Microbial dynamics of Swedish, industrially brewed kombucha

and variations between starter cultures

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Abstract

Kombucha is an acidic, slightly alcoholic beverage made by fermenting sweetened tea. Kombucha is rising in popularity worldwide as a result of the alleged health benefits. Common microbes involved has been found to vary, but include yeasts, acetic acid bacteria, and occasionally lactic acid bacteria. The aim of this thesis was to investigate how the microbial population differed between three starter cultures from one industrial brewery of kombucha, what microbes could be found in one cellulosic biofilm, and how the microbial population of one batch of industrially brewed combucha altered from day 2 to 21 of fermentation, using samples obtained from a Swedish brewery. This was done by plating sample dilutions on differential media and counting the colony forming units, as well as by partial amplification and sequencing of the 16S and 26S RNA gene respectively. Concentrations of sugars and end products were measured by high performance liquid chromatography. The starter liquids were found to consist of similar microorganisms but were not identical. The microbial population originating from the cellulosic pellicle was not as diverse as the most diverse starter liquid but had a greater microbial density. In the batch of industrially brewed kombucha, *Brettanomyces anomalus* was the dominating yeast throughout. *Hanseniaspora mollemarum* was found in samples from the first half of the fermentation period, and *Zymomonas mobilis* in the second. Sucrose equivalents decreased from 51.8 to 32.2 g L^{-1}, while ethanol increased from 3.5 to 15.4 g L^{-1}. The final concentration of acetic acid was found to be 3.0 g L^{-1}. No acetic acid bacteria or lactic acid bacteria were identified, and no lactate was formed.

Keywords: kombucha, microbial fermentation, yeast, lactic acid bacteria, acetic acid bacteria

Sammanfattning

Kombucha är en syrlig, något alkoholhaltig dryck tillverkad genom att fermentera te. Kombuchas popularitet ökar världen över, på grund av påstådda hälsofördelar. Mikrober delaktiga i fermentationen kan variera mycket, men innefattar ofta jäst, ättiksyrebakterier, och ibland mjölksyrebakterier. Syftet med denna avhandling var att undersöka hur den mikrobiella populationen skiljde sig åt mellan tre startkulturer från ett industriellt bryggeri av kombucha, vilka mikrober som kunde hittas i en biofilm, samt hur den mikrobiella populationen i en omgång industriellt bryggd kombucha förändrades från dag 2 till dag 21 av fermentationen, med prover inhämtade från ett svenskt bryggeri. Detta gjordes genom att sprida prover på differentierade medier och räkna koloniformande enheter, samt genom att partiellt amplifiera och sekvensera 16S och 26S RNA genen för jäst respektive bakterier. Koncentrationer av socker och slutprodukter mättes med högupplösande vätskekromatografi. Startvätskorna visade sig bestå av liknande, men inte identiska populationer av mikrober. Den mikrobiella sammansättningen från biofilmen var inte lika artrik som den mest artrika startvätskan, men hade en högre mikrobiell densitet. I omgången industriellt bryggd kombucha dominerade *Brettanomyces anomalus* vid alla tidpunkter. *Hanseniaspora mollemarum* kunde identifieras i prover från den första halvan av fermentationen, och *Zymomonas mobilis* i den andra. Sukrosekvivalenter minskade från 51.8 till 32.2 g L^{-1}, medan etanol ökade från 3.5 till 15.4 g L^{-1}. Den slutliga koncentrationen av ättiksyra uppmättes till 3.0 g L^{-1}. Inga ättiksyrabakterier eller mjölksyrabakterier identifierades, och ingen mjölksyra kunde uppmätas.

Nyckelord: kombucha, mikrobiell fermentering, jäst, mjölksyrabakterier, ättiksyrabakterier

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Abbreviations

AAB	Acetic Acid Bacteria
BCE	Before the Common Era
BRIX	Grams dissolved sugar per 100 mL aqueous solution
CAGR	Compound Annual Growth Rate
CFU	Colony Forming Unit
EPS	ExoPolySaccharides
GLP	Good Laboratory Practice
GRAS	Generally Regardes As Safe
LAB	Lactic Acid Bacteria
PCR	Polymerase Chain Reaction
RI	Refractive Index
RPM	Revolutions Per Minute
SCOBY	Symbiotic Consortium Of Bacteria and Yeasts
VBNC	Viable But Non-Culturable

1. Background

1.1 History

Kombucha is an acidic, slightly alcoholic beverage rising in popularity worldwide, due to its alleged health benefits. The drink consists of fermented sugared tea and its consumption is believed to date back to 220 before the common era (BCE). The tea originates from Manchuria in northeast China, and in year 414 it was used by the Emperor Inkyo as treatment for his digestive issues. As trade routes expanded, the consumption spread. In the 20th century, it reached Germany, and 50 years later France and northern Africa. By the end of the 20th century researchers found that the drink could potentially inhabit health promoting properties (Coelho et al. 2020).

1.2 Health benefits

Many health benefits have been attributed to the consumption of kombucha, but far from all are scientifically proven. Self-reported effects of consuming the beverage include reduced blood-pressure, reduced inflammation, reduced cholesterol, enhanced immune system, improved gut health, reduced stress, and enhanced metabolism. What is known is that the acetic acid in the beverage does exert antimicrobial properties against certain pathogenic bacteria, among other *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Campylobacter jejuni,* and *Salmonella enteritidis*. In addition, the antioxidant activity has been found to be higher in fermented tea compared to non-fermented tea. This could be a result of microbial enzymes and production of low-molecular weight components. In studies with rats and mice, kombucha has been found to enhance liver function, by suppressing liver toxins. This was established by measuring liver toxicity markers (Jayabalan et al. 2014).

1.3 Current market status

In 2021, the global market for kombucha was valued at 2.64 billion USD globally. Between 2022-2030 the market is expected to expand at a compound annual growth rate (CAGR) of 15.6%. Greater availability and a variety of flavours has and will continue to aid in the continuous growth. Producers will also benefit from spreading knowledge to consumers about potential health benefits that could arise from the consumption of the beverage (GVR 2021).

2. Common microbes involved

2.1 Yeasts

Yeasts are single-celled, eukaryotic microorganisms belonging to the fungal kingdom. They are non-motile, spherical, or oval shaped, and about 8 µm. Some yeasts are capable of producing biogenic amines. Yeasts are always involved in the fermentation of kombucha, and a plethora of yeasts have been encountered with great variation between fermentations. To mention a few, those from the genera of *Saccharomyces*, *Zygosaccharomyces*, *Pichia*, *Brettanomyces*/*Dekkera*, *Candida, Torulospora, Kloeckera, Mycotorula, Mycoderma, Saccharomycodes*, and *Schizosaccaromyces* (Jayabalan et al. 2014). Optimal temperature is between 20-30°C, and they can tolerate pH ranging between 2.5-7.0. Mentioned yeasts metabolise carbohydrates and synthesise ethanol and carbon dioxide. The mode of metabolism varies between yeasts, from obligate fermentative to obligate respiratory and facultatively fermentative to mention some. When carbohydrates are no longer available, some yeasts can produce carbon dioxide and water from ethanol if other nutrients needed are available. Other end products by yeasts include glycerol, acetic acid, esters, and higher alcohols. Lastly, dead yeast cells can provide other microbes with nutrients (Laureys et al. 2020).

2.2 Acetic acid bacteria

Acetic acid bacteria (AAB) are gram-negative, non-spore forming, obligate aerobic, acid-tolerant bacteria. They belong to the class Alphaproteobacteria, order *Rhodospirillales* and family *Acetobacteriaceae* (Qiu et al. 2021). Their optimal temperature is 25-30°C, pH can range from below 3.0 to 6.5. While being obligate aerobes, AAB can survive during long periods in environments with low oxygen. This can however lead to cells being viable but non-culturable (VBNC). AAB can be motile and are oval to rod shaped, 1-4 µm long and 0.5 µm wide (Laureys et al. 2020). In kombucha they are responsible for the low alcohol content, as they oxidise ethanol to acetic acid. AAB are commonly used in the food industry in the production of vinegar and fermented foods (Qiu et al. 2021).

Some of the frequently encountered AAB in kombucha include those from the genera of *Acetobacter*, *Gluconobacter, Gluconacetobacter*, and *Komagataeibacter*. *Komagataeibacter xylinus* is believed to synthesise the cellulosic biomass at the air-liquid interface in the fermenting container. However, other species of *Komagataeibacter* and *Gluconacetobacter* can also produce cellulose. Despite being acid-tolerant, the tolerance varies within the group,

between genera. *Komagataeibacter* can tolerate 10-20% whereas *Acetobacter* tolerates 8% (Laureys et al. 2020).

2.3 Lactic acid bacteria

Lactic acid bacteria (LAB) are gram-positive, round or rod-shaped, non-spore forming, non-motile and generally regarded as safe (GRAS) bacteria, despite some strains ability to produce biogenic amines (Laureys et al. 2020). LAB are facultatively fermentative, with some seemingly performing respiration (Müller 2024). They are 1-1.5 µm long and 0.7-1 µm wide (Schär-Zammaretti & Ubbink 2003). They belong to multiple families mainly within the order *Lactobacillales* (Mokoena 2017). Their optimal temperature is 25-40°C and grow in a pH range between 4.0-6.0. Some species are able to produce exopolysaccharides (EPS), which can be desirable or detrimental depending on production. LAB are classified as obligate homofermentors, obligate heterofermentors or facultative heterofermentors. Homofermentors produce lactic acid from glucose and heterofermentors produce lactic acid, acidic acid or ethanol, and carbon dioxide from glucose. With varying substrate, end products too vary. LAB are not always encountered in kombucha, but when they are they are often acid-tolerant species such as *Oenococcus oeni* and *Lactobacillus nagelii* (Laureys et al. 2020).

2.4 Obtaining kombucha

Kombucha can be made using any tea, but traditionally black or green tea from *Camellia sinensis* is used. Black, green, oolong and white teas only differ by processing method of the fresh leaves, resulting in different sensory experiences. The tea leaves are steeped for various times depending on kind and preference. Sugar is then added, usually between 5-10%. Sucrose is most common, but fructose or glucose are also used. Substrates other than teas have also been used, such as fruit juices and coconut water (Coelho et al. 2020). Altering raw materials and quantities thereof, will influence the composition of the microbial consortium (Laureys et al. 2020).

The fermentation begins by the addition of starter liquid from a previous batch. The main microbes involved in the fermentation are yeasts, AAB, and sometimes LAB (Figure 1). The microbial population has been found to vary greatly. During the fermentation a cellulosic, floating biomass is formed at the liquid-air interface in the fermenting container, which is called a symbiotic consortium of bacteria and yeasts (SCOBY). The top layer of the SCOBY provides AAB with oxygen, whereas the bottom part will serve as a means to achieve an anaerobic environment. The biomass can also be included whole or in part in following batches (Coelho et al. 2020).

