The Effect of Temperature and Fungal:Bacterial Ratio on Kombucha Culture Fermentation

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Abstract

The fermented tea beverage kombucha is a traditional drink that has been consumed for thousands of years in Asia. Over the past decades, it has gained greatly in popularity amongst consumers and researchers also in the rest of the world. Kombucha is produced through fermentation of sweetened tea by a complex community of bacteria, mainly dominated by acetic acid bacteria, and a variety of yeast. During the fermentation process, bacteria and yeast interact through forming compounds and metabolites that stimulate the growth of the other part. The aim of this project was to investigate how temperature and the fungal:bacterial ratio affects the kombucha fermentation process. First, I attempted to construct a gradient of fungal:bacterial biomass between inoculums based on filtration and sedimentation. However, as the visual evaluation as well as the PCR results implied a weak effectiveness of the method, it was decided not to continue with this approach. Instead, fermentation vessels were places in different temperature zones around 15.1 °C, 19.8 °C, 24.0 °C, 35.9 °C, and allowed to ferment for 20 days. Previous research have shown contrasting results between yeast and bacteria on optimal temperature and temperature sensitivity. Thus, given that the two groups are competing for resources, temperature conditions which favor one of the groups more would indirectly disfavor the other and an alteration of the fungal:bacterial biomass might be achieved by the temperature difference. During the 20 day fermentation time, pH and brix was measured continuously every second day and samples were saved and frozen for later analysis including qPCR and sugar content. The pH followed an expected pattern throughout the fermentation processes such as faster decrease associated with higher temperature. In contrast, the brix results were unexpected, not changing as much as expected for the three lower temperatures and increasing significantly for the highest temperature. A decrease in sucrose content and increase in glucose and fructose content could be observed from the sugar analysis.

Although few differences were significant, some tendencies could be observed when measuring the DNA amount and comparing amount of yeast and bacterial DNA between the different temperatures. Lower temperature seemed to favor the growth of both bacteria and yeast, although bacteria somewhat more. However, no evidence of a consistent effect of temperature on the fungal:bacterial ratio could be observed.

Overall, the observable effects of temperature on fungal:bacterial ratio and kombucha fermentation dynamics in this project was not as significant as was hypothesized when planning this project but further research on the subject is suggested before final conclusions are drawn.

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THE EFFECT OF TEMPERATURE AND FUNGAL:BACTERIAL RATIO ON KOMBUCHA CULTURE FERMENTATION **0**

1. Introduction

Kombucha is a traditional fermented tea beverage which has been consumed for thousands of years (Gaggìa *et al.*, 2019). The drink, originating from Asia, is fermented by a symbiotic culture of a variety of yeast and bacteria, mainly dominated by acetic acid bacteria, AAB (Coton *et al.*, 2017; Neffe-Skocińska *et al.*, 2017). Over the past decades, kombucha has become increasingly popular which has led to an increased interest in the beverage both by consumers and researchers. However, much about the fermentation process remains poorly understood and not yet well investigated.

Previous studies have shown that the temperature has a significant effect on the kombucha fermentation process and the composition of the microbial community (Neffe-Skocińska *et al.*, 2017; De Filippis *et al.*, 2018). However, to what extent it affects the fungal:bacterial ratio and the corresponding effect on the fermentation is not yet understood. Bacteria and yeast interact mutualistically and antagonistically in kombucha fermentation, and temperature effects on one group may indirectly affect the fermentation behavior of the other group. Generally, AAB thrive in temperatures in the range of 25 °C to 30 °C and fungi, such as members of the genera *Zygosaccharomyces,* grow in a wide range from 4 °C to 25 °C (Kurtzman and James, 2006; Gomes *et al.*, 2018). Studies that have examined the temperature sensitivity of fungi and bacteria have found contrasting results in regards to optimum temperature and temperature sensitivity of the two groups (Alster, Weller and von Fischer, 2018). How temperature affects the complex symbiotic relationship between these organisms in the kombucha culture, is still to be discovered.

2. Aim

The aim of this project is to investigate how the kombucha fermentation process is affected by fermentation temperature and the fungal:bacterial ratio of the microbial community.

3. Approach

To address this aim two different approaches will be employed. The first approach attempts to construct a gradient of fungal:bacterial biomass to examine the effect of different ratios on the kombucha fermentation process. A combination of filtration and sedimentation will be used with the aim of establishing a concentration gradient of yeast to bacteria microbial biomass ratio across different fermentations. By separation of the yeast and bacterial communities of the kombucha culture and addition of a yeast dense inoculum in specified amounts, a yeast concentration gradient will be created. Five different yeast concentrations will be tested ranging from low to high. In order to ensure the concentration differences and confirm the gradient, all samples will be tested and evaluated with qPCR.

The second approach will be to set up fermentation vessels in different temperature zones, thus favoring the growth of different organisms in the different fermentation trials. Much of the same analytical techniques will be used in the two different trial set ups such as PCR and qPCR. However, since the first method aims to alter the culture before the fermentation and investigate how that affects the fermentation parameters, and the latter method aims to alter the culture throughout, and with the help of, the fermentation process, the evaluating methods at the end differ somewhat. For the purpose of both experimental set ups, the kombucha will be fermented for around 20 days, where after metabolites such as acetic acid, sucrose, glucose and fructose will be analyzed together with other factors such as pH, brix, culture formation and taste.

3.1. Scope

The report aims to provide the reader with a general overview and background of kombucha, its microbial community and what can affect it. The methods and results of each experimental set up will be described and lastly discussed. The scope of the project was determined by the time limitation, set to 20 weeks in total, which determined how deeply the research questions could be investigated. Time, in addition to the available financial and equipment resources were factors when setting the boundaries of this project.

4. Background

Following below are a number of sections that highlight and expands on some of the previously presented information and other areas of importance for the purpose of the project.

4.1. Kombucha

With its origin in Asia, kombucha is a traditional fermented tea beverage that has been consumed for thousands of years (Coton *et al.*, 2017; Gaggìa *et al.*, 2019). As trade routes expanded, kombucha was introduced in Russia and later also further into eastern Europe, appearing in Germany early 20th century (Jayabalan *et al.*, 2014). Over the past couple of decades, kombucha has gained much popularity also in the rest of the world, creating an increased interest not only from consumers, but also researches. In order to produce kombucha, herbs, traditionally black or green tea, are steeped in hot water, which is then sweetened with sugar. This sweetened tea mixture is cooled and a mixed microbial community of bacteria, dominated by AAB, and a variety of yeasts is added and the fermentation begins (Neffe-Skocińska *et al.*, 2017). After a period of time, varying within a wide range from 8-25 days depending on several fermentation conditions, the beverage is ready to consume. If closed in an airtight vessel, the fermented product has the capacity to become carbonated due to residual sugars and yeast. Kombucha has a sweet-sour taste, somewhat resembling sparkling apple cider (Marsh *et al.*, 2014; Gaggìa *et al.*, 2019). As the fermentation period is prolonged, the fruity and sweet taste gives rise to drier, more sour flavors and the beverage becomes increasingly similar to apple cider vinegar. The final taste depends on the sugar, carbon dioxide and organic acid levels, especially acetic and gluconic acid (Abel and Andreson, 2020).

4.2. Kombucha brewing

The production process of kombucha generally consist of the following steps:

- Boiling water
- Mixing water with sugar and tea leaves
- Filtering to remove the tea leaves
- Cooling the tea and placing it in an open vessel
- Inoculating the liquid
- Covering the vessel with a cloth
- Allowing the beverage to ferment

The addition of sucrose and tea can be somewhere around 50 g and 5 g respectively per 1 L water (Jayabalan *et al.*, 2014). After about five minutes the tea leaves are filtered away. As a possible alternative, tea bags can be used instead of the leaves (Dutta and Paul, 2019). Cooling can be done until reaching room temperature and after this, the liquid is inoculated with starter culture. This starter culture typically consists of the cellulosic pellicle of a previous fermentation and about 0.2 L per 1 L water of previously fermented kombucha (Jayabalan *et al.*, 2014; Villarreal-Soto *et al.*, 2020). The addition of the kombucha lowers the pH of the liquid and thus prevents the growth of undesirable spoiler organisms and pathogens (Dutta and Paul, 2019; Tran *et al.*, 2020). A cloth or other suitable material is placed over the vessel to

cover and protect from contamination of airborne microbes and insects. The fermentation is most commonly performed at a temperature between 18 °C to 26 °C (Jayabalan *et al.*, 2014). After some time, often within a few days, a daughter culture will have formed, either as additional layer formed on the existing culture or as a new cellulose layer on top of the liquid surface if the old mother culture has sunk to the bottom of the container (Dutta and Paul, 2019). The process is usually ended sometime between 10 to 20 days (Gaggìa *et al.*, 2019). The pH of a finished kombucha can vary widely depending on fermentation time among other factors but is usually somewhere in the range of 2 to 3.5 (Jayabalan, Marimuthu and Swaminathan, 2007; Chakravorty *et al.*, 2016). If left too long, the acidic taste of vinegar can become too prominent. The final kombucha may be drunk directly, or stored cold, typically at 4 – 8 °C. It is common to flavor with other ingredients such as herbs or fruit. An additional option is to fill in air tight bottles which, when left out at room temperature, will cause the beverage to become carbonated, as the yeasts present consume the residual sugars and produce CO_2 (Coton *et al.*, 2017). For commercial purposes, some producers filter their product in order to remove the microbes and thus obtain a stabile product with longer shelf life. When deciding to keep the microbes present, the temperature decrease accompanied with storing the drink refrigerated slows down the process, however, it does not stop it completely. As a result, the production of for example CO_2 is to some extent still active, limiting the shelf life of the product. There are numerous ways to vary the recipe and the different steps of the process, which may lead to different results and effects on the finished product.

4.3. Kombucha biochemistry

Several studies on kombucha fermentation have shown that the process is carried by a very complex symbiotic relation between bacteria and yeast. Although much of the details are only proposed and in need of further research, a possible protocooperative process is described in several articles. During the fermentation process, bacteria and yeast interact through forming compounds and metabolites that stimulate the growth of the other part and which are necessary to achieve what is considered as kombucha. The sucrose that is added to the tea is broken down into monosaccharides by an enzyme, invertase, that is thought to be produced by yeast (Coton *et al.*, 2017; Neffe-Skocińska *et al.*, 2017). Yeasts are also responsible for the production of ethanol, CO_2 and organic acids with a preference for fructose as substrate. Bacteria, particularly AAB, convert primarily glucose, however not excluding fructose, into mainly gluconic and acetic acid as well as ethanol into acetic acid (Huang, Hu and Rohrer, 2016; Coton *et al.*, 2017). The increase of acetic acid throughout the fermentation process helps stimulate the ethanol production by yeast which is then converted to more acetic acid by AAB. Ethanol and acetic acid is accumulated in the liquid over time and has an antimicrobial effect, helping prevent contamination of spoilage or pathogenic organisms (Laavanya, Shirkole and Balasubramanian, 2021). When yeast cells die during the fermentation process and autolyze, the release of vitamins and other nutrients stimulate the activity of the bacterial cells (Chakravorty *et al.*, 2016). Furthermore, there are other compounds present in the sweetened tea solution that stimulates the fermentation process. Caffeine, theobromine and theophylline originate from the tea and stimulate the production of the below described pellicle, formed by mainly *K. xylinus* (Laavanya, Shirkole and Balasubramanian, 2021).

4.4. Kombucha microbial community

The microbial community of kombucha fermentation is diverse, typically consisting of dozens of yeast species and hundreds of bacterial species (Reva *et al.*, 2015; Leal *et al.*, 2018; Villarreal-Soto *et al.*, 2020). The microbial community taking part in the fermentation process is most commonly divided according to domain; bacteria and fungi, and according to the environment; one that is trapped in the cellulosic biofilm floating on top of the liquid, and the community present in the liquid. The biofilm, or pellicle, is mainly produced by an acetic acid bacteria called *Komagataeibacter xylinus*. As the pellicle is in direct contact with the surrounding air, it constitutes a microbial niche by maintaining aerobic conditions in the open tank for the strictly aerobic microorganisms, such as AAB (Marsh *et al.*, 2014; Coton *et al.*, 2017). During fermentation, new layers of this cellulosic pellicle are produced, which may or may not adhere to the existing pellicle. This pellicle can be, and is often, used as inoculum in a new fermentation (Coton *et al.*, 2017; Neffe-Skocińska *et al.*, 2017).

The bacterial composition is mainly dominated by species belonging to the genera *Acetobacter*, *Gluconacetobacter* and to some extent *Lactobacillus* (Marsh *et al.*, 2014; Reva *et al.*, 2015; Chakravorty *et al.*, 2016; Villarreal-Soto *et al.*, 2020). Since these studies were conducted, some of the species previously belonging to *Gluconacetobacter* have been reclassified to the genus *Komagataeibacter*. As microbial databases have not yet been completely updated to accommodate the reclassification of certain *Gluconacetobacter* to *Komagataeibacter*, researchers consider that some of the reads assigned to *Gluconacetobacter* may actually belong to *Komagataeibacter* (De Filippis *et al.*, 2018). The cellulose producing *K. xylinus* is one example of this. Compared to the bacterial composition, the yeasts have shown to display a larger variation in composition between different inoculums as well as a tendency to vary more throughout the fermentation process (Marsh *et al.*, 2014; Coton *et al.*, 2017). Studies on the microbial composition has identified yeasts species of the genera *Zygosaccharomyses*, *Candida*, *Kloeckera/Hanseniaspora*, *Torulaspora*, *Pichia*, *Brettanomyses/Dekkera*, *Saccharomyses* and *Saccharomyciodes* as some of the most abundant (Marsh *et al.*, 2014; Reva *et al.*, 2015; Chakravorty *et al.*, 2016; Abel and Andreson, 2020). Research has shown that the community may vary between different fermentations across the world, much depending on the source of inoculum (Jayabalan *et al.*, 2014; Chakravorty *et al.*, 2016). After ten days of kombucha fermentation the bacterial and yeast count generally reach between $10^4 – 10^6$ cfu/mL with yeast numbers usually slightly outnumbering the bacterial amount (Marsh *et al.*, 2014). The size of bacteria and yeast respectively varies between different taxonomies making it difficult to categorize them according to size, however, generally yeast cells are larger than bacterial cells. *K. xylinus,* that are usually considered one of the most important microorganisms for the kombucha fermentation process because of their cellulose producing properties, can be up to between 2 and 3 μm long (Campano *et al.*, 2016).

4.5. Temperature's effect on kombucha fermentation

There has been little research done on the effect of different fermentation temperatures on the kombucha fermentation process or on the microbial composition of the culture. De Filippis et. al. (2018) investigated changes in the bacterial population after fermenting in 20 °C compared

to 30 °C. With the aim of defining what conditions might boost gluconic and glucuronic acid production, they concluded that certain *Gluconacetobacter* species were selected at the different temperatures. The higher of the two temperatures promoted a higher production of organic acids. Also Neffe-Skocińska et. al. (2017) focused their work on determining the organic acid content, including glucuronic acid, but also sugar content compared between 10 days kombucha fermentation at 20 °C, 25 °C and 30 °C. They observed the greatest amount of glucuronic acid as well as the most rapid decrease in sucrose content associated with fermentation in 25 °C.

