Microbial characterization of Kombucha during brewing and storage

A comparison of luxury Kombucha, home-brewed and commercialized

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Abstract

Kombucha is a fermented, slightly acidic drink that has been consumed since 220 BC. It has gained much popularity worldwide, in recent years, partially because of its benefitial microroganisms and its potential health benefits. Although the microbial community in Kombucha is diverse and varies between products, the main composition entails of acetic acid bacteria and yeasts, lactic acid bacteria.

This study aims to characterize the composition of Kombucha; investigating Kombucha products from three different brands and using samples taken at different points of a homemade fermentation. With the variety of samples, an observation was done to understand if pasteurization and storage affect the microbial activity and composition.

The main samples were taken from Källsjö Kombucha brewery, which requested to investigate three different vintages of two different Kombucha flavours; Sencha and Oolong. Comparatively, two commercialized Kombuchas, Humm and Renee Voltaire, were also studied. Furthermore, the brewery was also interested in analyzing the difference in the composition of one of their pasteurized bottles. The method used was isolating colonies for DNA amplification and sequencing to identify the yeasts and bacteria. For a better comprehension of the Kombucha composition, organic acids, ethanol, and glucose were measured with high-performance liquid chromatography (HPLC).

The microbial activity in the different vintages of Sencha Kombucha, showed that the oldest vintage had the least microbial activity and diversity compared to younger products. Surprisingly, the Oolong vintage from 2022 showed the most microbial activity. Ethanol levels were lowest in the commercial Kombuchas. There was not much difference in the ethanol levels between pasteurized and unpasteurized. During the brewing process, the activity appeared to be the highest on days four and five, specifically in the selective media for yeast. It was apparent that there was little change in the glucose levels during the fermentation, whereas the acetic acid and ethanol increased with time. No lactic acid was found. In the Källsjö Kombucha, the dominant yeasts identified were *Brettanomyces anomalous* and *Brettanomyces bruxellensis*.

Keywords: Kombucha, Kombucha composition, fermentation, yeast, lactic acid bacteria, acetic acid bacteria, Kombucha characterization, microorganisms in Kombucha brewing

Sammanfattning

Kombucha är en fermenterad, lätt syrlig dryck som har konsumerats sedan 220 f.Kr. Det har vunnit mycket popularitet över hela världen, under de senaste åren, delvis på grund av dess mikrobiologiska aktivitet och dess potentiella hälsofördelar. Även om Kombuchas mikrobiella sammansättning är mångsidigt och varierar mellan produkterna, består den huvudsakligen av ättiksyrabakterier, jäst och mjölksyrabakterier.

Denna studie syftar till att karakterisera sammansättningen av Kombucha; genom att undersöka Kombucha-produkter från tre olika varumärken och prover tagna vid olika dagar av en hemgjord fermentering. Proverna observerades för att förstå om pastörisering och lagring påverkar den mikrobiella aktiviteten och sammansättningen.

Huvudproverna togs från Källsjö Kombucha bryggeri, som ville undersöka sammansättningen av tre olika årgångar av två olika Kombucha-smaker; Sencha och Oolong. Jämförelsevis undersöktes också två kommersiella Kombuchas, Humm och Renee Voltaire. Dessutom var bryggeriet också intresserade av skillnaden mellan deras pastöriserad Kombucha gentemot de opastöriserade. Metoden som användes var att stryka prover på selektiva medier för att sedan räkna kolonierna och isolera dem genom renstrykningar. De isolerade koloniers DNA blev amplifierad och sekvenserad för att identifiera. För en bättre förståelse av Kombucha-sammansättningen mättes organiska syror, etanol och glukos med högupplösande vätskekromatografi (HPLC).

Den mikrobiella aktiviteten i de olika årgångarna av Sencha Kombuchan, visade att den äldsta årgången hade minst aktivitet av mikroorganismer och mångfald jämfört med de yngre produkterna. Däremot visade Oolong årgången från 2022 mest mikrobiell aktivitet. Glukos nivåerna ökade med åren, däremot minskade etanolnivåerna, som var lägst i de kommersiella Kombuchas. Under bryggningsprocessen var mikrobiell aktiviteten högst dag fyra och fem, särskilt i de selektiva medierna för jäst. Dag två till fem observerades den högsta mikrobiella aktiviteten i det selektiva mediet för ättiksyrabakterier. Det skedde lite förändringar i glukosnivåerna under fermenteringen, medan ättiksyran och etanolen ökade med tiden. Ingen mjölksyra hittades. I Källsjö Kombucha var den dominerande jästen som identifierades *Brettanomyces anomalous* och *Brettanomyces bruxellensis*.