During fermentation, yeasts hydrolyse sucrose into glucose and fructose, and further to ethanol, glycerol, and carbon dioxide. This action enables AAB to convert glucose into gluconic acid and glucuronic acid. From the ethanol AAB makes acetic acid. AAB including some from the species of *Gluconacetobacter* and *Komagateibacter* produce cellulose from all sugars in Figure 1 but also from ethanol and glycerol (Laureys et al. 2020).

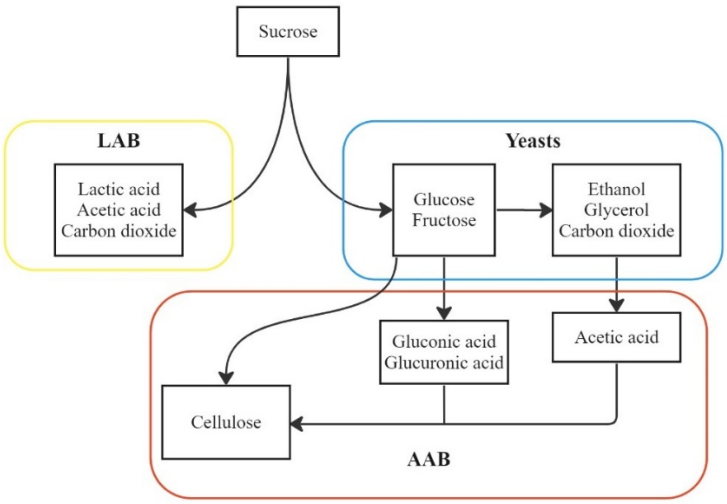

Figure 1. Main microbes and their end products. Adapted from (Laureys et al. 2020).

If conditions allow and LAB are present, they consume sugars and produce lactic acid, acetic acid, and carbon dioxide. As the fermentation progresses, pH decreases and prohibits growth of other microorganisms. Both the initial and final pH will vary depending on a multitude of process factors, but pH usually does not go below 2 (Laureys et al. 2020).

The fermentation container is of great importance. Hygiene of the container and all utensils involved in the preparation is needed to ensure product quality and safety. Appropriate materials like stainless steel should be used, and not ceramic containers. As plenty of organic acids are produced, heavy metals such as lead could migrate into the product (Jayabalan et al. 2014).

The container should be covered in gauze to enable gas exchange, while protecting from contamination by spores or pests. Fermentation times range between 3-60 days, while temperatures are most commonly around 20-30°C (Coelho et al. 2020) The time and temperature can be altered to increase or decrease the pace of the process or to promote growth of certain bacteria, and acquire their end products (Jayabalan et al. 2014).

2.5 Storage

Fu and co-workers investigated how microbes in kombucha changed over 14 days of storage at 4°C and found that 54.09% of AAB and 73.97% of yeasts where culturable after 10 days of storage. On the 8th day, 99.02% of all LAB where non-culturable (Fu et al. 2014). Mind that culture-dependent studies can be lacking as many microbes can be VBNC, meaning the cultured number does not reflect the actual number of viable cells (Laureys et al. 2020). Even if few probiotic microorganisms where to survive storage of kombucha, it does not convey that health benefits cannot be gained from the consumption. Parabiotics or postbiotics refer to the non-viable probiotics or their products that also hold nutritional value. These could be the microbially produced vitamins or other low-molecular weight components. This means pasteurisation can be performed to stop ongoing acid, gas, and alcohol formation, while keeping health promoting features of the beverage (Coelho et al. 2020).

3. Aim

3.1 Problem description

Despite industrially brewed kombucha being more strictly controlled than home brewed kombucha, inconsistencies can have a greater negative impact with larger volumes and financial input. Multiple microbes are involved in the fermentation, and the microbial consortium can vary widely with small deviations. This causes a problem for producers that strive towards a dependable and consistent process and product, to be able to meet customer demands and expectations, while also producing a safe product that follows legislative regulations.

3.2 Purpose and demarcation

The purpose of this master thesis was to investigate

- how the microbial consortium differed between three different starter cultures.
- what microbes could be found in one cellulosic biofilm.
- how the microbial population of one batch of industrially brewed kombucha altered between days 2, 5, 7, 10, 17, and 21 of fermentation.

This was done by plating sample dilutions on differential media and counting the colony forming units, as well as by partial amplification and sequencing of the 16S and 26S RNA gene respectively. Using high performance liquid chromatography (HPLC), concentrations of sugars were tested to detect potential conversions, along with end products. No exact numbers in population but merely estimates were made on microbial shares.

All literature used to construct this paper was retrieved from trustworthy sources, using search engines Primo, ScienceDirect, Wiley Online Library, and Google Scholar.

4. Material and methods

In this section the materials used, and methods applied will be described. Sterile technique and good laboratory practice (GLP) was applied throughout.

4.1 Media

Ingredients used to produce media can be seen in Table 1. All media and utensils used where autoclaved at 125℃ for 25 minutes. YPDC is a selective medium for yeasts, where $CACO_3$ was added to enable halo formation by members of the fungal genus *Brettanomyces*/*Dekkera*. MEAC and DRBC are selective media for moulds. DRBC aids enumeration of moulds by preventing overgrowth of colonies. Both moulds and yeasts can be cultivated on MEAC. Chloramphenicol was added to prevent bacterial growth. MRS is a selective medium for LAB. ABS is a selective medium for AAB, from the recipe by Kim and co-workers, with suggested modification (Kim et al. 2019). Delvocid or cycloheximide was used to prevent growth of eukaryotic organisms. When culturing microbes from the SCOBY and the starter liquids, Delvocid was used. When culturing microbes from the fermentation process, cycloheximide was used, as it was considered more effective. TGE was used to culture general microbes. Peptone water was used for the dilution series.

Table 1. Constituents of the media per one L deionized water.

Media	Constituents per one L deionized water
Yeast extract Peptone Dextrose Chloramphenicol (YPDC) agar	Yeast extract 10 g (Formedium, Hunstanton, United Kingdom) Bacterial peptone 20 g (Merck, Darmstadt, Germany) Agar 20 g (VWR Chemicals, Leuven, Belgium) D(+)-Glucose 20 g (Merck, Darmstadt, Germany) $CaCO_3$ 5 g (Merck, Darmstadt, Germany) Chloramphenicol 100 mg (Sigma-Aldrich, Saint Louis, USA) dissolved in 1 mL 70% EtOH (Solveco, Rosersberg, Sweden)
Malt Extract Agar with Chloramphenicol (MEAC)	Malt extract 30 g (Oxoid, Basingstoke, United Kingdom) Agar 15 g Bacterial peptone 5 g Chloramphenicol 100 mg dissolved in 1 mL 70% EtOH
Dichloran Rose Bengal agar with Chloramphenicol (DRBC)	DRB agar powder 31.5 g (Merck, Darmstadt, Germany)

	Chloramphenicol 100 mg dissolved in 1 mL 70% EtOH
De Man-Rogosa Sharpe (MRS) agar with Cycloheximide	MRS agar powder 62 g (Labkem, Barcelona, Spain) Delvocid Instant 100 mg (DSM, Delft, the Netherlands) dissolved in 1 mL 70% EtOH/Cycloheximide 5 mg (Sigma-Aldrich, Saint Louis, USA) dissolved in 10 mL pure EtOH
Acetic acid Bacteria Selective (ABS) agar	D(+)-Glucose 50 g Yeast extract 10 g Bromophenol blue 20 mg (Merck, Darmstadt, Germany) Agar 20 g Glacial acetic acid 1 mL (Sigma-Aldrich, Saint Louis, USA) Pure EtOH 40 mL (Solveco, Rosersberg, Sweden) Penicillin G sodium salts 100 mg dissolved in 1 mL sterile water (Sigma-Aldrich, Saint Louis, USA) Delvocid Instant 100 mg (DSM, Delft, the Netherlands) dissolved in 1 mL 70% EtOH/Cycloheximide 5 mg dissolved in 10 mL pure EtOH
Tryptone Glucose Extract (TGE) agar	Agar 15 g D(+)-Glucose 1 g Bacterial peptone 5 g Beef extract 3 g (Sigma-Aldrich, Saint Louis, USA)
0.1% peptone water	Bacterial peptone 1 g

4.2 SCOBY

The assay made from the SCOBY was performed in singlicate. The entire sampled SCOBY weighing 157.1 g was homogenized using a Stomacher 400 Circulator. The programme ran for 1 minute at 200 revolutions per minute (RPM), 1 minute at 230 RPM, 1 minute at 260 RPM and lastly 2 minutes at 230 RPM. From this sample, 110 g of the SCOBY and 55 mL of sterile peptone water was again homogenized using the Stomacher 400 Circulator. The programme ran at 230 RPM for 2 minutes.

The biomass was allowed to settle to the bottom of the bag for 2 minutes upon which 1 mL of the liquid sample was then pipetted from below the surface. A dilution series was made with peptone water in the dilutions 2:1, 2:10, 2:100, $2{:}10^3$, $2{:}10^4$, $2{:}10^5$, $2{:}10^6$, $2{:}10^7$ and $2{:}10^8$. The dilutions and a negative control consisting of peptone water were then spread on all media. Before pipetting, each sample container was vortexed to distribute cells evenly in the solution. DRBC, MEAC, and YPDC plates were incubated in 25°C for 7 days when colonies were numerable. ABS and TGE plates were incubated in 30°C for 7 days when colonies were numerable. MRS plates were incubated anaerobically (Anaerocult, Merck, Darmstadt, Germany) in 30°C for 3 days when colonies were numerable.

4.3 Starter liquids

The assay made from the starter liquids was performed in singlicate. A dilution series was made with peptone water from the sampled starter liquids in the dilutions 1, 1:10, 1:100 and $1:10^3$. Before pipetting, each sample container was vortexed to distribute cells evenly in the solution. The dilutions and a negative control consisting of peptone water were then spread on all media.

DRBC, MEAC, and YPDC plates were incubated in 25℃ for 7 days when colonies were numerable. ABS and TGE plates were incubated in 30℃ for 7 days when colonies were numerable. MRS plates were incubated anaerobically (Anaerocult, Merck, Darmstadt, Germany) in 30℃ for 7 days when colonies were numerable.

4.4 The fermentation process

The batch was prepared by the brewery by steeping tea for 23 minutes at 85-75℃. The tea was mixed with sucrose to a total concentration of 5%. When cooled, starter liquid was added, representing 20% of the total volume. The remaining volume consisted of water. The fermentation proceeded from the 31st of January to the 20th of February 2024. Samples were taken on the 2nd, 5th, 7th, 10th, 17th and 21st of February by the brewer. Two samples were taken each day, one was frozen to enable timepoint sugar analysis, and one was chilled to stop microbial growth.

During transport, chilled and frozen samples reached room temperature for 24 hours. Upon receival, they were placed in the refrigerator at 4±1℃.

The assays made from the different days were performed in triplicate. Dilution series were made with peptone water in the dilutions 1:100, $1:10^3$ and $1:10^4$. Before pipetting, each sample container was vortexed to distribute cells evenly in the solution. The dilutions and a negative control consisting of peptone water were then spread on all media.

