inoculum (Table 12). When compared to 10-day inoculum, GTK made with 15-day inoculum showed a significantly lower S/A ratio of 6.6 and an overall acceptance score of 26.8% (Table 12). The stark difference in flavor profiles of GTKs can only be partly explained by higher initial Day 0 concentration of acetic and gluconic acids in GTK made with 15-day inoculum (Figure 14).

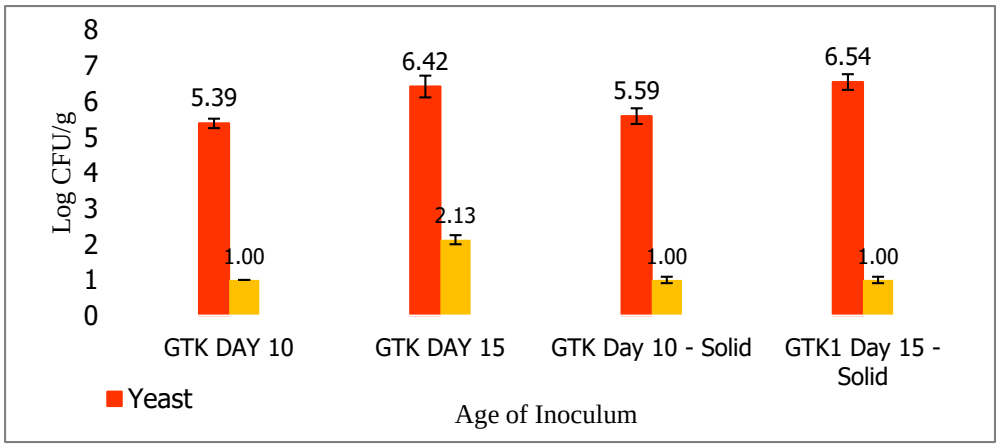


Figure 16: Effect of age of inoculum on bacteria and yeast counts in Kombucha after 9 days of fermentation

To fully explain the observed differences, we looked at the compositional differences in both starter cultures. While the bacteria profile between 10 and 15-day inocula remained mostly unchanged, the yeast composition predominantly shifted from *Brettanomyces spp* to *Lachancea fermentati*, previously known as *Zygosaccharomyces fermentati* (Table 14). *Lachancea thermotolerans*, a

close relative, when used as a co-starter culture imparted intense acidic notes which reduced perceived sweetness in wine (Gobbi et al., 2013). The change in taste of Kombucha from sour, fruity and lightly sparkling to a mild vinegary taste was attributed to prolonged incubation (Steels et al., 2002). From our data, we can further elaborate by saying that an older inoculum, essentially a product of prolonged fermentation, can result in a higher % of *Lachancea spp*. and reduced % of *Brettanomyces spp* (Table 14). Therefore, changing the age of inoculum can cause shifts in microbial composition that can alter or produce an undesired flavor profile.

Overall for both teas, 10-day inoculum produced better tasting Kombuchas'.

Table 14: Effect of age of inoculum on relative abundances (%) of major 16S bacterial and ITS fungal species in green tea Kombucha fermented for 9 days