4.6. Kombucha health claims

One reason as to why the popularity of kombucha has increased at such speed lately are the reported health benefits that are associated with the drink. Important to note is that the results are obtained from animal or in vitro studies and until 2019, no controlled studies on human subjects have been performed (Kapp and Sumner, 2019). The literature on non-human subject suggest that the health benefits are derived from either components of the tea, or products of the fermentation process. As a results of fermentation, kombucha contains organic acids, such as acetic acid, gluconic acid, glucuronic acid accompanied with several other (De Filippis *et al.*, 2018). Although not the most abundant one, glucuronic acid is the one most often linked to health benefits and therefore a research topic of interest (Nguyen *et al.*, 2015; Dutta and Paul, 2019). Glucuronic acid has shown detoxifying effects on pollutants, exogenous chemicals and bilirubin among others (Nguyen *et al.*, 2015). Additionally, the acid is a precursor to vitamin C and can be converted into glucosamine, a substance which is associated with treatment of osteoarthritis (Nguyen *et al.*, 2015; Neffe-Skocińska *et al.*, 2017). The organic acids, together with polyphenols from the tea, can, in addition to the aforementioned examples, contribute with even more health supportive effects. These include anti-carcinogenic and antioxidant properties (Kapp and Sumner, 2019). Moreover, it has been shown that they can contribute to the improvement of the immune response. (De Filippis *et al.*, 2018; Kapp and Sumner, 2019)

The possible probiotic effect of the drink is also a research area of current interest. To be able to classify a food or drink as a probiotic, according to WHO, the product must contain live microorganisms which contribute with a health benefit on the host, when consumed in adequate amounts (FAO/WHO, 2002). Although kombucha beverage has shown to confer health benefits to the consumer, as of today, no research has yet shown the direct association between health effects and the microorganisms of kombucha (Jayabalan *et al.*, 2014; Marsh *et al.*, 2014).

4.7. Methods for assessing microbial population - PCR and qPCR

PCR, Polymerase Chain Reaction, is a method of rapidly amplifying specific DNA fragments (Adams, 2020). It is a way of taking a small quantity of DNA and copying the genetic fragment exponentially until the amount of DNA is large enough to study the region of interest in detail. In research on kombucha, it has been extensively used to identify microbial communities (Marsh *et al.*, 2014; Reva *et al.*, 2015; Chakravorty *et al.*, 2016; Coton *et al.*, 2017; Gaggìa *et al.*, 2019; Tran *et al.*, 2020; Torán-Pereg *et al.*, 2021). If using PCR to examine the presence of a certain fragment, the evaluation of PCR is based on staining the DNA and separate fragments

through gel electrophoresis based on size. The expected size of bacterial and yeast fragment respectively is known.

Quantitative PCR, or qPCR, is a PCR amplification method of DNA in real time. The method of qPCR is based on real-time detection of a fluorescent reporter molecule (Fierer *et al.*, 2005). The process can be performed with one of two types of detection methods, both based on the aforementioned fluorescent light signal detection. The first one uses fluorescent dye that non-specifically binds to double-stranded DNA and emits light when intercalating with newly synthesized DNA. The second method uses hydrolysis probes that bind downstream of the qPCR primers and emits light as they are cleaved off the DNA and thus separated from the light signal inhibiting quencher. (Fierer *et al.*, 2005; Adams, 2020) For the purpose of this project, the firstly mention method will be used. SYBR Green is the most common intercalating dye for this purpose and will emit a green fluorescent light as it binds to double-stranded DNA (Adams, 2020). An image describing the SYBR green detection process can be seen in *Figure 4-1* below. When running the test, a detector measures the fluorescent light emitted after each amplification cycle which is then used to back calculate the amount of DNA, of the region of interest, that is originally present in the sample.

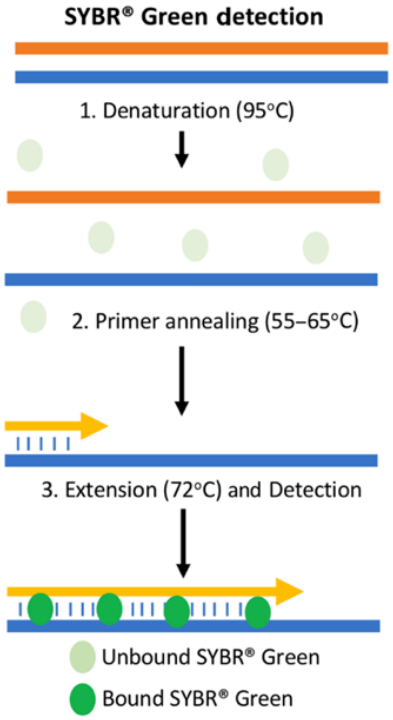

Figure 4-1. Descriptive image of SYBR green detection (Adams, 2020).

For the purpose of this particular project, the aim of using qPCR is to estimate the amount of DNA of the chosen region, that is present in the samples. To get an estimation of the total amount of bacteria and yeast present in the samples, the target regions are as general as possible for both yeast and bacteria. The bacterial primers target the 16S rRNA gene. Each bacterium contain somewhere between 5-10 copies of 16S rRNA, making the sensitivity of detection high (*16s rRNA, One of the Most Important rRNAs*, 2018). The fungal primers target the Internal

Transcribed Spacer 1 region, ITS1 region, found in rRNA genes. The length of the ITS1 gene can vary significantly between species or strains (Fierer *et al.*, 2005).

5. Materials and methods

5.1. Effect of a yeast gradient

In order to construct a series of several inoculums with a concentration gradient of yeast between each of them, filtration was performed with the aim of retaining the yeast and thus obtain a kombucha with as close to a yeast free community as possible. This liquid was to be mixed with a yeast dense liquid obtained through sedimentation. General knowledge in the brew industry is that cold temperatures and lack of mixing will facilitate the sedimentation of yeast, which is a widely used technique to reduce yeast loads in beer and kombucha production (Rosenstock, N., pers. comm.). In the kombucha industry this sedimentation is not thought to reduce bacterial population levels as much as yeast populations. For the purpose of this project it was hypothesized that after 3 days of sedimentation, yeast levels in the supernatant liquid would be significantly lower, while bacterial concentrations would be only moderately lower. Prokaryotic cells are commonly considered to be considerably smaller than eukaryotic cells and thus if the right pore size can be selected, the hypothesis was that yeast could be selectively eliminated by filtration.

5.1.1. Filtration

Because both the yeast and bacteria of interest have a size range, two different filter sizes were tested and compared. The first one was a Whatman 579 filter paper with a pore size of <2 μm. The second one was a Munktell grade 1F filter paper with a pore size of 5-6 μm. The filtration was performed using a Büchner funnel placed on a 1 L vacuum flask attached to a vacuum system. Samples of 100 mL kombucha were filtered. The flow through was poured into a sampling cup and stored in the fridge until analyzed. The filter was put in a glass beaker containing 100 mL of deionized water. After one minute, the filter was removed, and the sample was kept in the fridge until further analyzed. Both filters were tested on the same kombucha sample, later described as “regular”. Filtration was performed in triplicates and both filtrate samples and the diluted filter residue were saved in order to be examined with PCR.

5.1.2. Sedimentation

Six 25-liter aliquots of fully fermented kombucha were stored at 4 °C for 3 days. After this period of sedimentation, three buckets were mixed and two 1 L samples taken from each. These samples were noted as “regular” as they contained the fungal:bacterial biomass ratio of Roots of Malmö’s Naturell kombucha. From the remaining three buckets, first the top layer, or supernatant layer, was collected by ladling off 2 liters from the top of the bucket, taking care not to mix the sediment layer on the bottom. After this, all remaining liquid was poured off until only 1 liter remained in the bucket, and then this was collected. The bottom or “yeasty” layer was much darker and cloudier and clearly, according to the observation of Nicholas Rosenstock, brewmaster at Roots of Malmö, contained dramatically more yeast.

5.1.3. Microscopy and molecular analysis of filtered and sediment kombucha

To examine whether sedimentation or filtration was reducing the microbial population and to determine if a difference in microbial community composition was observable filtered and sedimented kombuchas were examined under a microscope for qualitative assessment as well

as cell density counting with a Bürker chamber (Kerl Hecht, Assistant, Germany). A small drop of the sample was placed on the Bürker chamber and a covered with a coverslip, then examined through a light microscope at 10 x, 50 x and 100 x magnification (Olympus 50, Olympus, Japan). The amount of microorganisms were counted in the same three squares for each sample and triplicates of all filtration samples were examined however only single samples of the sediment samples. The cell density in each sample was calculated based on the volume of each square. Unfiltered kombucha was compared to filtered kombucha and the diluted filter residues were compared between the filter sizes. As for the sediment samples, the supernatant, regular and yeasty samples were compared to each other.

To examine whether filtration significantly impacted the amount of yeasts relative to bacteria, DNA was extracted from the filtered kombuchas and amplified via PCR. Unfiltered kombucha was compared to the filtrate after 2 µm pore size and 5-6 µm pore size filtration as well as the filter residue of the same two filtration sizes. To start, one sample each of unfiltered kombucha, filtered through <2 µm pore size and through 5-6 µm pores, were analyzed in duplicates – once with bacterial primers and once with fungal primers. In the second round, samples of the unfiltered and filtered through <2 µm pores, were diluted 1:5 and 1:25 and analyzed for both yeast and bacteria. Comparing dilutions was done with the aim of being able to relatively quantify the yeast and bacterial amounts by investigating if the amount of DNA of either yeast or bacteria was too low to visualize at a certain dilution. Lastly, the filter residue samples were analyzed, undiluted and diluted 1:5, for both yeast and bacterial presence. All dilutions were done with milliQ purified water.

The DNA extractions of all samples were performed using the NucleoSpin® Soil extraction kit (Macherey-Nagel, Germany), following the protocol from the manufacturer. To ensure a high enough amount of acquired DNA, a sample of 50 mL was centrifuged at 5000 rpm, equivalent to 3214 g, for 10 minutes (Villarreal-Soto *et al.*, 2020). The supernatant was discarded and the pellet was resuspended in 700 µL of SL 2. 30 µL was decided as the volume of elution buffer in the final step. As the aim was to compare the total amount of bacteria and yeast present, primers targeting bacteria general- and fungi general regions were used. The bacterial primers (Eurofins, Denmark) were Eub338 (5'-ACT CCT ACG GGA GGC AGC AG-3') and Eub518 (5'– ATT ACC GCG GCT GCT GG – '3) and the yeast primers (Eurofins, Denmark) were ITS1f (5' – TCC GTA GGT GAA CCT GCG G – 3') and ITS5.8s (5'- CGC TGC GTT CTT CAT CG – 3') (Fierer *et al.*, 2005). 5 µL DNA sample was mixed with 15.875 µL water, 2.5 µL buffer, 0.5 µL dNTP, 0.5 µL of each primer (10 µM) and 0.125 µL TopTaq polymerase. For the occasions where dilutions of the samples were tested, they were diluted 1:5 and 1:25 with PCR water.

The PCR amplification was performed in an Eppendorf® Mastercycler® (Merck, Germany). The conditions used were 3 minutes of initial denaturation at 94 °C followed by 35 cycles of 1 minute denaturation at 94 °C, 45 seconds annealing at 50°C and 2 minutes extension at 72 °C then finishing with 10 minutes final extension at 72 °C. The samples were then cooled down to 4 °C. The finished PCR samples were evaluated by gel electrophoresis on agarose gel. The

samples were mixed with Orange DNA Loading Dye (6X, Thermo Scientific™, Lithuania) and the finished gel was stained with GelRed (Biotium, vwr, U.S.).

5.2. Effect of temperature on kombucha fermentation

5.2.1. Set up of temperature zones

In order to ensure that the temperature zones were stable enough over time, a first round of test fermentations was performed before the actual primary fermentation. Five different places at Roots of Malmö's facilities were selected with temperatures somewhere around 15 °C, 20 °C, 25 °C, 30 °C and lastly 35 °C. To prepare the tea, tap water was brought to a boil. Thereafter, 3.3 g of black tea/L water was added. When the tea had boiled for 10 minutes, 55 g sugar/ L water was added and the tea was brought back to a boil. The sweetened tea boiled for another 5 minutes, then it was rapidly cooled in a heat exchanger to a temperature of 25 °C. For the purpose of this test round, 2 liters of sweetened tea was mixed with 1 dL of kombucha in a 3.3 L glass container (365+, IKEA, Sweden). The containers were covered with a cloth, equipped with a temperature data logger and left for a period of 21 days. During this period of time, the temperature was checked continuously. Following these three weeks, the collected data from the temperature loggers were evaluated and it was ensured that the temperatures were stable within a ±1°C temperature span. Additionally, it was confirmed that a successful formation of a cellulose layer on top of the liquid had formed and that the pH, measured with a PCE-PH22 PEN pH meter (PCE Instruments, Germany), had dropped below 3, a typical pH of commercially available kombucha (Rosenstock, N., pers. comm.)

5.2.2. Establishment of fermentation vessels and sampling

With the basis of the results of the trial fermentations, which were considered successful, the main fermentation trial was set up. A total amount of 20 fermentation beakers were prepared. The tea was prepared according to the method described above, under *5.2.1. Set up of temperature zones*. Two batches of 24 kg sweetened tea were mixed with 1.26 kg of previously fermented kombucha, creating a 5 % kombucha addition. Ten 50 mL samples were taken from this blend and frozen for later analyses. pH was measured using the PCE-PH22 PEN pH meter. Brix was measured using an Extech® RF11 portable brix refractometer (Extech®, U.S) with automatic temperature compensator. 2200 g of the blend was transferred into each 3.3 L beaker which was covered with a cloth and secured with a rubber band. Four beakers were placed in each temperature zone.

Sampling of the kombucha was performed every two days. A 50 mL syringe was used to carefully reach about 5 cm below the cellulose layer on top of the liquid without disturbing the structure. To start, 15 mL of kombucha was sampled and used to measure pH and brix, then stored in the freezer until the decision was taken as to which samples would be further analyzed. When the mean pH in a temperature zone reached below 3.2, the amount of kombucha per sample was increased to 50 mL. After 20 days of fermentation, the process was terminated.

5.3. Evaluation of fermentation

5.3.1. Culture formation, evaporation and taste

When the fermentation process was ended on day 20, some additional factors were analyzed in addition to the routine sampling of pH and brix. Out of the five different temperature zones that had been used, the one supposed to be around 30 °C was chosen to be excluded from the final analysis. It was only possible to run analysis on four of the zones due to resource limitations and because the one aiming to be 30 °C one turned out to be almost 5 °C colder than aimed for, this was chosen as the one to exclude. From the remaining four treatment groups a total of two 50 mL and two 15 mL samples of kombucha were taken according to the same method performed every second day during fermentation and described under *5.2.2. Establishment of fermentation vessels and sampling*. Two additional 50 mL samples were taken after the kombucha had been properly mixed, which resuspended the sediment layer that had formed on the bottom of the fermentation vessel during the 20 days of fermentation. The mother culture, or the pellicle, of each fermentation was weighed. So was the remaining liquid in each beaker in order to evaluate the evaporation. Kombucha from each temperature zone was tasted by the report author and the supervisor from Roots of Malmö and evaluated on acidity, sweetness and tea taste. The different samples were compared and rated in relation to each other from less acidic to most acidic and in the same manner for both sweetness and taste of tea.

5.3.2. Sugar analysis

The sugar analyses were performed using the Megazyme Sucrose, D-Fructose and D-Glucose assay procedure (Megazyme, 2018). The principle of the kit was measuring D-glucose concentration before and after hydrolyzing sucrose by invertase and thereafter also determining the D-fructose content. Sucrose and D-glucose/D-fructose were measured through separate reactions in separate wells but during the same analysis run.