Nyckelord: Kombucha, Kombucha sammansättning, fermentering, jäst, ättiksyrabakterier, mjölksyrebakterier

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Abbreviations

SCOBY	Symbiotic culture of bacteria and yeast
AAB	Acetic acid bacteria
LAB	Lactic acid bacteria
CFU	Colony forming units
HPLC	High performance liquid chromatography
VBNC	Viable but not culturable
PCR	Polymerase chain reaction
RPM	Revolutions per minute
MRS	Man-Ragosa Sharpe
ABS	Acetic bacteria selective
TGEA	Tryptone glucose extract agar
YPD	Yeast Peptone Dextrose
DRBC	Dichloran Rose-Bengal Chloramphenicol
MEAC	Malt extract agar

1. Introduction

Kombucha is a fermented tea drink that has gained much popularity throughout the years, even though it has been consumed since 220 BC (Greenwalt et al.. 2000). It originated in China and travelled through Russia as trade routes expanded until it reached to Eastern Europe (ibid). One of the main reasons it spread and became a popular drink was due to its health benefits, which are the same reason it has gotten so much attention today.

The original productions of Kombucha were done at home by making a black tea infusion with sugar and a starter culture, which were left to ferment for 7-10 days. As the fermented drink has gained popularity commercialized products have been developed and the market has increased significantly in recent years, estimated to grow to around USD 5 billion by year 2025 (Wang et al. 2022). Diversifying from home-brewed products for private use and sold at farmer's markets to luxury Kombucha products becoming the new non-alcoholic trend.

Källsjö is a Kombucha brewery located in the south of Sweden, close to Ullared. They have been producing exclusive Kombucha products since 2020. Their high-quality products are produced locally and organically. Their focus is on craftmanship, which developed an original way to make their Kombucha resemble champagne, creating a festive non-alcoholic beverage (Källsjö). Having already received a reputation and confirmation of their work in such a short time - for example, being one of the chosen drinks at the Nobel dinner - they are now also interested in the final composition of their drink in order to understand what the consumers consume when drinking their products.

As the market grows, so do the producers of fermented tea, which leads to more varied fermentation processes and starter ingredients, all of which impact the final composition of the Kombucha and the potential health effects (Yang et al. 2022). Other important factors for a satisfactory end product are storage effects on the beverage and whether pasteurization is a possible solution for stabilisation. For this instance, more studies are needed to analyze and characterize the chemical and microbial profiles.

1.1 Aim and objective of this work

This study aims to investigate the microbial composition of Kombucha from different sources and assess how pasteurization and storage affect microbial activity.

The project was initiated in collaboration with Källsjö, who wanted to analyze the composition of three different vintages of their Kombucha to understand the effects of storage. As well as analyzing a pasteurized product to compare the microbial activity and effects of pasteurization. Further, two commercialized Kombuchas were also used for a comparison in the composition of the final products relative to what's available on the market. Additionally, samples of home-brewed Kombucha were monitored continuously throughout the brewing process to get a wider perspective of the microbial composition.

2. Background

2.1 Kombucha

The name Kombucha originates from Dr Kobu – a Korean doctor who brought tea to Japan - and cha which refers to the Japanese word for tea (Wang et al.. 2022). Kombucha goes by many names such as, Tea Fungus, Kargasok Tea, Manchurian Mushroom, and Haipo (Greenwalt 2000). This traditional drink was historically prepared at home and consumed as a remedy, originally coming from Manchuria, hence one of its names. With time, it reached Russia where it also became a popular medicinal beverage. During World War II it travelled further to Eastern Europe where it was often used for detox on the blood and digestive system. It was believed that Kombucha had magical properties and could cure illnesses (Greenwalt 2000).