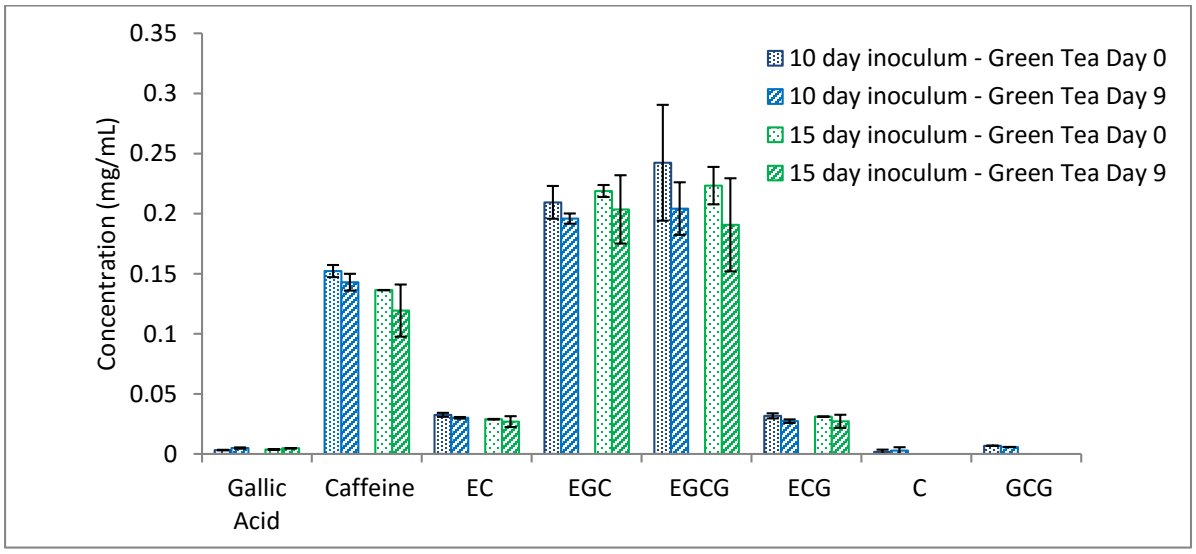
Bacteria	**GTK 10 day inoculum (%)**	**GTK 15 day inoculum (%)**
Komagataeibacter gluconacetobacter saccharivorans	66.53	65.98
Acetobacter tropicalis	9.62	10.51
Gluconacetobacter xylinus	3.58	6.95
Gluconacetobacter spp.	3.07	3.97
Acetobacter pomorum	7.50	8.39
Fungi	**GTK 10 day inoculum (%)**	**GTK 15 day inoculum (%)**
Lachancea fermentati	1.91	41.19
Brettanomyces dekkera bruxellensis	58.02	40.38
Brettanomyces dekkera anomala	39.07	17.08
Zygosaccharomyces bailii	0.47	1.049

	GTK (10 day inoculum)	**GTK (15 day inoculum)**	**BGTK (10 day inoculum)**	**BGTK (15 day inoculum)**
Catechins	No change	No change	No change	No change
Di/Tri hydroxylated catechins ratio	0.14 ± 0.01	0.14 ± 0.002	0.17 ± 0.009	0.2 ± 0.007
EGC+EGCG (mg/mL)	0.4 ± 0.02	0.39 ± 0.07	0.15 ± 0.07	0.15 ± 0.01

Table 15: Age of inoculum - Catechin Ratios

No observed change in catechin profile and total polyphenol content with either inocula.

Figure 17: Age of inoculum - GTK - Catechins and Caffeine



4.4 Presence or absence of solid inoculum on chemical, microbial and sensory profile

A blend of green and black teas (BGTK) was used in this study to overcome antimicrobial properties of green tea catechins while retaining some of its benefits. The resulting Kombucha made without solid inoculum fermented at a much slower rate. Without a cellulose pellicle the non-motile acetic acid bacteria, ranging from facultative to strictly aerobic types, settle at the bottom of the static fermenter where there is less oxygen. Titratable acidity and the concentration of metabolites produced during fermentation was 50-100% lower in Kombucha made without solid inoculum (Figure 12, Table 18).

	BGTK (With solid inoculum)	**BGTK (Without solid inoculum)**
pH	2.95 ± 0.03	3.1 ± 0.02
Titratable Acidity (acetic acid equivalent) g/100mL	0.42 ± 0.07	0.24 ± 0.03

Table 16: Presence or Absence of Solid Inoculum - pH and Titratable Acidity

As expected, the Kombucha made without solid inoculum was not nearly sour enough and significantly sweeter with an S/A ratio of ~28 (Table 17).

	With Solid Inoculum	**Without Solid Inoculum**
Description	Sweet, Sour, Yeasty	Much too sweet, not nearly sour enough, not nearly yeasty enough
Overall acceptance % (9-point hedonic scale)	55.4 ± 1.48	16.1 ± 7.63
Sugar/Acid ratio	12.61 ± 0.38	28.46 ± 1.70
Sugar (mg/mL)	25.62 ± 0.83	54.13 ± 0.13