Four replicate samples from day 0 and four replicates from each temperature zone on day 10 and day 20 were tested. After thawing the samples, 100 µL of each sample was diluted with 10 mL of milliQ water to achieve a 1:100 dilution. Standards were prepared from the D-Glucose, D-Fructose standard solution that was accompanied with the kit, containing 0.2 mg of each sugar per mL. Three additional standard data points were prepared with a threefold dilution between each.

The assay was performed on a plate reading spectrophotometer called SPECTROstar Nano (BMG LABTECH, Germany) in a 96 well Greiner F-bottom clear microplate. Instructions were followed according to kit protocol with the exception of 30 µL sample addition and 50 µL standard addition instead of 10 µL. The amounts were compensated by removing the corresponding sample increase volume from the water addition, resulting in same final sample volume of 250 µL. The incubation time before reading A2 was five minutes prolonged, from five to ten minutes in order to ensure completion of the reaction.

The different absorbance differences were calculated according to the instructions in the kit protocol however the final concentrations were obtained using the standard curve. Four samples

were removed from the data set due to the signals not being above the background and in five additional cases, where the calculated sucrose concentration was negative, the values for the sucrose concentration were set to zero.

5.3.3. DNA extraction, PCR and qPCR

For the purpose of primer validation, a qPCR test run was performed. A yeast sample of *Saccharomyces cerevisiae* was used. The DNA was extracted by transferring approximately 10 mg of *S. cerevisiae* into a sterile centrifugation tube together with 1 mL autoclaved water and 10 extraction beads. The tube was shaken for 30 minutes followed by three minutes centrifugation. For bacterial primer validation an already extracted sample of *Bifidobacterium T48727* was used. The qPCR was run with an undiluted sample, a ten times diluted sample and a negative control. The recipe for the qPCR contained 2 µL of the extracted DNA sample which was mixed with 6.5 µL of nuclease free water, 0.75 µL each of forward and backward primer (10 µM) and 10 µL of SYBR-green. The qPCR was run with a 15 minutes initial denaturation at 95 °C followed by 40 cycles of 1 minutes denaturation at 95 °C, 30 seconds of annealing at 53 °C and finished with 1 minutes elongation at 72°C (Fierer *et al.*, 2005). The qPCR was performed in a Rotor–Gene Q.

When it was confirmed that the primers worked well for the qPCR, thus that the final product concentration was ten times higher for the ten times less diluted sample, and the samples from the fermentation at Roots were gathered, the final testing started. Four samples from the original inoculated tea batch from day 0, four samples from when the pH dropped below 3.0 from each of the temperature zones, and four samples from each temperature zone on day 20 were used. The DNA from each sample was extracted according to the method previously described under *5.1.3. Microscopy and molecular analysis of filtered and sediment kombucha*.

Three samples from each temperature zone and sampling time points were chosen from the four that were extracted. All samples were analyzed using a Nano Drop. If the results indicated too low amount of DNA or if the sample was contaminated, this sample was taken away. If all four samples from the same sampling time point seemed successfully extracted, samples 1, 2 and 3 were tested with qPCR.

To be able to calculate the concentrations of yeast and bacterial DNA present in the samples during the qPCR, a standard curve for both bacteria and fungi was created. The aforementioned yeast and bacterial sample also used for primer validation, were amplified using PCR. Four bacterial samples and four yeast samples were prepared using the same recipe and primers as described under *5.1.3. Microscopy and molecular analysis of filtered and sediment kombucha.* The same PCR conditions were used. To purify the PCR product, mainly from residual primers, the MinElute® PCR Purification Kit was used. The four PCR products were mixed together for bacteria and fungi respectively and then the kit protocol was followed.

The data points used for the standard curve on the first run for bacterial quantification were done using 10–fold dilutions ranging from $0.2 \cdot 10^{-7}$ ng DNA/reaction to $0.2 \cdot 10^{-3}$ ng DNA/reaction with an additional data point at 0.2 ng DNA/reaction (Fierer *et al.*, 2005). For

the second run of the remaining bacterial samples, the standard curve was adjusted to ranging from $0.2 \cdot 10^{-4}$ ng DNA/reaction to $0.2 \cdot 10^{-1}$ ng DNA/reaction with a 10-fold dilution. The data points for the standard curve of the first run for fungal quantification contained $0.2 \cdot 10^{-6}$, $0.2 \cdot 10^{-4}$, $0.2 \cdot 10^{-3}$, $0.2 \cdot 10^{-1}$ and 0.2 ng DNA/reaction. For the second run, the concentrations were adjusted to contain $0.2 \cdot 10^{-4}$, $0.2 \cdot 10^{-3}$, $0.2 \cdot 10^{-1}$, 0.2 and 2.5 ng DNA/reaction All samples, both for standard curves and the unknown kombucha samples, were prepared in triplicates according to the qPCR recipe presented above, and the same running conditions were used.

The result from the qPCR was given in ng/µL. Since the DNA fragments of the bacteria and the yeast were of different size, the concentrations were converted from ng/µL to fragments/mL. This was done based on two main assumptions, the first one being that one base pair weighs 660 g/mole. The second assumption was that the bacterial DNA fragments were 200 base pairs long and the fungal DNA fragments were 300 base pairs long, based on an assumed 1:1 distribution of fungal fragments of 200 and 400 base pairs length respectively (Fierer *et al.*, 2005).

5.4. Statistics

All statistical analysis of the results were performed in the program SigmaPlot (Systat Software Inc, USA). Since no sample sizes for the purpose of this project's results were bigger than maximum four replicates, it was assumed that the samples were not normally distributed. Therefore, when comparing between independent groups, the Kruskal-Wallis One Way ANOVA on Ranks was used. The null hypothesis was that the means of the parameter in question were not different between the different samples. The significance level was decided as α=0.05, meaning that a p-value lower than α, would discard the null hypothesis with a 95 % certainty. If that was the case, at least two of the samples were significantly different. To find out which samples differed, a post hoc test was performed. Because normal distribution was not assumed, the Student-Newman-Keuls Method was used which compares the samples pairwise. An example of what the statistical results look like can be found in Appendix A – Statistical analysis. Figure A-1 shows the results of comparing pH between the different temperature zones on day 20.

When comparing samples that were dependent, for example when comparing a parameter in the beginning, middle and end within the same fermentation zone, the Friedman Repeated Measures ANOVA on Ranks was used. If $p<0.05$, a Tukey test was performed in order to find out which of the samples that differed significantly from each other. Figure A-2 shows the statistical results of comparing the amount of bacterial DNA in the lowest temperature compared between day 0, day 18 and day 20.

6. Results

6.1. Effect of a yeast gradient

In order to get a first indication of whether the filtration had the desired outcome or not, the samples were all investigated through a microscope. What could be observed was a decrease in microbes with filtration. The number of microorganisms present in the samples decreased with a smaller filter pore size. Mean concentration and standard error of mean from triplicate Bürker chamber count can be seen in *Table 6-1*. The raw data as well as information about calculations can be found in Table B-1 in *Appendix B – Microscopy examination of unfiltered and filtered kombucha.* As expected, with the aforementioned information in mind, the number of microorganisms present in the samples of diluted filter residue instead increased with a smaller filter pore size. Furthermore, the aim of the microbial count was to tell how much the yeast and the bacteria decreased respectively and to see if these results seemed to indicate that yeast was retained in the filter at the same time as the bacteria was passing through. However, already at an early stage it was observed that the size difference between the yeasts and bacteria was not as big as was expected and it appeared insufficient to differentiate between the groups based on size.

Table 6-1. Total microbial concentration in filtered and unfiltered samples.

	Unfiltered	**5-6 µm pore size**	**<2 µm pore size**
Total microbial concentration (psc/mL)	$1.5 \pm 0.1 \cdot 10^{10\,a}$	$6.0 \pm 0.8 \cdot 10^{9\,b}$	$3.5 \pm 0.3 \cdot 10^{9\,c}$

Reported are mean values and standard errors of 3 measurements. Values that are not sharing a common superscript letter differ significantly at $p < 0.05$.

An additional way of distinguishing between bacteria and yeast could be by shape, knowing that the most abundant bacterial species of kombucha are rod shaped, or slightly elliptic, and the most common fungal species are elliptical or round shaped (Kurtzman and James, 2006; Gomes *et al.*, 2018). The shape and size difference in combination would have been good to visually differentiate between the yeast and bacteria. However, since they in this particular case appeared so similar in size, the shape itself was not a sufficient factor of characterization as the definite limit between a rod and an oval is not always clear. An example of what this could look like is shown below in *Figure 6-1*. The microscopy image shows a 500x enlargement of an unfiltered kombucha sample. A range of sizes and shapes are displayed. Additional pictures that show a comparison of unfiltered and filtered samples are shown in Figure B-1, B-2 and B-3 in *Appendix B*. With the basis of this, it was concluded that microbial count using a Bürker chamber was not a certain enough method to give a reliable indication of the filtration outcome.

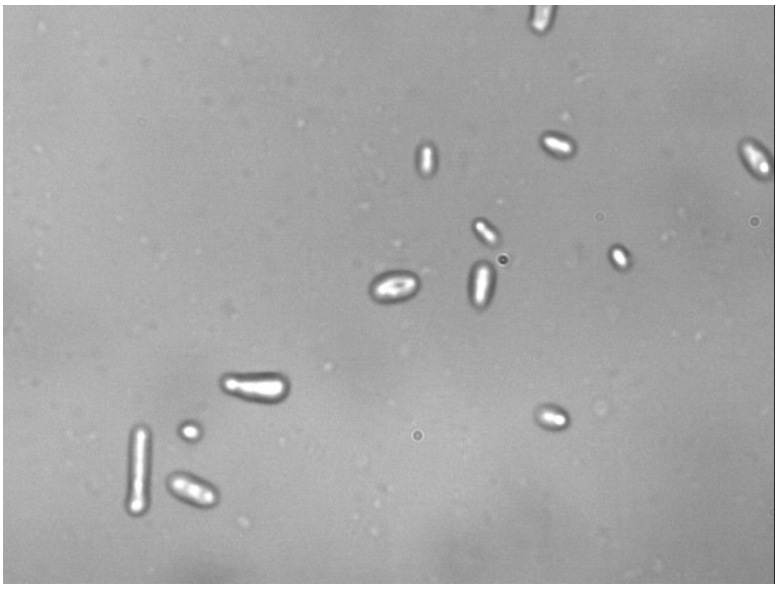

Figure 6-1. 500x enlarged microscopy image of unfiltered kombucha.

Also the sediment samples were visually examined. The amount of microorganisms in each sample was compared between the supernatant, the regular and the yeasty kombucha. As mentioned, only one replicate of these samples were counted, leading to low statistical reliability of the result. However, there was an indication from those that the amount of organisms decreased in order from the yeasty to the regular and lastly the sediment sample. Furthermore, the same observation as discussed above was made; the difficulty in distinguishing between bacteria and yeast. Thus making it difficult to evaluate if the yeasty samples truly contained a very high fungal:bacterial ratio.

To further demonstrate the outcome of the filtration, some samples were analyzed with PCR and evaluated with gel electrophoresis. The results of the first run showed that there were both bacteria and yeast in all samples: unfiltered kombucha, flow through from 5-6 μm pore size and <2 μm pore size. The strength of the DNA bands on the gel gave a possible indication that there could be a bigger retention of yeast with the smaller pore size than with the bigger, seeing that the bands for yeast were a little thinner. An image of this agarose gel can be seen in Figure C-1 in *Appendix C – PCR results from filtration*. This was then further examined by diluting the samples to relatively quantify the yeast versus the bacterial amount in the samples. After diluting the samples 1:25, they all still showed presence of both bacteria and yeast. When lastly analyzing the filter residue samples, both undiluted and with a 1:5 dilution, the results displayed presence of both bacteria and yeast also in these. These two agarose gels are also shown in *Appendix C*, Figure C-2 and C-3 respectively. With the basis of all of the aforementioned results, the filtration did not seem to be a sufficient way to separate the yeast from the kombucha.

6.2. Effect of temperature on kombucha fermentation

The mean temperature and standard error of each of the four zones that were chosen for further analysis is presented in *Table 6-2*. As can be seen, there was a difference of about 5 °C for the

three lower temperatures, however slightly more than 10 °C difference to the highest temperature.

Table 6-2. Temperature of each zone over time.

Temperature (°C)	15.1 ± 0.0	19.8 ± 0.0	24.0 ± 0.0	35.9 ± 0.0

Sampling of each kombucha fermentation was performed every second day. All raw data from the 20 days of sampling can be found in Table D-1 in *Appendix D – Raw data from fermentation*. Table D-2 and Table D-3 contain the raw data of the additional analyses that were performed as the fermentation was terminated. Since the sampling was performed every second day, the visual process similarities and differences could be followed throughout the fermentation. Already on the second day a thin cellulosic layer had formed on top of the liquid on each of the beakers placed in the highest temperature zone. Two days later, on the fourth day, the same could be observed for the kombucha placed in the two intermediate temperatures. On the eight day, a fully covering pellicle was observed for the kombucha with the lowest temperature. At this point, the pellicles present in all other temperature zones had started to develop a thicker, concentrated piece of cellulose as a small spot in the middle of the cellulosic film. Especially distinct were they on those pellicles fermenting in 35.9 °C. An example is seen in *Figure 6-2*. By day 20 these concentrated cellulose patches were visible on all mother cultures, with some variation in size and thickness.

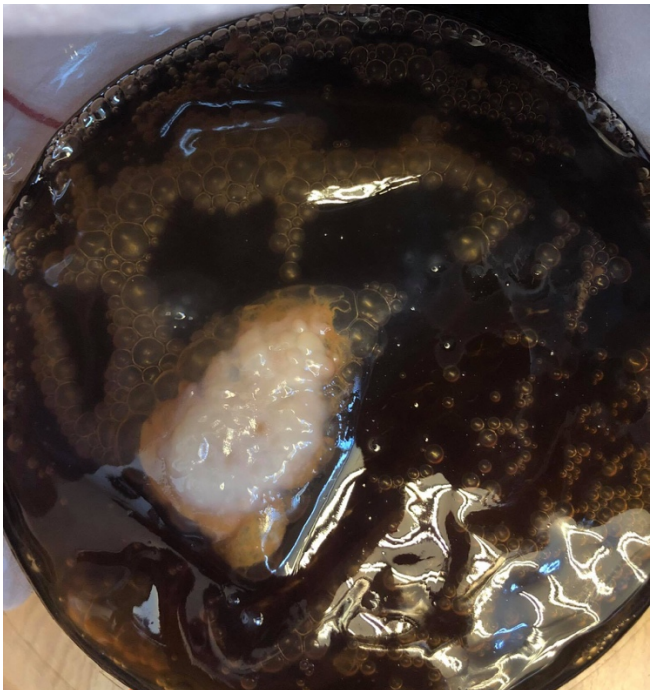

Figure 6-2. Picture of a pellicle formed on top of kombucha after eight days of fermentation in 35.9 °C

A general observation for all mother cultures throughout the fermentation process was the formation of air bubbles within the pellicle. This can also be seen in the figure above. At the final day, most of them were covered with air bubbles.

