
# Production of Kombucha with New Zealand Honeydew Honey

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## Abstract

Kombucha is a fermented tea drink using a Symbiotic Culture of Bacteria and Yeast (SCOBY) which is receiving increased research and development interest due to its increasing popularity, large market share in the beverage industry and numerous beneficial health claims. Typical kombucha utilises green or black teas as the base media with sucrose as the substrate for fermentation. In this study, a novel kombucha beverage utilising *Tie Guan Yin* (TGY), a Chinese oolong tea, and honeydew honey as a substrate for fermentation was developed. 2L of tea sweetened with 150g of honeydew honey was fermented using 50g of SCOBY at ambient temperature (20-25°C). TGY tea was selected for its complex aroma and flavour profile as well as the lack literature documenting the use of oolong teas for producing kombucha. Honeydew honey was selected for its high antioxidant activity, amino acid content and presence of oligosaccharide sugars.

The titratable acidity and concentration of kombucha's primary metabolites were assessed over the course of the fermentation period. The titratable acidity of the honey kombucha increased from 0.016 ± 0.003% acetic acid equivalent (ACE) on day 0, to 0.599 ± 0.0582% ACE after 14 days of fermentation. This was significantly higher than the titratable acidity from kombucha made from sucrose which increased from 0.011±0.007% ACE on day 0, to 0.199 ± 0.087% ACE after 14 days. This indicated that honeydew honey can accelerate the kombucha fermentation process when compared to sucrose as the substrate.

The primary kombucha metabolites consist of ethanol, acetic acid and gluconic acid. The concentration of these compounds increased with fermentation time and reached (in g/L kombucha) 1.325±0.469, 4.191±0.828, 4.890±1.256 for ethanol, acetic acid and gluconic acid respectively after 14 days of fermentation. In the case of ethanol, the concentration was below the 0.5% ABV threshold set by the New Zealand Ministry of Primary Industries, which allows the kombucha to be labelled as a non-alcoholic beverage in New Zealand.

The antioxidant activity was measured using three assays: phosphomolybdenum, ferric-ion reducing antioxidant power (FRAP) and cupric reducing antioxidant capacity (CUPRAC). All three assays showed that the antioxidant activity of the kombucha increased with fermentation time. The total phenolic content, determined using Folin-Ciocalteu reagent, also showed an increasing trend to increase with fermentation time.

The amino acid composition of the honeydew honey was predominantly proline; however, alanine, valine, leucine, isoleucine, phenylalanine, glutamic and aspartic acids were also detected. The concentration of all amino acids was found to decrease once the kombucha ferments. This is likely due to the amino acids being metabolised by the microorganisms in the SCOBY.

The sugar composition of the honeydew honey and kombucha was assessed. Glucose, fructose, maltose and melezitose were detected in the kombucha along with potentially palatinose and turanose. The glucose and fructose concentrations decreased with fermentation time as they are metabolised by the bacteria in the SCOBY. Melezitose, palatinose and maltose did not show any changes in concentration with fermentation time, indicating they are not metabolised by the bacteria and yeasts in the kombucha.

Microbiology analysis was carried out to identify the species of bacteria found in kombucha and to quantify their concentrations in the kombucha drink. DNA genomic sequencing on kombucha and SCOBY culture from the honey kombucha was conducted to elucidate the bacteria found in kombucha. The predominant species of bacteria found in the SCOBY were *komagataeibacter*

and *gluconobacter* species which combined to make up over 80% of the DNA sequenced in the samples. Plate count experiments quantified that bacteria cultured on agar plates increased with concentration with fermentation time, reaching 6.86 log(colony forming units/mL kombucha) which is above threshold criteria for a food to be considered probiotic. However, due to time constraint identification and DNZ sequencing of fungi and yeast in the honey kombucha were not carried out.

This thesis provides a comprehensive study on a kombucha beverage produced from TGY and honeydew honey. Solid foundations were also laid for further research into discerning the oligosaccharide composition of honeydew honey and kombucha microbiology analysis.

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| | |
|---|---|
| SCOBY | Symbiotic Culture of Bacteria and Yeasts |
| TGY | Tie Guan Yin |
| ACE | Acetic acid equivalent |
| TE | Trolox equivalent |
| GAE | Gallic acid equivalent |
| ISTD | Internal standard |
| GC | Gas chromatography |
| MS | Mass spectrometry |
| HPLC | High performance liquid chromatography |
| ELSD | Evaporative light scattering detector |
| MRS | De Man, Rogosa and Sharpe |
| NA | Nutrient agar |
| AAB | Acetic acid bacteria |
| LAB | Lactic acid bacteria |
| PDA | Potato dextrose agar |
| SDA | Sabourand dextrose agar |
| GYC | Glucose-yeast extract-calcium carbonate |

**Attestation of Authorship**

"*I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning"*

Name: Edward Quach

Signature

Date: 12/10/20

# 1 Introduction

Kombucha is a slightly sweet and acidic refreshing beverage produced by fermentation of sweetened tea liquid. The fermentation process involves the infusion of tea leaves, addition of sucrose and using a Symbiotic Culture Of Bacteria and Yeasts (SCOBY) (C. Chen & Liu, 2000). A typical batch of kombucha is depicted in Figure 1 which consists of a floating cellulosic pellicle layer and a sour liquid media. The fermented media can be consumed with minimal processing and tastes mildly sour with some sweetness and floral notes. While the exact origin of the beverage is unknown, it is believed that kombucha originated in China with its consumption spreading through Russia and then Europe (Dufresne & Farnworth, 2000).

Figure 1: Batch of Kombucha with fermented media and SCOBY

The kombucha market in New Zealand (NZ) is worth approximately 18 million NZD. The global kombucha market was worth approximately 1.67 billion USD in 2019 and projected to increase to 7.05 billion USD in 2027. Flavoured kombucha drinks make up 70% of the global kombucha market share, hence there is a strong push to develop new flavours by experimenting on different teas and substrates during the fermentation process (Grand View Research, 2020). As a fermented tea product, there have been many claims of therapeutic and health benefits from consumption of kombucha such as: promoting liver functions, detoxifying the body, protection against diabetes and improved resistance to cancer (Dufresne & Farnworth, 2000). Many of these health claims are currently unsubstantiated in scientific literature, therefore allowing room for kombucha research.

Honey is a viscous syrup produced by honeybee from sugary secretions found in nature such as floral nectar or honeydew. Honey is widely consumed and used in beverages for its distinct flavour and health benefits including antioxidant activity, polyphenols and inhibition of pathogens such as: *Listeria, Salmonella and Staphylococcus*. Honey can be used as a natural

sweetener in place of table sugars, the distinctive taste of honey can be used to complement the flavour and create new flavours in drinks such as honey lemon tea. In similar vein, honey have also been used as a fermentation substrate to produce mead and vinegar (Ilha, Sant'Anna, Torres, Porto, & Meinert, 2000).

Honeydew is a colourless, sugar-rich syrup secreted by the scale insects. As the scale insect feeds on phloem sap from southern beech (*Nothofagus. Spp*) trees, they secrete excess nutrients as a by-product in the form of honeydew (Crozier, 1981; Gaze & Clout, 1983) NZ's honeydew is unique from other countries as the species of scale insects and southern beech trees involved in producing honeydew are endemic to NZ. There are four main species of southern beech and one endemic sub-species can be found all over NZ. Pure beech forest with a single species of southern beech can be found in Fiordland, Taranaki and Southland. Forest with a mixture of beech trees can be found in East Cape and the Coromandel Peninsula (Orwin, 2007). In the absence of floral nectar, honeybees will take honeydew as a food source. The honeydew is processed with additional enzymes from the bees, which creates honeydew honey. Honeydew honey is distinguishable from floral honey by its darker colour, deeper flavour, higher sugar and dextrin content, greater free acidity and higher ash content (Kirkwood, Mitchell, & Ross, 1961). The price of dark honeys (including honeydew honey) when purchased in bulk from beekeepers in NZ can range between 3.50-6.00 NZD per kilogram. This price range is below the cost of production for many beekeepers and caused by an oversupply in the NZ market (Ministry of Primary Industries NZ, 2019). As such finding more ways to utilise honeydew honey is highly desirable.

Current research in kombucha largely uses green or black teas and sucrose as the substrates for fermentation. *Tie Guan Yin* (TGY) was selected as it possesses unique aromas and flavours, and its existing popularity as a drink. Experimentation into other sugar rich sources such as molasses and fruit juices as the substrate for fermentation have led to changes in flavours and chemical properties of the final beverage (Malbaša, Lončar, & Djurić, 2008; Sun, Li, & Chen, 2015). Research into the use of honey as the substrate for kombucha fermentation is sparse because of its reported anti-microbial properties. Honeydew honey was selected over other forms of honey because it is an under-utilised variety of honey. Additionally, honeydew honey has a deeper flavour, rich in oligosaccharides and higher amino acid concentration when compared to other honeys. The combination of TGY along with honeydew honey in kombucha is a novel combination. This study will highlight the effects of honeydew honey on the kombucha fermentation process.

# 2 Literature Review

## *2.1 Kombucha*

### *2.1.1 Preparation of kombucha*

Preparation of kombucha requires four primary components: water, tea leaves, sugar and a SCOBY culture. The process of brewing kombucha begins with steeping of the tea leaves in boiling water. The type of tea leaves used dictates the flavour of the kombucha by altering the final concentrations of polyphenols and organic acids (Jayabalan, Marimuthu, & Swaminathan, 2007). However, not all teas are suitable for making kombucha. For example, jasmine tea can greatly retard the growth of the SCOBY and potentially kill the culture (from our preliminary study not mentioned in this thesis). Once the tea has been steeped, a sugar is added into the tea to provide a carbon source for the SCOBY culture. Typically, sucrose are used for kombucha fermentation, however other substrates have been utilised such as molasses, fruit and wheatgrass juices (Malbaša et al., 2008; Sun et al., 2015). The use of different substrates can promote the growth of the culture, enhance the antioxidant activity, increase polyphenol and organic acid contents and alter the flavours of the kombucha. After the substrate is added, the sweetened tea is cooled down to around 20°C before the SCOBY is inoculated for fermentation. The fermentation process can last from 7 to 30 days depending on the desired acidity level, flavour and quality of the final kombucha product. The longest fermentation period that has been examined in literature was 60 days by Chen & Liu (2000), though this was far too acidic for regular consumption.

### *2.1.2 Microorganisms in kombucha*

The bacteria and fungi present in kombucha form a powerful symbiosis that can inhibit the growth of potential pathogenic bacteria. The main acetic acid bacteria found in the SCOBY are: *Komagataeibacter Xylinus* (formally known as *Acetobacter Xylinum), A. Xylinoides, Bacterium Gluconicum, A. Aceti, A. Pasteurianu*s (Reiss, 1994). Other bacteria species present include: *Gluconacetobacter, Lactobacillus* species, *Lactococcus* species (Marsh, O'Sullivan, Hill, Ross, & Cotter, 2014). A wide spectrum of yeasts are present including: *Saccharomyces Cerevisiae, Brettanomyces Bruxellensis, B. Lambicus, B. Custersii, Schizosaccharomyces Pombe, Saccharomycodes Ludwigii, Kloeckera Apiculata, Zygosaccharomyces Bailii* and *Pichia* species (Liu, Hsu, Lee, & Liao, 1996; Mayser, Fromme, Leitzmann, & Gründer, 1995; Reiss, 1994; Sievers, Lanini, Weber, Schuler-Schmid, & Teuber, 1995). It should be noted that the exact microbial composition of each individual SCOBY may vary depending on its origin and how it was prepared (Reiss, 1994).

*Komagataeibacter Xylinus* can synthesize a cellulose network or biofilm from glucose. The floating cellulosic biofilm at the top of the kombucha enhances the association between bacteria and fungi (Sievers et al., 1995). The biofilm holds a variety of bacteria, yeasts and the liquid

media within the cellulose network, this allows it to be inoculated into subsequent batches of kombucha. The cellulose network also aids the fermentation by allowing aerobic bacteria to have ample oxygen supply as the pellicle layer floats at the media-air interface (Villarreal-Soto, Beaufort, Bouajila, Souchard, & Taillandier, 2018)**.** The function of yeast cells is to hydrolyse the sucrose to form fructose and glucose and subsequently converting the sugars into ethanol. The acetic acid bacteria converts the resulting glucose to gluconic acid and glucuronic acid, while fructose is converted into acetic acid (Villarreal-Soto et al., 2018). The resulting acetic acid stimulates the production of ethanol by the yeast, which in turn can help the acetic acid bacteria grow and produce additional acetic acid (Liu et al., 1996). Both ethanol and acetic acid possess antimicrobial properties against pathogenic bacteria, thereby protecting the SCOBY from contamination. This symbiotic process between bacteria and yeasts are stimulated by the caffeine and xanthine compounds from the tea infusion (Fontana, Franco, De Souza, Lyra, & De Souza, 1991).

### *2.1.3 Chemical Composition of kombucha*

Many studies have been conducted to identify active compounds in tea and to elucidate their chemical and biological properties. It should be noted that the exact composition of kombucha will vary with fermentation time, contents of the tea used, and the individual SCOBY used for the ferment (Reiss, 1994).

Several compounds have been found to be common in all kombucha samples reported in literature such as: sucrose, glucose, fructose, carbon dioxide, ethanol, and acetic, gluconic and glucuronic acids. Sucrose is the primary substrate used in most kombucha batches for fermentation. Sucrose is hydrolysed into glucose and fructose by the yeasts within the SCOBY. The production rate and final concentration of glucose and fructose were found to be different, which suggests that the sugars serve slightly different purposes in the fermentation process (C. Chen & Liu, 2000). While both sugars can be used to produce ethanol and carbon dioxide, their usage by the *Komagataeibacter Xylinum* differ. Glucose is used by the *K. Xylinum* to biosynthesise cellulose for the daughter culture and produce gluconic and glucuronic acids. *K. Xylinum* predominant use for fructose is to produce acetic acid. Ethanol produced by yeast was found to reach a maximum concentration during the fermentation process before further decline (C. Chen & Liu, 2000; Reiss, 1994; Sievers et al., 1995). The decline is attributed to the oxidation of ethanol by *Acetobacter* species to produce more acetic acid and the lack of sugars for the yeast to produce ethanol towards the end of fermentation. Acetic acid is an organic acid which is consumed at low concentrations in the form of vinegar. Acetic acid is the pre-dominant flavouring acid in kombucha and has many therapeutic effects such as: lowering blood pressure, antioxidant activity, inhibit growth of cancerous cells and anti-diabetic effects (Budak, Aykin, Seydim, Greene, & Guzel-Seydim, 2014; Murooka & Yamshita, 2008). Gluconic acid is the

secondary organic acid which imparts a milder sour taste to the kombucha compared to acetic acid. Gluconic acid has been shown to promote gut health by encouraging the growth of probiotic bacteria in the large intestine (Asano, Yuasa, Kunugita, Teraji, & Mitsuoka, 1994). Glucuronic acid is the last common organic acid and provides a wide variety of health benefits such as: detoxifying xenobiotics from the liver, aid bioavailability of polyphenols, improve steroid hormone balance and is a precursor of L-ascorbic acid (vitamin C) (Vina, Linde, Patetko, & Semjonovs, 2013).

Although acetic, gluconic and glucuronic acids are the three primary organic acids found in kombucha, several other organic acids may also be present. Such acids include; tartaric, lactic, succinic, citric, pyruvic, oxalic, malic, and quinic acids (Blanc, 1996; Neffe-Skocińska, Sionek, Ścibisz, & Kołożyn-Krajewska, 2017; Reiss, 1994). These lesser organic acids also contribute to the overall flavour of the kombucha, giving it subtle flavour notes. There are conflicting accounts on whether some of these organic acids have been found. For instance the two studies conducted by Sievers et al (1995) and Neffe-Skocińska et al. (2017) showed conflicting results on the presence of lactic and malic acids in their final kombucha samples. This may be attributed to the individual SCOBY used between the two experiments. Typically, these minor organic acids were found in low concentrations of less than 1g/L.

Several components associated with health benefits can also be found in kombucha are vitamins and tea polyphenols. The concentration of vitamins $B_1$, $B_6$, $B_{12}$, and C, increased over the course of the fermentation process compared to their prepared tea decoction (Bauer-Petrovska & Petrushevska-Tozi, 2000). The mean concentration for those vitamins after 15 days of fermentation were: vitamin $B_1$ 0.74mg/mL, vitamin $B_6$ 0.84mg/mL, vitamin $B_{12}$ 0.52mg/mL and vitamin C 1.51mg/mL. Chu & Chen (2006) observed a linear increase in total phenolic content with fermentation time. They also noted that their kombucha broth becoming progressively lighter in colour over time. As the black tea colour is attributed to the presence of thearubigins, the change in colour is likely due to the polyphenols undergoing some alteration as the kombucha ferments. It is believed that depolymerisation of thearubigins into simpler phenolic compounds may have occurred, which also explains the increased total phenol content (Chu & Chen, 2006).