Table 17: Presence or absence of solid inoculum - Flavor Profile

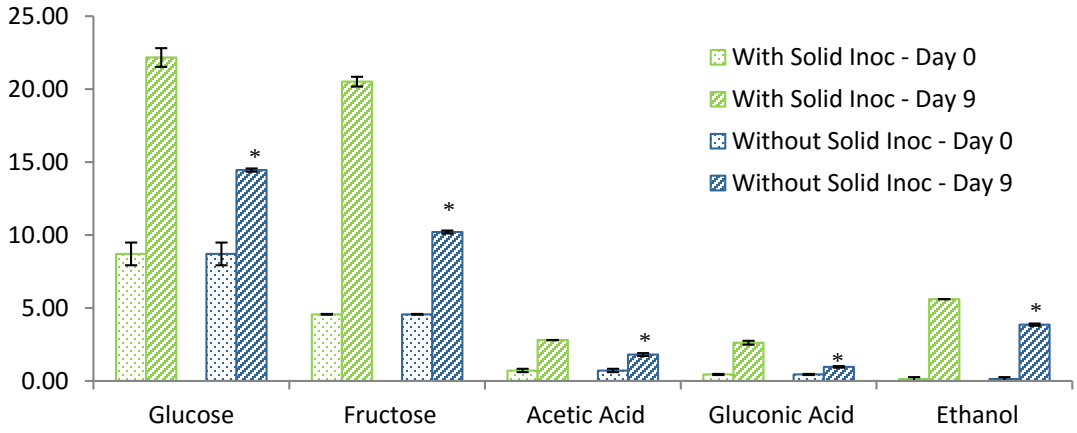


Figure 18: Solid inoculum - Sugars, Acids and Alcohol Concentration in BGTK Blended Tea (mg/mL)

'*' $p \leq 0.05$ between with and without solid inoculum fermented kombucha after 9 days

There were no statistically significant differences in individual catechins before or after fermentation (Figure 19). An unexpected result was the EGC+EGCG concentration and total polyphenol concentration at the end of fermentation was significantly higher in Kombucha made without solid inoculum (Table 18, Figure 20). This is similar to an earlier report where the increased polyphenol level was speculated to be a result of conversion of one catechin to another, perhaps a case

of theaflavins or thearubigins converting to green tea catechins with help of certain starter cultures (Jayabalan *et al.*, 2007).

Figure 19: Solid inoculum - Catechins and Caffeine

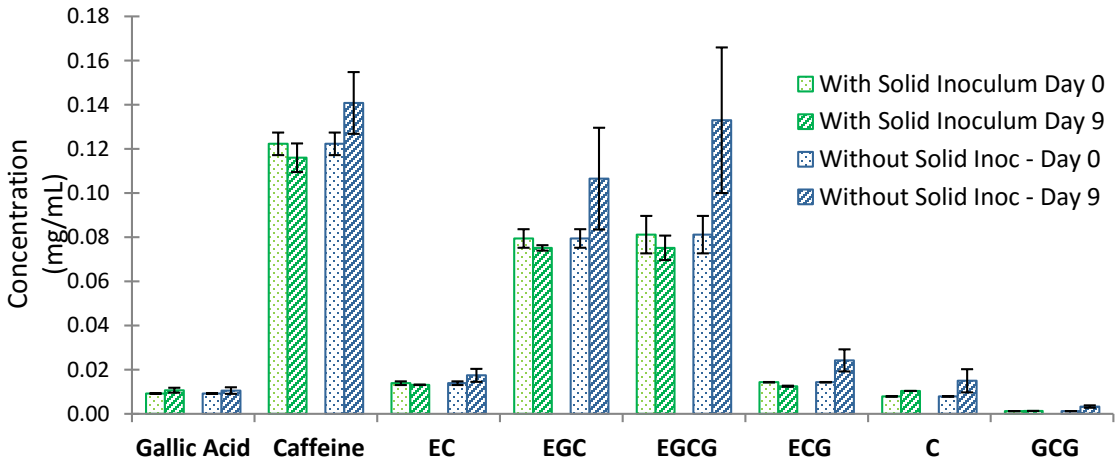


Table 18: Solid inoculum - Catechins ratio

Category	Blend Tea Kombucha (With solid inoculum)	Blend Tea Kombucha (Without solid inoculum)
Catechins	No change	No change
Di/Tri hydroxylated catechins ratio	0.17±0.01	0.17±0.01
EGC+EGCG (mg/mL)	0.15±0.01	0.24±0.06*