6.2.1 pH

The pH was measured in each beaker every second day during the fermentation process and the result can be viewed in *Figure 6-3*. As seen when looking at the four graphs, the pH associated with the two higher temperatures, 24.0 °C and 35.9 °C, started lowering faster than the ones associated with the two colder temperatures, and remained below them until day 14. In comparison to each other, the pH of the fermentations in 24.0 °C decreased slightly slower than in 35.9 °C during the first half of the process, while after 10 days the two graphs crossed each other and the decrease in pH in 35.9 °C reach a slow-decreasing part of the curve. By the end of the fermentation, 24.0 °C had the lowest pH at 2.72. Until day 6, the pH of the kombucha fermented in 15.1 °C dropped faster than 19.8 °C. However, after that point, the pH of 19.8 °C started to decrease faster and when the fermentation was terminated the pH it seems as though the acidification in 19.8 °C is still active, displaying a steeper curve than the two higher temperatures. The pH of the highest temperature dropped relatively even throughout the process, somewhat faster in the beginning than in the end. By day 20, 35.9 °C has the second highest pH at 2.80. Furthermore, comparing between all temperatures, the rate of the pH drop varied to a larger extent during the first half of the fermentation time. In the end of the process, several of the graphs seem to converge and reach towards a similar final pH, although it appears that the two lower temperatures would still decrease somewhat more if the process was not terminated.

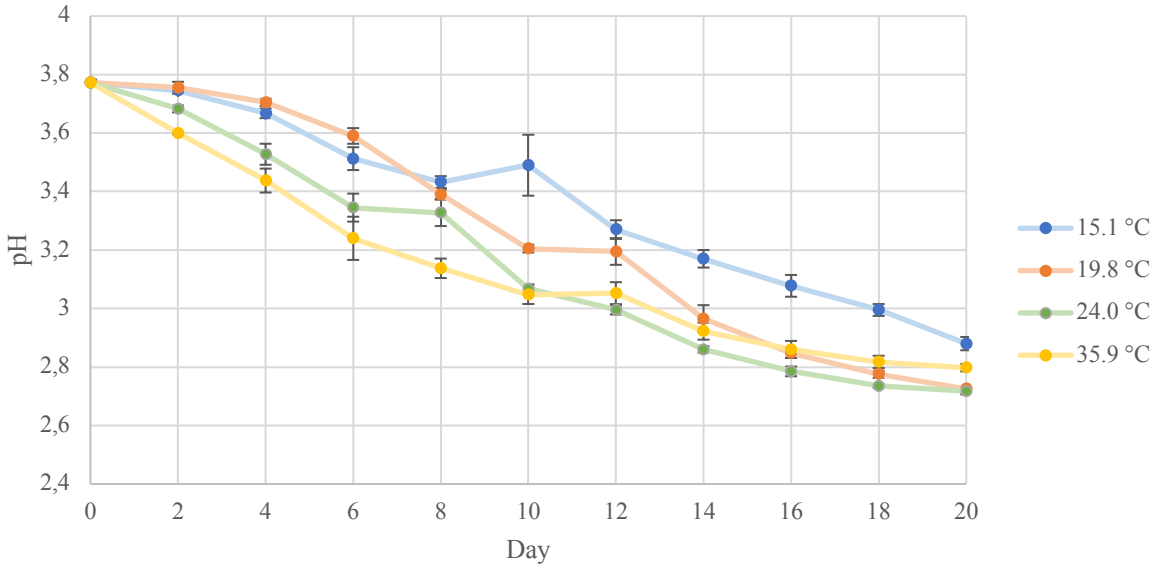

Figure 6-3. The pH in each temperature zone throughout the fermentation process presented as mean value ± standard error of mean.

The pH values on the final fermentation day are presented in *Table 6-3*. As can be seen on the superscripted letters in the table, there was no significant difference in the final pH between 19.8 °C and 24.0 °C, who have the two lowest pH values. pH 2.88, corresponding to the fermentation in 15.1 °C, was significantly higher than the rest. Also the pH associated with 35.9 °C fermentation temperature differ from the rest.

Table 6-3. pH in each temperature zone on day 20.

	15.1 °C	19.8 °C	24.0 °C	35.9 °C
pH	2.88 ± 0.02^{a}	2.73 ± 0.00^{b}	2.72 ± 0.01^{b}	2.80 ± 0.01^{c}

Values that are not sharing a common superscript letter differ significantly at $p < 0.05$.

6.2.2. Brix

Measurements of brix were also done in each beaker every second day during the fermentation. The results are displayed in *Figure 6-4*. The brix of the three lower temperatures followed about the same pattern during the fermentation process. They all fluctuated somewhat up and down but all go from 5.5 °Bx at day 0 to approximately 5.2 °Bx at day 20. There was no significant difference in brix between those three temperatures on the final day of fermentation, as can be seen in Table 6-4.

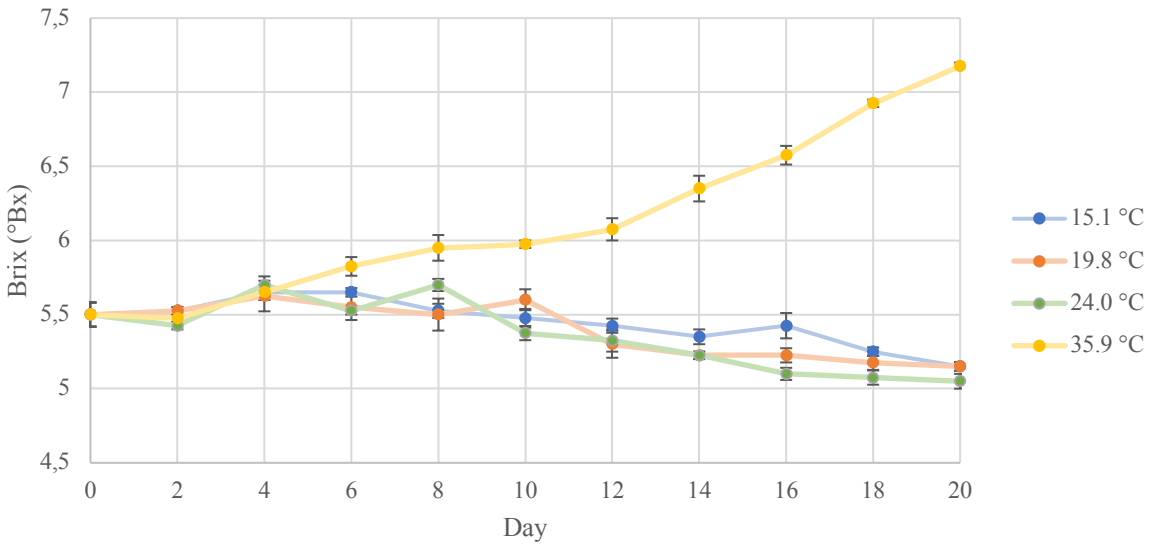

Figure 6-4. Mean brix ± SE of mean for each temperature throughout the fermentation process.

The brix of the kombucha fermented in the highest temperature, 35.9 °C, displayed a different behavior from the rest. Already early in the process the brix started to increase and it continued to do so throughout the entire duration of the fermentation, with a drastically increase in speed after day 12. On the last day of fermentation, the final value was 7.2 °Bx, which was significantly higher than the rest.

Table 6-4. Brix in each temperature zone on day 20.

	15.1 °C	19.8 °C	24.0 °C	35.9 °C
Brix (°Bx)	5.2 ± 0.0^{a}	5.2 ± 0.0^{a}	5.1 ± 0.1^{a}	7.2 ± 0.0^{b}

Values that are not sharing a common superscript letter differ significantly at $p < 0.05$.

6.2.3. Sugar analysis

The results of the sugar content in the samples on day 0, day 10 and day 20 are shown in *Figure 6-5*. The raw data, calibration curve and calculations are described in *Appendix E – Sugar analysis*. A general observation, although with some exceptions, is that the sucrose content decreased over time, while the fructose and glucose content increased, though the variance was high for some of the sampling times.

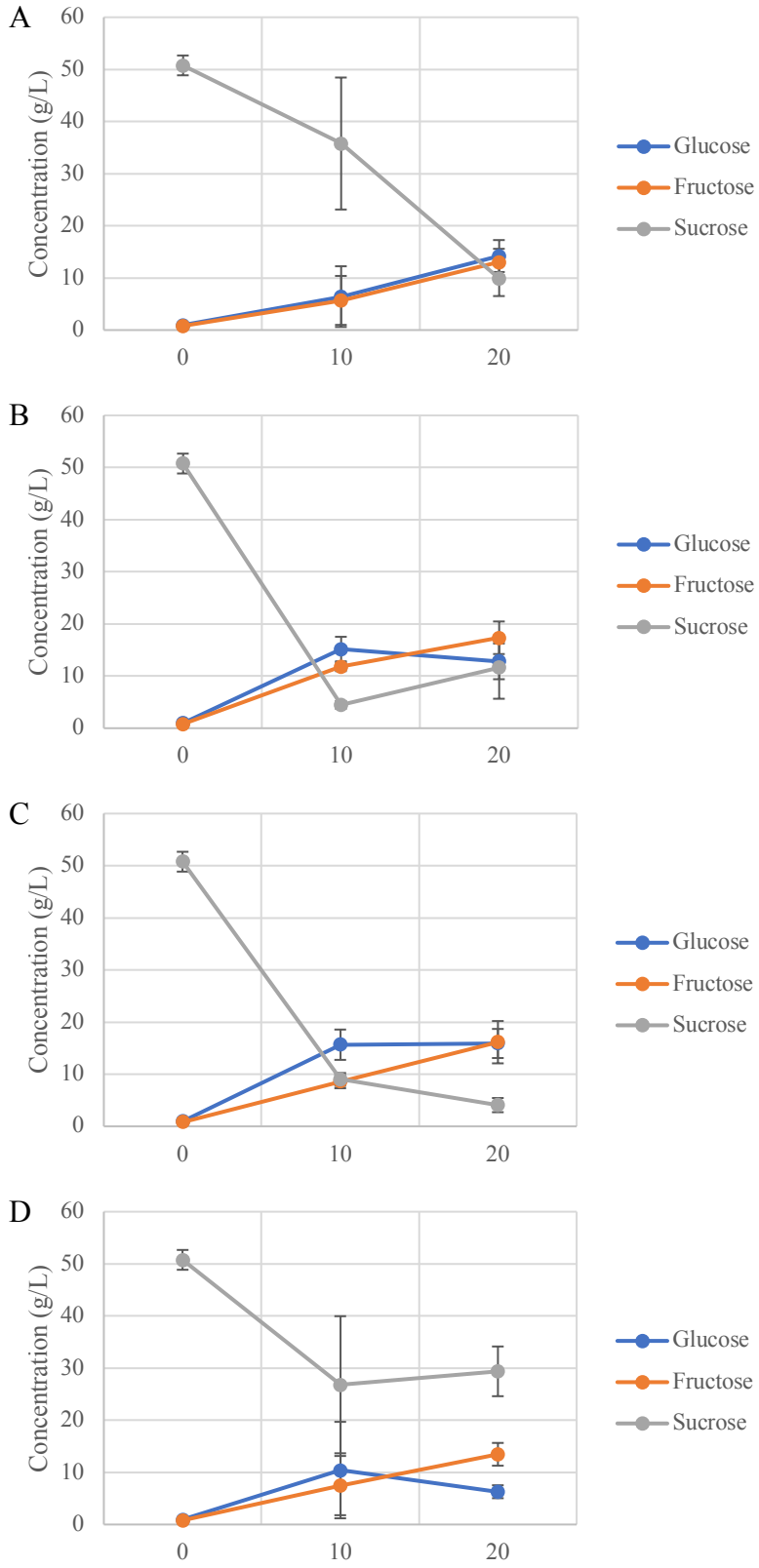

Figure 6-5. Sugar concentrations on day 0, day 10 and day 20 in kombucha fermented in 15.1 °C (A), 19.8 °C (B), 24.0 °C (C) and 35.9 °C (D)

At the lowest temperature, the sucrose decreased over time and the glucose and fructose concentration increased. For the two intermediate temperatures, the decrease in sucrose from day 0 to day 10 was very large and by this time point, the sucrose concentration had reached below the glucose and fructose. At the highest temperature the decrease in sucrose content was more moderate between day 0 and 10 but with no further changes between day 10 and day 20. The sucrose content in the samples fermented at 15.1 °C and 24.0 °C continued to decrease over time, between day 10 and 20, while the sucrose content in the samples fermented at 19.8 °C and 35.9 °C did not change in that same time period. Glucose and fructose content in the samples fermented at the three higher temperatures all increased significantly from day 0 to day 10, and then no more change could be observed between day 10 and day 20. When comparing each sugar content between the different temperature zones from the final day, found in *Table 6-5*, there were no significant differences except for sucrose, which was significantly lower at 24 °C than at 35 °C.

Table 6-5. Sugar content on day 20.

	15.1 °C	**19.8 °C**	**24.0 °C**	**35.9 °C**
Glucose (g/L)	$14.2^{a} \pm 3.1$	$12.8^{a} \pm 3.4$	$15.9^{a} \pm 2.8$	$6.3^{a} \pm 1.2$
Fructose (g/L)	$13.0^{a} \pm 2.5$	$17.3^{a} \pm 3.1$	$16.1^{a} \pm 4.1$	$13.5^{a} \pm 2.2$
Sucrose (g/L)	$9.9^{ab} \pm 3.4$	$11.6^{ab} \pm 6.0$	$4.1^{a} \pm 1.4$	$29.4^{b} \pm 4.8$

Values that are not sharing a common superscript letter within each row differ significantly at $p < 0.05$.

The results are further visualized in *Table 6-6* and *Table 6-7* where it can be observed that neither the total sugar concentration nor the fraction of sugar present in the sample as fructose differ significantly over time. The only exception is the fraction of sugar present as sucrose in the second highest temperature which showed a significant decrease between day 0 and day 20.

Table 6-6. Total sugar concentration in sample.

	15.1 °C (g/L)	**19.8 °C (g/L)**	**24.0 °C (g/L)**	**35.9 °C (g/L)**
Day 0	$52.5^{a} \pm 1.6$	$52.5^{a} \pm 1.6$	$52.5^{a} \pm 1.6$	$52.5^{a} \pm 1.6$
Day 10	$47.9^{a} \pm 4.7$	$31.4^{a} \pm 3.2$	$33.2^{a} \pm 3.8$	$44.7^{a} \pm 1.8$
Day 20	$37.2^{a} \pm 3.6$	$38.9^{a} \pm 6.5$	$36.1^{a} \pm 5.5$	$49.1^{a} \pm 4.1$

Values that are not sharing a common superscript letter within each column differ significantly at $p < 0.05$.

Table 6-7. Fraction of sugar present as sucrose in sample.

	15.1 °C	**19.8 °C**	**24.0 °C**	**35.9 °C**
Day 0	97^{a} %	97^{a} %	97^{a} %	97^{a} %
Day 10	75^{a} %	14^{a} %	27^{ab} %	60^{a} %
Day 20	27^{a} %	28^{a} %	11^{b} %	60^{a} %

Values that are not sharing a common superscript letter within each column differ significantly at $p < 0.05$.

6.2.4. Culture formation, evaporation and taste

One of the extra measurements that were performed on the final day of fermentation was the weighing of the mother culture. All four pellicles were weighed and in *Table 6-8* below, the mean weight of the pellicles from each temperature zone is presented. As seen on the

superscripted letters, only the pellicles from the 35.9 °C kombuchas weighed significantly more than the rest.

Table 6-8. Pellicle weight and evaporation in each temperature zone on day 20.

	15.1 °C	**19.8 °C**	**24.0 °C**	**35.9 °C**
Pellicle weight (g)	52 ± 2.5^{a}	39.0 ± 1.5^{a}	47.5 ± 5.0^{a}	84.3 ± 5.4^{b}
Evaporated liquid (g)	176.0 ± 6.8^{a}	197.8 ± 7.0^{a}	255.5 ± 6.9^{b}	915.5 ± 19.0^{c}

Values that are not sharing a common superscript letter in each row differ significantly at $p < 0.05$.