### *2.1.4 Beneficial health claims of kombucha*

Numerous health benefits have been claimed to be associated with consumption of kombucha from personal account and testimonies (Greenwalt, Steinkraus, & Ledford, 2000). However, majority of these health claims are unsubstantiated scientifically so the credibility of these claims are questionable. Dufresne & Farnworth (2000) collected many claimed beneficial health effects from drinker's testimonies and Russian researchers. Such claims include reduction in

cholesterol, blood pressure, inflammation problems and atherosclerosis; promote liver functions, improvement of eyesight, detoxify the body, protection against diabetes, improve resistance to cancer, relieve symptoms of bronchitis, asthma arthritis and gout; counteract aging problems, enhance the immune system, normalise intestinal activity, and curing haemorrhoids.

Kombucha has been reported to exhibit *in vitro* antimicrobial activity against several pathogenic microorganisms. Kombucha with low fractions of tea (4.4g dry weight /L) have reported antimicrobial activity against *Helicobacter pylori, Escherichia coli, Staphylococcus aureus* (Steinkraus, Shapiro, Hotchkiss, & Mortlock, 1996). A similar result was observed by Greenwalt, Ledford, & Steinkraus (1998) with kombucha containing 7g/L of acetic acid inhibiting *Bacillus Cereus, Salmonella Choleraesuis Serotype Typhimurium,* and *Agrobacterium Tumefaciens*, in addition to the previously mentioned pathogens. Varying the concentration of tea infusions, between 4.4g tea/L and up to 70g tea/L, showed minor increases in inhibiting the growth of the tested microorganisms. Additionally, when the fermented kombucha samples were pH neutralised back to 7, the observed antimicrobial effects returned to similar levels of the unfermented tea samples (Greenwalt et al., 1998). Therefore, the antimicrobial activity of kombucha may be primarily attributed to the acidity of the kombucha.

The antioxidant properties of kombucha have seen variations depending on the way kombucha are prepared. *In vitro* free radical scavenging assays conducted by Chu & Chen, (2006) on kombucha from different origins, found variations in the scavenging activity between the kombucha samples. The average antioxidant potentials of the kombucha samples increased by 70%, 40% and 49%, for 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzothiazoline- 6-sulfonic acid) (ABTS) radical scavenging, and inhibition of linoleic acid peroxidation assays respectively after 15 days of fermentation. Another study comparing free radical scavenging abilities of kombucha prepared with different tea infusions found that kombucha prepared with green tea generally exhibited better free-radical scavenging ability than black tea and tea waste material (Jayabalan, Subathradevi, Marimuthu, Sathishkumar, & Swaminathan, 2008). Similar trends were observed in the DPPH scavenging and inhibition of linoleic acid oxidation assays. Both studies concluded that, in general, the antioxidant properties of kombucha tend to increase with fermentation time. However extremely long periods of fermentation is not recommended due to organic acid concentration reaching potentially harmful levels for consumption (Chu & Chen, 2006).

### *2.2 Tie Guan Yin (TGY) tea*

Tie Guan Yuan, which literally translates to Iron Goddess of Mercy, is a variety of oolong tea, made from the *Camellia Sinensis* leaves, originating from Anxi in the Fujian Province of China. It is one of the most well-known and popular teas which can be found in many Asian groceries

shop and is a staple tea served in Chinese restaurants all over the world. TGY's popularity is owed to its distinctive and complex aroma profile with over 43 aroma active volatile compounds present (Zhu et al., 2015). Existing studies involving kombucha tend to use black teas or green teas which are also derived from *Camellia Sinensis* buds and leaves. The difference is having varied degrees of oxidation during processing of the tea. Green teas have minimal oxidation, whereas black teas are fully oxidized. Oolong teas lie in the middle of these two extremes, with the degree of oxidation ranging from 12 to 85% depending on the style of oolong tea (Shi, 2007). As there are very few research publications conducted in producing kombucha from an oolong tea, the use of TGY to produce kombucha is quite novel.

### *2.2.1 Health benefits of consuming tea*

Tea consumption has been proven to have several beneficial health effects such as: antiatherogenic, antioxidant and anti-inflammatory activities. However, the health benefits from tea consumption should not necessarily be associated with kombucha. This is because the compounds associated with the described health benefits can be altered during the fermentation process, though not fully understood (Dufresne & Farnworth, 2000). Additionally, the quantity of tea components consumed from kombucha may not be the same as typical tea consumption (Stagg & Millin, 1975) .

Tea contains a wide variety of phenolic compounds, the most abundant of which are catechins such as epigallocatechin gallate. Catechins haves been correlated to human health benefits such as: antioxidant activity, maintaining cardiovascular and metabolic health, prevention of cancer and anti-bacterial activities (Brown, 1999; Cushnie & Lamb, 2005; Wolfram, 2013). During the oxidation process, catechins are oxidised into other polyphenols such as thearubigins, theaflavins and bisflavanols which give the oolong black teas a red-brown colour and added complexity to the flavour profile of the tea (Graham, 1992). Despite this, many of the health benefits are retained in the new forms of tea. At high concentrations of unfermented black tea (200g dry weight/L), antimicrobial activity was detected against *Vibrio cholerae, Staphylococcus Aureus, Salmonella Typhi and Vibrio Parahaemolyticus* (Toda, Okubo, Hiyoshi, & Shimamura, 1989; Toda et al., 1991).

## *2.3 Honeydew honey*

The composition of honeydew honey is dependent on the species of scale insect and sap used to produce the honeydew. In New Zealand the honeydew is produced by the scale insect *Ultracoelostoma Assimile*, which feed on phloem sap from southern beech trees (*Nothofagus. spp)*. The *U. Assimile* and varieties of *Nothofagus. spp* involved in producing the honeydew honey are endemic to New Zealand. As an approximation, honeydew honey is composed (in w/w percentages): 16% moisture, 57% monosaccharide sugars in the form of glucose and

fructose, and 12.5% complex mixture of disaccharide and trisaccharide sugars(more explanation on this can be found in Section 2.3.1), 0.9% minerals, 0.6% amino acids and proteins and 1.1% acids (Bogdanov, Jurendic, Sieber, & Gallmann, 2008). The honey contains a wide variety of minerals which can be utilised by the human body such as: calcium, copper, boron, iron, potassium, phosphorus, magnesium, molybdenum, nickel, sodium, silicon and zinc (Madejczyk & Baralkiewicz, 2008; Vanhanen, Emmertz, & Savage, 2011). The composition of minerals found in honey is predominantly potassium with the remaining mineral existing in trace amounts.

### *2.3.1 Oligosaccharides Sugars*

Oligosaccharides are sugars composed of two to ten monosaccharide units linked together with glycosidic bonds. Honeydew honey differs from other varieties honey by having a higher concentration of oligosaccharides present as well as a more complicated oligosaccharide composition (Weston & Brocklebank, 1999). Blossom honeys contain approximately 10% (w/w) oligosaccharides compared to honeydew honey being made up of around 17% oligosaccharides, especially in oligosaccharides with a higher chain order. The oligosaccharides found in honeydew honey are largely dependent on the species of sap insect and the composition of the sap in the trees. Oligosaccharides that can be found in honeydew honey include, but are not limited to: erlose, melezitose, turanose, palatinose and raffinose (Astwood, Lee, & Manley-Harris, 1998; Bogdanov et al., 2008; Weston & Brocklebank, 1999). Within New Zealand honeydew honey, the presence of four tetrasaccharides, one pentasaccharide and one hexasaccharide have also been detected. Many difficulties have been encountered in attempting to identify oligosaccharides as longer chain carbohydrates (more monosaccharides monomers) become less readily amenable. This is why despite numerous studies on the carbohydrate composition of honey, some of the minor oligosaccharides have yet to be identified (Sanz et al., 2005).

There are various health benefits to human health with the consumption of oligosaccharide sugars. Oligosaccharide sugars can be broadly split into two categories: digestible and functional. Digestible oligosaccharides can be fully metabolised in the small intestine. Functional oligosaccharides are resistant to hydrolysis from enzymes in the human digestive system and therefore remain whole until they reach the colon. This allows them to provide health benefits by acting as dietary fiber as the oligosaccharides are fermented by the micro-organisms in the colon promoting their growth (Cheng et al., 2017). Xylo-oligosaccharides, which can be found in honey and fruits, consumed by elderly people showed an increase in *Bifidobacterium* species, a probiotic, without any changes to the growth of *Clostridium Perfringens*, which is a cause of food poisoning, in their faeces (Chung, Hsu, Ko, & Chan, 2007). Digestible oligosaccharides also provide health benefits in other ways. Consumption of

palatinose, a digestible oligosaccharide, prior to an endurance exercise resulted in better performances and enabling higher rate of fat oxidation. This was attributed to a better sustained blood glucose level due the slow absorption rate of palatinose in the small intestine (König, Zdzieblik, Holz, Theis, & Gollhofer, 2016). Other structural isomers of sucrose such as turanose and trehalulose are sweeteners which are not metabolised by tooth decay causing bacteria in the mouth (Hamada, 2002).

### *2.3.2 Health benefits of honeydew honey*

Honeydew honey have shown to promote the growth of probiotic strains which are beneficial to human health. *In vivo* experiments found that the use of honeydew honey and oligosaccharides separated from the honey increased the populations of *Bifidobacteria* and *Lactobacilli*, although to a lesser extent than using purely fructo-oligosaccharides (Sanz et al., 2005). The growth of two strains of lactic acid bacteria, from *in vitro* and *in vivo* studies in the small and large intestines of rats, increased 10 to 100 fold in the presence of honey when compared to sucrose (Shamala, Shri Jyothi, & Saibaba, 2000). Similarly, lactic acid production in skim milk fermented with lactic acid bacteria and *Bifidobacterium* species were enhanced with honey (Chick, Shin, & Ustunol, 2001). Growth of commercial strains of *Bifidobacteria*, in pure culture, were also shown to be enhanced with honey. These results were comparable to the use of commercial prebiotic oligosaccharides such as fructooligosaccharide, galactooligosaccharide and inulin (Chick et al., 2001; Kajiwara, Gandhi, & Ustunol, 2002). The use of honeydew honey has also been shown to inhibit the growth of pathogens like: *Listeria, Salmonella and Staphylococcus* due to its acidic conditions and presence of hydrogen peroxide (Molan, 1992).

The polyphenols found in most honeys are flavonols, which are typically yellow in colour, such as catechins, lutelolin, kaempferol, apigenin and galangin (Bogdanov et al., 2008). Phenolic acids such as courmaric, gallic, caffeic and ferulic acids were also detected in honeydew honey (Silici, Sarioglu, & Karaman, 2013). Using the Folin-Ciocalteu method on honeys from Burkina Faso, honeydew honey exhibited the highest phenolic content of 113.9mg gallic acid equivalent (GAE)/ 100g honey. This was higher than floral nectar honey which typically does not exceed 100mg GAE/100g honey (Meda, Lamien, Romito, Millogo, & Nacoulma, 2005). In contrast, the total phenolic content from Turkish honeys measured by liquid chromatography was found to have been lower with 72.6mg/kg in honeydew honey compared to 161.71mg/kg in nectar honeys (Silici et al., 2013). This difference can be attributed to the botanical origin of the honeys. Honeydew honeys from various origins were analysed by Otilia Bobiş, Daniel Severus Dezmirean, Oltica Giorgiana Stanciu (2011) using a modified Folin-Ciocalteu method which found that the total polyphenol contents in Romanian, Bulgarian, Croatian, Greece and Turkish honeydew honeys were (in GAE/100g honey): 173.33, 125.790, 116.37, 80.85 and 70.45 respectively.

The amino acid composition of honeydew honey consists of a variety of amino acids including but not limited to: proline, lysine, valine, leucine, isoleucine, serine, glutamine, arginine, aspartic and glutamic acids (Pérez, Iglesias, Pueyo, González, & De Lorenzo, 2007). Of all the amino acids, proline was present in the highest concentrations while the remaining amino acids were significantly lower. Pérez et al., (2007) reported the following concentrations of amino acids in honeydew honey: proline 72.68 mg/100g honey; aspartic acid 13.22 mg/100g honey; glutamic acid 27.60 mg/100g honey; phenylalanine at 8.42 mg/100g honey; valine 2.69 mg/100g honey to name a few. The concentration of proline in honeydew honey varies between literature; Pérez et al., (2007) reported a mean concentration of 72.68mg/100g in Spanish honeydew honey , Meda et al. (2005) reported up to 121.6mg/100g in Burkina Fasan honeydew honey and Purcărea et al. (2014) documented an average of 98.3mg/100g and 66.2mg/100g from Polish and Romanian respectively. This indicates variation in the amino acid concentration and composition depending on the botanical origin of the honey.

# 3 Research aims and hypothesis

This thesis will present a comprehensive study on a kombucha product made from fermenting TGY tea and honeydew honey as the substrate for fermentation. The research objectives for this study are as follows:

- Understand the fermentation process of kombucha by monitoring: the titratable acidity, concentrations of ethanol, acetic and gluconic acids
- Measure the antioxidant activity and total polyphenol content of the final kombucha product so assess potential health benefits.
- Characterise the chemical composition of the kombucha in terms of amino acids, organic acids and sugars and how they change with fermentation time.
- Identify the species of micro-organisms present in the kombucha and SCOBY through DNA sequencing.
- Evaluate the concentration of bacterial species in the kombucha through plate count experiments.

Preliminary testing of the honeydew honey kombucha showed that the SCOBY cellulose biofilm formed at a faster rate compared to sucrose-based kombucha during fermentation. This finding aligns with the study by Shamala et al., (2000) who demonstrated that it was possible to accelerate probiotic bacteria growth by using honey solution as the media. The following hypotheses were generated from these initial findings and will be verified by this study:

- The functional oligosaccharides found in honeydew honey may be metabolised by the bacteria in the SCOBY at an accelerated rate when compared to sucrose. This will be verified by determining the sugar compositions of the honeydew honey kombucha prior to and post fermentation.
- Selective growth of certain bacterial strains in the SCOBY may have occurred to adapt to the new environment provided by the honeydew honey used in the culture media. This can be verified through DNA sequencing of the kombucha and the SCOBY from the two kombuchas prior to and post fermentation.

A sensory analysis to observe the sensory attributes and acceptability of the honey kombucha was not conducted due to the safety concerns which arose from the Covid-19 pandemic. A sensory experiment may be conducted in the future when physical distancing requirements are eased and risk of potential spread of the virus can be properly managed.

Control experiments utilising sucrose and other forms of tea were not carried out during this study due time constraints which arose from the Covid19 pandemic.

# 4 Methods and materials

## *4.1 Materials*

### *4.1.1 Preparation of kombucha*

Iodophor sanitiser used for sterilising utensils and container for kombucha brewing was purchased from Five Star Chemicals & Supply. Inc, USA. The TGY tea leaves were purchased from Hiland Tea Ltd, NZ. Honeydew honey has been generously provided by Steamland Honey Group Ltd, NZ. White sugar (sucrose) was purchased from Countdown, NZ. The original mother SCOBY used for this project was purchased from Get Cultured Limited, NZ.

### *4.1.2 Chemical characterization of kombucha*

Trolox, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), neocuproine, gallic acid, Folin-Ciocalteu reagent, methanol, gluconic acid and trimethylsilyl-imidazole (TMSI) were all sourced from Sigma-Aldrich, US. Hydrochloric acid (37%), sulphuric acid (97%), ammonium heptamolybdate, sodium carbonate and pyridine were purchased from Ajax Fine Chemicals, Australia. Ammonium acetate, glacial acetic acid, chloroform, acetone, butan-1-ol, toluene and acetonitrile were all obtained from Fisher Scientific, US. Monopotassium phosphate was purchased from Interchem, NZ. Iron (III) chloride and anhydrous sodium sulfate were sourced from Scharlau, Spain. Methyl chloroformate were obtained from Merck Millpore, US. Copper (II) chloride was purchased from VWR International, US. Sodium acetate trihydrate was sourced from LabServ, UK. Sodium bicarbonate was obtained from Panreac, Spain. Ultrapure water (MilliQ water) was produced using a Purite Select Fusion water deionisation unit from Suez Water Technologies & Solutions, US.

Amino and organic acid standards were sourced from as follow: amino acid standard solution, L-amino acids standards kit, malonic acid, maleic acid was sourced from Sigma Aldrich, US. Citric, tartaric and malic acids were purchased from VWR International, US. Succinic acid was acquired from Scharlau, Spain. Caffeine was purchased from Acros Organics, US. Maleic acid was purchased from Ajax Fine Chemicals, Australia.

Sugar standards were sourced from as follows: glucose was acquired from Fisher Scientific, US. Sucrose and galactose were sourced from VWR International, US. Fructose was obtained from Panreac, Spain. Lactose purchased from Ajax Fine Chemicals, Australia. Maltose was obtained from Scientific Supplies Ltd, NZ. Raffinose and erlose were purchased from Sigma Aldrich. Turanose and palatinose were sourced from Alfa Aeser, US. Melezitose and mannose were acquired from Acros Organics, US.

### *4.1.3 Microbiology*

For kombucha metagenomics: Proteinase K was purchased from Invitek, Germany. InstaGene Matrix was obtained from Bio-rad, US. AMPure XP Beads were obtained from Beckham Coulter, US. For conducting the DNA amplification steps: KAPA HiFi HotStart ReadyMix was acquired from Roche, Switzerland. The 16S and ITS1 rRNA forward and reverse primers were purchased from Integrated DNA Technologies, US. For quality control during the procedure, a Qubit dsDNA High Sensitivity Assay kit was purchased from Invitrogen and the Bioanalyzer DNA High Sensitivity Kit was purchased from Agilent, US. For preparing the amplicon library for sequencing, a MiSeq Reagent Kit v3 was obtained from Illumina, US.