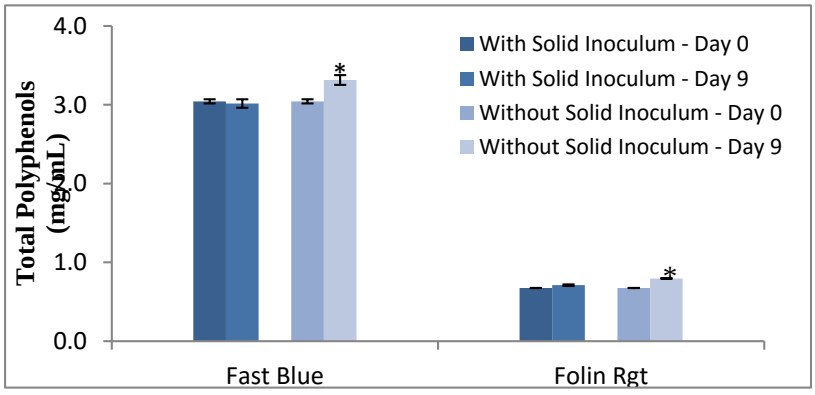


Figure 20: Total Polyphenols - BGTK

4.5 Effect of specific interfacial area on chemical, microbial and sensory profile

Specific interfacial area was suggested as a key process variable in scaling up Kombucha fermentation, however no relationship with metabolite production was identified (Cvetkovic *et al.*, 2008). SA/V ratios and corresponding fermentation times were chosen based on this earlier study. Concentration of glucose, fructose and gluconic acid increased by increasing specific interfacial area. We expected larger surface area to facilitate more dissolved oxygen in Kombucha, hence preferentially enhance the growth of acetic acid bacteria that produced acetic acid, gluconic acid and cellulose via aerobic fermentation. Kombucha made in a vessel with SA/V 0.16 produced glucose and fructose 5-7 times as much as the one with lowest SA/V of 0.09 (Figure 21). Acetic acid and ethanol levels were not significantly different between samples. However, glucose and

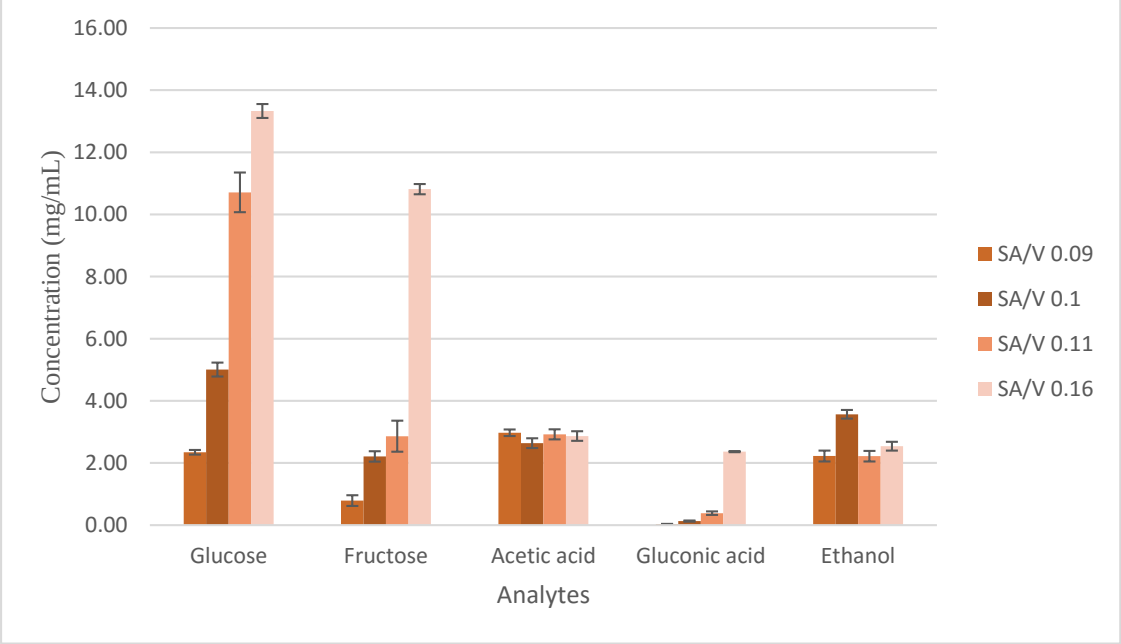


Figure 21: Effect of specific interfacial area on sugar, acid and alcohol profile in black and green blended tea Kombucha and made with 15 day inoculum (SA/V 0.09-0.11 – 7 day fermentation, SA/V 0.16 – 4 day fermentation)

fructose are produced during anaerobic yeast fermentation of sucrose to ethanol. So, another explanation is increased surface area also increased the amount of yeast that settles at the bottom of the container hence also promoting anaerobic fermentation.