The amount of liquid left in the containers was also noted upon harvest on day 20. By subtracting the amount of liquid left in container as well as the amount of sampled kombucha during the experiment from the 2200 g of inoculated tea that was put in each beaker on day 0, the amount of evaporated liquid was calculated. The results are also presented in *Table 6-8* where it can be observed that the evaporation between the two colder temperatures did not differ from each other. However, both of the warmer temperatures, and particularly the warmest one, displayed a significantly higher evaporation. The amount of evaporated liquid from the beakers placed in 35.9 °C was more than three times as high as the amount of the beakers in 24.0 °C, which has the second largest evaporation.

To ease the comparison during the taste evaluation performed on day 20 the samples were called sample 1 to sample 4, starting from the coldest temperature to the warmest. When comparing the sweetness, it was distinctively higher in sample 4 compared to the other three samples. Sample 2 and 3 had a comparable sweetness, somewhat lower than sample 4 but stronger than the last sample, that had a very low sweetness. The acidity was highest in the samples that were fermented in the two highest temperatures. It was somewhat lower in sample 2 and lowest in sample 1. When comparing the taste of black tea, the only sample that had a very clear taste of black tea was sample 1. In the other three kombuchas, the taste was not very prominent in any of them. Overall, sample 4 tasted “more fermented”, sample 1 tasted “less fermented”, and samples 2 and 3 tasted similarly.

6.2.5. Microbial content

The amount of DNA in each sample was, after the qPCR, given in ng/µL. The results are displayed as a graph of the increase in fluorescent emission each cycle, as a graph of the plotted standard curve and as a table with the calculated DNA concentrations. An example of what these look like can be seen in Figure F-1 and Figure F-2 in *Appendix F – qPCR result examples*.

Table 6-9 and *Table 6-10* show the mean amount of DNA fragments per mL ± standard error of mean from the qPCR results. Comparisons were made between the three sampling time points within each temperature zone and between different temperature zones at a given sampling time point for both bacteria and yeast. Overall, there was a consistent pattern that microbial density, both for bacteria and yeast, increased from the start of the fermentation (day 0) to the end of fermentation (day 20). However, due to large variance in the data, the only significant difference in amount of DNA between day 0 and day 20 was for bacteria in 15.1 °C and between day 14 and day 20 for yeast in 35.9 °C.

Table 6-9 Amount of DNA in the two colder temperature zone at the three sampling time points.

	15.1 °C		19.8 °C	
Day	Bacteria (pcs/mL)	Yeast (pcs/mL)	Bacteria (pcs/mL)	Yeast (pcs/mL)
0	$2.95 \pm 0.57 \cdot 10^{7\ a}$	$1.15 \pm 0.30 \cdot 10^{9\ a}$	$2.95 \pm 0.57 \cdot 10^{7\ a}$	$1.15 \pm 0.30 \cdot 10^{9\ a}$
14/18	$8.40 \pm 3.6 \cdot 10^{7\ ab}$	$1.59 \pm 1.3 \cdot 10^{9\ a}$	$6.89 \pm 2.8 \cdot 10^{7\ a}$	$6.46 \pm 3.9 \cdot 10^{8\ a}$
20	$5.30 \pm 2.4 \cdot 10^{9\ b}$	$1.16 \pm 0.31 \cdot 10^{11\ a}$	$2.19 \pm 1.6 \cdot 10^{9\ a}$	$6.98 \pm 1.9 \cdot 10^{10\ a}$

Values within each column that are not sharing a common superscript letter differ significantly at $p < 0.05$.

Table 6-10 Amount of DNA in the two warmer temperature zone at the three sampling time points.

	24.0 °C		35.9 °C	
Day	Bacteria (pcs/mL)	Yeast (pcs/mL)	Bacteria (pcs/mL)	Yeast (pcs/mL)
0	$2.95 \pm 0.57 \cdot 10^{7\ a}$	$1.15 \pm 0.30 \cdot 10^{9\ a}$	$2.95 \pm 0.57 \cdot 10^{7\ a}$	$1.15 \pm 0.30 \cdot 10^{9\ ab}$
14/18	$6.17 \pm 3.7 \cdot 10^{7\ a}$	$1.08 \pm 0.67 \cdot 10^{9\ a}$	$3.23 \pm 1.9 \cdot 10^{7\ a}$	$3.26 \pm 1.2 \cdot 10^{8\ a}$
20	$1.21 \pm 0.77 \cdot 10^{9\ a}$	$4.55 \pm 1.0 \cdot 10^{10\ a}$	$1.23 \pm 0.56 \cdot 10^{9\ a}$	$4.30 \pm 1.7 \cdot 10^{10\ b}$

Values within each column that are not sharing a common superscript letter differ significantly at $p < 0.05$.

When looking closer at the concentrations of bacterial DNA on day 20, no observation of an effect by temperature, on the amount of microbial DNA present can be observed. A common feature that can be seen in both of the tables above, is the large standard errors. That means that the samples within each group are widely spread around the mean and thus increasing the risk of the mean not being representative, which is something that needs to be taken into consideration when interpreting the results.

Figure 6-6. Fungal:Bacterial ratio for each temperature on each sampling time point. 1=Day 0, 2=Day 14 or 18, 3=Day 20.Figure 6-6 show the fungal:bacterial ratio for each temperature and sampling time. The mean values describe how many folds more yeast DNA is present in comparison to bacterial DNA. Observed in the figure is that the ratios all decrease somewhat from day 0 to the middle sampling point (day 14, 18) and then increase again on day 20. Significance was tested between each temperature on day 20, thus if the fungal:bacterial ratio differs depending on fermentation temperature. None of the samples differ significantly. Also in this case the standard errors were relatively high.

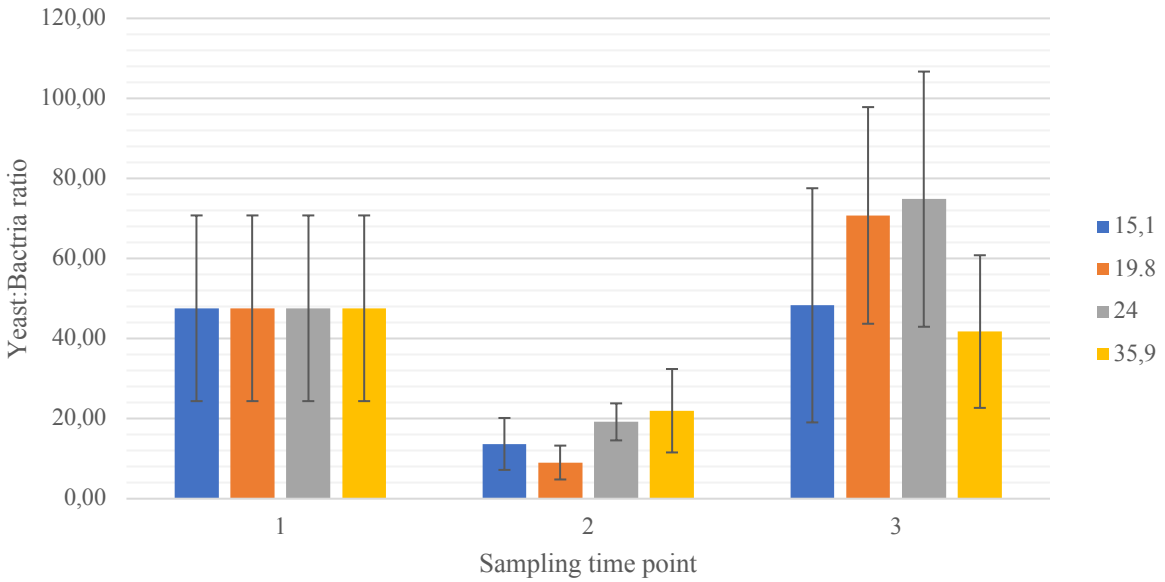

Figure 6-6. Fungal:Bacterial ratio for each temperature on each sampling time point. 1=Day 0, 2=Day 14 or 18, 3=Day 20.

7. Discussion

7.1. Effect of a yeast gradient

The basis of the filtration and sedimentation approach was to a large extent based on the previously assumed significant difference in size between yeast and bacteria, as well as the assumption that yeast would settle to the bottom of a cooled solution more rapidly. As mentioned in the beginning of this report, under *Kombucha microbial community*, the size difference between bacteria and yeast is often distinct, with yeast being larger than bacteria. Thus, filtration was considered a potential approach for separating the two different microorganisms. Already at the first stage of method evaluation, when observing the microbes through a microscope, the assumption of a significant size distinction between the yeasts and bacteria appeared weak. This weakness was further highlighted when little variation in the sizes and gross morphology of organisms was observed when comparing the filter flow through with the diluted filter residue. The expectation was that by retaining almost exclusively large organisms, either larger than 5 or 2 μm depending on which filter was used, and letting only smaller ones through, a selection against yeasts could be made. If the filtration would have been successful in selecting against yeast, this would be notable by significantly larger and differently shaped cells in the concentrate of yeast collected on the filters, however, this was not observed. The same observations were done when comparing the supernatant and the "yeasty" sample where the expectations were both a decrease in total microbial count but also that the "yeasty" sample would contain significantly larger and differently shaped cells than the supernatant. This was not observed in this case either.

The expected results were hypothesized based on prior research on kombucha. Villarreal-Soto et. al. (2018) displayed the morphology of yeast species commonly present in kombucha, all roughly within the range of 5 – 8 μm long and one of the most abundant bacterial species, *K. xylinus,* is generally around 2 μm long (Campano *et al.*, 2016). Based on the observations in this experiment, it seems as though the microbial community in Roots of Malmö's kombucha differ in morphology. *K. xylinus* appears, according to the visual examination, to be longer than expected and several yeast species appear smaller.

The lack of effectiveness of the filtration process was further validated when the PCR treatment of the samples was evaluated. When comparing the filtered samples to unfiltered, they all showed the presence of both yeast and bacteria, with very little difference independently of filter treatment. One possible factor that could have had in impact on the aforementioned outcome is if the pores of the filter paper were clogged by the larger organisms and as a consequence also retained smaller ones that otherwise would have passed through. This could be one of the explanations for why there was bacteria retained also with 5-6 μm pore size, a size that would normally allow the flow through of bacteria. As a next step, the unfiltered and 2 μm filter flow through were diluted with the hopes of being able to interpret band intensity after PCR and gel electrophoreses as a semi-quantitative indication of extract DNA concentration, a relative quantification of the microbes. Had the DNA concentration of either bacteria or yeast become markedly fainter or disappeared after a certain dilution, this could have been seen as an indication of a lower presence. Although the indication would have been

weak, as PCR conducted in this manner is not a truly quantitative method, it would have provided motivation to continue testing the filtration methods to develop a fungal:bacterial biomass gradient in inoculum sources and evaluate further with more reliable quantitative methods such as qPCR.

With the basis of the results from the microscopy count and PCR, it was decided not to move forward with filtration as an approach to create a treatment gradient. One possible source of error that might have had an impact on some of the results is the sensitivity of the filtration method and the risk of the filter not being closely attached to the bottom of the funnel.

7.2. Fermentation in temperature zones

The general observations of the fermentation vessels throughout the 20 days of the main experiment confirmed that the fermentation processes seemed to proceed successfully. Mother cultures formed on top of the liquid and kept increasing in thickness with time. The temperatures of the different zones were as earlier described with a 5 °C gap between the three lower ones and then a 10 °C gap to the highest one. These were created to the best possible ability taking in to account the conditions that were available at Roots' facilities. As can be seen in *Table 6-2* presenting the temperatures over time, the standard errors were low, meaning that the zones were kept stable throughout the process. The goal, during creation of the temperature zones was to cover a range of 15 °C to 35 °C, with intervals of 5 °C. Due to lack of resources and changing heat patterns in the building, the intermediate and high-intermediate zones, which were designed to be 25 °C and 30 °C, respectively, were both very stable at around 24 °C. Given the lack of temperature difference and resource limitations only one was retained. This is why a 12 °C gap exists between the two higher temperature zones. Given additional resources, it would have been worthwhile to have evenly spaced temperature zones from 15 °C to 35 °C or, given four treatments, have the highest temperature being 30 °C instead of 35 °C, creating an even distribution between the temperature zones.

The lowering of the pH followed to a large extent an expected pattern. A more rapid lowering was observed under higher temperatures, indicating a higher activity rate and a faster acidification. The activity was lower and acidification slower for those batches of kombucha that were fermented in colder temperatures. During the end of the experiment, somewhere between day 14 and day 20, the pH of all different temperature zones started to converge at around pH 2.8.

In contrast to pH, the brix did not exhibit an expected behavior throughout the experiment. As explained early on, under *Kombucha biochemistry*, the basics of the process is that the sucrose is converted to glucose and fructose which then are consumed. The consumption leads to production of ethanol followed by consumption of ethanol, glucose and fructose and production of organic acid and carbon dioxide. The brix did however not markedly decrease in any of the treatments. Since the pH showed that the process was active and that the kombucha was acidifying, something else was likely affecting the outcome of the brix measurements. The refractometer measures the refraction in the liquid which is affected by the sugar concentration, but can also be affected by other compounds. With the results in *Figure 6-4* in mind, one

possible explanation for what has happened in this case is that other compounds in the kombucha started to affect the refractive index as they increase in amount. Two likely interferers could be the organic acids and ethanol. Ethanol, however, will decrease the refractive index, not increase it. An increase in organic acids, acetic acid and gluconic acid in particular being the most dominant organic acids in kombucha fermentation (Chen and Liu, 2000), could explain why the values are quite stable for the three lower temperatures throughout the process. As the sugar content goes down, the concentration of organic acids increases at a relatively even pace. However, the brix in the kombucha fermented in 35.9 °C does not follow the same pattern. Already early in the process it increases fast and continues to do so throughout the fermentation time. If considering the evaporation results that were presented, the amount of evaporated liquid from these particular beakers were about three to five times as high as from the other temperature zones. The evaporation of water can have had an impact on the brix by relatively increasing the concentration of sugar and organic acids as the liquid they are present in decreases. However, while the final sugar concentrations in the 35.9 °C fermentation were highest amongst these temperature treatments, they were not higher than in the beginning of the experiment (day 0). Thus, it appears that something else was also likely contributing the steep rise in brix other than only evaporative concentration of sugar. Nonetheless, in future permutations of this experiment, if higher temperature treatments are to be used, some compensation for evaporation by adding water may be warranted. The uncertainty of the measurements was +/- 0.1 °Bx. A more accurate, digital, refractometer may have allowed for observation of treatment specific trends, although minor ones.

The sugar analysis showed overall few significant changes in sugar content over time. Comparing the different treatments on day 20, the only difference found was the significantly higher sucrose content in 35.9 °C than in 24.0 °C. Over time, within each temperature zone, it was expected to see a continuous decrease in sucrose and an initial increase in fructose and glucose that with time would start to decrease again. This would have been in line with the general understanding of how kombucha fermentation proceeds; sucrose is cleaved into fructose and glucose, which are then consumed in favor of acetic acid and ethanol production.