For plate count experiments: nutrient agar (NA) and MRS agar powders were purchased from BD, US. Glucose and yeast extracts used for nutrient agar enrichment were both sourced from Fisher Scientific, US.

## *4.2 Preparation of kombucha*

Containers and utensils were firstly sterilised using Iodophor sanitiser to prevent contamination from airborne moulds or pathogenic organisms. The kombucha recipe in this study prepares a 2L batch based on Greenwalt et al.(2000) and Neffe-Skocińska et al.(2017) with slight modifications. 16g of TGY tea leaves were infused in 1L of boiling distilled water and steeped for 5 minutes. The brewed tea was rapidly cooled down to room temperature in an ice bath before 150g of honeydew honey was dissolved into the tea. The sweetened tea was then strained to remove the TGY leaves and mixed with 1L of pre-boiled distilled water in a sanitised glass jar. 30mL aliquots of the sweetened tea mixture were taken as Day 0 samples from each batch for kombucha characterisation (pre-fermentation). 50g of SCOBY was then inoculated into the vessel and covered with a cotton cheese cloth (Mad Millie, NZ) to prevent entry of foreign matter and allow the mixture to ferment aerobically at ambient temperature (22-25°C). Samples for monitoring the titratable acidity, major chemical products and microbiology were taken fresh and processed on the designated fermentation periods. Samples for characterising the kombucha were taken on the days 0, 10 and 14 of fermentation and stored at -20°C when not in use.

When not in use, SCOBYs were maintained in a “SCOBY Hotel”. The purpose of the SCOBY Hotel is to store and maintain the health of the SCOBYs when not in use, as well as to grow additional SCOBYs for kombucha samples preparation. A SCOBY hotel is made using the same steps as above with the following changes: 40g of TGY was infused in 2.5L of freshly boiled distilled water for 5 minutes. After 5 minutes have elapsed, 500g of sucrose was dissolved into the brewed tea and allowed to settle for another 5 minutes. The sweetened tea was then cooled down to ambient temperature using an ice water bath. Once cooled down, the

tea was strained and mixed with 2.5L of pre-boiled distilled water in a sanitised glass jar. Any SCOBY not in use were then inoculated into the vessel and covered with a cheese cloth. The SCOBY hotels were left to ferment ambient temperature. SCOBY hotels were remade with fresh sweetened tea once a month to ensure they are kept alive and healthy. SCOBYs were visually inspected and discarded if they appear dark brown and/or appear wrinkly.

### *4.3 Monitoring kombucha properties during fermentation.*

The progress of the kombucha's fermentation was monitored by measuring the titratable acidity and the concentrations of ethanol, acetic acid and gluconic acids of the kombucha. These variables were measured on days 3, 7, 10 and 14 of fermentation.

#### *4.3.1 Titratable acidity*

The acidity of the kombucha will be expressed as a percentage of titratable acidity (weight/volume). 10 ml aliquots of kombucha sample will be titrated with a standard alkali solution of 0.05M NaOH until pH 7 (endpoint) is reached. The pH was monitored using an Oakton pH 700 Bench Meter (Oakton Instruments, USA). The titratable acidity is then calculated using the following equation (Nielsen, 2010):

$$\%\ acid\left(\frac{wt}{vol}\right) = \frac{N \times V_1\ \times Eq\ wt}{V_2\ \times 10}$$

Where $N$ is the normality of titrant (mEq/mL), $V_1$ and $V_2$ are the volume of titrant required to reach the endpoint and volume of sample respectively (mL), and $Eq\ wt$ is the equivalent weight of the predominant acid which is assumed to be acetic acid ($Eq\ wt = 60.05$) from Nielsen, (2010)

#### *4.3.2 Concentration of ethanol, acetic acid and gluconic acid*

The concentration of ethanol, acetic and gluconic acids were monitored using a GC-FID. 1µl of kombucha samples in triplicate were analysed using a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu, Japan) equipped with a split/splitless injector, a flame ionisation detector (FID) and fitted with an Agilent Durabond – Fatwax Ultra Inert column which has 30m length x 0.25mm i.d. x 0.25µm film thickness (Agilent, USA). The temperature of the inlet and detector were both set to 250°C and a split ratio of 1:80. The temperature program starts at 40°C which is held for 10 min before increasing the temperature to 240°C at the rate of 10°C/min and held for 10 min. Nitrogen was used as the carrier gas supplying at a constant flow rate of 0.4 mL/min. Bench marking against standard curves was done to determine the concentrations of ethanol (0 to 5g/L, $R^2$=0.998), acetic acid (0 to 10g/L, $R^2$=0.998) and gluconic acid (0 to 5 g/L,

$R^2$=0.994). The alcohol concentration of the kombucha will be reported in percentage alcohol by volume (ABV).

## *4.4 Characterising the kombucha*

### *4.4.1 Antioxidant analysis*

Antioxidant analysis were carried out using three different assays to check for false positives in the changes to the antioxidant properties of kombucha over time.

#### *4.4.1.1 Phosphomolybdenum assay*

Phosphomolybdenum assay was performed as outlined in Ivanišová, Kačániová, Petrová, Frančáková, & Tokár (20s16). 1mL of kombucha diluted by a factor of 100 was mixed with 2.8mL $KH_2PO_4$ (0.1 M), 6 mL $H_2SO_4$ (1 M), 0.4 mL $(NH_4)6Mo_7O_{24}$ (0.1 M) and 0.8 mL of distilled water in glass vials. Once vortexed, the samples were incubated at 90°C for 120 mins. After incubation the samples were rapidly cooled in an ice water bath, then the absorbance measured at 700nm against a reagent blank using a GE Ultrospec 7000 Spectrophotometer. Results were expressed in g Trolox Equivalents (TE)/L kombucha through comparison to a standard curve (0 to 400mg TE/L, $R^2$=0.993)

#### *4.4.1.2 Ferric-ion reducing antioxidant power (FRAP)*

The FRAP assay involves the following procedures according to Benzie & Strain (1996) with slight modifications. Every 12mL of FRAP reagent was composed of 10mL acetate buffer solution (0.3M), 1mL of $FeCl_3$(0.02M) and 1mL of TPTZ (0.01M) dissolved in HCl (0.04M). 0.1mL of kombucha was diluted by a factor of 25, mixed with 2mL of FRAP reagent and 0.9mL of distilled water and then left to react for 5 minutes at ambient temperature. The absorbance of the samples was measured at 593nm against a reagent blank. Results were expressed in g TE/L kombucha through calibration against a standard curve (0 to 170mg TE/L, $R^2$ = 0.999).

#### *4.4.1.3 Cupric reducing antioxidant capacity (CUPRAC)*

The CUPRAC method is described in Özyürek, Güçlü, & Apak (2011) with slight modifications. 1mL of kombucha diluted by a factor of 50 was added to 1mL $CuCl_2$(0.01M), 1mL of $NH_4Ac$(1M), 1mL of neocuproine (0.0075M) and 0.1mL of distilled water. The solution was left to react for 5 mins at ambient temperature and its absorbance was measured at 450nm against a reagent blank. Measurements of antioxidant activity were expressed in g TE/L kombucha, by means of a standard curve (5 to 160 mg TE/L, $R^2$=0.993)

#### *4.4.1.4 Folin-Ciocalteu (FC) total phenolic assay*

The FC assay was carried out according to the method reported in Singleton, Orthofer, & Lamuela-Raventós (1999) with slight modifications. 1mL of diluted kombucha at a ratio of 1:50 was mixed with 500µL of FC reagent in a glass vial and left to react for 5 minutes at ambient

temperature. 1.5mL of 20%(m/v) $Na_2CO_3$ was then added to the solution and vortexed for 10 seconds. The glass vial was then wrapped in aluminium foil and the sample was left to react for 2 hours in the dark. Samples were then transferred into cuvettes and their absorbance was measured at 765nm against a reagent blank. Total phenolic content was expressed in mg gallic acid equivalents (GAE)/L kombucha, by means of a standard curve (0 to 40mg GE/L, $R^2$=0.999).

### *4.4.2 Colour*

Colour of the kombucha was measured according to the European Brewery Convention (EBC) method (Villa, 2019) with slight modification. Undiluted kombucha is centrifuged at 3000rpm at 4°C for 10 mins to remove suspended solids. The absorbance of the kombucha is then measured at 430nm against a water blank. Colour of the kombucha was expressed in EBC units and calculated according to the following formula:

$$C = A_{430} \times f \times 25$$

Where $C$ denotes the colour (EBC units), $f$ denotes the dilution factor (in this case 1), and $A_{430}$ is the absorbance at measured at 430 nm.

### *4.4.3 Amino acids and organic acids*

The alkylation derivatisation technique using methyl chloroformate (MCF) was employed to identify and quantify the amino and organic acid content of the kombucha. The procedure was based on the optimised protocol described (Villas-Bôas, Smart, Sivakumaran, & Lane, 2011). 1mL of kombucha sample was spiked with 20µL of $D_4$-alanine (0.001M) solution as internal standard (ISTD) inside silanised vials, and then freeze dried using a VirTis AdVantage Pro Freeze Dryer (SP Scientific, USA). The dried sample was resuspended in a mixture of 200µL of NaOH (1M) solution, 34µL of pyridine and 167µL of methanol. The amino and organic acids are then derivatised using 20µL of MCF which was added to the reagent mixture and vortexed for 30 seconds, this step was repeated to ensure all desired compounds have been derivatised. The MCF derivatives were suspended in 400µL of chloroform and 400µL of $NaHCO_3$ (50mM) solution. The aqueous phase was discard followed by the addition of 0.3g of anhydrous sodium sulphate to remove any remaining moisture. The remaining chloroform phase was analysed using a gas chromatogram coupled with a mass spectrometer (GC-MS). The organic acids and Sigma Aldrich amino acid standards were treated in the same manner and used to quantify their concentrations in kombucha by using standard curves.

GC-MS analysis was performed using an Agilent 7980B Gas Chromatograph System coupled with an Agilent 5977B Mass Spectrometer (Agilent, USA) with an Extractor Electron Ionization (EI) source. Samples were loaded onto an automated Gerstel Multipurpose Sampler (Gerstel,

Germany) for injection into the chromatograph. 1µL of the derivatised sample was injected with an inlet temperature of 290°C with a split ratio of 1:20. Separation of MCF derivatives was achieved using an Agilent DB-1701 column, 30m x 0.25mm i.d x 0.25µm film thickness (122-0732; Agilent USA), with helium as carrier gas at a flow rate of 1.1mL/min. The oven temperature was initially set at 45°C and held for 2 mins, this is followed by increasing the temperature to 180°C at a rate of 9°C/min and then held for 5 mins. Afterwards, the temperature was raised with a gradient of 40°C/min to 220°C and held for 5 mins, followed by increasing temperature to 240°C at the same rate and held for 11.5 minutes. Finally, the oven temperature was increased to 280°C at a rate of 40°C/min and held for 2 mins. The transfer line temperature to the MS detector was 250°C.

Concentrations of the amino and organic acids are expressed in µg/mL of kombucha and calculated by use of standard calibration curves. Compounds generally showed a linear relationship between the concentration of analyte and the electrical signal observed. $R^2$ values ranged between 0.990 to 0.999. Limit of detection (LoD) and limit of quantification (LoQ) was carried out for amino acids but not on organic acids. Calculations for the LoD and LoQ of amino acids are given in Appendix A

### *4.4.4 Sugar Analysis*

#### *4.4.4.1 Silylation for GC-MS*

The silylation derivatisation technique was used to analyse and quantify oligosaccharides sugars and content of the kombucha. The method utilised was based on the protocol from the following three papers: Garland, Goheen, Donald, McDonald, & Campbell (2009); Rodríguez-Sánchez, Hernández-Hernández, Ruiz-Matute, & Sanz (2011); and Troyano, Olano, Fernández-Díaz, Sanz, & Martínez-Castro (1991). 300µL of kombucha sample was spiked 20µL of xylitol solution (0.03M) as ISTD in a silanised vial and then freeze dried. Anhydrous sodium sulphate was then added to the dried solids to ensure all moisture has been removed. 500µL of TMSI is then added to the dried sample and vortexed gently such that the liquid does not touch the lid of the vial. 1mL of toluene is then added to the sample and lightly mixed. Samples were then left to incubate for 1 hour at 55°C to initiate the derivatisation process. After incubation, the samples were left to cool down to ambient temperature and the trimethylsilyl (TMS) derivatives were analysed by GC-MS.

The components of the GC-MS system and MSD parameters used analyse silylation derivatisation compounds are identical to the alkylation (Section 4.4.3), with several changes in processing parameters. 1µL of derivatized sample was injected with the inlet at 230°C and a 1:25 split ratio. Helium gas was the carrier gas with a flow rate of 1.1mL/min. The temperature program of the oven was identical to the one used in Villas-Bôas, Noel, Lane, Attwood, &

Cookson, 2006. The initial temperature of the oven was 70°C and held for 5 mins. The temperature was then increased to 179°C at a rate of 10°C/min followed by increasing the temperature to 180°C with a gradient of 0.5°C/min and held for 2 mins. This was followed by raising the temperature with a gradient of 10°C/min to 220°C which is held for 1 minute. Thereafter, the temperature was increased to 265°C at a rate of 2.5°C/min and then held for 1 minute. The temperature was then increased at a rate of 10°C/min to 280°C, held for 1 min, and then raised to 290°C at a rate of 1°C/min. The final temperature of 300°C was achieved by one final increase in temperature at a rate of 10°C/min.

#### *4.4.4.2 Major Sugars using high performance liquid chromatography (HPLC)*

Quantification of sugars found in kombucha, were carried out using HPLC in conjunction with an evaporative light scattering detector (ELSD).

Extraction of non-polar organic compounds was carried out prior to sample analysis. 1mL of kombucha was diluted by a factor of 25 using MilliQ Water. A second set of samples for identifying oligosaccharides present in the kombucha was made at a dilution factor of 10. Honeydew honey samples were also prepared by diluting 1g of honeydew honey in 50mL of MilliQ water. 3mL of the diluted sample was spiked with 60µL of 0.05g/mL xylitol solution as ISTD. Non-polar organic material was removed from the kombucha samples using three washes with 3mL of chloroform and vortexed for 30 seconds before they were centrifuged at 4000rpm for 10 minutes at 10°C. The bottom chloroform phase was removed between each wash cycle. 1.2mL of the final aqueous phase is then centrifuged at 10000rpm to separate any remaining solids in the aqueous phase. 1mL of the aqueous phase was used for HPLC analysis.

Analysis of major sugars were carried out using a Shimadzu LC-10AT$_{vp}$ HPLC (Shimadzu, Japan) equipped with a Shimadzu FCV-10AL solenoid valve (Shimadzu, Japan), Phenomenex Degassex Model 4400 Degasser (Phenomenex, USA) and a Shimadzu SIL-20AC autosampler (Shimadzu, Japan). 10µL of each sample was injected into a 50µL sample loop (228-21038-91; Shimadzu, Japan). Analytes were eluted under isocratic conditions using a mobile phase of 80:20 v/v acetonitrile: MilliQ water solution at a flow rate of 0.5mL/min. Separation of the sugars was achieved using a Phenomenex Luna Omega Sugar column (3µm particle size, 100Å pore size, 250mm long, 4.6mm I.D; Part# 00G-4775-E0; Phenomenex, USA), equipped with a Security Guard Cartridge (AJ0-4495; Phenomenex, USA) held at ambient temperature. Detection of analytes accomplished using an Agilent 385-ELSD. The nebulizer and evaporator temperatures were 50°C and 80°C respectively with the nebulizing gas ($N_2$) flow rate of 1.2 standard litre per minute (SLM). Concentrations of sugars were expressed in mg/mL kombucha by means of a standard curve.

The HPLC-ELSD method for quantification of sugars were validated by determining the linearity, precision and accuracy of the method. Quantification the sugar concentration was accomplished by means of a standard curve between 250 to 2500µg/mL. All sugars examined exhibited a linear response within the concentration range with $R^2$ values ranging between 0.9945 to 0.9981. The recovery values for the extraction process method ranged between 100.8% and 120.7%.

## *4.5 Microbial community analysis on fermented kombucha*

### *4.5.1 Kombucha metagenomics*

Next-Generation Sequencing (NGS) was conducted on the SCOBY and the kombucha beverage to identify the bacteria and yeasts present. DNA samples were extracted on days 7, 10, 14, 17 and 21 to monitor any changes to the microbial community in the kombucha as it ferments.