2.1.1 Fermentation Process

Even though the process today can vary, the most common and original recipe is the following: black tea is infused in boiling water which is sweetened with 5-15% sugar and let to rest for about 10 minutes. The tea leaves are then removed, and the infusion is left to cool down. When toom temperature is reached 100 ml of the starter culture is added. The started culture is usually kept from a previous batch of kombucha. This is usually prepared in a glass jar covered with a cotton cloth and left to ferment for around 7 to 10 days (Greenwalt 2000). The starter culture is added to help start the fermentation and lower the pH to avoid pathogens and unwanted microorganisms (Wang et al. 2022).

During fermentation, a symbiotic culture of yeast and bacteria is formed called SCOBY, which mainly contains Acetic acid bacteria (AAB) and yeasts (ibid). Yeast undergoes fermentation in anaerobic conditions at the bottom of the fermentation tank, while the AAB stays at the top close to the SCOBY (Zailani & Adnan 2022). Throughout the fermentation, the taste of the Kombucha changes from fruity to vinegar like flavours (Wang et al. 2022). The product turns out to be slightly carbonated and filled with organic acids, vitamins, minerals, and tea components, which are often compared to the taste of cider (Greenwalt 2000). The

organic acids produced during fermentation lower the pH, and one of the major products of fermentation is ethanol (Yang et al. 2022). The pH values commonly decrease to values under three, which is a favourable pH range for Kombucha (Li et al. 2023).

The sweetened brewed tea is the substrate in which the microorganisms thrive in during Kombucha production. Sugar is essential for the colonies to grow and for the fermentation process to happen (Greenwalt 2000). Different substrates can be used, such as green tea, oolong tea, medicinal herbs, and black tea, which is the most common one (Wang et al. 2022). The chemical composition of the substrate will impact the quantity of the compounds in the Kombucha produced (Bishop et al. 2022). Caffeine is an important nitrogen source during Kombucha production as it seems to stimulate the growth of microorganisms. Therefore, since green tea contains more caffeine than black tea, using it as a substrate will promote greater fermentation (Coelho et al. 2020). Other parameters such as temperature and time during the fermentation process, will also affect the final chemical composition (Bishop et al. 2022).

The contents of polyphenols and flavonoids increase after fermentation. This is due to the many enzymes produced, which help to reduce the polyphenols of large molecular compounds from the tea leaves into smaller polyphenol monomers. The tea leaves also contain large amount of insoluble bound phenols which are released into the Kombucha with the help of the acids, alcohols and esters produced during (Wang et al. 2022).

2.2 Composition of Kombucha

Kombucha is produced by the SCOBY and its main components, Acetic Acid bacteria (AAB) and yeasts, and sometimes Lactic acid bacteria (LAB). Nevertheless, studies have shown there is a high variability in the composition, not only comparing between countries or regions but also among the different Kombucha products (Greenwalt 2000). This is the result of the large variety of ingredients and recipes used (Laureys et al. 2020). Besides the main components, Kombucha also contains polyphenols coming from the tea, which undergo enzymatic transformations such as oxidation and partial polymerization. These biological activities give the Kombucha unique profiles, which are reflected in the final chemical composition (Yang et al. 2022). Sugars, organic acids, and ethanol such as fructose and glucose, acetic acid, and gluconic acid are also primary constituents of the fermented beverage (Greenwalt 2000). Fructose is the most popular source of carbon during fermentation (Wang et al. 2022).

2.2.1 Symbiotic culture of yeast and bacteria - SCOBY

The SCOBY goes by many names, such as tea fungus, cellulosic pellicle, or consortium. For the synthesis of cellulose, the precursor-uridine diphosphate-glucose (UDPGc) is needed, which is produced from carbon sources such as ethanol, sucrose, fructose, and glycerol. The specific acetic acid bacterium. *Komagataeibacter xylinus* creates the cellulose network, with the help of caffeine, theophylline, and theobromine from the tea. Additionally, the type and concentrations of sugar, as well as the pH, have an important effect (Wang et al. 2022).