On day 0, a total sugar concentration of 52.5 g/L was measured, corresponding well with the 55 g/L that was added when preparing the kombucha blend. Thereafter, the only significant difference was in the fraction of sugar present as sucrose which had decreased over time in 24 °C. The lack of a decrease in sugar throughout this experiment is contradicted by the pH results that indicate significant fermentation activity during the process. As kombucha fermentation proceeds and sugars are consumed, other compounds including ethanol and organic acids are produced. These may interfere with either the enzymatic chemistry of the sugar assays or the absorbance readings that they are based on, though the contents of acetic acid and ethanol were within the parameters recommended by the manufacturer of the kits. The apparent lack of decrease in sugar, despite consistent increase in acidity may reflect the multistep nature of kombucha fermentation; sugars initially being consumed by the microbes to produce organic acids and ethanol, then ethanol being oxidized into organic acids. One possibility is that the ethanol oxidation to organic acids is the primary process behind the acidification observed during later parts of the experiment, which may be one of the reasons why no reductions in

sugar contents were observed. As mentioned in the introduction, Neffe-Skocińska et. al. (2017) compared the sugar content between kombuchas fermented in 20 °C, 25 °C and 30 °C for a total amount of ten days. The sucrose content in their samples decreased for all temperatures although most intense when fermented in 25 °C and by far the least when fermented in 30 °C, which does correspond well with the results given by this analysis as well. Invertase activity in kombucha fermentation is generally attributed to yeasts, and it may be that invertase production or activity is inhibited by higher temperatures.

Variations in the microbial community composition, induced by either the temperature treatments or progressing over time, may additionally have had an impact on the fermentation process. As described early on in the report, the community fermenting kombucha is very complex and can vary throughout the process and depending on different factors. In connection to the brix result discussed above, Villarreal-Soto et. al. (2020) found when comparing the fermentation kinetics, metabolite production and microbiome of different kombucha consortia that the sugar consumption between different samples varied a great deal. In addition, they found that the yeast *Schizosaccharomyces pombe* was present in samples with higher sugar conversion. Without knowing what the microbial community composition is in Roots of Malmö's kombucha and how it was affected by temperature, or changed over time, it is not possible to understand the role of community composition on fermentation kinetics. In addition, as concluded in a research project comparing the microbial population of pellicles derived from different geographical location, the origin of the inoculum plays a very important role in what species or genera that dominates the fermentation (Marsh *et al.*, 2014).

As has been briefly discussed already is the evaporation and what possible impact that could have had on some of the other measurements. The three lower temperatures had roughly the same amount of evaporation with no significant difference between them. The higher temperature differed a lot. In addition to likely contributing to an effect on the brix, also the taste results could be impacted by this. As expected with regards to the brix measurements and the effect on sugar concentration from evaporation as well as, although not significant, the results on total sugar concentration in the samples, the kombucha fermented in the highest temperature was the one with the most observable sweetness. What was not as expected was that the kombuchas from the two highest temperatures had about the same level of acidity even though the pH associated with 35.9 °C was not as low as the pH in the kombucha from 24.0 °C. As the warmest fermented kombucha had both the highest sweetness and pH in comparison to the second highest, the acidic taste was expected to be somewhat lower. In the same way as the sugar concentration went up, also the concentration of organic acids could be expected to increase with the water evaporation, but considering the higher pH, this does not seem to have been affected as much. That the taste of black tea was only strong in the kombucha fermented in the lowest temperature is in line with the rest of the results, for example that the fermentation process had been the slowest and had come the least far when considering the pH as an indication of fermentation activity.

The weight of the microbially formed pellicle, which according to *Table 6-8* did not differ between the three lower temperatures, was significantly heavier in the highest temperature. As

mentioned in the beginning of the report under *Kombucha microbial community*, the pellicle is mainly formed by cellulose producing *K. xylinus* bacteria. Considering what other factor differ between the fermentation processes, what is driving the increase in pellicle production is likely either the increased concentration of sugar or the temperature itself. De Filippis et. al. (2018) characterized and compared the bacterial population of kombucha after fermenting in either 20 °C or 30 °C. They found that the prevalence of *Gluconacetobacter xylinus* was strongly affected by the fermentation temperature, being present in higher amounts at 20 °C (De Filippis *et al.*, 2018). Furthermore, the observations in this experiment, although not significant, imply greater cell density at lower temperatures. With the basis of this, it appears more likely that the high sugar levels, not temperature, could be the factor driving the increased pellicle growth at the highest temperature. However, the biomass growth measured in this experiment may not be the same as cellulosic activity, and it is possible that under the highest temperatures, bacteria, which do not grow and replicate more, produce more cellulose. There has been an increasing amount of research lately on the cellulosic activity of *K. xylinus* (Nguyen *et al.*, 2008; Campano *et al.*, 2016; Laavanya, Shirkole and Balasubramanian, 2021; Torán-Pereg *et al.*, 2021) but it remains poorly understood what factors contribute to greater cellulosic production.

7.2.1 Microbial content

As a consequence of the resources available it was possible to analyze the microbial content at three timepoints. In addition to day zero and day 20, it was decided to take the time point of each temperature where the pH dropped below 3.0. This resulted in three samples from day 14 and one sample from day 18. The decision to take them at a specific pH instead of one specific time point was made in order to investigate and compare the microbial content at the same state of the fermentation process. pH 3.0 was chosen as a typical pH of a commercially available kombucha (Rosenstock, N., pers. comm.). If they would have been chosen based on one and the same time point, the results would to a larger extent have reflected how the temperature affects the speed of the process. It was, however, known that the speed of the process would differ depending on fermentation temperature. Thus, by choosing what time points to analyze based on pH, a measure of the fermentation activity, it was possible to do a comparison of the microbial load at the same point of the process. By choosing sampling time points that represent the same degree of fermentation, the results would give a better view of what effect temperature has on the fungal:bacterial ratio, which was the aim of the project. It is also important to note that the day 0 and day 20 sampling points represent the total community in the fermentation vessel while the second sampling time point represents only the community present in the supernatant liquid. Knowing this, a drop of microbial density from day 0 to day 14 or 18 does not necessarily indicate a total decrease in microbial population size, as there may have been a higher microbial density at the bottom of the vessel. A sediment layer could be observed on the bottom of the beaker which is the reason why the liquid was vigorously stirred before sampling on day 20, until no bottom residue remained. The intermediate sampling event is valid for comparisons between the different treatments, however less so for comparison over time.

Since three time points ended up being on the same day, and the fourth one only two days before the end on the experiment, perhaps the choice of a higher pH at which to take the intermediate sampling, thus at a time point earlier than day 14, could also have yielded interesting

information. As previously discussed, and as can be observed in *Figure 6-3*, the main activity of the fermentation processes occurred prior to day 10 or 12 after which the pH of all the temperatures began to even out and eventually converge. At day 14 some of the higher temperatures have started to reach what looks like a plateau in pH where perhaps other factors begin to have a greater influence than temperature in determining the fungal:bacterial biomass ratio. According to pH, treatment differences appeared greatest between day 6 and day 10.

The quantitative PCR measurements were all associated with a large standard error and few of the results display any significant difference between temperature treatment or time points. Yeast and bacterial DNA concentrations consistently increased by one or two orders of magnitude over the course of the total fermentation time. However, this was only significant for bacteria in the lowest temperature. Yeast in the highest temperature showed an increase between the intermediate and the second time sampling point but not over the entire course of the fermentation. This behavior is likely a reflection of the manner in which the intermediate sample was gathered. As previously noted, this sampling was made from the supernatant liquid and if a significant amount of microbes had settled at the bottom of the container, which could visually be observed, their DNA would be missed by only sampling the liquid without stirring the beaker. It is interesting to note that this effect was only significant for yeast, which could possibly reflect a greater tendency for yeast to settle out of solution during fermentation.

Given that bacteria and yeast are competing for resources, conditions which favor one of the groups more would indirectly disfavor the other. Thus, it was expected that temperature would affect the fungal:bacteria ratio. However very little emerged by examining the effect of temperature on fungal:bacterial ratio and nothing was significant.

One thing that is important to consider regarding the qPCR results is that all concentrations are given as amount of DNA fragments per mL. This means that the results display how much DNA is present, but these numbers cannot be directly translated to how many organisms that are present. The regions that are copied when using the chosen primers are the 16s rRNA gene in bacteria and the Internal Transcribed Spacer region 1, ITS1, for yeast. The number of copies of aforementioned regions per genome, varies from species to species, as does the ratio of genomic DNA:microbial biomass. As this is an investigation of a diverse microbial community which varies, it is not possible to convert gene copies to biomass. However, these communities are diverse enough, the primers general enough and the expected differences in community composition small enough, that differences in gene copy density between treatments should be able to be interpretable as differences in microbial biomass and growth.

Another important aspect to take into consideration is the impact of DNA from non-viable microbes. Throughout the process, microorganisms will die and their DNA will stay present in the solution for a certain amount of time. When extracting the DNA and amplifying it through qPCR, the method does not discriminate between dead and viable cells. When investigating the viability of yeast and bacterial cells in prolonged kombucha fermentation up to 60 days, Chen and Lui (2000) found that all samples tested reached a maximum of both yeast and bacteria after between 6-14 days, after which the viable count of the two started to gradually decrease.

This indicates that the results presented from the qPCR in this report is likely to some extent also include cells that are no longer active in the fermentation process. With the basis of the information given in this segment and the segment above, the amount of DNA does not necessarily represent the amount of bacterial or yeast cells present and active but gives a picture of how they change in relation to each other.

In addition, there are some general limitations associated with the methods chosen that may can have impacted both the PCR results early on during the project as well as the results of the qPCR. During the DNA extraction process there is a risk that the kit works better on some species than others or compared between yeast and bacteria. During both the PCR and qPCR process, the affinity of the primers on the target region is of high importance and if this works better on some groups than others, this might also have an effect on the result. As seen on the pictures of the PCR results in Appendix C, there are two bands, meaning two sizes of DNA fragment present after the amplification. As mentioned under *Methods for assessing microbial population* - PCR and qPCR the length of the ITS regions can vary a lot between different species. In this case, the PCR results show that among the yeasts in the kombucha samples, there are species that have different lengths on their ITS1 region. If the affinity for one or the other is stronger this could also have an effect on the final results.

8. Conclusion

It was determined early on that sedimentation and filtering were not viable techniques to separate the yeasts and bacteria in the kombucha microbial community. Instead the focus was placed on fermentation in different temperature zones and it was, by using qPCR, possible to track the growth of bacteria and yeasts in the kombucha during the fermentation process. No observation of a consistent effect of temperature could be made, though both yeasts and bacteria seemed to prefer cooler temperatures, bacteria somewhat more than yeast. Furthermore, no evidence for competition between bacteria and yeast was observable from the groups' responses to temperature.

9. Future work

The first recommendation for future permutations of this experimental set up would be to increase the amount of replicates in each temperature zone for all analyses. In addition, since the zone aiming to be around 30 °C was the least temperature stable over time and that one zone had to be excluded from analyzes due to resource limitations, there are no results at all for this temperature zone. As there were several results in the 35.9 °C that were difficult to explain, it would be interesting to include a sample at around 30 °C in future studies to see how they correlate. Furthermore, the ethanol content would also be an interesting factor to study to see how it is affected by temperature over time.

As mentioned in the discussion, the qPCR results are likely impacted by DNA from dead organisms which stays present in the kombucha after the cell dies. By complementing the qPCR test with a viability test, the effect of this impact could be excluded. Culturing the cells or using a suitable staining technique are possible options. Exchanging qPCR with reverse transcription PCR could also be an alternative, were the rRNA instead of DNA is detected in the sample which is less prone to stay present after the cells die.

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Appendix

Appendix A – Statistical analysis

Figure A-1 and A-2 show the results given by SigmaPlot when running a Kruskal-Wallis One way ANOVA on Ranks test on independent samples or a Friedman Repeated Measurement ANOVA on Ranks on depend samples. Both of these examples had a $p<0.05$ and thus also show the results from the following post hoc test, either Student-Newman-Keuls or Tukey.

Kruskal-Wallis One Way Analysis of Variance on Ranks Wednesday, May 05, 2021 1:37:48 PM

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk): Passed (P = 0.437)

Equal Variance Test (Brown-Forsythe): Passed (P = 0.212)

Group	N	Missing	Median	25%	75%
L	4	0	2.725	2.720	2.730
H	4	0	2.710	2.703	2.740
VH	4	0	2.810	2.773	2.810
VL	4	0	2.870	2.842	2.928

H = 13.195 with 3 degrees of freedom. (P = 0.004)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.004)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) :

Comparison	Diff of Ranks	q	P	P<0.050
VL vs H	44.000	4.621	0.006	Yes
VL vs L	36.000	4.992	0.001	Yes
VL vs VH	16.000	3.266	0.021	Yes
VH vs H	28.000	3.883	0.017	Yes
VH vs L	20.000	4.082	0.004	Yes
L vs H	8.000	1.633	0.248	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Figure A-1: Example of significance test for pH between all temperatures on day 20.

Friedman Repeated Measures Analysis of Variance on Ranks Wednesday, May 05, 2021 3:57:58 PM

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk): Passed (P = 0.101)

Equal Variance Test (Brown-Forsythe): Passed (P = 0.798)

Group	N	Missing	Median	25%	75%
Before	3	0	1627732.910	924910.768	1874529.880
middle	3	0	2625455.140	2211228.220	7757234.740
after	3	0	369389617.000	26528826.100	399680838.000

Chi-square= 6.000 with 2 degrees of freedom. P(est.)= 0.050 P(exact)= 0.028

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.028)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P	P<0.050
after vs Before	6.000	3.464	0.038	Yes
after vs middle	3.000	1.732	0.438	No
middle vs Before	3.000	1.732	0.438	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

Figure A-2. Example of significance test for bacterial DNA concentration in 15.1 °C between day 0, 18 and 20.

Appendix B – Microscopy examination of unfiltered and filtered kombucha

Table B-1 below show the mean amount of microorganisms per sample that was counted with a Bürker chamber. For each sample, three squares were counted. Each square was 0.02 mm^2 in area and 0.001 mm deep, thus the number of microbes was divided with the volume of the square and lastly unit converted.

Table B-1. Mean amount of microorganisms in each sample.

	Unfiltered	**5-6 µm pore size**	**<2 µm pore size**
Sample 1	61	19	14
Sample 2	54	24	12
Sample 3	68	30	16

Below are some examples of pictures taken on unfiltered and filtered kombucha. The squares that were counted were the largest square that can be seen in Figure B-1. All images are taken at 200 x enlargement.