#### *4.5.1.1 DNA extraction*

DNA extraction followed the method outlined in the Zymo Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, 2019) with slight modifications. 0.1g of SCOBY in triplicate were taken for DNA extraction on days 7, 10, 14, 17 and 21 of fermentation. The 1mL kombucha sample was centrifuged at 15000RPM for 3 minutes to generate a pellet, the supernatant was discarded. Samples were suspended in 200µL of Instant Gene Matrix (Bio-rad, USA) along with 20µL of Proteinase K (Invitek, Germany) for lysis. The mixutre was vortexed and then incubated at 55°C overnight. After incubation the samples were vortexed and incubated at 100°C for 8 minutes to denature the Proteinase K. Finally samples were centrifuged at 15000RPM for 3 minutes, and the DNA supernatant was transferred to a new tube leaving behind the Chelex resin pellet. The concentration of the extracted DNA samples were then measured using a Qubit Fluormeter following standard procedures.

#### *4.5.1.2 DNA amplification*

The DNA amplification and indexing for NGS follows the protocol outlined in Illumina, (2014), with some slight modifications. Target genes were amplified through polymerase chain reaction (PCR). PCR amplification was conducted using 16S and ITS DNA gene specific primers in order to identify bacteria and yeast respectively in the kombucha. Each PCR mix consists of 12.5µL of KAPA Hifi Hotstart Readymix, 2.5µL of the forward and reverse primers (1µM), 5µL of MilliQ water and 2.5µL of DNA sample. Primer sequences are as follows: 16S forward CCTACGGGNGGCWGCAG; 16S reverse GACTACHVGGGTATCTAATCC; ITS1 forward CTTGGTCATTTAGAGGAAGTAA; ITS1 reverse GCTGCGTTCTTCATCGATGC. The PCR amplification was conducted using Eppendorf Mastercycler Pro (Eppendorf, Germany) following the thermal cycle:. initialization temperature of 95°C, 30 cycles of: denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and elongation 72°C for 30 seconds,

final elongation step at 72°C for 5 minutes and 4°C PCR products were purified using AMPure XP beads following a modified version of the standard procedure as detailed in Section 4.5.1.3.

#### *4.5.1.3 Purification of DNA samples*

20µL of room temperature AMPure XP beads was mixed with each amplified DNA sample (25µL) by pipetting up and down 10 times on the PCR plate. The DNA samples were left for 5 minutes to allow the DNA to bind onto the magnetic beads. The PCR plate was then transferred onto a magnetic stand to separate the beads from the supernatant, after which the supernatant is discarded. 80µL of 80% ethanol was then added into each sample to remove excess primers, TAQ mix and polyethylene glycol (from AMPure XP solution which helps precipitate the DNA out of solution) from the DNA sample. The ethanol was left for one minute and then removed, the ethanol wash step was then repeated to ensure DNA samples were properly cleaned. The magnetic beads were left to air dry under ambient conditions for 5 minutes. The PCR plate was then taken off the magnetic stand, and the DNA eluted in 17.5µL of MilliQ water. Mixing of the water and beads was accomplished by pipetting the water over the beads repeatedly until the solution turns brown, the solution was then left for 3 minutes to ensure proper re-suspension of the DNA in solution. The PCR plate was transferred back onto the magnetic stand to separate the beads from the MilliQ water. Once separated, 15µL of each DNA sample was removed for further processing. Concentration of the purified amplicon libraries were measured using a Qubit Fluorometer following standard procedures.

#### *4.5.1.4 Sequencing Preparation*

Sequencings adapters and index tags for sample identification were added onto the amplicons by a second PCR. Samples were pooled together by taking a 5µL aliquot of each amplicon library. The pooled library was then purified using AMPure XP beads and quality checked using a Qubit Fluorometer and Agilent BioAnalyser High Sensitivity DNA Kit to quantify the concentration of the pooled libraries. This pooled library was diluted to 4nM and denatured following standard protocol A of the Illumina *MiSeq Denature and Dilute Libraries Guide*. The denatured library was then loaded onto an Illumina MiSeq system at a concentration of 10pM with 15% PhiX control using a MiSeq Reagent Kit v3 (600-cycle) (MS-102-3003)

### *4.5.2 Kombucha plate count*

Plate count experiments for kombucha were conducted to quantify the probiotic content of the kombucha drink. The spread plate technique was selected in favour of the pour plate technique due to the described presence of obligate aerobic bacteria in the kombucha from literature and possible killing of the bacteria from contact with molten agar. Pre-pouring of agar plates, serial dilution and spread plating were all carried out using aseptic technique and in a laminar flow cabinet.

Serial dilutions of the kombucha were made by diluting 1mL of kombucha in 9mL of 0.1% (w/v) Peptone Water. Serial dilutions up to $10^{-4}$ were prepared by taking 1mL of the higher dilution and adding it to 9mL of 0.1%(w/v) peptone water for each subsequent dilution. Two types of plates were used for quantifying the probiotic content of the kombucha, De Man, Rogosa and Sharpe (MRS) agar and a modified Nutrient Agar (NA) plate. The MRS agar was prepared using Difco Lactobacilli MRS Agar (BD, 288210) according to their instructions. The modified NA was prepared by dissolving 23g of NA powder (Difco, 234000), 2g yeast extract and 100g of glucose into 1L of de-ionised water. Sterilisation of the plates was achieved by autoclaving. NA agar was autoclaved at 118°C for 10 minutes, whereas the MRS agar is autoclaved at 121°C for 15 minutes. 0.1mL of each kombucha dilution from $10^{0}$ to $10^{-4}$ was inoculated onto prepared agar plates in triplicate using spread-plate technique. Plates were then inverted and incubated at 25°C for 72 hours. Probiotic concentrations were expressed in log number of colony forming units per litre of kombucha (log(cfu)/ mL kombucha).

To determine the species of bacteria cultured on the plate, the cultured bacteria from counted plates was isolated by streak plating on fresh agar plates. Once isolated to single colonies, a single colony was taken for Gram staining and observed under a light microscope at 100x magnification under immersion oil. Observations made under the light microscope are compared to accounts of shape and stain colour of expected species found in literature.

### *4.6 Data collection and statistical analysis*

All chemical analysis data were processed using R version 3.6.3. One-way analysis of variance (ANOVA) test with Tukey pairwise comparison was used to confirm statistical differences for all the chemical analysis of the kombucha samples. The level of significance was set to p-value <0.05. All GC-MS data was processed using Agilent MassHunter Quantitative Analysis MS vB.07.01, quality of the data was checked using Agilent MassHunter Qualitative Analysis vB.07.01. All DNA sequence data were analysed following the MiSeq Illumina pipeline.

# 5 Results and discussions

## *5.1 Kombucha properties during fermentation*

### *5.1.1 Titratable acidity*

The titratable acidity of the honeydew honey kombucha compared to kombucha made with sucrose over the fermentation period of 14 days were plotted on Figure 2.

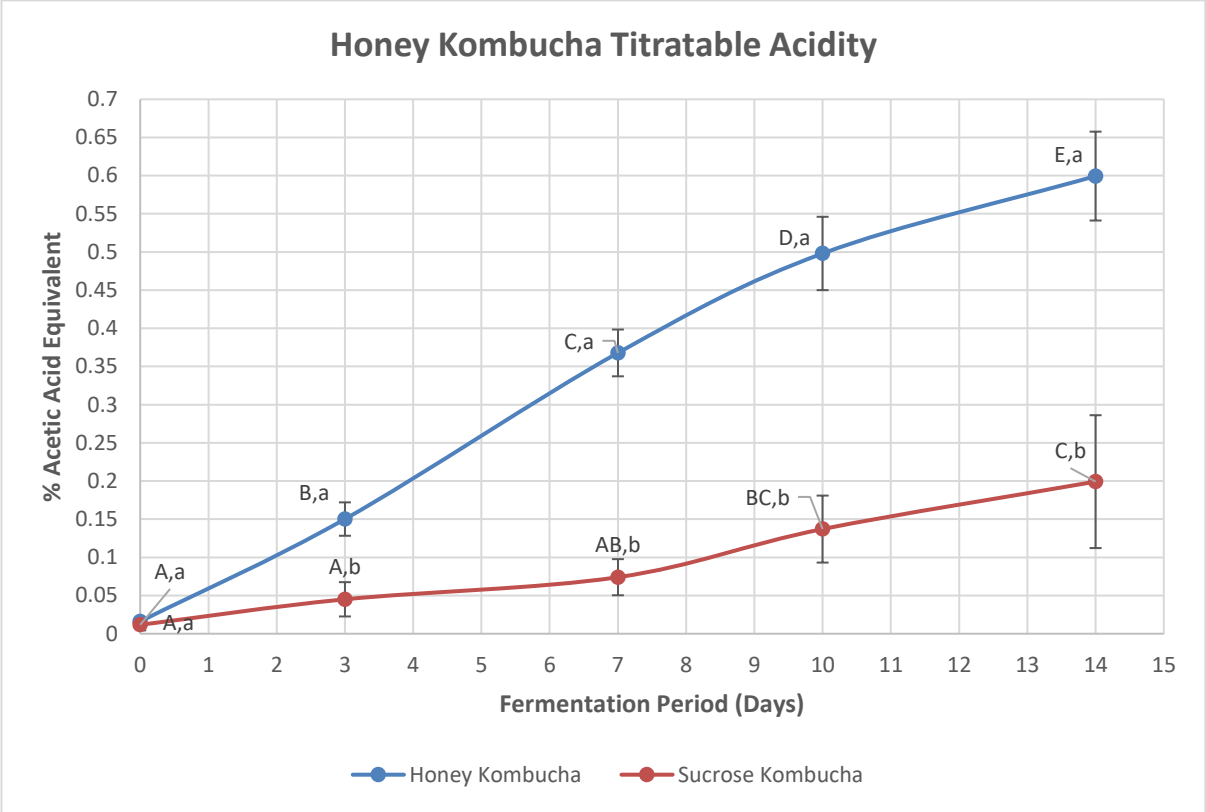


Figure 2: Change in Titratable Acidity of Kombucha with Fermentation Time
Common capitalized letters within each type of kombucha which do not differ statistically by Tukey's test (p-value < 0.05). Common lower-case letters from the same fermentation period do not differ statistically by Tukey's test (p-value < 0.05). Error bars represent standard deviation of the means.

The initial titratable acidity of the liquid before inoculation of the SCOBY was 0.016 ± 0.003% acetic acid equivalent (ACE) in the honey kombucha, which is slightly but not significantly higher than 0.011±0.007% ACE in the sucrose kombucha. The slightly higher titratable acidity in the honey kombucha can be attributed to the presence of organic acids found in the honeydew honey (Da Silva, Gauche, Gonzaga, Costa, & Fett, 2016). The average titratable acidity of honey kombucha increased at a much higher rate compared to the sucrose kombucha. Starting at day 3 of fermentation, significant difference in the titratable acidity was observed between the honey and sucrose kombuchas. This difference continued to increase as the kombucha fermented until day 14 when titratable acidity measured were 0.599 ± 0.0582% ACE and 0.199

± 0.087% ACE for the honey and sucrose kombucha respectively. The increase of titratable acidity is indicative of the formation of acidic compounds as the kombucha ferments. This is mainly in the form of acetic acid and gluconic acid which give the beverage it's sour taste (C. Chen & Liu, 2000). This suggests that the kombucha fermented with honeydew honey progress much faster compared to the kombucha prepared with sucrose. The presence of these acetic and gluconic acids are discussed in Section 5.1.2. Other organic acids were also produced during the kombucha fermentation, as shown in Section 5.2.4. The increase in titratable acidity indicates that the kombucha takes on a progressively sour taste as it ferments.

### *5.1.2 Major fermentation products*

The main metabolites from the kombucha fermentation process are ethanol, acetic acid and gluconic acid. These compounds were monitored using a GC-FID and the results are recorded in Table 1.

Table 1: Concentration of major metabolites in honeydew honey kombucha.

| Sample | Ethanol (g/L Kombucha) | Alcohol % (v/v) | Acetic Acid (g/L Kombucha) | Gluconic Acid (g/L Kombucha) |
|---|---|---|---|---|
| **Day 3** | $0.314 \pm 0.271^{A}$ | $0.039 \pm 0.034$ | $1.693 \pm 0.476^{A}$ | $1.769 \pm 0.808^{A}$ |
| **Day 7** | $0.777 \pm 0.302^{B}$ | $0.098 \pm 0.038$ | $2.292 \pm 0.866^{AB}$ | $3.509 \pm 1.561^{AB}$ |
| **Day 10** | $1.242 \pm 0.430^{BC}$ | $0.157 \pm 0.054$ | $2.969 \pm 0.851^{B}$ | $4.017 \pm 1.944^{AB}$ |
| **Day 14** | $1.325 \pm 0.469^{C}$ | $0.168 \pm 0.059$ | $4.191 \pm 0.828^{C}$ | $4.890 \pm 1.256^{B}$ |

Values represent the mean of triplicates, ± depicts standard deviation of the means.
Different superscript letters in the same column for each compound have statistically different means by Tukey's test, p-value < 0.05

The concentration of all major metabolites was found to increase as the kombucha ferments. This result was expected as the metabolic pathways of the yeasts and bacteria found in the SCOBY produce these compounds (Reiss, 1994). The yeasts found in the SCOBY consume the sugars, provided by the honeydew honey to produce ethanol and carbon dioxide. The *Komagataeibacter* and other acetic acid bacteria metabolize the fructose and glucose to produce into acetic acid and gluconic acid respectively. The alcohol content of the kombucha increased over time to 1.325g/L kombucha. Assuming the density of ethanol to be 789g/L, this gives an alcohol volumetric percentage of 0.168%. This is below the 0.5% threshold set by Ministry of Primary Industries NZ (2018), which allows the kombucha to be labelled as a non-alcoholic within New Zealand. The concentrations of the acetic and gluconic acids concentrations measured were lower for the same fermentation period than those reported by Chakravorty et al., (2016) however the ethanol concentrations measured was significantly higher for the same fermentation periods. This may be attributed to different compositions of bacteria and yeasts

species present in the SCOBYs utilised between the two studies, however this study was unable to complete a microbial community analysis for yeast species. Chen & Liu (2000) reported that ethanol tends to reach a maximum value between 10 and 15 days of fermentation before decreasing as it is further hydrolysed by bacteria present to produce more acetic acid. A similar trend was observed where the honey kombucha had no significant increase in ethanol concentration from day 10 to day 14, along with a larger increase in acetic acid concentration. Gluconic acid formation in the kombucha was found to be quite slow, with no significant difference between adjacent measurements. This could be attributed to the lower fraction of *Gluconobacter* species present in the SCOBY as discussed in Section 5.3.1. Chen & Liu (2000) reported that gluconic acid was not produced until after six days of fermentation had passed. Since the day 0 data point was not measured, we cannot discern whether this is the case for the honeydew honey kombucha as gluconic acid is also present in honey (Pita-Calvo & Vázquez, 2017).

## *5.2 Characterisation of Kombucha*

### *5.2.1 Antioxidant Properties*

The three assays conducted were CUPRAC observes the reduction of Cu(II) ion to Cu(I) ion, whereas the FRAP assay involves the reduction of Fe(III) ion to Fe(II) ion by the antioxidant compounds in the kombucha. The phosphomolybdenum assay looks at the total antioxidant activity though the reduction of Mo(VI) ions to Mo(V) ions. The FC assay is used to quantify the total phenolic content of the kombucha. The antioxidant activities of the honeydew honey kombucha is presented in Table 2.

Table 2: Antioxidant activity of honeydew honey kombucha.

| **Sample** | **CUPRAC** **g TE/L Kombucha** | **FRAP** **g TE/L Kombucha** | **Phosphomolybdenum** **g TE/L Kombucha** | **Folin-Ciocalteu** **mg GAE/L Kombucha** |
|---|---|---|---|---|
| **Day 0** | $1.75\pm0.46^{A}$ | $0.86\pm0.22^{A}$ | $19.9\pm4.6^{A}$ | $0.141\pm0.026^{A}$ |
| **Day 10** | $2.74\pm0.38^{B}$ | $1.05\pm0.31^{B}$ | $30.9\pm4.9^{B}$ | $0.227\pm0.032^{B}$ |
| **Day 14** | $3.63\pm0.68^{C}$ | $1.15\pm0.30^{B}$ | $40.9\pm6.2^{C}$ | $0.243\pm0.033^{B}$ |

Values represent the mean of triplicates, ± depicts standard deviation of the means.
Different superscript letters in the column for each assay have statistically different means by Tukey's test, p-value < 0.05
Units: Trolox equivalent (TE). Gallic acid equivalent (GAE)

The antioxidant activity of the honeydew honey kombucha was found to generally increase with kombucha fermentation times. Similar observations were shared in studies conducted by Chu & Chen (2006), however their study used different assays such as DPPH radical and linoleic acid oxidation to assess the antioxidant activity of the kombucha. Some degree of variance in the

antioxidant activities were detected in kombucha fermented for the same duration. This is likely due to variations in the fermentation progress of each kombucha batch as they were produced at different times throughout the year. The variation in how the fermentation proceeds between different batches can also be observed in the changes to the sugar composition of the kombucha over time as detailed in Section 5.2.5.2.

The total phenolic content was found to increase with fermentation time, this observation is shared with several other studies (Chu & Chen, 2006; Sun et al., 2015). The explanation to the increase of phenolic content involved tea polyphenols being broken down into smaller molecules. From literature, catechins which are the primary polyphenols found in honey and tea and are stable under acidic conditions (Z. Y. Chen, Zhu, Tsang, & Huang, 2001). Chu & Chen, (2006) theorised that the phenomena shared similarity to the enzymatic degradation of phenolic compounds in the colon by bacteria action. The metabolic pathway for bacteria in the colon, cleaves the carbon ring in polyphenols to release phenolic acids. Bacterial species in the SCOBY can secrete a similar enzyme capable of catalysing the degradation of catechins, theaflavins and thearubigins into phenolic acids which are associated with the increase in phenolic content.