DRBC, MEAC, and YPDC plates were incubated aerobically in 25℃. TGE and ABS plates were incubated aerobically in 30℃. MRS plates were incubated anaerobically (Anaerocult, Merck, Darmstadt, Germany) in 30℃. Plates of all media with samples from day 2 were incubated for 7 days. Plates of all media with samples from day 5-21 were incubated for 6 days.

4.5 Isolation

To achieve pure cultures of the different microbes, 4 colonies of equal morphology were randomly chosen from each media and sample and restreaked using a loop onto new plates of the same media as the initial. The procedure was done twice, to ensure a pure culture.

4.6 Polymerase chain reaction

Upon establishing if the microbial colonies were yeasts or bacteria by microscopy, taxonomic identification of microbes was achieved by partial amplification and sequencing of the 16S and

26S RNA gene respectively. For bacteria the partial amplification of the 16S RNA gene resulted in a 1420 bp fragment (Pedersen et al. 2004), for yeasts the partial amplification of the D1/D2 region resulted in a 600 bp fragment (Kurtzman & Robnett 1997).

PCR-tubes were placed in a holder. DNA templates were prepared by taking a small amount of material from a well isolated colony with a sterile toothpick. The material was dissolved in 20 µL of sterile Milli-Q water for bacteria, 20 µL of 0.02 M NaOH for yeasts. The templates were heated at 95℃ for 10 minutes.

PCR-tubes were placed in a holder. In all tubes 12.5 µL DreamTaq Green Master Mix (Merck) and 8.5 µL sterile Milli-Q water. For yeasts, 1 µL primer NL1 with the sequence GCATATCAATAAGCGGAGGAAAAG and 1 µL primer NL4 with the sequence GGTCCGTGTTTCAAGACG was pipetted. For bacteria, 1 µL primer 16S with the sequence AGAGTTTGATCCTGGCTC and 1 µL primer 16Sr with the sequence CGGGAACGTATTCACCG was pipetted. 2 µL DNA template was added to the appropriate tube and mixed by inversion.

The tubes were placed in the PCR-machine. The amplification condition differed slightly between bacteria and yeasts. For yeasts it was one cycle of initial denaturation at 94℃ for 2 minutes, then followed 35 cycles of denaturation at 94℃ for 30 seconds, annealing at 50℃ for 30 seconds and extension at 72℃ for 1 minute. The final extension was set to 72℃ for 10 minutes upon which the samples were held at 16℃. For bacteria, the programme started with one cycle of initial denaturation at 94℃ for 2 minutes, then followed 30 cycles of denaturation at 94℃ for 30 seconds, annealing at 50℃ for 30 seconds, and extension at 72℃ for 2 minutes. The final extension was set to 72℃ for 10 minutes upon which the samples were held at 16℃.

4.7 Gel electrophoresis

1.5 % agarose gel was prepared in 1x TAE with GelRed Nucleic Acid Stain 10000x. GeneRuler Plus 1 kb was used as the size standard. The gel was cast and allowed to set. 5 µL of the samples were pipetted into separate wells, and the electric current was applied, approximately 90 V/cm gel for 45 minutes. The results were read under a UV-light.

4.8 Sequencing

To ensure sufficient concentration of DNA, a Qubit broad range fluorometer was used to measure randomly chosen samples and establish a perception of the general concentration. PCR-products that resulted in a clearly visible band under the UV-light and a perceived sufficient concentration of DNA were packaged and sent to Macrogen Europe for purification and sequencing. All samples were sequenced unidirectionally. A total of 192 samples were submitted.

The resulting chromatograms and sequences were inspected using the app FinchTV. Selected base sequences were 550-600 bases long for yeasts, 900-1100 bases long for bacteria. Selected base sequences were then compared with the NCBI GenBank database using the BLASTn search tool to identify similar sequences.

4.9 High performance liquid chromatography

Samples were prepared for HPLC as follows. To achieve standard curves for sugars and acids, 10 g/L was calculated for pure ethanol, lactic acid, acetic acid, glucose, and fructose.

The solid sugars were weighed, and a 100 mL stock solution was prepared for each sugar. The densities of the liquids were used to calculate volume needed. 0.94 mL of acetic acid was added to 100 mL of Milli-Q water. 1.25 mL of pure ethanol was added to 100 mL of Milli-Q water. 0.83 mL of lactic acid was added to 100 mL of Milli-Q water.

The sugars, acids and ethanol were then diluted with Milli-Q water in Falcon tubes to a final concentration of 7.5, 5.0, 2.5, 1.0 and 0.5 g/L respectively.

From the Falcon tubes, 3 mL from each vial was pipetted into 5 mL Eppendorf tubes. 300 µL of 5 M H_2SO_4 was added to each Eppendorf tube.

The samples from the process used to test for sugars were day 2, 5, 7, 10 and 17. The samples were pipetted into separate Falcon tubes and diluted with Milli-Q water to a ratio of 1:10 and 1:4. The samples were then centrifuged using a Sorvall LYNX 4000 Centrifuge and the programme ran for 10 minutes at 22℃ with G-force 5000 x g. From the supernatant, 3 mL was pipetted into a 5 mL Eppendorf tube. 300 µL of 5 M H_2SO_4 was added to each Eppendorf tube.

All samples were then filtered and transferred into HPLC glass vials, which were then put separately into the HPLC machine. The programme ran for 53 minutes for each sample with both a refractive index (RI) detector and a UV-detector separately.

5. Results

In this section the results will be presented. Colony forming units (CFU)/mL were calculated by multiplying the number of colonies with the dilution factor and dividing the sum with the volume added to the culture plate. Colony numbers used where preferably between 30-300. If possible, a mean value was calculated. YPDC is a selective medium for yeasts. MEAC and DRBC are selective media for moulds. MRS is a selective medium for LAB. ABS is a selective medium for AAB, TGE is used to cultivate general microorganisms. A hyphen in cells of tables indicates no results.

5.1 SCOBY and starter liquids

In this section, colony forming units per millilitre, colony morphology, and identified microorganisms from the SCOBY and starter liquids will be presented.

5.1.1 Colony forming units per millilitre

Cell counts, mean values, and standard deviations from the plates for the SCOBY and starter cultures are available in Appendix I. The SCOBY had the greatest CFU/mL values when compared to those of the starter liquids (Table 2). There was also growth on all media. Greatest growth on YPDC, followed by MEAC, DRBC, MRS, TGE, and finally ABS.

The nantou starter culture had the highest CFU/mL calculated from the MEAC plates, same as the other starter cultures. Second highest number was on YPDC, DRBC, and TGE. Low growth of suspected LAB and no growth on ABS.

The black tea starter liquid CFU/mL values were greatest at MEAC, YPDC, TGE and DRBC. Small number of suspected AAB and no growth on MRS.

Similarly to the black tea starter culture, the rosé starter culture also had the greatest CFU/mL calculated from the MEAC plates. Next greatest growth was at YPDC. The same number of colonies at TGE and DRBC. Small amount of suspected LAB grew on MRS and there was no growth on ABS.

Table 2. CFU/mL SCOBY, nantou, black tea, and rosé starter liquids.

Media	**CFU/mL** SCOBY	**CFU/mL** Nantou	**CFU/mL** Black tea	**CFU/mL** Rosé
YPDC	3.9×10^6	3.0×10^5	3.6×10^5	1.1×10^6
MEAC	3.7×10^6	4.2×10^5	4.9×10^5	1.6×10^6
DRBC	1.5×10^6	3.8×10^4	2.1×10^5	4.9×10^5

ABS	2.2×10^2	-	4.3×10^2	-
MRS	3.7×10^5	0.5×10^2	-	1.7×10^3
TGE	2.1×10^5	2.7×10^4	2.6×10^5	4.9×10^5
Total	9.7×10^6	7.9×10^5	1.3×10^6	3.7×10^6

5.1.2 Colony morphology

All descriptions of colony morphology are compiled in Table 3.

Table 3. Colony morphology descriptions for the SCOBY, nantou, black tea, and rosé starter liquids grown on all media.

Media	**SCOBY**	**Nantou**	**Black tea**	**Rosé**
YPDC	1. Small, white, round, shiny, with a peak.			
	2. Large, light beige, wrinkled with uneven edges.			
MEAC	1. White, shiny, small, round, with a peak.			
	2. Large, beige, spread out, flat, with irregular edges.		-	2. Large, beige, spread out, flat, with irregular edges.
DRBC	1. Small, round, evenly pink, shiny, with a peak.			
	2. Large, light pink to pink, inflated, creamy, round.			
ABS	1. Small, black, shiny, round.	-	1. Small, black, shiny, round.	-
MRS	1. Very small, white, round.	1. Very small, white, round.	-	1. Very small, white, round.
	2. Small, spread out, white, round, irregular edges.			2 Small, spread out, white, round, irregular edges.
	3. Large, white with yellow centre, creamy, round.			
TGE	1. Round, white, shiny, and creamy.			

Two visually different colonies grew on YPDC, where the description was equal from all samples. One was round, smaller, shiny, and white, with a peak. The other was larger, light beige, wrinkled and had uneven edges (Figure 2).

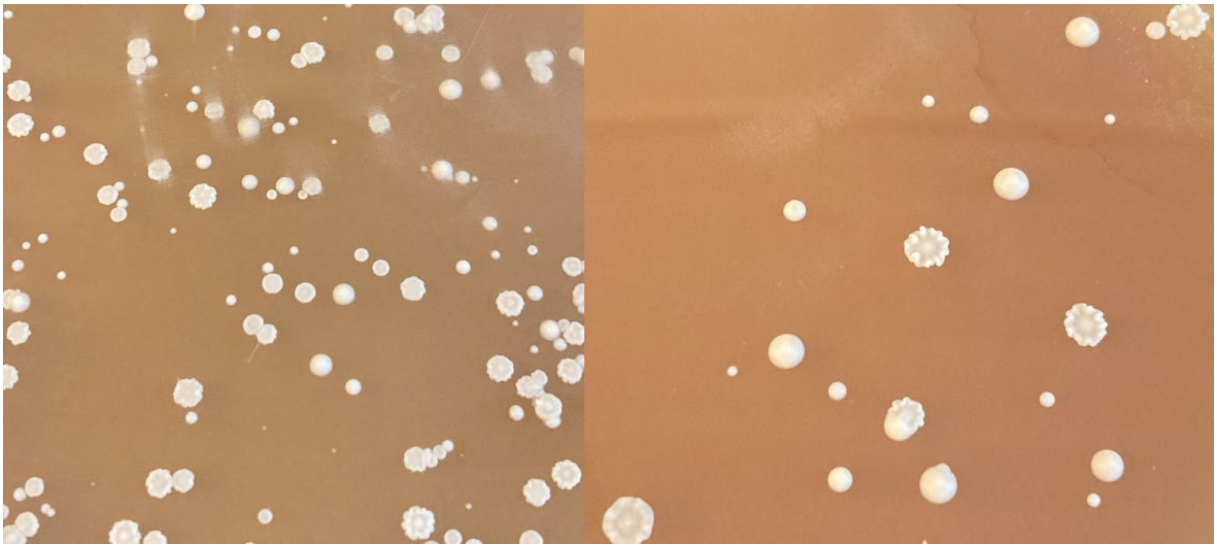

Figure 2. Colonies on YPDC. SCOBY dilution 1:1000 to the left, nantou dilution 1:100 to the right.