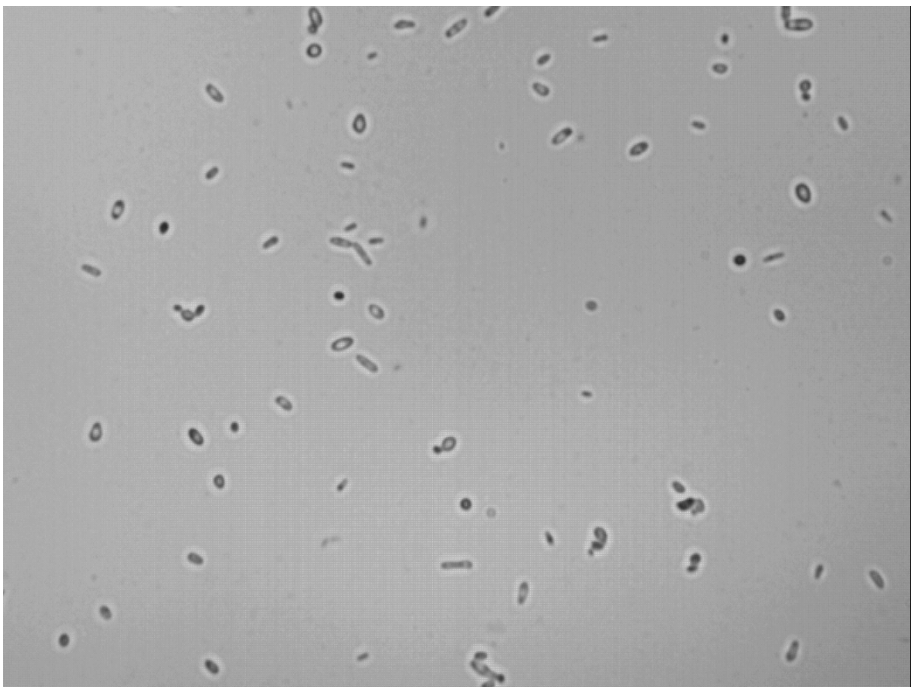

Figure B-1. Unfiltered kombucha

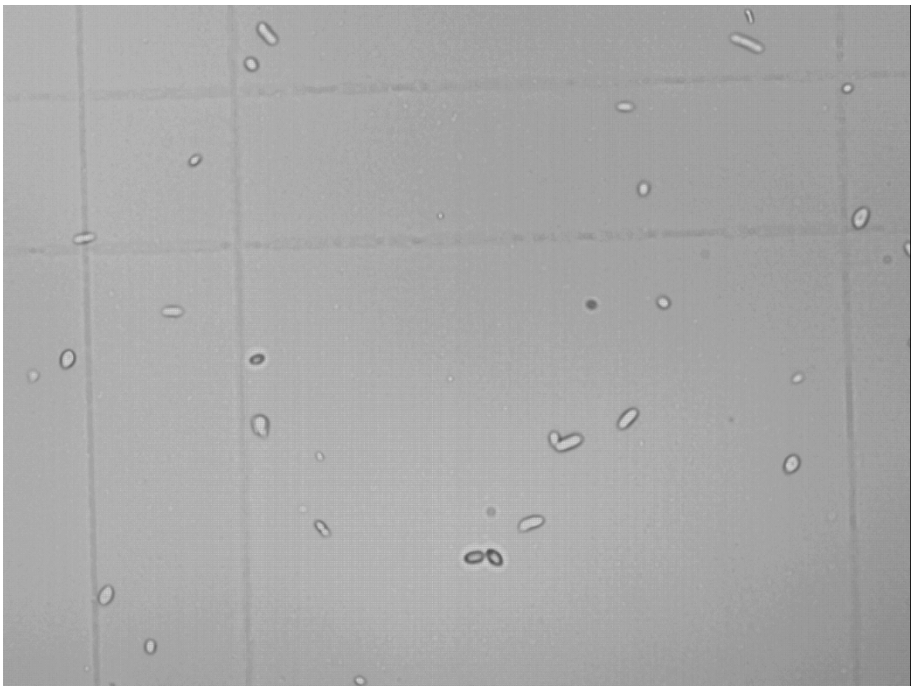

Figure B-2. Kombucha filtered through 5-6 μm filter pore size.

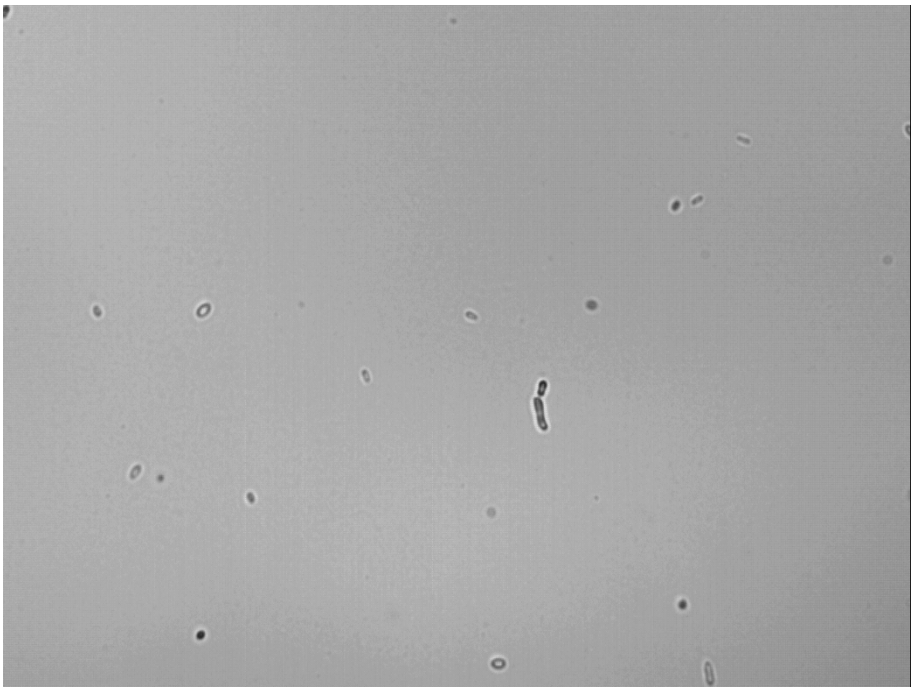

Figure B-3. Kombucha filtered through <2 μm filter pore size.

Appendix C – PCR results from filtration

The images in Appendix B display some examples of the gel electrophoresis done to evaluate the PCR results. All samples were placed in wells present in the lower part of the pictures. As a result of the electric current the DNA fragments have traveled upwards in the picture. Smaller fragments travel further then large ones and thus the fragments get separated by size. In each picture there is a ladder to use a size reference with the relevant sizes marked in the first picture.

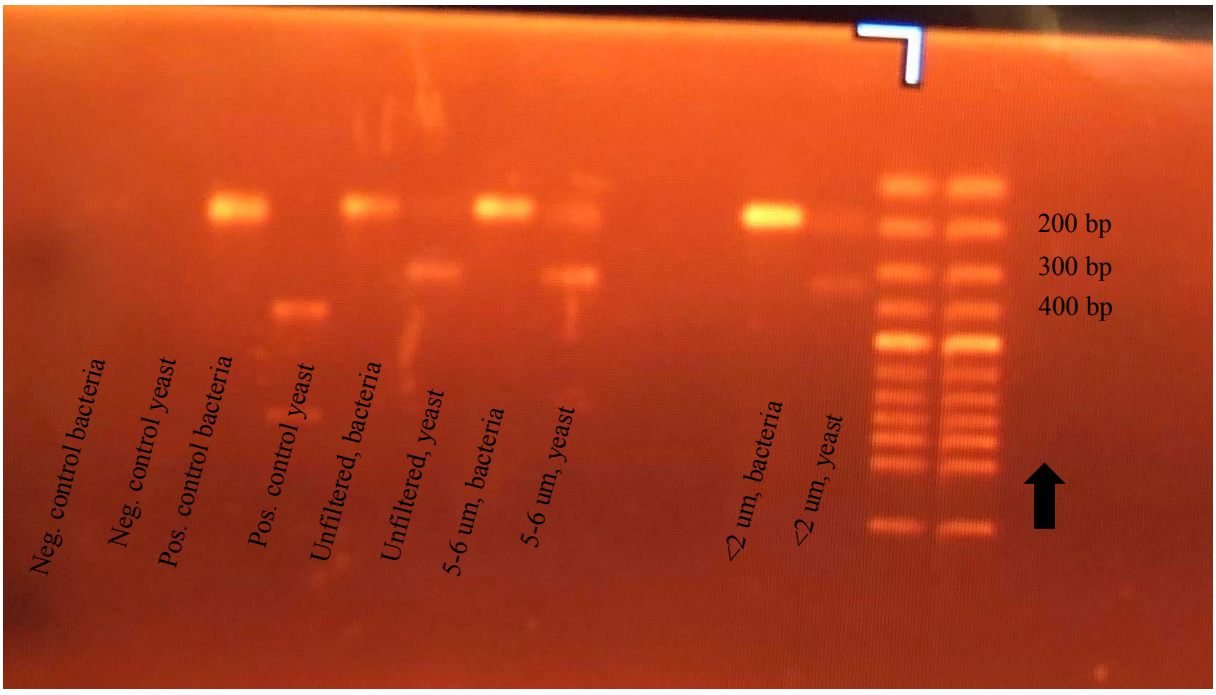

Figure C-1. PCR results comparing unfiltered and filtered kombucha using pore sizes 5-6 μm and <2 μm.

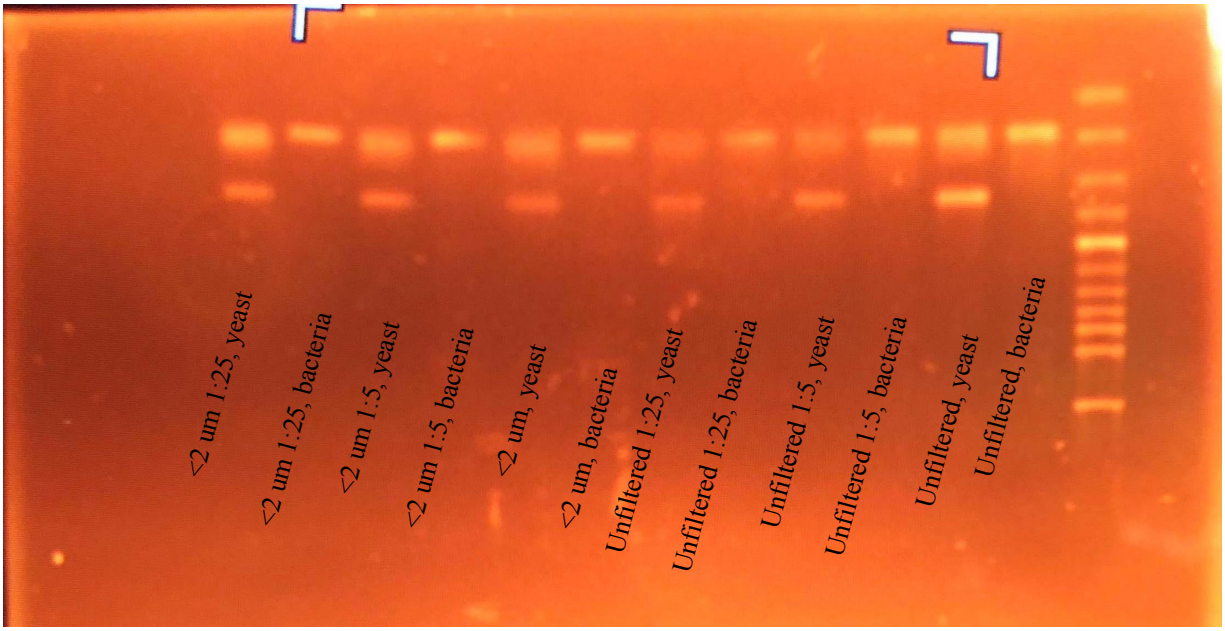

Figure C-2. PCR results comparing unfiltered and <2 µm filtered kombucha. Also diluted 1:5 a 1:25.

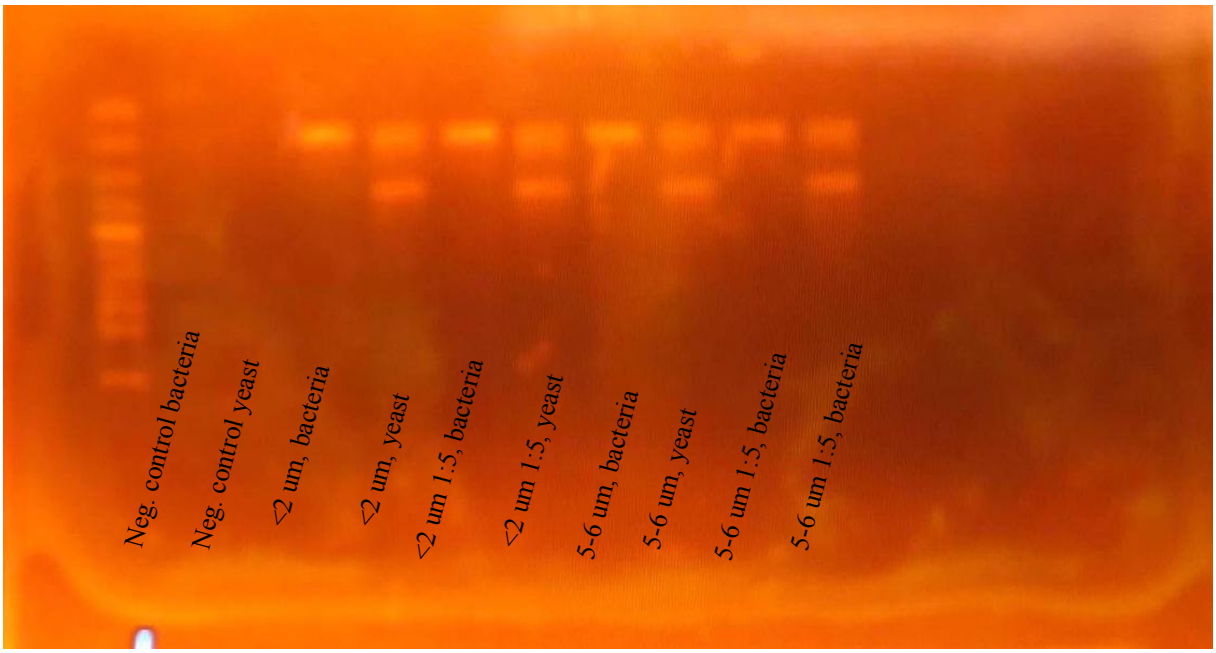

Figure C-3. PCR results comparing filter residues from <2 μm and 5-6 μm pore sizes. Both also diluted 1:5.

Appendix D – Raw data from fermentation

Table D-1. Raw data from fermentation.