Overall, it seems larger surface area to volume can favor growth of bacteria and yeast, hence increase microbial fermentation. A SA/V ratio range of 0.09-0.11, even after 7 days of fermentation, delivered an under fermented Kombucha with a titratable acidity of 2.6-3.1 g/L acetic acid. Kombucha made in a vessel with SA/V ratio of 0.16, fully fermented with a lower sugar/acid ratio and delivered a desired flavor profile only after 4 days of fermentation (Table 19). Within the test range of 0.09-0.16, the rate of fermentation was directly proportional to the SA/V ratio.

	SA/V 0.09	**SA/V 0.10**	**SA/V 0.11**	**SA/V 0.16**
	7 days of fermentation			**4 days of fermentation**
Description	Much too sweet, not nearly sour enough, hint of fermentation	Much too sweet, Not nearly sour enough	Much too sweet, Not nearly sour enough	Sweet, sour, slightly over fermented
Overall acceptance % (9-point hedonic scale)	31.5 ± 5.66	18 ± 3.55	30.1 ± 7.59	63.2 ± 0.49
Sugar/Acid ratio	25.68±1.263	28.15±0.79	25.94±1.69	14.23±0.126

Table 19: Effect of specific interfacial area on sensory characteristics of black and green tea Kombucha

5 SUMMARY AND CONCLUSIONS

A comprehensive analysis was performed to provide a better understanding of relationship between few key process parameters, microbial activity and quality parameters. Analytical methods and testing approaches developed in this study can be used to monitor Kombucha quality. Recording the rate of fermentation in terms of sugar/acid ratio and relating that to a pre-calibrated sensory score of S/As and corresponding 9-point hedonic scale can be a useful semi-quantitative measure and potentially a good quality indicator.

We confirmed an antimicrobial effect of green tea on Kombucha starter cultures. Selective growth of bacteria and yeast based on tea type is novel and interesting. Antimicrobial catechins in green tea not only affect the amount of bacteria and yeast in starter cultures, but also can alter their composition in a strain dependent manner. Currently accepted two step mechanism of Kombucha fermentation i.e. yeast mediated conversion of sugar to alcohol followed by a bacteria mediated conversion of alcohols to acid is over-simplified and the role of tea also needs to be taken into account. A tea blend of black and green teas (BGTK) delivered a good balance of bioactives and flavor profile without compromising on rate of fermentation, making this a better candidate for a commercial application over green tea alone.

Alternatively, age of inoculum can also be a useful tool in combating this antimicrobial property. While an older/15-day inoculum has produced more organic acids, from an overall quality standpoint younger/10-day inoculum has delivered a more balanced Kombucha. This harsher taste with 15-day inoculum was due to higher microbial activity that has resulted from a shift in dominant yeast species. Older inoculum need not have either higher or lower bacteria and yeast, but their composition itself could be different. Based on this novel finding, we may also conclude that the

change in sensory characteristics of Kombucha is not only linked to the number of microbes in the starter culture but also strongly influenced by shifts in microbial population.

Kombucha fermented at a much slower rate in the absence of solid inoculum. While handling solid inoculum in a manufacturing environment can be quite difficult, these findings support the need to use both types of inocula. However, manipulating the specific interfacial area can reduce the fermentation time without compromising on flavor quality. This can be a useful scale up tool to improve rates of fermentation and gain efficiencies.

While our data offers meaningful insights into Kombucha fermentation, the complex interplay between process parameters and Kombucha fermentation makes it impossible to offer just one strategy to produce a consistent product. The path to consistent quality will involve closely monitoring and controlling the key process parameters. Also, some level of standardization of starter cultures, most preferably using pure cultures, will be useful. One other key recommendation would be to profile the microbiota in starter cultures under various conditions to draw any meaningful relationships with microbial activity and flavor profile.

We have not been able to identify any proven probiotic bacteria in our Kombucha starter cultures. Therefore, until the dominant species in Kombucha starter cultures belonging to *Zygosaccharomyces, Brettanomyces,* Komagataeibacter and *Gluconacetobacter* genera are qualified as probiotics, we should not categorize Kombucha as a probiotic beverage. Prebiotic-like properties of tea polyphenols along with antimicrobial properties of tea catechins and organic acids can modulate the composition of good and bad bacteria in the gut. Hence, it is reasonable to consider Kombucha as a functional beverage with a great potential to promote gut health.