Colony appearance on MEAC differed slightly between the samples. All samples had one colony that was small, round, white, and shiny, with a peak. This colony looked very similar to the colony growing on YPDC. But only the SCOBY, nantou and rosé starter liquid had an additional visually different colony. That colony was large, flat, beige and spread out with irregular edges (Figure 3).

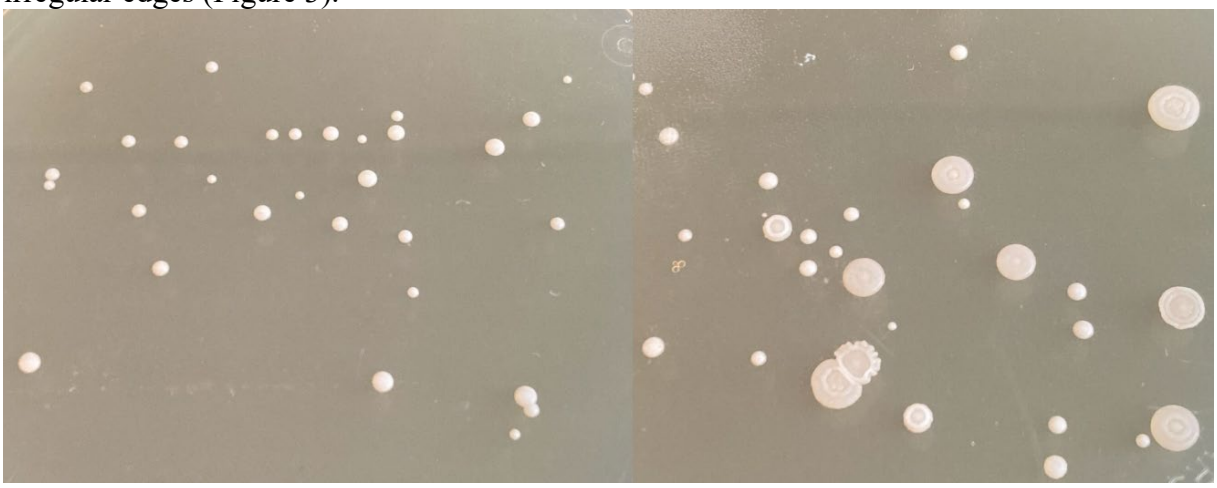

Figure 3. Colonies on MEAC. Black tea dilution 1:1000 to the left, nantou dilution 1:100 to the right.

Two visually different colonies grew on DRBC (Figure 4), with equal appearance from three of the samples. The smaller colonies were bright pink, round, and shiny. The larger, round, and creamy colonies were pinker at the top, but whiter underneath. The larger colony was not present on the black tea plates, but instead a colony that looked similar but wrinklier.

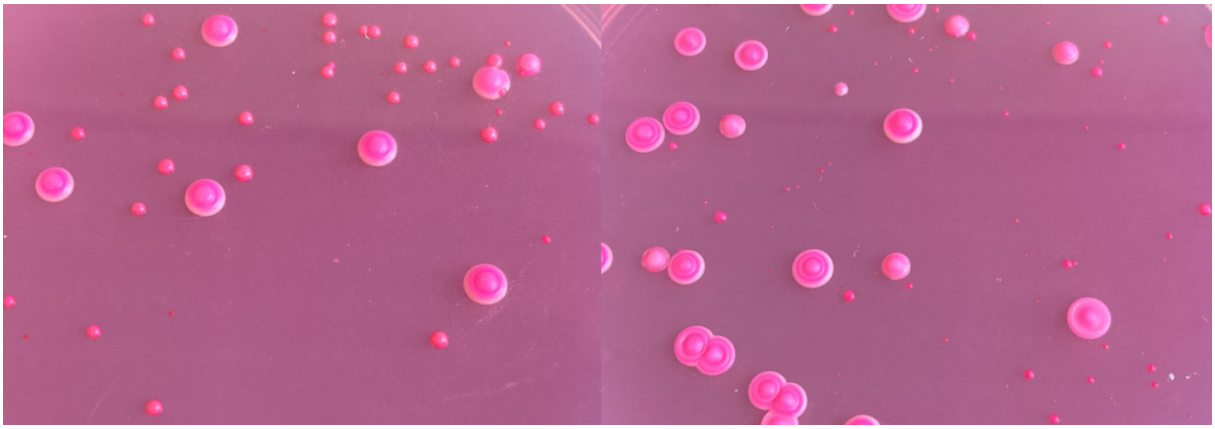

Figure 4. Colonies on DRBC. Rosé dilution 1:1000 to the left, SCOBY dilution 1:1000 to the right.

Colonies from the SCOBY assay and the black tea starter liquid growing on ABS agar were observed as having equal morphology. Small, black, shiny, and round colonies. The media contains a pH indicator and was blue initially, but turned green over time, indicating acid formation. Figure 5 shows a photo of colonies growing from the non-diluted black tea starter liquid.

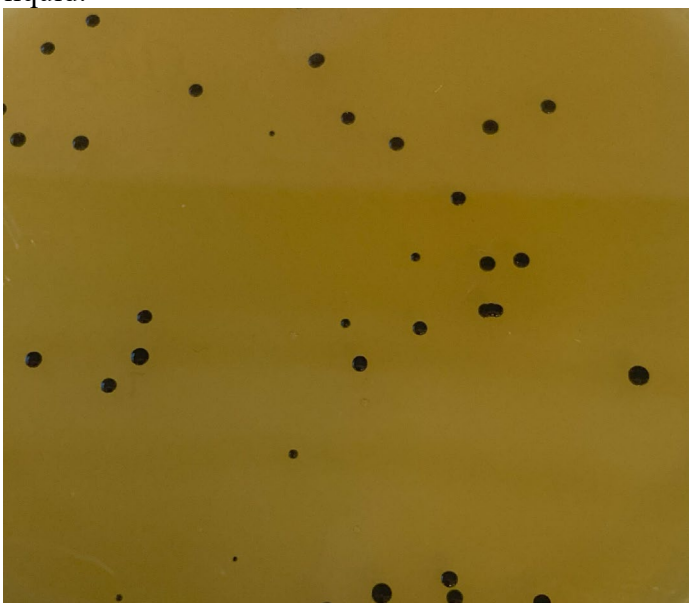

Figure 5. Suspected AAB from the non-diluted black tea starter liquid on ABS.

Colony appearance on MRS varied between samples. One colony from the nantou starter liquid, which were small, cloudy, and round. From the rosé sample two colonies were visually different. One very small, white, and round and another which was round and white but flatter and more spread out. The plates with the SCOBY assay looked similar to the rosé but with an additional colony which was large, creamy, and round with a peak and yellow centre (Figure 6).

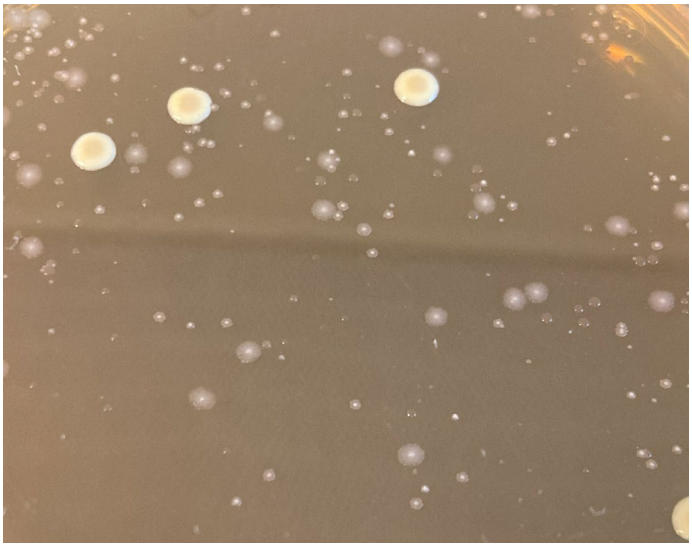

Figure 6. Colonies on MRS, from the SCOBY dilution 1:100.

One morphologically identical colony grew on TGE. Appearance was the same for all samples. Colonies growing looked identical to those growing on MEAC from black tea dilution 1:1000. Colonies were round, white, shiny, and creamy.

5.1.3 Identified microorganisms

All of the mentioned microorganisms had minimum 98% query coverage and 98% percentage identity when compared using the BLASTn search tool. Of the amplicons submitted for sequencing, the following where found from the SCOBY and the starter liquids (Table 4). Half of the amplicons retrieved from the SCOBY were identified as *Brettanomyces anomalus*. One fourth was identified as *Zygosaccharomyces lentus* and the last fourth as *Brettanomyces bruxellensis*. The amplicons retrieved from the nantou starter culture were represented by equal parts *Z. lentus* and *B. anomalus*. The identified microorganisms in black tea were equal parts *Z. lentus, B. anomalus, B. bruxellensis* and *Kregervanrija fluxuum*. The samples from the rosé starter liquids were identified as 43% *Z. lentus, 52% B. anomalus,* and 5% *B. bruxellensis.*

Table 4. Identified microorganisms SCOBY, nantou, black tea, and rosé starter liquids.

Sample	MEAC	YPDC	DRBC	TGE
SCOBY	*Zygosaccharomyces lentus* *Brettanomyces anomalus* *Brettanomyces bruxellensis*	*B. bruxellensis*	*Z. lentus* *B. anomalus* *B. bruxellensis*	*B. anomalus*
Nantou	*Z. lentus* *B. anomalus*	*Z. lentus*	*Z. lentus*	*B. anomalus*
Black tea	*B. anomalus*	*B. bruxellensis*	*Z. lentus* *Kregervanrija fluxuum*	*B. bruxellensis* *B. anomalus*
Rosé	*Z. lentus* *B. anomalus* *B. bruxellensis*	*Z. lentus* *B. anomalus*	*Z. lentus* *B. anomalus*	*B. anomalus*

5.2 The fermentation process

In this section, colony forming units per millilitre, colony morphology, identified microorganisms, and values from HPLC retrieved from the days of the fermentation will be presented.

5.2.1 Colony forming units per millilitre

Values displayed in Table 5 were measured and provided by the brewer. Initial temperature was at 17.4℃ and reached a peak on day 5 at 22.5℃ to then decrease until fermentation was considered complete. BRIX value started at 48.1 and was lowered to 29.8 at the end of the fermentation. pH was initially 3.63 and went below 3.49 after day 5.

Table 5. pH, BRIX, and temperature (℃) during the fermentation.