### *5.2.2 Colour Changes*

The colour change of the kombucha from the start and finish of fermentation in given on Figure 4. The colour of the honeydew honey kombucha starts out brown and becomes progressively lighter turning into a yellow colour after 14 days of fermentation. On day 0 of fermentation, the colour of the kombucha had an average of 10.46 EBC units. The initial brown colour of the kombucha likely came from the amber colour of the honeydew honey. The colour of the honey is attributed to the phenolic compounds, mineral content and Maillard rection products found in the honey (Can et al., 2015). The brewed TGY tea colour is a subtle brown which is attributed to polyphenols in tea leaves such as catechins, thearubigins, theaflavins and bisflavanols (Graham, 1992). Following 10 days of fermentation the mean colour measurement of the kombucha decreased to 6.16±1.69 EBC units. On day 14 the mean colour measurement of the kombucha decreased further to 5.79±1.09 EBC units, however this was not statistically different from the measurements for day 10 of fermentation. It should be noted that the EBC method is primarily used for determining the colour of beer, therefore a valid colour comparison to other kombucha drinks could not been found in literature.

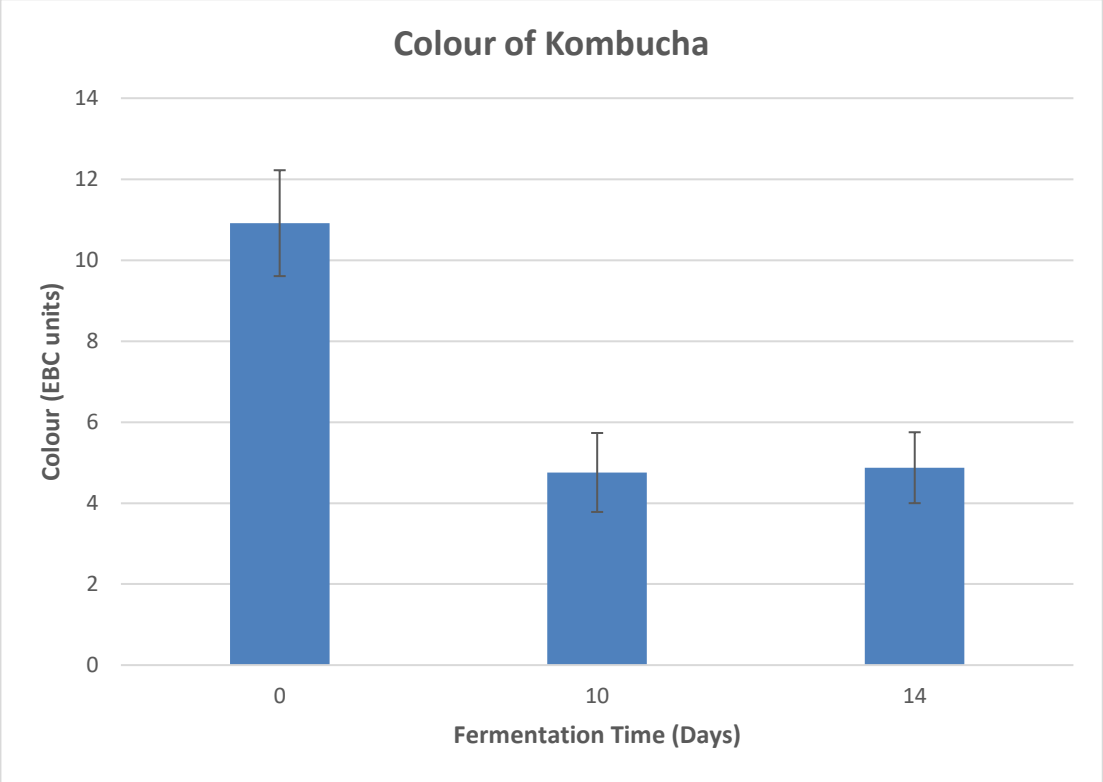


Figure 3: Colour change of honeydew honey kombucha.
Measurements of each sample was carried out in triplicates across 12 replicates of the sample. Error bars represent standard deviation of the means. Common capitalized letters do not differ statistically using the Tukey's test (p-value < 0.05)

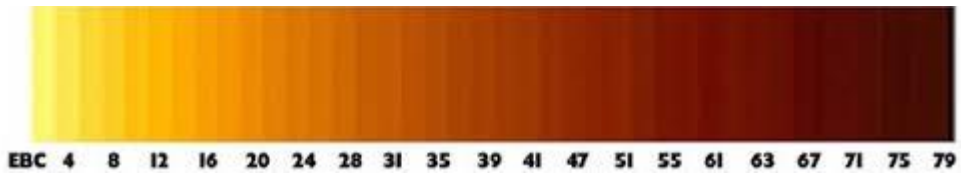


Figure 4: Colour scale of EBC units.

The discolouration of the kombucha appears to conflict with the total polyphenol contents assay conducted from Section 5.2.1, as the total polyphenol content increased despite the kombucha became lighter. The colour of the kombucha is attributed to the phenolic compounds provided by the tea and honey such as catechins, thearubigins, theaflavins, lutelolin, kaempferol, apigenin. As detailed previously, it is theorised that the phenolic compounds are broken down into smaller molecules by some unknown enzyme secreted by the bacteria present in the SCOBY resulting in phenolic acids (Chu & Chen, 2006). As these resulting compounds are colourless, the kombucha becomes lighter in colour and the measured intensity of the colour will decrease.

*5.2.3 Amino acid composition*

The free amino acids found in the honeydew honey and kombucha, using the MCF method are presented in Table 3. From our results: glycine, threonine, serine, methionine were not detected in neither the honeydew honey nor the kombucha. Lysine, histidine and tyrosine responses obtained from MCF was inconsistent. While the peaks were discernible, the calibration curve did not give a linear response meaning they could not be quantified.

Table 3: Amino acid composition of honeydew honey and kombucha.

| Amino Acid | Honeydew Honey (mg/100g Honey) | Honey Kombucha (µg/mL Kombucha) | | |
|---|---|---|---|---|
| | | Day 0 | Day 10 | Day 14 |
| **Alanine** | 0.00±0.00* | 3.608±0.582$^{A}$ | 1.465±0.900$^{B}$ | 0.972±1.084$^{B}$ |
| **Glycine** | ND | ND | ND | ND |
| **Valine** | 1.329±0.067 | 1.273±0.246$^{A}$ | 0.157±0.264$^{B}$ | 0.154±0.207$^{B}$ |
| **Leucine** | 0.00±0.00* | 0.479±0.135$^{A}$ | 0.061±0.076$^{B}$ | 0.054±0.047$^{B}$ |
| **Isoleucine** | 0.00±0.00* | 0.733±0.158$^{A}$ | 0.107±0.158$^{B}$ | 0.103±0.124$^{B}$ |
| **Proline** | 36.086±2.084 | 31.840±7.042$^{A}$ | 21.934±3.848$^{B}$ | 21.174±4.284$^{B}$ |
| **Threonine** | ND | ND | ND | ND |
| **Aspartic Acid** | 0.00±0.00* | 2.038±0.956$^{A}$ | 0.111±0.132$^{B}$ | 0.112±0.126$^{B}$ |
| **Serine** | ND | ND | ND | ND |
| **Glutamic Acid** | 6.116±0.057 | 2.190±1.597$^{A}$ | 0.062±0.061$^{B}$ | 0.082±0.059$^{B}$ |
| **Methionine** | ND | ND | ND | ND |
| **Phenylalanine** | 6.095±0.0816 | 1.202±0.810$^{A}$ | 0.061±0.041$^{B}$ | 0.045±0.022$^{B}$ |
| **Lysine[1]** | ND | ND | ND | ND |
| **Histidine[1]** | ND | ND | ND | ND |
| **Tyrosine[1]** | ND | ND | ND | ND |

Values represent the mean of triplicates, ± depicts standard deviation of the means.

Superscripts with the different letters within the same row have statistically significant differences, p-value < 0.05.

ND = Not detected in the sample.

* Peak was detected in extracted ion chromatogram but below the range of the standard curve.

[1] Denotes compounds with poor responses in standard curve, low or non-linear regression.

The amino acids composition of the honeydew honey is primarily made up of proline with a concentration of 36.086mg/100g honey. This result is consistent with literature in which proline is the predominant amino acid found in honey (Pérez et al., 2007). The concentrations of proline measured from our experiment tended to be lower than values found in literature. Pérez et al., (2007) reported a mean concentration of 72.68mg/100g in Spanish honeydew honey , Meda et al. (2005) reported up to 121.6mg/100g in Burkina Fasan honeydew honey and Purcărea et al.,

(2014) documented a proline concentration of 98.3mg/100g from Polish honeydew honey. As such the lower proline concentration can be explained by regional variations between the honeys.

The other amino acids quantified in the honeydew honey were valine, glutamic acid and phenylalanine. Amino acids which were detected in the honeydew honey, with the appropriate target ion, qualifier ion ratio and retention times, were alanine, leucine, isoleucine and aspartic acid. These could not be quantified by the standard curve as the concentration used for the analysis is likely below the limit of quantification. Nevertheless the presence of these amino acids were also found in Pérez et al. (2007), although in much lower concentrations. Glutamic acid concentration from our experiments was found to be 6.12mg/100g compared to 27.6mg/100g reported; phenylalanine was 6.09mg/100g compared to 8.42mg/100g; and valine was 1.33mg/100g compared to 3.75mg/100g reported by Pérez et al. (2007).

The concentration of all amino acids was found to have decreased significantly from day 0 to day 10 of the fermentation. Majority of the amino acids were contributed into the kombucha by honeydew honey. For instance, the theoretical proline concentration in the kombucha at day 0 would be 27.79µg/mL kombucha, which is a under the measured 31.84µg/mL kombucha. The decrease in amino acid concentration with fermentation time can be attributed to the metabolization of the amino acids by the bacteria in the SCOBY through the tricarboxylic cycle (TCA). TCA is a metabolic pathway that bacteria cells use to store and release energy (Krebs & Johnson, 1937). In particular for Gram negative bacteria, they employs a bifunctional enzyme called proline utilization A which is capable of metabolising proline into glutamate (Arentson, Nikhilesh, & Becker, 2012). As observed in Section 5.3.1, *Komagataeibacter.spp* are the primary genus of bacteria found in the SCOBY and are known to be Gram negative (Komagata, Iino, & Yamada, 2014). Glutamate can be oxidised into α-ketoglutarate (Moreadith & Lehninger, 1984) which is an intermediate compound of the TCA. The additional intermediate compounds for TCA leads can accelerate the metabolism of the bacteria in the SCOBY thereby increasing the rate of fermentation. Matsuoka, Tsuchida, Matsushita, Adachi, & Yoshinaga, (1996) reported that a corn steep liquor media containing amino acids, increased the cellulose production from *Komagataeibacter Sucrofermentans* by 50%. This effect was most prominent with methionine in their study; however, methionine was not detected in the honeydew honey or the kombucha in this study. As discussed in Section 5.3.1, *komagataeibacter* was the primary genus of bacteria found in the SCOBY, which metabolises the proline and other amino acids to form glutamate. The concentration of all amino did not differ significantly between day 10 and day 14 of fermentation. This can be due to the decreased rate of fermentation.

### *5.2.4 Organic acid composition*

The minor organic acids that were detected in the honeydew honey kombucha were malonic, maleic, lactic, succinic, malic, citric and tartaric acids. Other minor organic acids that were also detected but not quantified in this study are lactic, fumaric, benzoic, citraconic, aconitic and syringic acids because the reference standards were not available. In the honeydew honey, the following acids were detected fumaric, lactic and quinic acids which have also been reported in literature (Brugnerotto et al., 2019). The concentrations of minor organic acids analysed and caffeine are presented in Table 4.

Table 4: Organic compound composition of honeydew honey and kombucha.

| **Organic Compound** | **Honeydew Honey (mg/100g Honey)** | **Honey kombucha (µg/mL kombucha)** | | |
|---|---|---|---|---|
| | | **Day 0** | **Day 10** | **Day 14** |
| **Malonic Acid** | 6.64±0.12 | $2.21\pm0.50^{A}$ | $5.57\pm1.63^{B}$ | $6.55\pm1.97^{B}$ |
| **Maleic Acid** | 28.77±1.57 | $6.71\pm0.95^{A}$ | $7.89\pm2.01^{A}$ | $7.83\pm2.44^{A}$ |
| **Succinic Acid** | $0.00\pm0.00^{*}$ | $39.56\pm7.25^{A}$ | $134.96\pm41.98^{B}$ | $190.88\pm88.30^{C}$ |
| **Malic Acid** | 99.98±7.23 | $69.03\pm14.52^{A}$ | $110.47\pm38.55^{B}$ | $121.04\pm53.12^{B}$ |
| **Citric Acid** | 6.54±4.85 | $29.12\pm16.72^{A}$ | $70.31\pm36.34^{B}$ | $76.42\pm49.12^{B}$ |
| **Tartaric Acid** | ND | ND | $28.51\pm22.03^{A}$ | $17.49\pm20.41^{A}$ |
| **Caffeine** | ND | $341.39\pm337.15^{A}$ | $539.09\pm549.74^{A}$ | $426.44\pm590.13^{A}$ |

Values represent the mean of triplicates, ± depicts standard deviation of the means.
Superscripts with the different letters within the same row have statistically significant differences, p-value < 0.05.
ND = Not detected in the sample.
* Peak was detected in extraction ion chromatogram but below the range of the standard curve.

The mean concentration of organic acids tended to increase as the kombucha ferments, with succinic acid having the highest concentration after 14 days (190.88µg/mL kombucha). Malonic, succinic malic and citric acids experience significant increases in concentration from day 0 to day 10 during fermentation. This indicates that these compounds were produced during the fermentation process except for maleic acid. These organic acids are known to be intermediate compounds produced within TCA (Krebs & Johnson, 1937), which explain the increase in concentration of these acids. No significant changes were observed to the concentration of maleic acid as it is not an intermediate within TCA. All organic acid compounds did not exhibit significant increase in concentration between fermentation day 10 and day 14 except for succinic. This indicates that succinic acid is the primary metabolite for the kombucha as far more succinic acid were produced within a smaller duration of fermentation. There was extremely high variability in the measurements of caffeine concentration using this technique, so no conclusions can be made on its contribution to kombucha fermentation.

The concentrations of organic acids found in this study vary from the values found in literature. Kaewkod, Bovonsombut, & Tragoolpua (2019) reported that succinic acid was detected in kombucha made from black tea, but not from green or oolong tea, though the exact type of tea leaf used was not specified. In contrast, succinic acid could be detected in kombucha prepared in our experiment which used TGY, which is a type of oolong tea. Neffe-Skocińska, Sionek, Ścibisz, & Kołożyn-Krajewska (2017) reported higher citric acid concentrations between 44µg/mL kombucha to 86µg/mL kombucha from day 0 to day 10 of fermentation, utilising a mixture of green and black teas, 100g/L sucrose and incubation temperature of 20, 25 and 30°C. Jayabalan, Marimuthu, & Swaminathan (2007), could not detect citric acid in majority of their kombucha samples. The large disparity in concentration of organic acids from this study and literature can be attributed to different substrates used during kombucha fermentation and the microbiological difference between kombucha starter cultures.

The maleic acid concentration in the honeydew honey was higher than what can be considered reasonable. Maleic acid found in food primarily comes from an unapproved food additive, maleic anhydride. The tolerable daily intake (TDI) of maleic acid for 60kg adult is 30mg/day (Baoren, 2014). Exceeding these limits leads to risk of kidney damage. At the concentration measured, consuming around 105g of the honeydew honey would exceed the TDI. Furthermore, when utilising the concentration of maleic acid found in the honeydew honey, the theoretical concentration in the kombucha on day 0 would be at least 22.1µg/mL kombucha. This is three times larger than the concentration of maleic measured on day 0 in the honeydew honey kombucha. As such we believe that it is likely experimental error, possibly contamination, to be the cause of the high maleic acid concentration found in the honeydew honey. The experiment on honeydew honey and the kombucha should be repeated to verify the concentration of maleic acid.

A succinic acid peak was detected within the honeydew honey with the correct target ion, qualifier ion ratios and retention time. However, the succinic acid peak area ratio with the internal standard in the honey sample was lower than ratio found in lowest point of the calibration curve. Due to time constraints, the limit of detection and quantification for the organic acids were not conducted. As such we can infer that succinic acid is likely present in the honeydew honey, however only in a trace amounts.