6 FUTURE WORK

I. Study the effect of other key process parameters on chemical, microbial and sensory properties

1. Type of carbohydrate

   Majority of Kombucha is produced with refined cane sugar but across different beverage categories natural sweeteners such as evaporated cane juice, turbinado sugar, agave nectar, honey and fruit nectars are increasingly used in product formulations to further clean label efforts. Prebiotics such as short chain fructo-oligosaccharides may be added to Kombucha during the fermentation process, to increase its health benefits. The effect of different carbon sources on Kombucha fermentation and overall quality needs to be understood.

2. Standardized bacteria and yeast cultures

   There are advantages and disadvantages to using standardized cultures. Defined amounts of single strain cultures when inoculated into each batch of Kombucha can theoretically produce a safer and more consistent quality Kombucha. Malbasa *et al*. (2011) reported higher amounts of total acids, antioxidant activity and vitamin c content with black tea when standardized mixture of acetic acid bacteria and *Zygosaccharomyces* was used as the inocula. However, it is unknown if single strain cultures can produce a complex flavor profile of Kombucha. The same study showed the opposite result with green tea with more metabolite production using unstandardized mixed cultures. It is important to perform a comprehensive study to understand the effect of pure cultures on bioactive production and flavor profile.

3. Scale up factors - specific interfacial area, headspace and oxygenation

   Scale up parameters are still being explored. Most acetic acid bacteria are obligate aerobes and need a constant supply of oxygen. A larger fermentation vessel without any means to distribute air uniformly will cause oxygen deprivation. Hence, oxygen transfer rates have to be maintained to balance with anaerobic yeast fermentation. Viability of sparging air or oxygen

through different sections of fermentation vessel needs to be explored. Lack of a balance can lead to uncontrolled production of alcohol.

Cvetkovic *et al*., 2008, showed the importance of keeping specific interfacial area, a ratio of surface area to liquid volume, in maintaining the quality of fermentation. While this is an interesting finding, these design limitations could be quite restrictive for sourcing fermentation tanks and ease of manufacturing. Data from our study showed an increased rate of fermentation (both aerobic and anaerobic types) with increase in specific interfacial areas, but headspace was not taken into consideration. Studying effect of headspace could help understand other micro-environmental triggers for aerobic or anaerobic fermentation.

4. Secondary (2') fermentation

   It is important to maintain the quality of Kombucha, including levels of alcohol, after it is packaged in a retail container. The closed anaerobic environment will favor secondary yeast mediated fermentation converting any residual sugars into alcohol. Screening yeast strains that do not produce alcohol under refrigeration conditions will be a novel way to address secondary alcohol production. This approach would also require standardizing Kombucha cultures.

II. Explore mechanisms and analytical tools for quality control

1. Mechanism

   The proposed simplified two step mechanism of Kombucha fermentation is not sufficient to explain all observations. While findings from this research are novel and will help expand our understanding of Kombucha fermentation, there is a need to further explore the strain dependent growth inhibition or promotion with green and black teas. Using data from this research, two or more pure single cultures of bacteria and yeast may be isolated to confirm their interactions with tea. Also, the Kombucha cultures should be checked for interdependency

and co-evolution characteristics as detailed in the cheese rind study in Section 2.1.3 (Wolfe et al., 2015).

2. Metagenomic sequencing

   The following sequencing efforts will offer additional insights into the mechanism:

   i. Bacteria and yeast compositions when black and green tea are blended together.

   ii. SCOBY cultures under various process conditions.

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8 Appendix

8.1 Sensory Calibration Curve

Table 20: Sensory Calibration

			OVERALL LIKING										
Black Tea Kombucha acetic acid (mg/ml)	**Standardized Sucrose (mg/ml)**	**S/A**	**T1**	**T2**	**T3**	**T4**	**T5…**	**..T18**	**T19**	**T20**	**9 point scores**	**Descriptors**	**Comments**
3.6	25	6.94	1	3	2	3	2	2	5	2	2.40	Not nearly sweet enough, much too sour	Harsh, acidic, too vinegary
3.6	35	9.72	3	4	4	4	3	3	6	4	3.68		Slightly harsh, acidic
3.6	45	12.50	5	6	5	5	6	5	5	6	5.23	Sweet/Not nearly sweet enough, much too sour	Good sweetness, acidic
3.6	55	15.28	7	7	8	7	7	7	3	4	5.80	Sweet, Sour/much too sour	Good sweetness, Good acidity/ acidic, fruity
3.6	65	18.06	8	8	8	8	9	8	2	5	6.70	Sweet, Sour	Good sweetness, Good acidity
3.6	75	20.83	5	6	6	7	5	4	1	4	5.05	Sweet/Too sweet, Sour/Not nearly sour enough	Good sweetness/too sweet, Good acidity/not enough acidity