Day	pH	BRIX	Temperature (℃)
0	3.63	48.1	17.4
2	3.58	47.5	16.6
5	3.49	44	22.6
7	3.48	40.9	22.5
9	3.46	38.3	21.5
16	3.43	32	21.9
20	-	29.8	-

Cell counts, mean values, and standard deviations from the plates for day 2, 5, 7, 10, 17 and 21 are available in Appendix II. As can be seen both in Figure 7 and Table 6, the microbial growth declines with pH. CFU/mL on MEAC and ABS are the only media where growth increases from start to end. The CFU/mL from ABS plates were calculated as greatest on day 7, but then lowers while remaining higher than the initial concentration. Suspected LAB on MRS increases significantly on day 7, when the smallest colonies were countable, but then decreases.

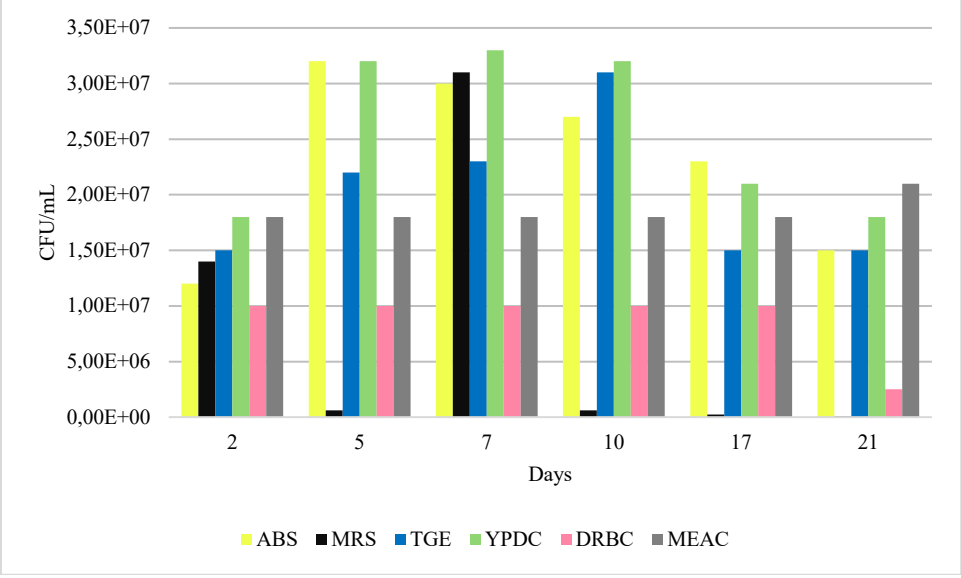

Figure 7. CFU/mL during the fermentation.

CFU/mL on TGE and YPDC remains the same from start to finish with a small growth curve in between. CFU/mL calculations from DRBC plates is lower at the end point compared to the start point.

Table 6. CFU/mL during the fermentation.

Day	ABS	MRS	TGE	YPDC	DRBC	MEAC
2	1.2×10^7	1.4×10^7	1.5×10^7	1.8×10^7	1.0×10^7	1.8×10^7
5	3.2×10^7	6.1×10^5	2.2×10^7	3.2×10^7	-	-
7	3.0×10^7	3.1×10^7	2.3×10^7	3.3×10^7	-	-
10	2.7×10^7	6.0×10^5	3.1×10^7	3.2×10^7	-	-
17	2.3×10^7	2.5×10^5	1.5×10^7	2.1×10^7	-	-
21	1.5×10^7	6.2×10^4	1.5×10^7	1.8×10^7	2.5×10^6	2.1×10^7

5.2.1 Colony morphology

Colonies growing on MEAC were not distinguishable between. All were small, white, round, and shiny. Colonies from day 2 and day 21 was observed as of equal morphology (Figure 8).

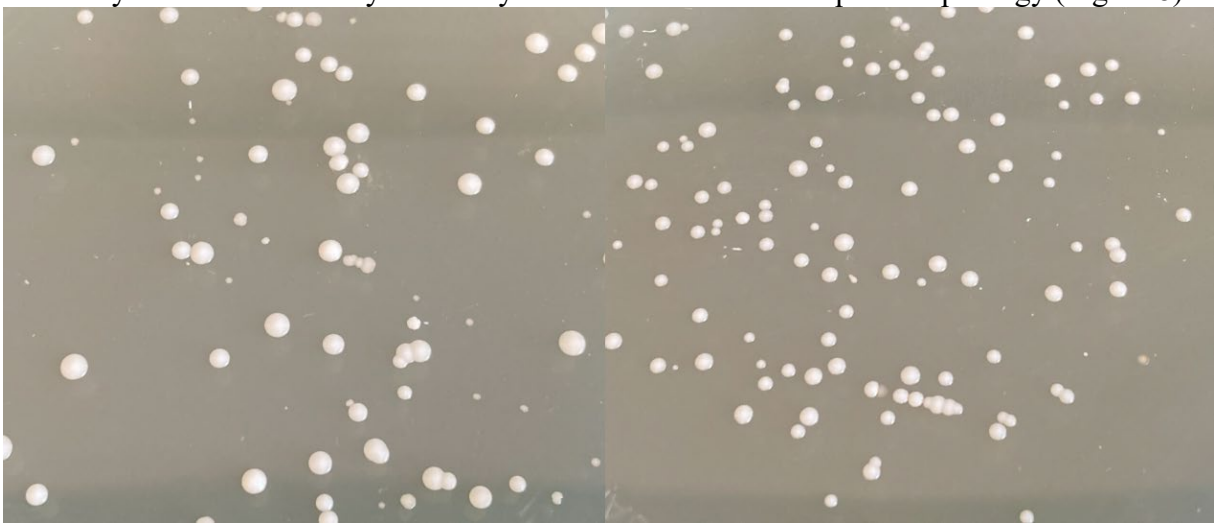

Figure 8. Colonies on MEAC. Day 2 to the left, day 21 to the right, both diluted 1:10000.

Colonies growing on YPDC looked similar to those growing on MEAC, however, they grew larger occasionally. An additional colony was observed that looked deflated, small, white, and round, but with irregular edges (Figure 9).

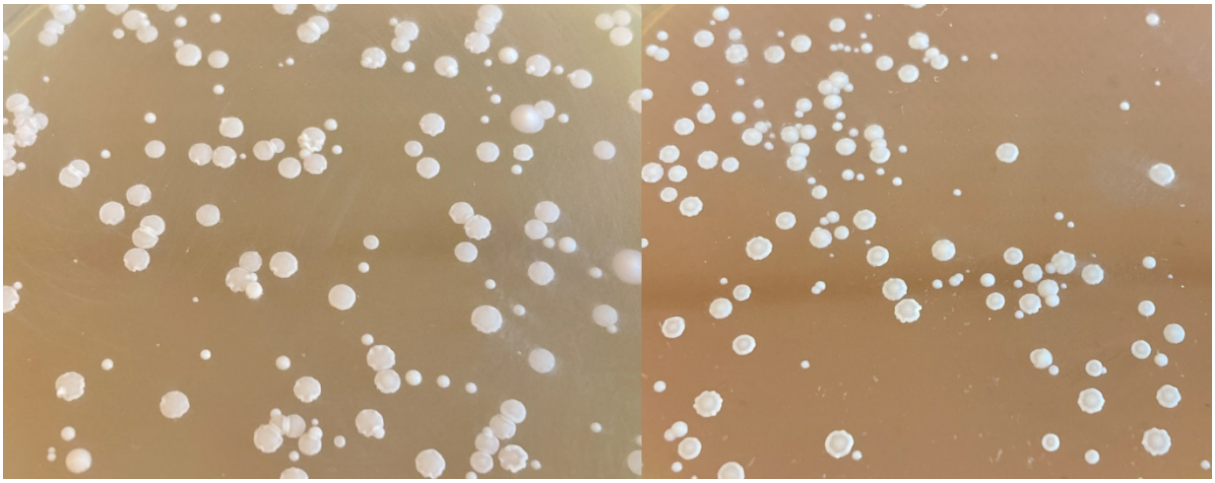

Figure 9. Colonies on YPDC. Day 5 to the left, day 17 to the right, both diluted 1:10000.

Three types of colonies were distinguishable on DRBC (Figure 10). One large, round, creamy, white below, and pink at the top. One round, shiny, creamy, and light pink. The last was small, round, shiny, and bright pink. All three were present on plates from day 2 and 21, in varying degree.

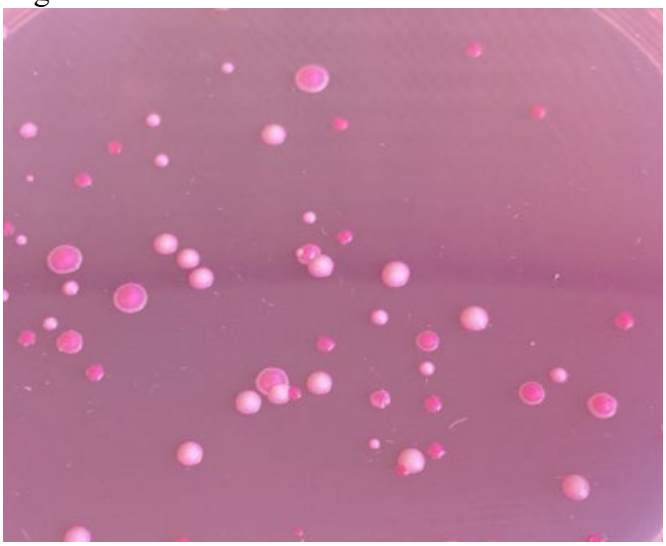

Figure 10. Colonies on DRBC. Day 2, dilution 1:10000.

Two different colonies grew on MRS (Figure 11). One was very small, white, and round, the other was white, cloudy, round, and flat.

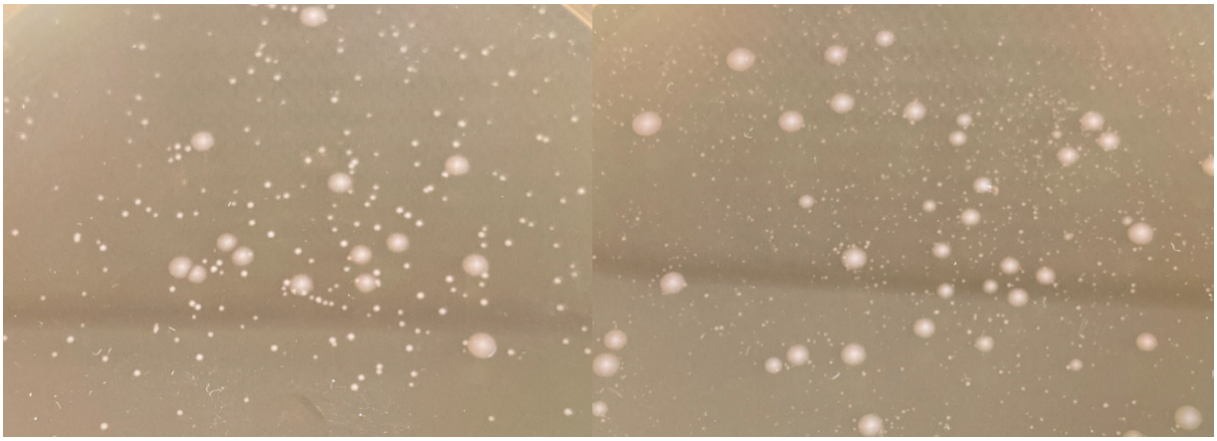

Figure 11. Colonies on MRS. Day 7 to the left, dilution 1:10000, day 10 to the right, diluted 1:1000.