### *5.2.5 Sugar Composition*

#### *5.2.5.1 Silylation for GC-MS*

The silylation technique using TMSI coupled with GC-MS was initially used to test for the sugar composition and glucuronic acid in the honeydew honey and kombucha. This method

proved difficult particularly with reliability. The first problem encountered was eventual loss of signal from chromatograms generated by the method after several successful experimental runs. The solvent used to dissolve the TMS-derivatives was changed from heptane to toluene, as both solvents are non-polar, and this resolved the problem of loss of signal. However, after this change was made, carry over peaks for TMSI, imidazole and TMS-derivatives were found. The carry-over of TMS-derivatives will lead in inaccurate results in terms of what sugars are in the samples and the concentration of the samples, while the imidazole and TMSI carry over would interfere with other experiments conducted on the GC-MS. As a solution for eliminating the carry over peaks could not be found within the time constraints of the study, all sugar analysis was placed onto the HPLC-ELSD system. Full details with chromatograms explanation of the issues encountered are found in Appendix B

#### *5.2.5.2 HPLC-ELSD*

The major sugars typically found in kombucha are typically fructose, glucose and sucrose. The concentration of these sugars in the honeydew honey kombucha are presented in.

Table 5: Major sugars in honeydew honey analysed on HPLC-ELSD.

| **Sugar** | **Honeydew Honey (g/100g honey)** | **Sugar concentration in kombucha (mg/mL Kombucha)** | | |
|---|---|---|---|---|
| | | **Day 0** | **Day 10** | **Day 14** |
| **Fructose** | 32.33±4.24 | $25.21\pm1.93^{A}$ | $21.40\pm4.25^{B}$ | $18.91\pm4.63^{B}$ |
| **Glucose** | 19.69±2.63 | $15.71\pm1.24^{A}$ | $8.68\pm3.11^{B}$ | $6.50\pm2.21^{C}$ |
| **Sucrose** | ND | ND | ND | ND |

Values represent the mean of triplicates, ± depicts standard deviation of the means.

Superscripts with the different letters within the same row have statistically significant differences, p-value < 0.05.

Retention times are taken from calibration standard run with kombucha standards.

ND = Not detected in the sample.

The concentrations of glucose and fructose shown on Table 5 were found to decrease over time as the fermentation of the kombucha progresses. This was expected as fructose and glucose are metabolised by the bacteria and yeasts in the SCOBY. The acetic acid bacteria utilise both fructose and glucose. *Komagataeibacter* utilises the fructose and glucose to form acetic acid and cellulose respectively, while the *Gluconobacter* species metabolise glucose into gluconic acid (Reiss, 1994). The HPLC chromatograms for sugar analysis for the honeydew honey kombucha over time, are depicted on Figure 5. The fructose (peak B) and glucose (peak C) peaks decrease in peak height and area as the fermentation proceeds, meaning the concentration of these sugars are decreasing with fermentation time.

Sucrose was not detected in the honey kombucha as no sucrose peak was found in the honey kombucha chromatogram in Figure 5. A sucrose peak could not be found in the honeydew honey chromatogram (Figure 6) as well. From this we can infer that, similar to other studies on honeydew honey conducted by Astwood et al.(1998) and Sanz et al. (2005), honeydew honey only contains little to no sucrose. As such, no sucrose could be detected in the honey kombucha as well. This means the honey provides glucose and fructose as the main carbohydrate substrate for the SCOBY growth during kombucha fermentation.

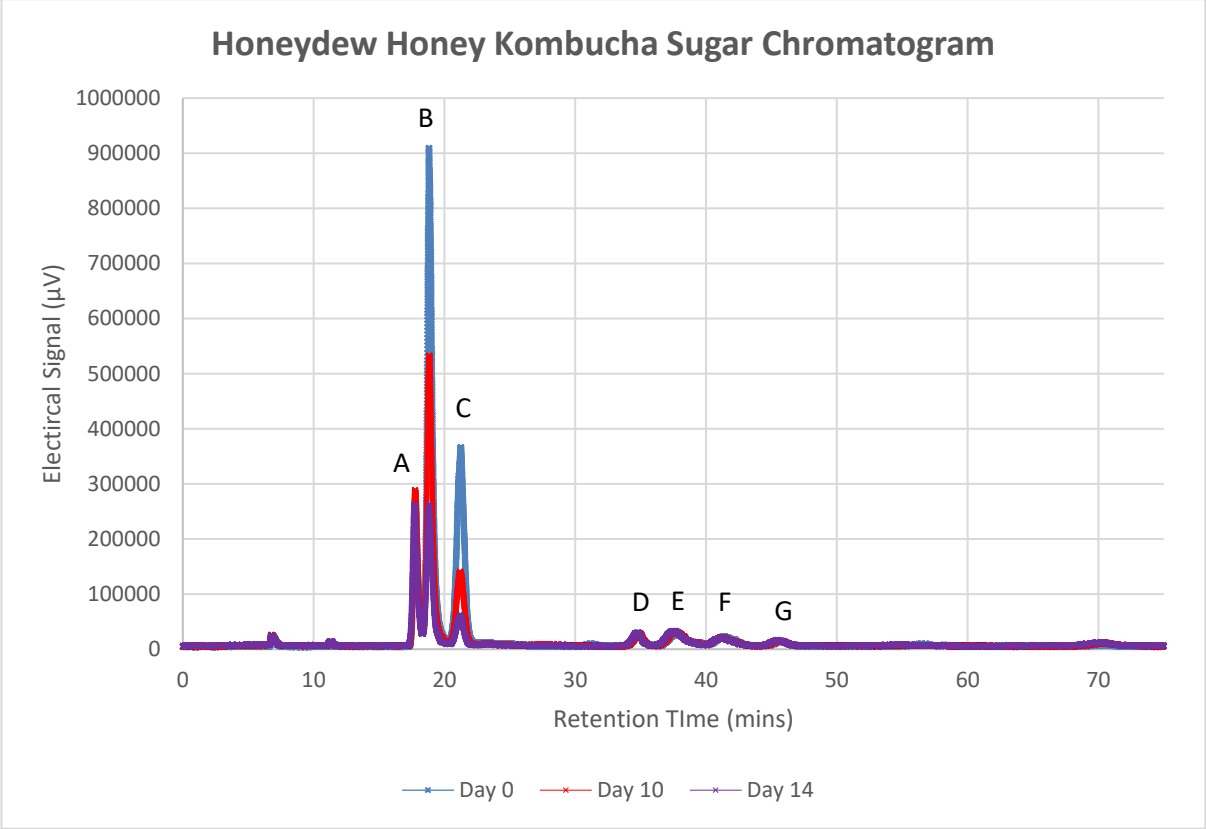


Figure 5: Sugars Chromatogram of Honeydew Honey Kombucha from HPLC-ELSD
A: Xylitol (ISTD), B: Fructose, C: Glucose, D: Turanose/Palatinose, E: Maltose, F: Unidentified, G: Unidentified

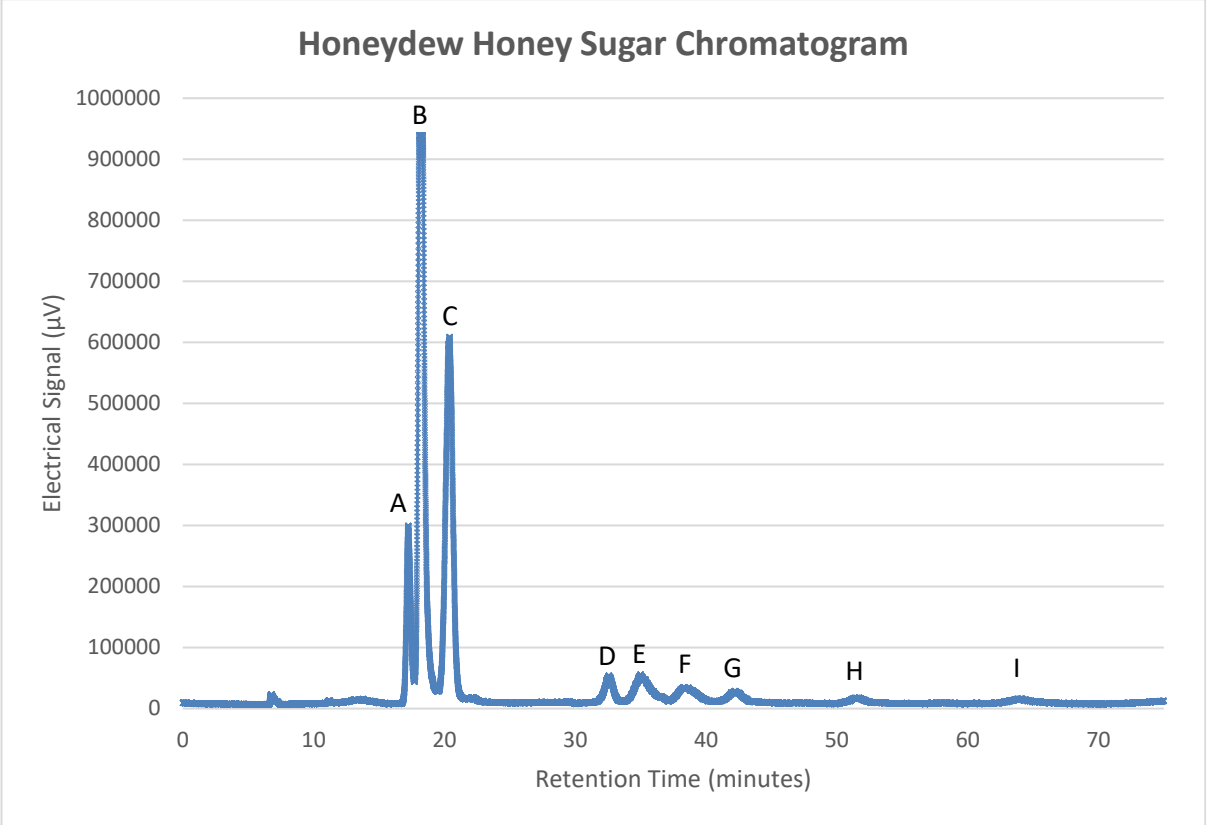


Figure 6: Sugars Chromatogram of Honeydew Honey obtained from HPLC-ELSD
A: Xylitol (ISTD), B: Fructose, C: Glucose, D: Turanose/Palatinose, E: Maltose, F: Unidentified, G: Unidentified, H: Melezitose, I: Unidentified

#### *5.2.5.3 Oligosaccharides and other sugars*

As mentioned previously the silylation technique had issues with reliability and not enough time was available to refine the experimental protocol. As such the HPLC-ELSD was used to obtain a surface level understanding of the oligosaccharide sugars present in the honeydew honey and the kombucha. Suggested methods for further analysis of oligosaccharide sugars are given in Section 6.1.2.

The small peaks after 30 minutes on the chromatograms depicted in Figure 5 and Figure 6, are likely to be the oligosaccharide sugars that could be found in the kombucha. The retention times of the sugar peaks in kombucha (given in Table 6) and honey are slightly different as they were conducted on different batches with slight variations in temperature, pump pressure and mobile phase composition. The retention times of the sugars were re-calibrated at the beginning of each run for sugar identification purposes. The concentrations of each sugar during each stage of fermentation are presented on Table 6.

Table 6: Oligosaccharides and other sugars in honeydew honey kombucha analysed on HPLC-ELSD.

| Sugar | Fresh honeydew honey (g/100g honey) | Kombucha Sugar Concentration (mg/mL Kombucha) | | |
|---|---|---|---|---|
| | | Day 0 | Day 10 | Day 14 |
| **Galactose** | ND | ND | ND | ND |
| **Turanose*** | 2.28±0.32 | 1.53 ±0.14$^{A}$ | 1.58±0.21$^{A}$ | 1.70±0.13$^{A}$ |
| **Palatinose*** | 4.25±0.70 | 2.13±0.12$^{A}$ | 2.18±0.1$^{A}$ | 2.28±0.12$^{A}$ |
| **Maltose** | 1.56±0.34 | 3.66±0.16$^{A}$ | 4.45±0.4$^{B}$ | 4.52±0.23$^{B}$ |
| **Lactose** | ND | ND | ND | ND |
| **Erlose** | ND | ND | ND | ND |
| **Melezitose** | 0.11±0.02 | ND | ND | ND |

Values represent the mean of triplicates, ± depicts standard deviation of the means.

Superscripts with the different letters within the same row have statistically significant differences, p-value < 0.05.

Retention times are taken from calibration standard run with kombucha samples.

* Obtained from same peak due to overlap.

ND = Not detected in the sample.

The oligosaccharides detected were palatinose, or turanose and maltose. The concentration of oligosaccharide sugars quantified through HPLC-ELSD did not show any statistically significant changes from day 0 of fermentation to day 10 or 14 of fermentation. This trend is also observed on the kombucha chromatograms, depicted on Figure 5, where the peaks past 30 minutes from different fermentation periods overlap. These results indicate that the oligosaccharide sugars present in honeydew honey were not metabolized by the SCOBY during the kombucha fermentation process. Previous literature has found that oligosaccharides are metabolised by and promoted the growth of probiotic bacteria such as *Bifidobacterium* and *Lactobacillus* species (Chung et al., 2007; Sanz et al., 2005). As observed in Section 5.3.1 and Appendix C, *Lactobacillus* and *Bifidobacterium* species did not make up a significant portion of the bacterial species in the SCOBY samples analysed by DNA extraction. Therefore, it is theorized that the predominant bacterial species in the SCOBY, *Komagataeibacter* and *Gluconobacter,* do not metabolise the oligosaccharides in the honeydew honey which explains why the oligosaccharide concentrations do not change during the kombucha fermentation. Additionally, the yeasts in kombucha are not able to break down the oligosaccharides into their monosaccharide constituents.

Palatinose and turanose, under these experimental conditions, were not properly separated as they had similar retention times of 34.0 and 34.3 minutes respectively. This led to the two sugars forming a single peak (peak D in Figure 6) within the honeydew honey and the

kombucha chromatograms. This single peak cannot be used to discern whether either palatinose, turanose or both sugars are present. The concentration of these sugars was calculated by applying the same peak to the respective standard curves, which was measured in separate solutions, however theses value should not be taken as an accurate representation. Melezitose, a carbohydrate that is unique to honeydew honey (Pita-Calvo & Vázquez, 2017), was detected in the honeydew honey but not within the kombucha. This was mostly likely due to the concentration of melezitose in the kombucha being below the limit of quantification for this HPLC-ELSD set-up as no peak for melezitose could be found in any of the kombucha chromatograms. Peak G in Figure 6, had a very similar retention time to lactose at around 45.0 minutes. Lactose is a sugar that is find primarily in milk samples, and no literature has documented the presence of lactose in honey. As such this peak was concluded to be an unidentified sugar which has a retention time that is very close to that of lactose.

## *5.3 Kombucha microbiology*

### *5.3.1 Kombucha metagenomics*

A total of 63 samples consisting of: three samples from the mother culture and 30 samples of honey and sucrose kombucha each. From each form of kombucha samples were taken in triplicate from the SCOBY and fermented media on day 7, 10, 14, 17, 21 days of fermentation. All samples were processed and amplified on the16S rRNA and ITS1 gene regions. In our experiments only two 16S rRNA honey kombucha SCOBY samples at 21 days fermentation were successfully sequenced out. No DNA samples produced from the fermented media, SCOBY produced from sucrose kombucha or ITS1 amplicons libraries were sequenced due to insufficient DNA concentrations post amplification. The extra ordinary circumstance (pandemic) this year prevented the further processing of the remaining samples. Only the bacterial composition from two honey kombucha SCOBY samples fermented for 21 days are presented. The bacterial community composition is presented in pie charts identified at the genus level (Figure 7).