		Very low T		Low T		High T		Very high T	
		pH	Brix	pH	Brix	pH	Brix	pH	Brix
Dag 2 2021-04-01	1	3,77	5,5	3,79	5,5	3,65	5,4	3,61	5,5
	2	3,74	5,5	3,78	5,5	3,70	5,4	3,60	5,4
	3	3,74	5,6	3,75	5,6	3,68	5,5	3,60	5,5
	4	3,73	5,5	3,70	5,5	3,70	5,4	3,59	5,5
	Mean	3,745	5,525	3,755	5,525	3,6825	5,425	3,6	5,475
Dag 4 2021-04-03	1	3,70	5,6	3,72	5,4	3,44	5,8	3,50	5,8
	2	3,69	5,6	3,69	5,5	3,60	5,6	3,34	5,6
	3	3,65	5,8	3,68	5,8	3,50	5,6	3,51	5,6
	4	3,63	5,6	3,73	5,8	3,57	5,8	3,40	5,6
	Mean	3,6675	5,65	3,705	5,625	3,5275	5,7	3,4375	5,65
Dag 6 2021-04-05	1	3,52	5,6	3,57	5,3	3,48	5,5	3,46	5,7
	2	3,56	5,6	3,56	5,6	3,34	5,6	3,19	5,8
	3	3,57	5,7	3,56	5,6	3,30	5,5	3,15	6,0
	4	3,40	5,7	3,67	5,7	3,26	5,5	3,16	5,8
	mean	3,5125	5,65	3,59	5,55	3,345	5,525	3,24	5,825
Dag 8 2021-04-07	1	3,43	5,4	3,37	5,3	3,40	5,7	3,22	6,0
	2	3,41	5,6	3,38	5,5	3,41	5,8	3,07	6,1
	3	3,40	5,6	3,44	5,4	3,27	5,6	3,16	5,7
	4	3,49	5,5	3,37	5,8	3,23	5,7	3,10	6,0
	mean	3,4325	5,525	3,39	5,5	3,3275	5,7	3,1375	5,95
Dag 10 2021-04-09	1	3,35	5,3	3,20	5,3	3,11	5,5	3,13	5,9
	2	3,80	5,5	3,23	5,3	3,05	5,3	2,98	6,0
	3	3,40	5,6	3,22	5,4	3,04	5,4	3,02	6,0
	4	3,41	5,5	3,17	5,6	3,07	5,3	3,06	6,0
	mean	3,49	5,475	3,205	5,4	3,0675	5,375	3,0475	5,975
Dag 12 2021-04-11	1	3,18	5,4	3,15	5,1	2,99	5,5	3,15	6,0
	2	3,29	5,3	3,31	5,2	2,98	5,2	2,98	6,3
	3	3,28	5,5	3,22	5,4	2,97	5,2	3,01	6,0
	4	3,33	5,5	3,10	5,5	3,04	5,4	3,07	6,0
	mean	3,27	5,425	3,195	5,3	2,995	5,325	3,0525	6,075
Dag 14 2021-04-13	1	3,1	5,2	2,97	5,2	2,88	5,2	3	6,4
	2	3,22	5,4	3,09	5,3	2,83	5,2	2,91	6,5
	3	3,14	5,4	2,93	5,2	2,86	5,3	2,86	6,4
	4	3,22	5,4	2,87	5,2	2,87	5,2	2,92	6,1
	mean	3,17	5,35	2,965	5,225	2,86	5,225	2,9225	6,35
Dag 16 2021-04-15	1	2,99	5,2	2,87	5,1	2,78	5,2	2,94	6,7
	2	3,09	5,6	2,88	5,3	2,78	5	2,85	6,6
	3	3,06	5,5	2,82	5,2	2,75	5,1	2,8	6,6
	4	3,17	5,4	2,82	5,3	2,83	5,1	2,85	6,4
	mean	3,0775	5,425	2,8475	5,225	2,785	5,1	2,86	6,575
Dag 18 2021-04-17	1	2,96	5,2	2,8	5,1	2,74	5	2,86	6,9
	2	3	5,3	2,79	5,3	2,73	5	2,82	7
	3	2,97	5,3	2,76	5,1	2,72	5,2	2,76	6,9
	4	3,05	5,2	2,75	5,2	2,75	5,1	2,83	6,9
	mean	2,995	5,25	2,775	5,175	2,735	5,075	2,8175	6,925
Dag 20 2021-04-19	1	2,89	5,1	2,73	5,1	2,71	5,2	2,81	7,2
	2	2,85	5,2	2,72	5,2	2,71	5	2,81	7,1
	3	2,84	5,2	2,73	5,1	2,7	5	2,76	7,2
	4	2,94	5,1	2,72	5,2	2,75	5	2,81	7,2
	mean	2,88	5,15	2,725	5,15	2,7175	5,05	2,7975	7,175

Table D-2 Weight of pellicle on day 20.

	15.1 °C	19.8°C	24.0 °C	35.9 °C
Sample 1 (g)	56	40	41	82
Sample 2 (g)	45	35	39	100
Sample 2 (g)	50	39	49	77
Sample 4 (g)	55	42	61	78

Table D-3 Liquid left in the beaker on day 20.

	15.1 °C	19.8°C	24.0 °C	35.9 °C
Sample 1 (g)	1478	1467	1390	636
Sample 2 (g)	1498	1478	1420	705
Sample 2 (g)	1490	1459	1396	709
Sample 4 (g)	1467	1445	1412	648

Appendix E – Sugar analysis

Table E-1 and table E-2 contain the raw data from the sugar analysis. Reading A1 is a measure of the background. In the sucrose samples, invertase has been added and converted sucrose into glucose and fructose. Reading A2 measures absorbance equivalent to d-glucose content, which in the case of sucrose samples corresponds to both d-glucose and sucrose. A3 in the sucrose samples is supposed to be equivalent to A2. In those samples where they are not, the incubation time before reading A3 was likely not enough. A3 in the glucose/fructose measurements measures the signal equivalent to d-glucose and d-fructose content.

Absorbance of glucose was calculated according to following equation:

$$A_g = A2_g - A1_g$$

Absorbance of fructose was calculated according to following equation:

$$A_f = A3_g - A2_g$$

Absorbance of sucrose was calculated according to following equation:

$$A_s = A3_s - A2_g$$

The standard curve for glucose is presented in Figure E-1 below. The intersect is forced through origo in order to increase the strength of the readings with very low absorbance signals.

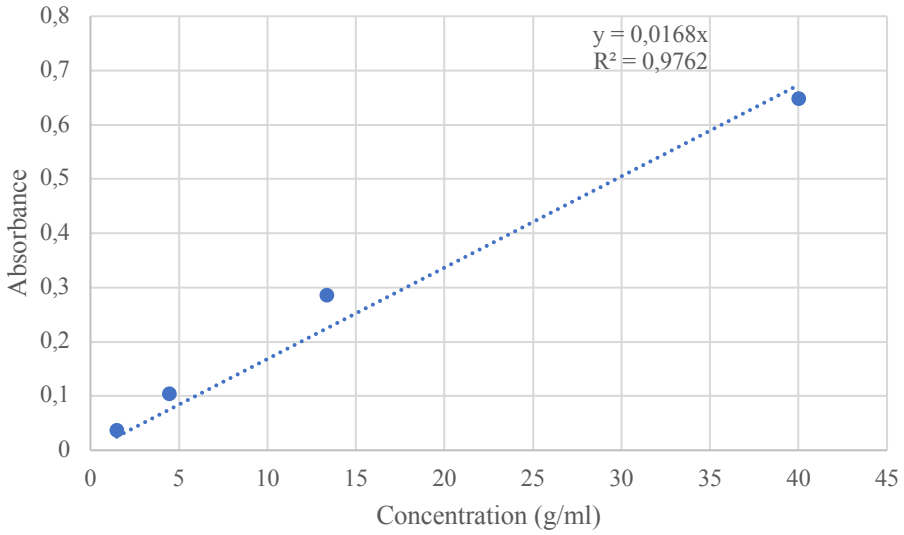

Figure E-1: Standard curve for glucose absorbance.

The concentration of glucose and fructose was calculated according to the following equation:

$$c\left(\frac{g}{ml}\right) = \frac{A}{Slope} * \frac{V_{tot}}{V_{sample}} * F$$

Where A is the absorbance for either glucose of fructose, Slope is the slope of the standard curve. V_{tot} is the total volume of liquid in the wells, V_{sample} is the volume of sample in each well and F is the dilution factor, in this case 100.

As the slope of the standard curve converts units of absorbance to units of mass and is based on glucose, the size difference between sucrose and glucose was taken into consideration when calculating the sucrose concentration. Thus it was calculated according to the equation below.

$$c\left(\frac{g}{ml}\right) = \frac{A}{Slope} * \frac{V_{tot}}{V_{sample}} * F * \frac{M_{sucrose}}{M_{glucose}}$$

Table E-1: Raw data of sucrose analysis

		A1	A2	A3
		0.157	0.842	0.936
		0.162	0.642	0.753
		0.165	0.571	0.685
D0		0.155	0.62	0.73
		0.16	0.466	0.641
		0.177	0.18	0.181
		0.185	0.507	0.584
	15.1 ℃	0.176	0.595	0.736
		0.159	0.454	0.487
		0.157	0.51	0.644
		0.164	0.331	0.471
	19.8 ℃	0.158	0.291	0.401
		0.159	0.438	0.491
		0.151	0.454	0.509
		0.161	0.624	0.714
	24.0 ℃	0.163	0.425	0.582
		0.163	0.525	0.604
		0.175	0.6	0.701
		0.181	0.613	0.92
D10	35.9 ℃	0.182	0.176	0.177
		0.162	0.401	0.496
		0.159	0.398	0.491
		0.162	0.416	0.585
	15.1 ℃	0.168	0.368	0.505
		0.166	0.559	0.637
		0.154	0.157	0.158
		0.164	0.38	0.572
	19.8 ℃	0.163	0.452	0.55
		0.156	0.659	0.681
		0.147	0.487	0.51
		0.154	0.445	0.517
	24.0 ℃	0.164	0.321	0.401
		0.169	0.482	0.633
		0.168	0.365	0.575
		0.164	0.316	0.47
D20	35.9 ℃	0.173	0.56	0.76

Table E-1: Raw data of glucose and fructose analysis

		A1	A2	A3
		0.2	0.215	0.223
		0.168	0.188	0.198
		0.16	0.186	0.206
D0		0.166	0.178	0.196
		0.15	0.514	0.818
		0.139	0.144	0.145
		0.148	0.157	0.174
	15.1 ℃	0.136	0.151	0.174
		0.176	0.381	0.569
		0.154	0.56	0.805
		0.164	0.529	0.786
	19.8 ℃	0.168	0.416	0.677
		0.163	0.376	0.521
		0.173	0.439	0.554
		0.155	0.638	0.859
	24.0 ℃	0.16	0.461	0.672
		0.156	0.18	0.216
		0.159	0.556	0.821
		0.159	0.157	0.157
D10	35.9 ℃	0.157	0.216	0.287
		0.16	0.376	0.581
		0.144	0.327	0.551
		0.161	0.447	0.863
	15.1 ℃	0.16	0.62	0.827
		0.17	0.61	0.808
		0.187	0.321	0.703
		0.157	0.327	0.827
	19.8 ℃	0.162	0.452	0.771
		0.171	0.622	1.091
		0.16	0.452	0.626
		0.163	0.519	0.713
	24.0 ℃	0.162	0.345	0.81
		0.172	0.284	0.493
		0.17	0.349	0.583
		0.172	0.236	0.638
D20	35.9 ℃	0.17	0.322	0.563

Appendix F – qPCR result examples

Below are examples of results given after the qPCR. Figure F-1 show the concentration of DNA in each sample during a qPCR run. The y-axis shows fluorescent light detection and the x-axis shows amount of cycles. In this case the green graphs are samples, the yellow ones are the standards and the red ones are the negative controls. The second picture, Figure F-2, shows the standard curve as well as all the samples and negative control plotted after the run is finished.

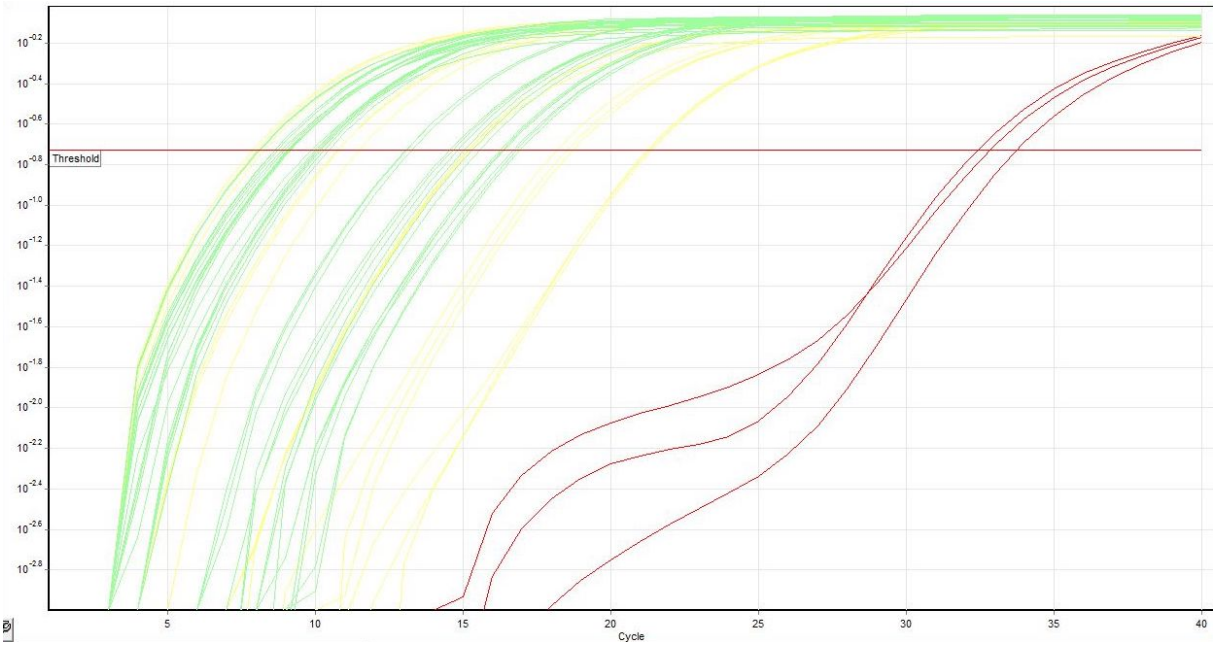

Figure F-1: Graphs showing the fluorescent light emission of each sample, standard and negative controll throughout the second yeast run.

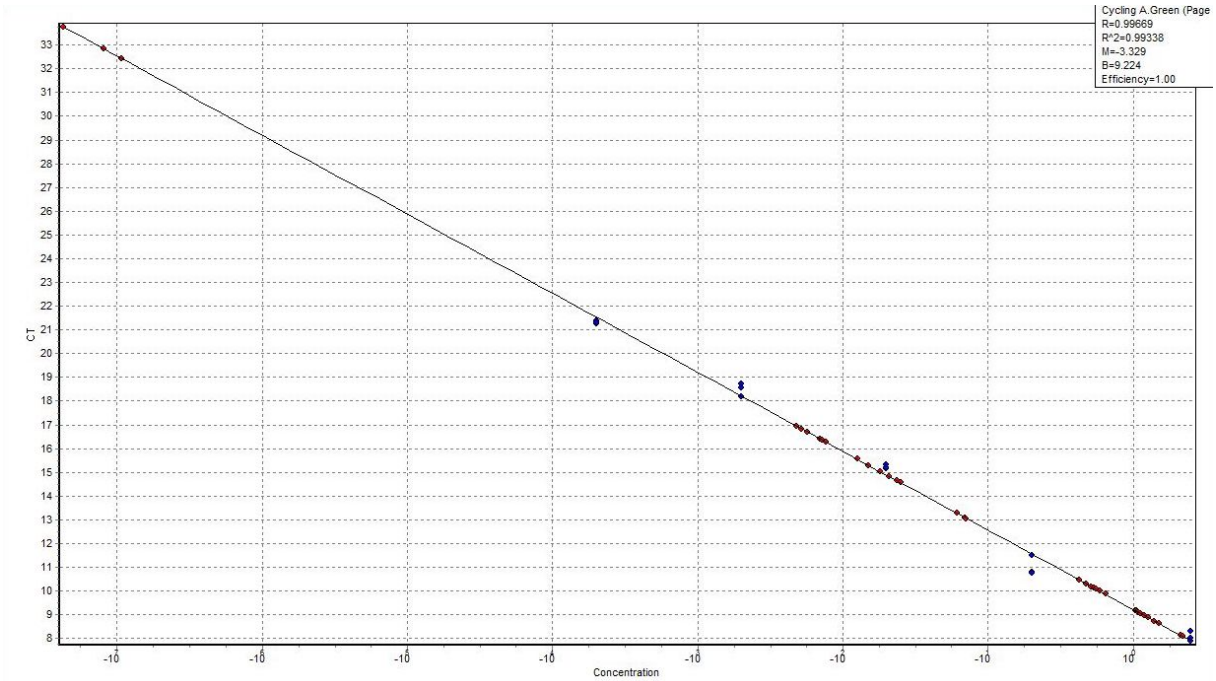

Figure F-2. The standard curve, negative controls and samples for the second yeast run.