Three different colonies grew on ABS. A small amount was black, round, and shiny. The vast majority were either light or dark blue, round, and dull. In Figure 12 to the right a stack can be seen, were acid formation is visible. Day 5, 7, and 10 has most acid formation. The media contains a pH indicator and is initially blue but turns green when pH is lowered.

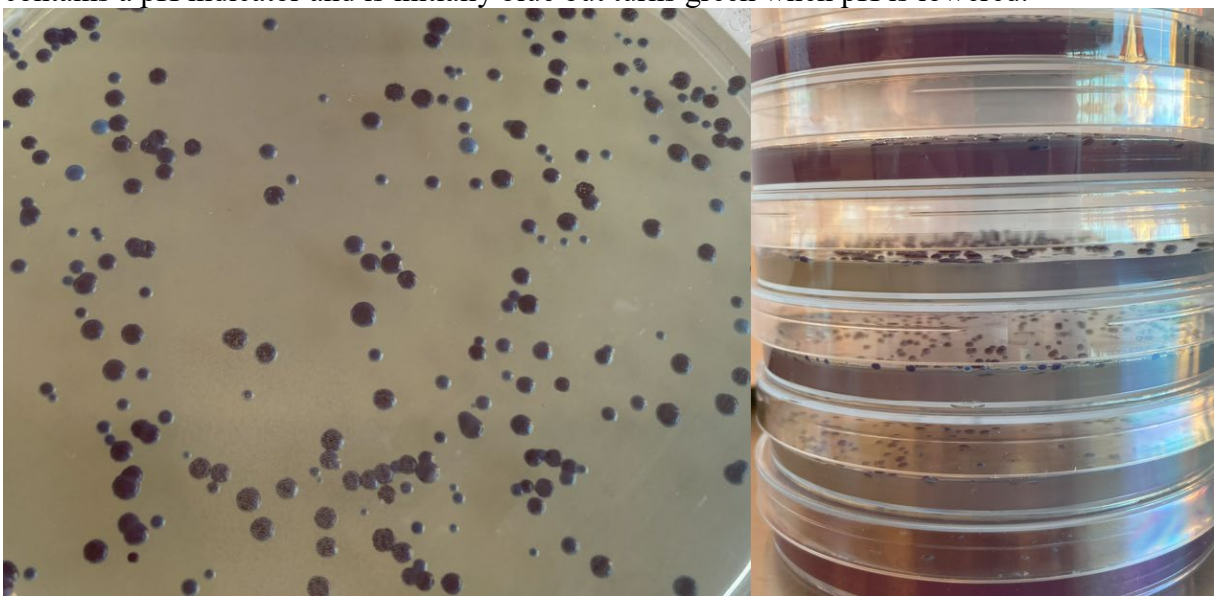

Figure 12. Colonies on ABS. Day 5 to the left, dilution 1:10000. To the right, plates stacked 2-21 from the bottom up.

Colonies growing on TGE were observed as identical to those growing on MEAC.

5.2.2 Identified microorganisms

All of the mentioned microorganisms had minimum 98% query coverage and 98% percentage identity when compared using the BLASTn search tool. Of the amplicons submitted for sequencing, the following where found from the fermentation (Table 7).

Table 7. Identified microorganisms during the fermentation. D = day.

Sample	MEAC	YPDC	DRBC	MRS	ABS	TGE
D2	*B. bruxellensis*	*Hanseniaspora mollemarum*	*H. mollemarum*	*B. anomalus*	*B. bruxellensis* *B. anomalus*	*B. anomalus*

		B. bruxellensis *B. anomalus*	*B. bruxellensis*			
D5	-	*H. mollemarum* *B. bruxellensis* *B. anomalus*	-	*B. bruxellensis* *Zymomonas mobilis* subsp. *pomaceae*	*B. anomalus*	*B. anomalus*
D7	-	*H. mollemarum*	-	*B. bruxellensis* *B. anomalus* *Z. mobilis* subsp. *pomaceae*	*B. anomalus* *B. bruxellensis*	*B. anomalus*
D10	-	*B. anomalus*	-	*B. bruxellensis* *B. anomalus*	*B. bruxellensis* *B. anomalus*	*B. anomalus*
D17	-	*B. anomalus*	-	*B. bruxellensis* *Z. mobilis* subsp. *pomaceae*	*B. anomalus*	*B. anomalus*
D21	*B. bruxellensis* *B. anomalus*	*B. bruxellensis* *B. anomalus*	*B. anomalus*	*B. bruxellensis* *Z. mobilis* subsp. *pomaceae*	*B. anomalus*	*B. bruxellensis* *B. anomalus*

From day two, the microbes that were possible to identify included *B. anomalus, B. bruxellensis*, and *Hanseniaspora mollemarum*. From day five, *Zymomonas mobilis* subspecies *pomaceae* was also identified. These four microbes remain among the identities from day seven. From day 10, no *Z. mobilis* subsp. *pomaceae* or *H. mollemarum* was identified. From day 17 and 21, the identified microbes include *B. anomalus, B. bruxellensis* and *Z. mobilis* subsp. *pomaceae.* Figure 13 illustrates the microbes as percentages of total amount of sequencable samples from day 2-21 of fermentation. *B. anomalus* represented the greatest share in all samples, with varying shares of the other microorganisms. *B. bruxellensis* increased towards the end of the fermentation, while *H. mollemarum* was not among the identified microorganisms from day 10 onwards.

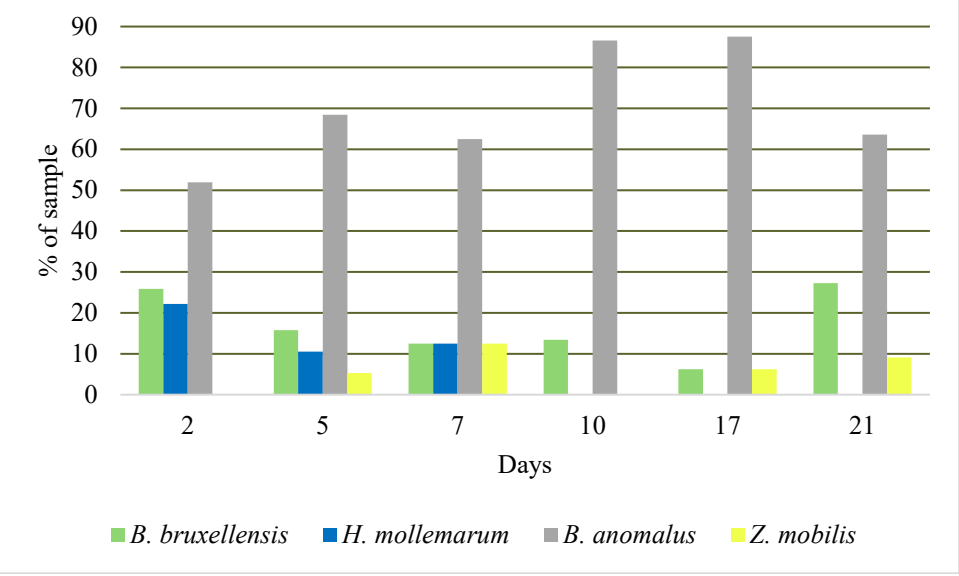

Figure 13. B. anomalus, B. bruxellensis, H. mollemarum, and Z. mobilis illustrated as percentages of total amount of sequencable samples from day 2-21.

5.2.3 High performance liquid chromatography concentrations

The numbers included in Table 8 are mean values from two dilutions, rounded to the closest decimal. As can be seen in Figure 14, the standard deviation was low. All obtained data calculated to g/L can be found in Appendix III.

Table 8. Acetate, ethanol, glucose, and fructose concentrations (g/L) on day 2, 5, 7, 10, and 17.

Day	Acetate	Ethanol	Glucose	Fructose	Fructose -Glucose
2	1.6	3.5	25.9	29.2	3.3
5	1.9	5.6	24.0	28.4	4.4
7	2.1	8.0	22.1	26.6	4.5
10	2.6	10.0	19.4	22.0	2.6
17	3.0	15.4	16.1	20.3	4.2

When starting the batch, the brewer reported adding 50 g L^{-1} sucrose. Sugars could also have originated from the starter liquid. As sucrose is a disaccharide consisting of one glucose molecule and one fructose molecule linked with covalent bonds that can break in acidic, aqueous solution, what was left to observe was glucose and fructose. Examples of sucrose equivalents will be provided, based on the lowest amount of the two sugars doubled.

After 48 hours of fermentation, sucrose equivalents were at about 50 g. More glucose than fructose was consumed, with a difference of about 3 g L^{-1}. After an additional 3 days, the trend continued with glucose being preferred over fructose. The first 7 days of fermentation, sugar consumption was slow with a total of less than 8 g L^{-1} sucrose equivalents consumed. From day 7 to day 17 of the fermentation, sugar was consumed faster compared to the first 7 days, with an additional total of 4 g L^{-1}. Despite fluctuations, 4 g L^{-1} of fructose remained on day 17.

Initial acetate production is fast as the concentration on day two is at about 1.5 g L^{-1}, which doubles during the course of the following two weeks.

On day two, ethanol is at 3.5 g L^{-1}, and rises quickly to 15.4 g L^{-1} on day 17. The final mass percentage of ethanol on day 17 would be about 1.2%, calculated by using the density of ethanol and water.

No lactate, propionate, isobutyrate, butyrate, isovalerate, or valerate was found in the samples.

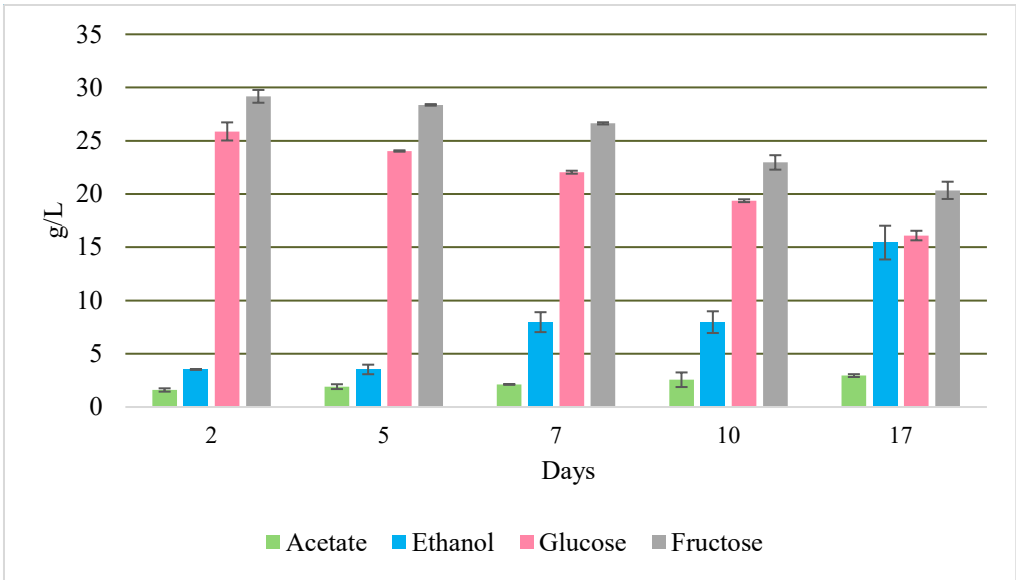

Figure 14. Acetate, ethanol, glucose, and fructose concentrations (g/L) on day 2, 5, 7, 10, and 17.