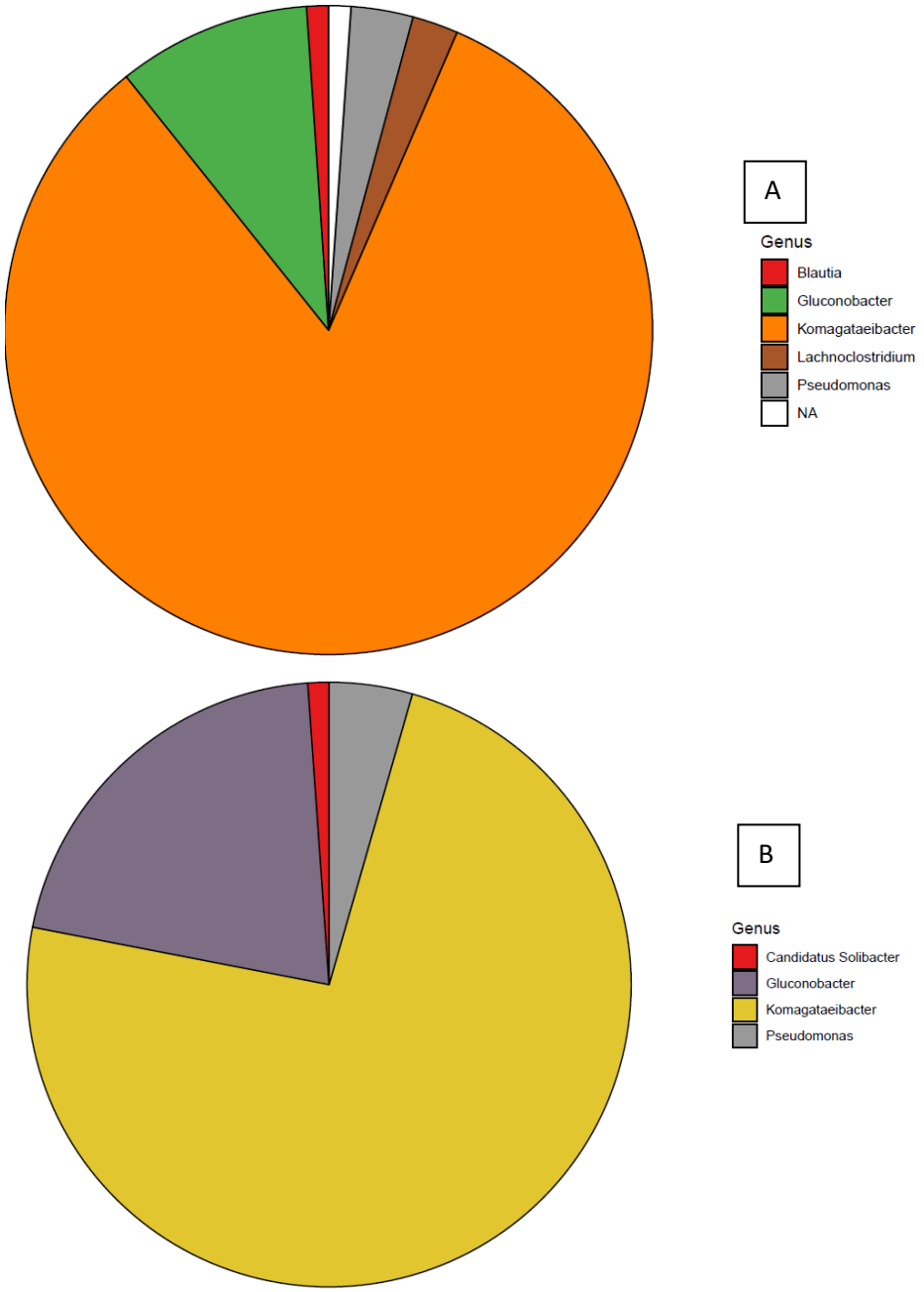


Figure 7: Genus Level Bacteria Distribution of Two Honey Kombucha SCOBY Samples

The predominant genus of bacteria found in the SCOBY was *Komagataeibacter* making up over 86.3% and 76.9% of the bacteria sequenced in samples A and B respectively. This result is largely expected as *Komagataeibacter* are from the *Acetobactearceae* family which is known to metabolise glucose and produce acetic acid and cellulose in the process. The cellulose produced by the *Komagataeibacter* binds the bacteria and fungi together and forms the pellicle layer (daughter culture) which floats at the top of the kombucha as it ferments (Sievers et al., 1995). The next most prominent genus of bacteria the *Gluconobacter*. This was also expected as *Gluconobacter* species form gluconic acid from glucose and oxidize ethanol into acetic acid (Komagata et al., 2014), which are characteristic to the system of kombucha fermentation. The last major genus of bacteria is the *Pseudomonas*, which cover an extremely wide spectrum of

bacterial species and some are known to be pathogenic. *Pseudomonas* species are commonly found in water, soil and surfaces and is therefore likely a contamination during the NGS processing or brewing of the kombucha rather than the SCOBY itself. A negative control using an unfermented tea mixture could be conducted to confirm this theory, however this was not done during the project. These results conflict with a study conducted by Marsh, O'Sullivan, Hill, Ross, & Cotter (2014), which found that around 85% of the bacteria present in the SCOBY are from the *Gluconoacetobacter* with the *Lactobacillus* as the next most common genus. The difference in results can be attributed to regional differences in the SCOBY starter culture (Reiss, 1994) for which the study conducted by Marsh et al.(2014) also found.

From the two samples, A and B, 200 and 108 unique sequences of bacteria were identified in the two samples respectively. The Shannon equitability of the of A and B were 0.511 and 0.517 respectively, indicating most of the bacteria species present come from the a few genera and a similar diversity of bacteria species between the two samples. The Shannon index of samples A and B was 1.53 and 1.59 respectively, indicating the diversity of bacterial community in the sample of which are higher than those found by Marsh et al.(2014). However, their study sequenced SCOBYs produced from kombucha made from black tea and sucrose. It is possible that the different conditions of the culture media generated by the honeydew honey has enabled favourable growth condition for more microorganisms. As such further investigation in replicating the experiment and successful sequencing of the kombucha made from sucrose is extremely desirable.

The results cannot be compared statistically due to limited sample size. This was due to the extremely low concentration of DNA eluted from the post PCR purification step which was below detectable limits on the Qubit Fluorometer for most of the samples. Assuming the purification step was done correctly, we can infer that the PCR amplification for all except the two samples sequenced was unsuccessful. There are several possible reasons for unsuccessful PCR amplification. There may have been insufficient template DNA for PCR amplification due to flawed DNA extraction process. As the dye used for Qubit Fluorometer will fluoresce on nucleotides regardless of the DNA strands length, this could have misled us in believing enough template DNA was extracted from the SCOBY. Successful ITS amplification would have been ideal in order to discern the fungal species present in the kombucha.

### *5.3.2 Probiotic concentration*

The concentration of probiotics found in the honeydew honey kombucha was assessed using two different agar plates. The two plates used were De Man, Rogosa and Sharpe (MRS) agar and a Nutrient Agar (NA) enriched to 10% glucose. The NA agar is a non-selective culture, so most forms of bacteria and fungi will be able to grow on it. The MRS agar is an enriched

medium in which lactic acid bacterial (LAB) species grows favourably, and therefore used to assess the concentration of lactic acid bacteria in the kombucha which was detected by Marsh et al. (2014). The log(cfu/ml kombucha) of the kombucha at different stages of fermentation are depicted in Figure 8. There are two considerations which should be kept in mind regarding these results. Firstly, the sample at day 0 was not analysed as it was assumed that no target micro-organisms would be cultured on the plate. Future plate count experiments on kombucha should have a day 0 data point to establish the baseline concentration of bacteria in the kombucha. Secondly, only two sample replicates were assessed during the experiment. This was because pathogenic mould was found to have contaminated one batch of the honeydew honey kombucha prepared for this experiment. Due to time constraints, a replicate of the experiment could not be conducted in time. As such the results presented here is only a snapshot of the possible probiotic concentration of the kombucha.

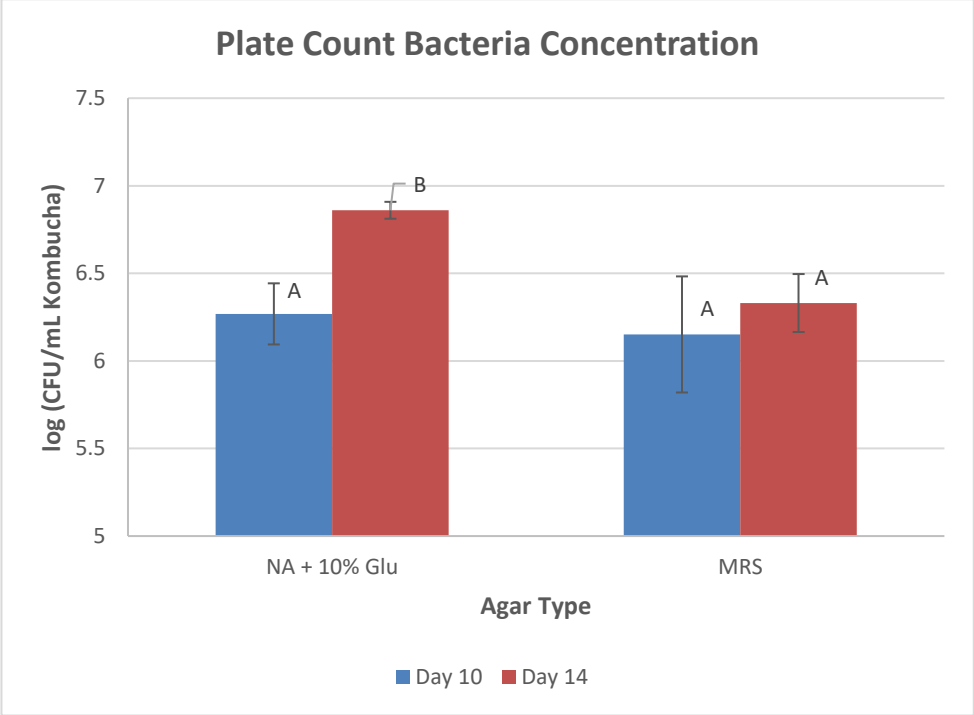


Figure 8: Probiotic Concentration Plate Count of Honeydew Honey Kombucha Measurements of each sample was carried out in triplicates. Error bars represent standard deviation of the means. Common capitalized letters do not differ statistically using a Two-sample t-test (p-value > 0.05)

On the nutrient agar (NA) enriched with 10% glucose, the concentration of bacteria increased from 6.29 log (CFU/mL Kombucha) to 6.86 log (CFU/mL Kombucha) at day 10 and day 14 respectively. This is a significant increase in bacteria concentration by approximately $5.7x10^6$ cfu/mL kombucha. In contrast the bacteria grown on the MRS agar which did not show a statistically significant increase in the concentration in the kombucha. The mean log CFU/mL kombucha on for the MRS agar on day 10 was 6.15 with a small increase to 6.33 on day 14. A

higher variation of the probiotic concentration was observed on plates culturing day 10 of the kombucha fermentation, the exact reason for this is unclear and further investigation is warranted.

The species growing on the plate are not been fully known, especially when the NA agar is non-selective. The cultured bacteria on the MRS and NA+10% glucose plates were isolated by streak plate technique on fresh MRS and NA+10% glucose plates respectively. Gram staining of the isolated colonies was conducted, and microscope images are presented in Figure 9.

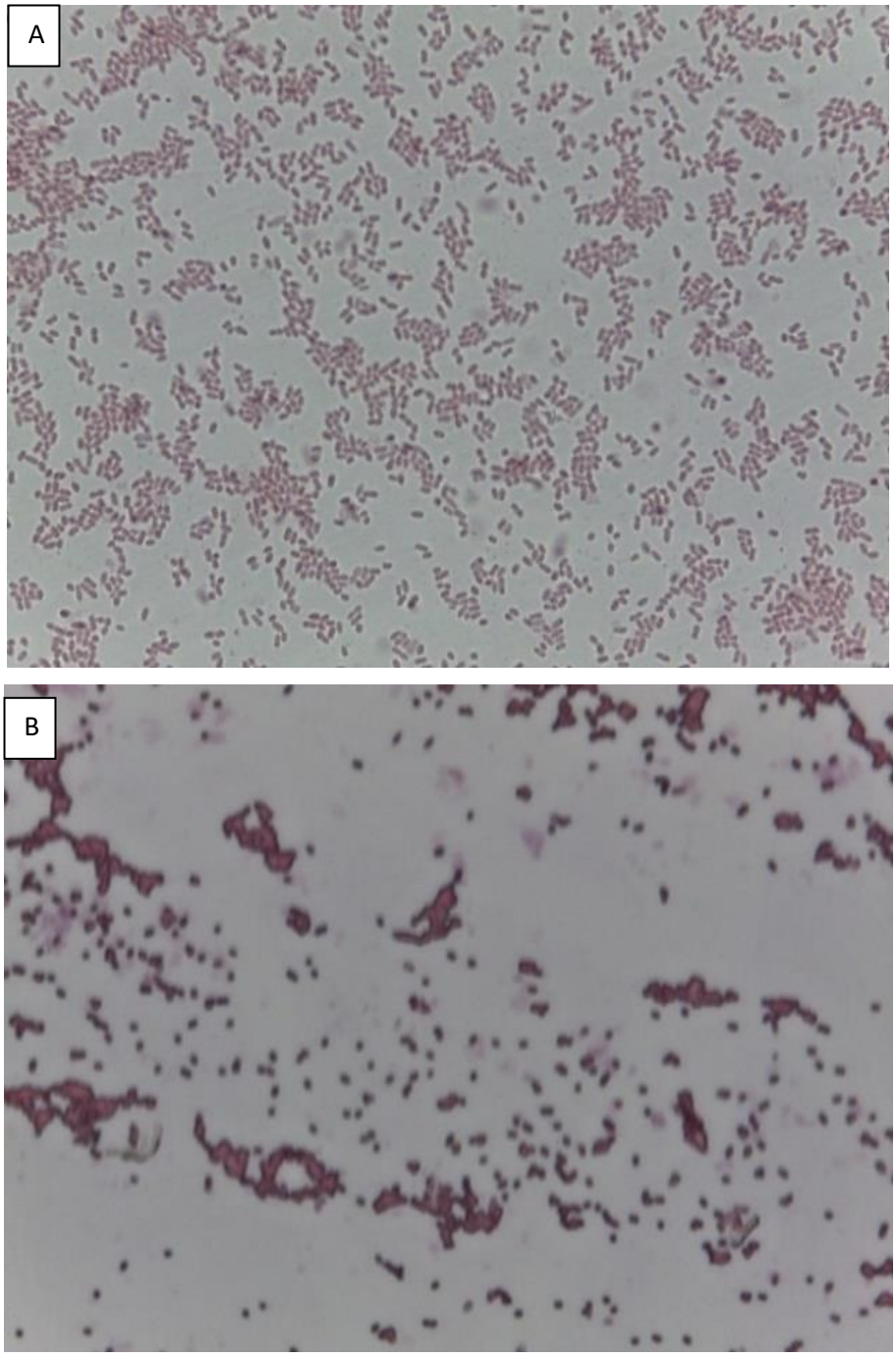


Figure 9: Gram Stain Images Honeydew Honey Kombucha, magnification 100X
A: MRS agar plate B: NA+10% glucose plate

The Gram staining of bacteria cultured on the MRS agar, depicted on Figure 9A, showed red stain indicating Gram-negative bacteria. This was unexpected as lactic acid bacteria (LAB) are known to typically be Gram-positive (Mokoena, 2017) which comes up as a violet stain. This indicates that some other form of bacteria within the honey kombucha is capable of being cultured on the MRS agar. Though not explicit, this may explain why no LAB was reported to have grown on the MRS plate count experiments by Neffe-Skocińska et al. (2017). On the NA + 10% glucose agar plate, Figure 9B, spherical Gram-negative bacteria were observed. While it cannot be confirm what bacteria species were cultured as the agar plate is non-selective, the appearance and stain colour match that of *Komagataeibacter* and *Gluconobacter* (Komagata et al., 2014). This means that the cultured bacteria on the plate is consistent with the kombucha metagenomics results described in Section 5.3.1. Due to time constraints, only preliminary work towards identifying species of bacteria cultured on the plate was completed.

# 6 Recommendations for Further Research

### *6.1.1 Extending the Fermentation Period*

According to the sugar results found in Section 5.2.5.2, the presence of fermentable sugars was still found in the kombucha after 14 days of fermentation. This implies that the fermentation of the kombucha was not fully completed and could still be left for a longer period. Extending the fermentation further will give us a more complete picture of changes in kombucha metabolites and chemical properties. For instance, the ethanol concentration was found to decrease after reaching a maximum value as it is hydrolysed to form acetic acid when sugars when no longer available for the AAB (C. Chen & Liu, 2000). This was not observed in this study but may occur if the fermentation could proceed for a longer period.

### *6.1.2 Oligosaccharide Sugars*

The current method for detecting oligosaccharides on the HPLC-ELSD has two main problems: separation and sensitivity. Separation of the oligosaccharide sugars proved to be insufficient, particularly for sugars which have common monosaccharide constituents (more details). The second issue of sensitivity can be seen in the detection on melezitose in the honey sample but not within the kombucha sample. This likely due to the amount of melezitose injected is below the HPLC-ELSD systems limit of detection, although the limit of detection and quantification could not be assessed due to time constraints. Shifting the analysis of oligosaccharides to a more sensitive system is preferable, such as GC-MS or Liquid Chromatography–Mass Spectrometry (LC-MS).

Future work on identifying and quantifying oligosaccharides can include further work to optimize troubleshooting of the silylation technique for the GC-MS, which has been successfully used to characterise melezitose as demonstrated by Swears & Manley-Harris (2020). Alternatively use of LC-MS with pre-column derivatization using 1-Phenyl-3-methyl-5-pyrazonlone (PMP) may yield better separation of the oligosaccharides. The PMP method is also able to derivatize uronic acids (Dai et al., 2010), such as glucuronic acid, which is another important metabolite found in kombucha but was not able to be characterized over the course of this project. The LC-MS also benefits from increased sensitivity compared to the ELSD. There may be potential issues caused by the high concentration of fructose and glucose in the kombucha, which are derivatised using PMP.

### *6.1.3 Kombucha metagenomics*

There are several recommendations which can be implemented to increase the likelihood of successful NGS DNA sequencing. The first is the introduction of more quality control steps between each step of the NGS workflow. This comes primarily in the form of using electrophoresis gels in addition to the Qubit Fluorometer tests. This enables confirmation of the

presence of proper template DNA, successful amplicons and primer dimers in the sample. The use of other DNA extraction protocols can be experimented on, as certain matrices are more resistant to different methods of cellular lysis. Possible alternative DNA extraction protocols are the use of cetyltrimethylammonium bromide (CTAB) described by Archer, McDonald, Herbold, Lee, & Cary (2015) or the protocol used by Marsh et al. (2014). can be replicated as they have successfully sequenced both bacterial and yeast species present in the kombucha and SCOBY. Successful ITS amplification would be desirable to discern the fungal species present in kombucha. Another possible avenue of kombucha metagenomics is sequencing of the cells cultured on the agar plates through Sanger sequencing. This involves isolating the bacteria to single cultures rather than attempting to sequence the entire community of bacteria present. The method will provide the full 16S rDNA and ITS gene region which would allow the identification of the microorganism to species level. The sequence data may be used for further phylogenetic analysis that can delineate the species in relation to other known microorganisms. Preliminary work on this has been undertaken, however could not be completed within the timeframe of this project.