3.6	85	23.61	4	3	3	4	4	3	1	4	3.95	Too sweet, Not nearly sour enough	Too sweet, like sweet tea

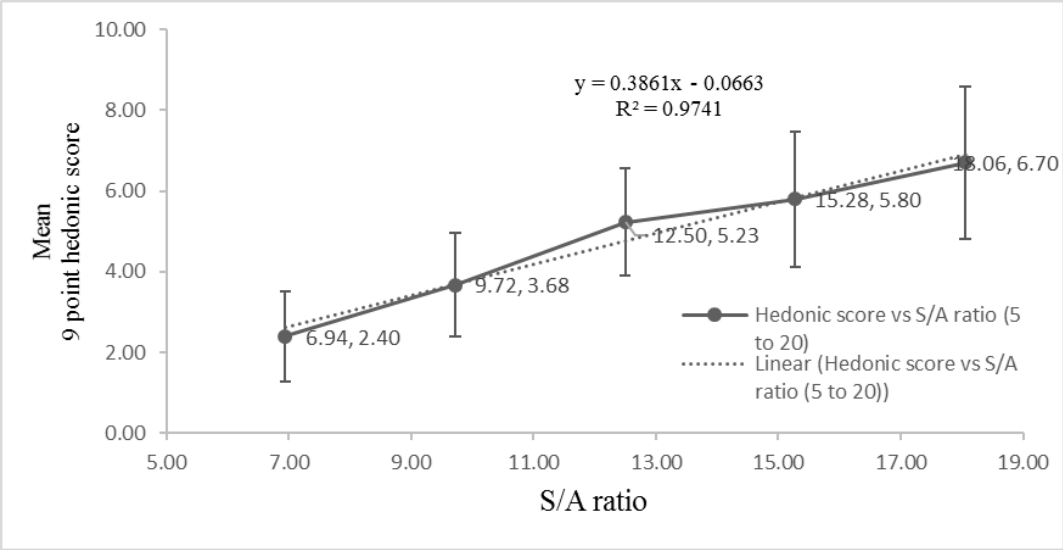

Figure 24: Sensory Calibration Curve (S/A ratio range: 5 to 20)

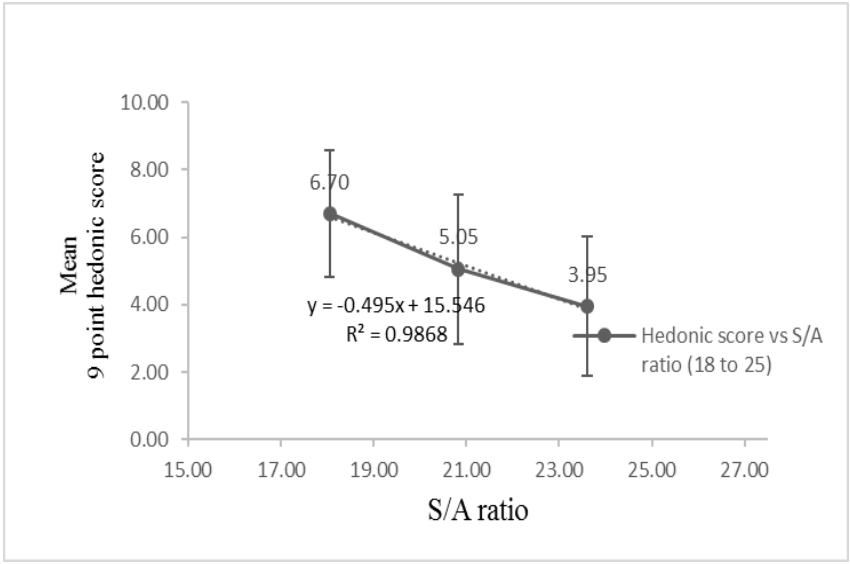

Figure 22: Sensory Calibration Curve (S/A ratio range: 18 to 25)

8.2 Glucose, Gluconic Acid calibration curves