6. About the identified microorganisms

In this section a brief overview of identified microorganisms will be provided.

6.1 Brettanomyces species

B. anomalus and *B. bruxellensis* are two of five described species in the genus of *Dekkera/Brettanomyces*. *Dekkera* is used to describe the teleomorph, and *Brettanomyces* the anamorph. Yet, the terms are used interchangeably. This species can grow in acidic environments in the presence of carbon dioxide, ethanol and sorbic acid. They are thus commonly encountered in wine, beer and soft drinks (Hulin et al. 2014).

Their mode of respiration is facultatively anaerobic, meaning the fermentation can be carried out with or without oxygen, with varying byproducts. If conditions are anaerobic, ethanol is produced (Feldmann 2012). Multiple factors influence the preferred growth temperature, including concentration of ethanol, but growth can occur within the range of 10-35°C (Oswald & Edwards 2017).

In terms of morphology, the most typical form is ogival, but can also be cylindroid to elongated, with occasional formation of pseudo mycelium. Production of acetic acid from glucose is characteristic for this species, along with resistance to a concentration of 0.01-0.1% cycloheximide. They grow more slowly compared to *Saccharomyces cerevisiae* but have been found to possess a resistance to ethanol that is close to that of *S. cerevisiae.* Some nutrients are required, such as thiamine (Batt 2014).

Sulphite and SO_2 is commonly used in the wine industry to prevent growth of unwanted yeasts. However, evidence has been presented that demonstrates a growing tolerance among *Brettanomyces* species towards these compounds, up to 2.1 mg l^{-1} SO_2 (Agnolucci et al. 2017).

What distinguishes these species from each other is genetic sequence and fermentation characteristics, resulting in varying flavour compounds (Cocolin et al. 2004).

6.2 *Hanseniaspora* species

H. mollemarum is a novel ascomycetous species belonging to the genus of *Hanseniaspora*. *Hanseniaspora* is used to describe the sexual or teleomorph life stage, and *Kloeckera* the asexual or anamorphic life stage (Chen et al. 2023). The apiculate, ovoid to elongate cells proliferate by bipolar budding and can be observed as single, in pairs or short chains. Growth occurs around 25°C (Groenewald et al. 2018). The species has been encountered throughout

Europe, from garden soil in the Netherlands, to elderberry syrup in Hungary and cider in England. (Čadež et al. 2021).

Species of *Hanseniaspora* have been frequently encountered both on mature fruits but also in fermented beverages. They seemingly play an important role during the initial fermentation, producing enzymes and flavour compounds (Chen et al. 2023). *Hanseniaspora* species could however slow down fermentation, due to the consumption of thiamine, which is also required by other yeasts (Phister et al. 2007).

6.3 Kregervanrija fluxuum

The teleomorph life stage of this thought heterothallic yeast is referred to as *K. fluxuum and* was previously classified as a member of the genus *Pichia*. The anamorphic life stage is represented by *Candida vini*. The yeast cells are ovoid to elongate, with formation of ascospores. If pseudo hyphae is formed it is not pronounced, and no true hyphae is formed. A pellicle is often formed in the growth media (Kurtzman 2006). Studied species have been found in wine, cider, kombucha, and other substrates. Growth occurs at 15-25°C, with no growth at 37°C (Kurtzman 2011). One strain of *K. delftensis* exhibit the ability of weak glucose fermentation, but other species of the genus are non-fermentative. The genus consists of three closely related species, where the D1/D2 region varies by 15 substitutions, not including gaps (Kurtzman 2006).

6.4 Zygosaccharomyces lentus

Members of the *Zygosaccharomyces* genus are often considered as spoilage yeasts, due to their osmotolerance and ability to persist in environments with low pH. Their metabolic activity can create unpleasant flavour compounds, and generate CO_2, causing exploding glass bottles.

Z. lentus can grow in temperatures 4-25°C with pH values ranging between 2.2-7.0. However, growth will not occur at 25°C in the presence of 1% acetic acid, which distinguishes this species from *Z. bailii. Z. lentus* has also been found to prefer static cultures (Steels et al. 1999).

Ascosporous yeast cells are spherical, ovoid, or elongate, without formation of true hyphae. If pseudo hyphae is formed, it is not pronounced. Their mode of respiration is facultatively anaerobic, but growth is very slow in anaerobic conditions (James & Stratford 2011).

Z. lentus is not affected by food preservatives such as benzoic (900 mg l^{-1}) and sorbic acid (400 mg l^{-1}). Fermentable sugars include glucose, sucrose and fructose, but fermentation is significantly weaker compared to *S. cervisiae* (Steels et al. 1999).

In a recent study, *Z. lentus* was found in botrytised Hungarian dessert wine. Tolerance was established to 900 mg l^{-1} sulphur and an alcohol concentration of 8% (Csoma et al. 2023).

6.5 *Zymomonas mobilis* subspecies *pomaceae*

Z. mobilis is a motile, rod-shaped, gram-negative, anaerobic bacterium, belonging to the group Alphaproteobacteria. Tolerant to high ethanol and sugar concentrations, the species has been commonly encountered in tropical fruit saps and spoiled ciders. Optimal growth temperature is at 30°C, but some species can operate as high as 36°C. (Agrawal et al. 2017). The pH tolerance ranges between 3.5-7.5. However, tolerance can vary between strains, with some might tolerating even lower pH (Ley & Swings 1976).

Z. mobilis employs the Entner-Doudoroff pathway, aided by the enzymes pyruvate decarboxylase and alcohol dehydrogenase, to produce ethanol and CO_2. Fermentable sugars include sucrose, glucose, and fructose (Kalnenieks 2006).

Z. mobilis subspecies *pomaceae* is distinguishable from other subspecies by genetic sequence and morphological tests. For an example low salt tolerance compared to the subspecies *mobilis* (Ley & Swings 1976).

7. Discussion

The microbial populations found from the starter liquids were similar but not identical, despite these starter liquids originating from the same brewery.

The black tea starter liquid was found to be most diverse, alone in containing *K. fluxuum*. This could be a result of multiple factors, including age and ingredients. *K. fluxuum* has been encountered in kombucha previously (Kurtzman 2011), and could have been present in the other starter cultures. At sampling point *K. fluxuum* could have been VBNC in those samples, as a result of long-term exposure to low pH or other stressors.

The second most diverse microbial population was found from the rosé starter culture, and was represented by *B. anomalus, B. bruxellensis*, and *Z. lentus*. That *B. anomalus* and *B. bruxellensis* grows on all plates is explained by resistance to cycloheximide (Batt 2014). Despite addition of $CaCO_3$ no halo formation was observed on YPDC plates by *Brettanomyces* species.

The same composition of microbes was found in the SCOBY, however at a greater density. Looking at the values for CFU/mL, total CFU/mL was almost four times greater in the SCOBY than in the rosé starter culture. Greater cell growth in the SCOBY could be a result of greater access to oxygen, which is still required by facultatively anaerobe yeasts for cell growth.

The nantou starter culture was composed of two microbes, namely *Z. lentus* and *B. anomalus*. Both of these microbes are known to be acid- and osmotolerant, which is the reason they are present in all starter cultures that should have a pH closer to 3.0. The nantou starter culture had the lowest total value of CFU/mL.

What distinguishes the starter cultures from the fermentation in terms of microbial consortium is the presence of *Z. lentus*, which is not encountered during the fermentation. Storage of starter cultures could be more static compared to the batch fermentation, thus favouring growth of *Z. lentus* (Steels et al. 1999).

Microbes found from day two include *B. anomalus, B. bruxellensis*, and *H. mollemarum*. Another possible explanation to the absence of *Z. lentus* could be the presence of *H. mollemarum*, which is known to utilize thiamine in early fermentation, which is also required by other yeasts (Phister et al. 2007). It could also be a result of fermentation temperature differing from the temperature in which the starter cultures are stored. The optimal temperature of *Z. lentus* is lower (Steels et al. 1999) compared to *H. mollemarum* (Groenewald et al. 2018). *H. mollemarum* has been found in ongoing fermentations previously (Čadež et al. 2021). Overall microbial consortium will be determined by how well suited the microorganisms are to the current conditions.

On day 5, *Z. mobilis* was also found among the microbes, but represents a far smaller share compared with the yeasts. As it is highly tolerant to high sugar and acid and has been

encountered in cider fermentation previously, it is not surprising. However, this microbe can cause severe problems when striving towards a low to non-alcoholic product. Ethanol production can be reduced by lowering the temperature (Agrawal et al. 2017), or the finished product could be dealcoholized.

H. mollemarum is found among the microorganisms until day 7, but not in subsequent time points. *B. anomalus* and *B. bruxellensis* represents the majority in the microbial population on day 10, 17 and 21. *Z. mobilis* is not found in samples from day 10 but from day 17 and 21, this is more likely to be due to a laboratory error and not reflective of reality.

Looking at total values of CFU/mL, they increase until day 7, but then decreases as pH drops below 3.48. Final values of CFU/mL are lower on day 21 than on day 2. As pH drops exceptionally low, fewer microorganisms are sufficiently tolerant to continue metabolic activity. The pH is low even initially at the fermentation, which is probably why no LAB are active in this fermentation. Few LAB grow below pH 4.0 (Laureys et al. 2020). As no lactate was found, this claim is further supported. With no LAB or lactate found, no health benefits associated with the consumption of pro-, para-, or postbiotics can be assumed from the consumption of this specific batch. However, the drink is very low in pH and contains acetic acid and will thus exert an antimicrobial effect on common pathogens such as *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Candida albicans, Campylobacter jejuni,* and *Salmonella enteritidis* (Jayabalan et al. 2014).

In addition, the low pH of all timepoints could explain the slow pace of the microbial activity. But fermentation could also be slowed by the presence of *H. mollemarum* in early fermentation (Phister et al. 2007). Thiamine which is required by most yeasts could be reduced to a level where only the most competitive yeasts survive, which would be represented by *B. anomalus* and *B. bruxellensis* in the second half of the fermentation.

It is not clear whether or not AAB were present in the fermentation. Acetic acid is produced to a final concentration of 3 g L^{-1}. However, multiple yeasts were found, including those from the genus *Brettanomyces*, that produce acetic acid (Batt 2014). The optimal temperature of AAB is higher than the temperature of the fermentation, and the liquid in the tank is quite static, which might not be favoured by obligately aerobic AAB (Qiu et al. 2021). AAB could be VBNC, which might be why this culture-dependent method failed to identify them. It is also possible that they are present in a layer on the top of the fermenter liquid. A formation of a SCOBY in this fermentation is unknown.