### *6.1.4 Kombucha probiotic assessments*

The enumeration viable yeasts and acetic acid bacteria (AAB) was not conducted within this study due to time constraints. These are both micro-organisms widely accepted to be present in kombucha and therefore worth further investigation. The use of potato dextrose agar (PDA) or sabouraud dextrose agar (SDA) should be used to quantify the yeasts in kombucha. Both PDA and SDA have successfully been used to culture yeast species in kombucha from studies conducted by Chen & Liu (2000) and Neffe-Skocińska et al. (2017) respectively. Glucose - yeast extract - calcium carbonate (GYC) agar or the AAB selective media developed by Kim, Chon, Kim, & Seo, (2019) may be used to culture AAB found in kombucha. GYC agar has been successfully used to be used to find the concentration of AAB from wine vinegar (Vegas et al., 2010), whereas the AAB selective media is recently developed media which will require further investigation.

Further experimentation should be undertaken to kombucha follows World Health Organization (WHO) and the International Probiotic Association (IPA) guidelines as a probiotic beverage. Additional plate count experiments should be undertaken to ensure that the concentration of colony forming units is accurate, as only a small sample size of the experiments was conducted by this study (International Probiotics Association, 2017). Stability testing to assess the concentration of viable bacteria at the kombuchas storage conditions for the duration of its expected shelf-life (FAO/WHO, 2002; International Probiotics Association, 2017). Finally, identifying the taxonomy of the cultured bacteria and it’s the health benefits needs to be investigated. This can be accomplished by sanger sequencing of the bacteria cultured on the

plate after isolation as mentioned in Section 6.1.3. Once the strain is identified and metabolism establish, further research can be conducted to ascertain the health benefits that come with consumption the bacteria and the amount of kombucha required to achieve that health benefit.

# 7 Conclusions

This study has developed a novel kombucha utilising TGY tea and honeydew honey as the substrate for fermentation of the SCOBY. A comprehensive analysis of the honey kombucha products was carried out to assess the kombucha antioxidant activity, total phenolic content, colour changes, and the composition of amino acids, organic acids and sugars. The micro-organisms found within the kombucha was also assessed through DNA sequencing to identify micro-organisms present and their concentration found through plate count experiments.

The acidity of the honey kombucha increases at a much higher rate compared to the sucrose kombucha indicating an increased rate of fermentation. The main metabolites of kombucha, namely ethanol, acetic acid and gluconic acids, were found to increase with fermentation time. Sugar compositional analysis indicated that oligosaccharides found in honey was not the cause of this observation as they were not metabolised during the fermentation. From our kombucha metagenomics experiments, *Komagataeibacte*r and *Gluconobacter* were the predominant genus of bacteria present in the kombucha. Neither of these bacterial species have been documented to metabolise oligosaccharides sugars. Literature has shown that *Komagataeibacter* is able to metabolise amino acids to increase the rate of production of cellulose and acetic acid.

The antioxidant activity and total phenolic content were all found to increase with fermentation time. Contrary to this result the colour of the kombucha becomes progressively lighter as the kombucha ferments.

This study provides a solid foundation for further research in kombucha micro-biology. While successful DNA sequencing has elucidated *Komagataeibacter* and *Gluconobacter* as the primary genus of bacteria present. This result cannot be considered statistically significant due to lack of samples being successfully sequenced. Regional variations in how the SCOBY culture is produced also means this not representative of all SCOBY cultures. The concentration of bacteria assessed by plate count experiments indicate that ample bacteria is present in the kombucha to be considered probiotic. Further research is required to determine the species of bacteria cultured and assess further conditions laid out by WHO to determine whether kombucha can be considered a probiotic drink.

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# Appendices

## *Appendix A: Amino Acids Limit of Detection and Quantification*

The calculations for the amino acid limits of detection (LoD) and limit of quantification (LoQ) were obtained from equations from in Little, (2015). The equations are as follows

$$LoD = 3.3\frac{\sigma}{Gradient} \qquad (A1)$$

$$LoQ = 10\frac{\sigma}{Gradient} \qquad (A2)$$

$\sigma$, is the standard deviation of the residuals from the lowest point of the calibration curve of the compounds. Gradient, is the slope of the standard curve of each component. The LoD and LoQ for each amino acid quantified by MCF are given in Table A1

Table A1: Amino acid limits of detection and quantification.

| Amino Acid | Limit of Detection (µg/mL) | Limit of Quantification (µg/mL) |
|---|---|---|
| **Alanine** | 0.0414 | 0.1256 |
| **Glycine** | 0.0475 | 0.1439 |
| **Valine** | 0.0572 | 0.1735 |
| **Leucine** | 0.0683 | 0.2071 |
| **Isoleucine** | 0.0711 | 0.2156 |
| **Proline** | 0.0362 | 0.1097 |
| **Threonine** | 0.1473 | 0.4464 |
| **Aspartic Acid** | 0.06418 | 0.1945 |
| **Serine** | 0.9378 | 2.8418 |
| **Glutamic Acid** | 0.1017 | 0.3083 |
| **Methionine** | 0.1359 | 0.4119 |
| **Phenylalanine** | 0.0688 | 0.2086 |
| **Lysine**[1] | - | - |
| **Histidine**[1] | - | - |
| **Tyrosine**[1] | - | - |

[1] Denotes compounds with poor responses in standard curve, low or non-linear regression

## *Appendix B: Issues Encountered with Silylation of Sugars*

The first problem was encountered after several experimental runs where the responses obtained from the silylation technique returned close to no signal. Depicted on Figure B1 are four injections of the honey kombucha on day 0, derivatised using the same reagents and run on the same batch within one day, run from top to bottom. Initially the expected response was obtained, however subsequent injections of the batch gave a decreased response until the fourth day 0 sample which gave the same response as a reagent blank. The solvent used to suspend the TMS-derivatives was changed from heptane to toluene, as toluene is another non-polar solvent, and this issue was resolved.

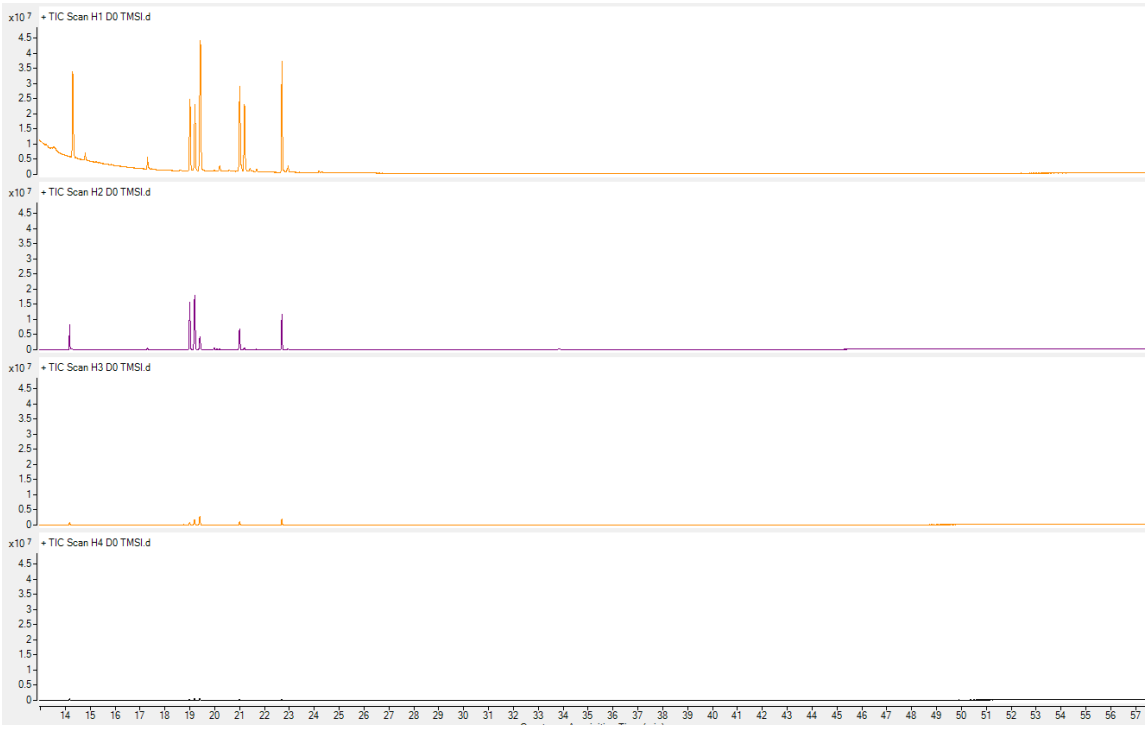


Figure B1: Chromatograms of Four Day 0 Homey Kombuchas by TMSI Derivatization

The second problem encountered was of carry over target analyte peaks into subsequent injections. The effect of this is observed on three chromatograms in Figure B2. Glucose (Figure B2A), sucrose (Figure B2B) and a toluene matrix blank (Figure B2C) were injected in this order in one batch. The two major peaks on Figure B2A at 21 and 23 minutes are the TMS-derivative peaks of α-D-glucopyranose and ß-D-glucopyranose respectively. Carry over of the two glucose peaks were found in the along with the sucrose-TMS derivative peak at 34 minutes in Figure B2B. On Figure B2C, while the carry-over from the glucose peaks has been significantly reduced, a sucrose-TMS derivative peak was found. This problem was particularly tricky as the carry over peak always had the same retention times and only appears when the injection method is ran. The peak would appear without the syringe injecting anything into the inlet. Troubleshooting on all parts of the GC-MS system was undertaken such as, cleaning of the

mass spectrometer, cleaning and replacing inlet consumable, replacement of the syringe, wash solvents and cutting of the column though none of these were effective in reducing the carry over within a reasonable timeframe. As carry over peaks could not be eliminated within the time frame of this project all sugar composition experiments were shifted on to the HPLC-ELSD.

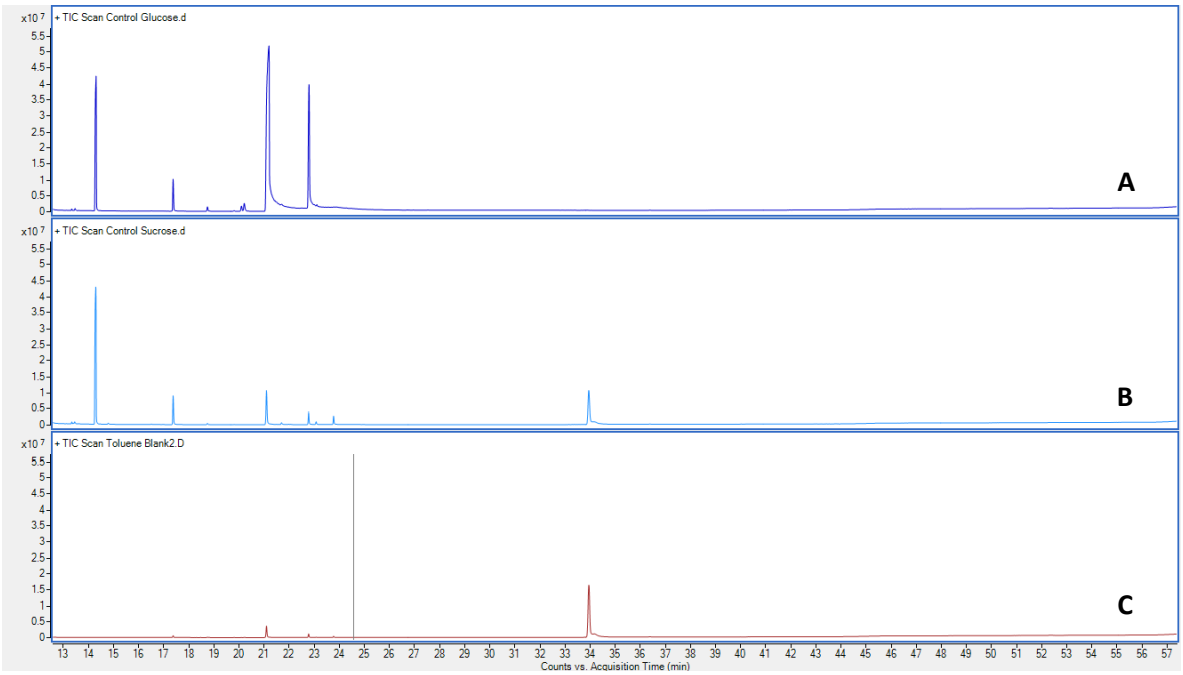


Figure B2: Chromatogram of Silylation of Sugar Standards

A: Glucose Chromatogram. B: Sucrose Chromatogram, C: Toluene Blank at End of Run

*Appendix C: Taxonomy Table of SCOBY*

The taxonomy table presented was generated from one of the two successfully sequenced samples of honey kombucha SCOBY. Species below 250 reads were removed. The taxonomy table elucidates some genus of bacteria that were not shown on the pie chart within Section 5.3.1 due to making up lower fraction of the total reads.

Table C1: Taxonomy Table of SCOBY Produced by Honey Kombucha

| Number of Reads | Kingdom | Phylum | Class | Order | Family | Genus |
|---|---|---|---|---|---|---|
| 164336 | Bacteria | Proteobacteria | Alphaproteobacteria | Acetobacterales | Acetobacteraceae | Komagataeibacter |
| 32035 | Bacteria | Proteobacteria | Alphaproteobacteria | Acetobacterales | Acetobacteraceae | Komagataeibacter |
| 22908 | Bacteria | Proteobacteria | Alphaproteobacteria | Acetobacterales | Acetobacteraceae | Gluconobacter |
| 7420 | Bacteria | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Pseudomonadaceae | Pseudomonas |
| 5485 | Bacteria | Firmicutes | Clostridia | Lachnospirales | Lachnospiraceae | Lachnoclostridium |
| 2673 | Bacteria | Proteobacteria | Gammaproteobacteria | Enterobacterales | Yersiniaceae | NA |
| 2313 | Bacteria | Proteobacteria | Alphaproteobacteria | Sphingomonadales | Sphingomonadaceae | Novosphingobium |
| 2207 | Bacteria | Gemmatimonadota | Gemmatimonadetes | Gemmatimonadales | Gemmatimonadaceae | Gemmatimonas |
| 1623 | Bacteria | Firmicutes | Clostridia | Lachnospirales | Lachnospiraceae | Blautia |
| 1008 | Bacteria | Proteobacteria | Gammaproteobacteria | Diplorickettsiales | Diplorickettsiaceae | Aquicella |
| 980 | Bacteria | Firmicutes | Clostridia | Lachnospirales | Lachnospiraceae | Blautia |
| 968 | Bacteria | Proteobacteria | Alphaproteobacteria | Caulobacterales | Caulobacteraceae | NA |
| 909 | Bacteria | Firmicutes | Bacilli | Lactobacillales | Streptococcaceae | Streptococcus |
| 854 | Bacteria | Firmicutes | Clostridia | Lachnospirales | Lachnospiraceae | Catonella |
| 804 | Bacteria | Actinobacteriota | Actinobacteria | Bifidobacteriales | Bifidobacteriaceae | Bifidobacterium |
| 789 | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Xanthobacteraceae | Afipia |
| 636 | Bacteria | Actinobacteriota | Actinobacteria | Corynebacteriales | Corynebacteriaceae | Corynebacterium |
| 539 | Bacteria | Proteobacteria | Alphaproteobacteria | Acetobacterales | Acetobacteraceae | Komagataeibacter |
| 467 | Bacteria | Firmicutes | Bacilli | Bacillales | Planococcaceae | Domibacillus |
| 463 | Bacteria | Proteobacteria | Alphaproteobacteria | Acetobacterales | Acetobacteraceae | Komagataeibacter |
| 443 | Bacteria | Proteobacteria | Alphaproteobacteria | Acetobacterales | Acetobacteraceae | Komagataeibacter |
| 417 | Bacteria | Proteobacteria | Alphaproteobacteria | Acetobacterales | Acetobacteraceae | Komagataeibacter |
| 406 | Bacteria | Proteobacteria | Alphaproteobacteria | Paracaedibacterales | Paracaedibacteraceae | Candidatus Finniella |
| 397 | Bacteria | Firmicutes | Bacilli | Erysipelotrichales | Erysipelotrichaceae | [Clostridium] innocuum group |
| 360 | Bacteria | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | Acinetobacter |
| 328 | Bacteria | Proteobacteria | Alphaproteobacteria | Sphingomonadales | Sphingomonadaceae | Sphingomonas |
| 265 | Bacteria | Actinobacteriota | Actinobacteria | Bifidobacteriales | Bifidobacteriaceae | Bifidobacterium |