PCR was not successful for all colonies, but for the vast majority of them. Despite no microorganisms found from the fermentation are known to form a pellicle, *K. fluxuum* from the black tea starter culture is known to do so (Kurtzman 2006). Nevertheless, it is possible that a small amount of AAB was viable but not possible to sequence due to sequence length, low DNA concentration, improper lysing, or other laboratory error.

Throughout, this fermentation was dominated by yeasts. *B. anomalus* represented over half of all samples possible to sequence. Looking at colony morphology, *B. anomalus* and *B. bruxellensis* were not distinguishable. Grassi and co-workers also found *B. anomalus* to be the dominant yeast species, as it is resilient in tough environments (Grassi et al. 2022).

Ethanol production in the fermentation was significantly higher than expected and reached a concentration of 15.4 g L^{-1} on day 17.

A key factor to consider is that the fermentation is conducted in large tanks, in a total volume of about 1000 L. The opening in the top of the tank is quite small and covered with gauze, so gas exchange is possible, but aeration is limited. The result is a highly anaerobic process, which will favour ethanol production (Kalnenieks 2006; Feldmann 2012; Oswald & Edwards 2017). The tap were fermentation samples were taken is located in the middle of the tank, between the bottom and the surface. It is likely that samples were not representative of the entire liquid, as gradients can be formed in the tank, of both microbes and measured substances.

In this study, sucrose equivalents decreased from 51.8 g to 32.2 g L^{-1} on day 17. More fructose was left compared with glucose. As no AAB were found yet acetic acid concentration rises, the greater amount of glucose consumed was likely converted by *B. anomalus* and *B. bruxellensis* to acetic acid (Batt 2014). Both *Brettanomyces* species and *Z. mobilis* found in this study are able to use all of the sugars available as a carbon source (Kalnenieks 2006; Batt 2014).

No microbes found are of clinical importance, as none of those found grow above 36°C. Suggestions for improvements include bidirectional sequencing to achieve sufficient gene coverage, which was not possible due to time restrictions. In addition, taking samples from multiple locations in the tank could provide greater coverage of microorganisms involved in the fermentation, in particular from the top to verify or confirm the presence or absence of AAB. This, too, was not done as the time reserved for the project was not sufficient for a greater quantity of samples. It should however be considered that the microbial consortium will vary between each batch. As it is highly dependent on multiple factors, achieving identical circumstances is difficult when fermenting with few interventions. However, further studies of microbial behaviour during brewing of kombucha will aid in gaining the knowledge needed to be able to obtain a dependable and consistent process and product. Also, to be able to meet customer demands and expectations, while producing a safe product that follows legislative regulations.

8. Conclusion

The microbial consortium from the starter liquids consisted of similar microorganisms, but were not identical, despite coming from the same brewery. The black tea starter culture was most diverse and the nantou starter culture the least. This could be a result of any number of factors including freshness, temperature, acidity, and ingredients. The microbial consortium originating from the SCOBY consisted of three main microbes, namely *B. anomalus, B. bruxellensis*, and *Z. lentus.* The total value of CFU/mL was more than double in the SCOBY than the highest total value of CFU/mL from the rosé starter culture.

The microbial population in the batch of industrially brewed combucha altered from day 2 to 21 by fewer live microorganisms in the end compared to the beginning. Initially, *H. mollemarum* was present, but after day 7 it was completely replaced by *Z. mobilis*.

The fermentation was dominated by yeasts, with *B. anomalus* representing over half of all samples sequenced.

Sucrose equivalents decreased from 51.8 g to 32.2 g L^{-1}, with glucose and fructose being converted to ethanol and acetic acid. Glucose was preferred throughout the fermentation, with 4.2 g L^{-1} fructose left on day 17. Acetate production was fast initially but slows after day 7 to reach a concentration of 3 g L^{-1} on day 17. Ethanol increased fast from 3.5 g L^{-1} on day two to 15.4 g L^{-1} on day 17. No lactate was found, which agrees with the lack of identified LAB.

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Popular science summary

Kombucha is a slightly acidic, slightly alcoholic drink made by fermenting sweetened tea. Kombucha originates from Manchuria in northeast China, and in year 414 it was used by the Emperor as treatment for his stomach issues. As trade routes spread, so did the consumption of this drink (Coelho et al. 2020).

As kombucha is high in acetic acid, it is scientifically proven to protect against common pathogens. In addition to acetic acid, is contains more antioxidants than non-fermented tea. Some of the self-reported effects of consuming the beverage includes enhanced immune system and metabolism, improved gut health, and reduced stress and blood pressure (Jayabalan et al. 2014). It is not difficult to understand why the popularity of this drink is rising and spreading worldwide.

When kombucha is made, microorganisms are involved. Yeasts often dominate the population, along with bacteria that make acetic acid. The acetic acid bacteria are often responsible for producing a pellicle on the surface of the liquid. Sometimes, bacteria that make lactic acid are also present. The microbial diversity vary greatly between batches, as a result of ingredients, temperature, time, and other factors (Laureys et al. 2020).

In this study, three starter liquids and one pellicle were tested to see what microbes could be found. One batch was also tested on day 2, 5, 7, 10, 17 and 21 to see if the microbes changed as the fermentation progressed. Sugars, ethanol, and acids were also measured.

The starter liquids were found to consist of similar microbes but were not identical. The microbial population originating from the pellicle was not as diverse as the most diverse starter liquid but had a higher number of microbes per millilitre of sample. In the batch of industrially brewed kombucha, *Brettanomyces anomalus* was the dominating yeast throughout. *Hanseniaspora mollemarum* was found in samples from the first half of the fermentation period, and *Zymomonas mobilis* in the second half. Sucrose equivalents decreased from 51.8 to 32.2 grams per litre, while ethanol increased from 3.5 to 15.4 grams per litre. The final concentration of acetic acid was found to be 3.0 grams per litre. Multiple yeasts found produce acetic acid, however, no acetic acid bacteria were identified. No lactic acid bacteria were identified, and no lactate was formed.

Studying the microbes involved in the fermentation of kombucha is important to increase the knowledge about microbial dynamics in kombucha. In addition, to develop the methods used to identify the microbes.

Microbial knowledge can be of particular interest for brewers to ensure consistent product behaviour when brewing. Inconsistencies can have great negative impact with large volume and economic input. It is also of great importance to produce a safe product while meeting customer demands and expectations.

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Appendices

Appendix I

SCOBY total colony count								
Medium	C	ND	-1	-2	-3	-4	MV	SD
MEAC	0	>300	>300	>300	248	20	224	33.94113
DRBC	0	>300	>300	>300	99	12	109.5	14.84924
YPDC	0	>300	>300	>300	259	18	219.5	55.86144
MRS	0	>300	>300	307	18	0	243.5	89.80256
ABS	0	15	10	3*	0	0	57.5	60.10408
TGE	0	>300	>300	143	17	0	156.5	19.09188

Nantou total colony count							
Medium	C	ND	-1	-2	-3	MV	SD
MEAC	0	>300	272	56	5	164	203.6468
DRBC	0	>300	320	38	6	350	42.42641
YPDC	0	>300	173	43	6	301.5	181.7264
MRS	0	5	0	0	0	-	-
TGE	0	>300	356	18	0	268	124.4508

Black tea total colony count							
Medium	C	ND	-1	-2	-3	MV	SD
MEAC	0	>300	>300	486	49	488	2.828427
DRBC	0	>300	>300	208	19	199	12.72792
YPDC	0	>300	>300	308	36	334	36.76955
ABS	0	43	5	0	0	46.5	4.949747
TGE	0	>300	>300	261	29	275.5	20.5061

Rosé total colony count							
Medium	C	ND	-1	-2	-3	MV	SD
MEAC	0	>300	>300	>300	155	-	-
DRBC	0	>300	>300	290	68	485	275.7716
YPDC	0	>300	>300	>300	108	-	-
MRS	0	168	11	0	0	139	41.01219
TGE	0	>300	>300	446	49	468	31.1127

Appendix II

Day 2 total colony count								
Medium	-3			-4			MV	SD
MEAC	>300	>300	>300	146	183	211	180	32.60368
DRBC	>300	>300	>300	70	140	92	100.6667	35.79572
YPDC	>300	>300	>300	136	160	130	142	15.87451
MRS	>300	>300	>300	126	138	170	144.6667	22.74496
ABS	>300	>300	>300	115	128	112	118.3333	8.504901
TGE	>300	>300	>300	231	134	166	177	49.42671
Day 5 total colony count								
Medium	-3			-4			MV	SD
YPDC	>300	>300	>300	327	297	348	324	25.63201
MRS	55	50	74	9*	6*	4*	59.66667	12.66228
ABS	>300	>300	>300	329	329	292	316.6667	21.36196
TGE	>300	>300	>300	251	215	184	216.6667	33.53108
Day 7 total colony count								
Medium	-3			-4			MV	SD
YPDC	>300	>300	>300	308	300	373	327	40.03748
MRS	>300	>300	>300	295	338	308	313.6667	22.05297
ABS	>300	>300	>300	321	259	333	304.3333	39.71566
TGE	>300	>300	>300	247	217	232	232	15
Day 10 total colony count								
Medium	-3			-4			MV	SD
YPDC	>300	>300	>300	333	318	309	320	12.12436
MRS	49	56	55	3*	10*	7*	53.33333	3.785939
ABS	>300	>300	>300	162	317	337	272	95.78622
TGE	>300	>300	>300	332	296	293	307	21.70253
Day 17 total colony count								
Medium	-3			-4			MV	SD
YPDC	>300	>300	>300	223	213	185	207	19.69772
MRS	20	38	23	2*	0*	5*	27	9.643651
ABS	>300	>300	>300	210	253	217	226.6667	23.07235
TGE	>300	>300	>300	161	140	153	151.3333	10.59874
Day 21 total colony count								
Medium	-3			-4			MV	SD

MEAC	>300	>300	>300	206	210	225	213.6667	10.01665
DRBC	351	465	351	0*	9*	21*	389	65.81793
YPDC	>300	>300	>300	184	190	178	184	6
MRS	6	8	5	1*	1*	0*	6.333333	1.527525
ABS	>300	>300	>300	149	127	162	146	17.69181
TGE	>300	>300	>300	130	171	157	152.6667	20.84067

Appendix III

Concentrations in g/L from dilutions A and B				
Day	Acetate	Glucose	Fructose	Ethanol
2A	1.488945	26.4806	29.6003	3.52206
2B	1.70923	25.2773	28.7504	3.53292
5A	1.751565	24.0876	28.42425	5.27495
5B	2.07087	23.9981	28.3218	5.9101
7A	2.097865	21.95275	26.70955	7.30785
7B	2.14468	22.1494	26.5651	8.63038
10A	2.076145	19.46825	23.4489	10.76015
10B	2.07087	23.9981	28.3218	5.9101
17A	2.85691	15.7848	19.7747	14.319
17B	3.04399	16.4191	20.9214	16.5618

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