Its appearance resembles mould or mushroom, hence the name, however the actual composition is a cellulose mat (Greenwalt 2000). As the fermentation process starts a new fungus grows, which covers the surface of the 'mother' SCOBY to gain the most access to oxygen. For the formation to happen the ethanol needs to be kept at a low level (Wang et al. 2022). The cellulose produced in Kombucha is not just interesting for fermentation but also as a material for various applications, including artificial corneas, wound dressings, and biodegradable packaging (Landis et al. 2022).

2.2.2 Yeast

Yeast is a unicellular fungi, which in some cases can grow facultatively anaerobic. They are non-motile, with cells of a diameter around 8 µm and spherical or oval-shaped. The optimal conditions for growth of most known yeast species are 20-30°C and a pH between 4.5-7.0. Nevertheless, many yeasts are capable of growth at pH 2.5 (Laureys et al. 2020). Yeasts are often used for the fermentation of foods and beverages due to their ability to hydrolyse substrate into valuable final products. In aerobic conditions, they often convert sugar to carbon dioxide and energy, whereas in anaerobic conditions, the sugars will mostly be converted into ethanol and glycerol, and carbon dioxide (Wang et al. 2022). This depends of which strain is used. When carbohydrates are consumed, yeast is capable of metabolizing ethanol into carbon dioxide and water, when oxygen is present. When an internal redox imbalance is reached, yeasts can produce glycerol or acetic acid to get it back in balance (Laureys et al. 2020).

Kombucha yeast has been reported to mainly include members of the *Zygosaccharomyces* and *Brettanomyces* genera (Yang et al. 2022). However, commonly found are also yeasts from the *Saccharomyces* and *Pichia* genera (Laureys et al. 2020). *Saccharomyces cerevisiae* is often preferred in alcoholic fermentation as it manages osmotic stress by synthesising glycerol, whereas *Brettanomyces bruxellensis* thrives in aerobic conditions. In altered ethanol levels,

S. cerevisiae outcompetes other fungal diversity, such as *B. bruxellensis* and *B. anamalus* (Harrisson et al. 2023).

Yeast species will vary between Kombuchas due to the environmental factors playing a key role. The autolysis of yeast provides vitamins and other nutrients to support the growth of AAB, and possibly impact the aroma and flavour of the beverage. Generally, yeast populations outnumber the bacteria present in Kombucha (Laureys et al. 2020).

2.2.3 Acetic Acid Bacteria and Lactic Acid Bacteria

AAB

Acetic acid bacteria are gram-negative bacteria, which can be motile and around 0.5 um wide and 1-4um long with cell-shaped ellipsoidal or rod-shaped. They are obligately aerobic; however, they can survive longer periods in low oxygen environments. As a result of this, the AAB enter a viable but non-culturable (VBNC) state, which lowers the chances of recovery on cultural media. The optimal environment for AAB growth is 25-30 °C and a pH between 5.0-6.5, however, many can grow in low pH, such as 3.0-4.0 or even lower (Laureys et al. 2020).

Acetic Acid bacteria belong to the Acetobacteriaceae family, which possesses the ability to oxidize alcohols or sugars to form organic acids (Kim et al. 2019). AAB metabolize glucose for instance into acetic acid, gluconic or glucuronic acids (Wang et al. 2022). The reported acetic acid bacteria in Kombucha include the genera *Gluconacetobacter, Gluconobacter, Komagateibacter,* and *Acetobacter. Komagataeibacter* species are associated with the formation of the SCOBY (Wang et al. 2022). Species of *Gluconobacter* oxidize glycerol and glucose, whereas in alcoholic drinks, you find the genera *Acetobacter* and *Gluconacetobacter,* which have species that oxidize ethanol rather than glucose.

There has been a drawback with analyzing AAB due to the lack of a representative environment in the media available (Kim et al. 2019). Nevertheless, studies confirm that acetic acid bacteria are more commonly found in Kombucha than lactic acid bacteria (Yang et al. 2022). They are primarily responsible to produce acetic acid, gluconic acid, and cellulose (Greenwalt 2000).

LAB

Lactic Acid bacteria are Gram-positive, non-spore forming, motile and gas-tolerant microorganisms, which belong to the phylum of the Firmicutes. Morphologically, they can be recognised by their rod-shapes or spherical shapes. LAB are

facultatively anaerobic but do not need oxygen for their metabolism. Their optimal growth happens at 24°C – 40°C and at a pH between 4.0 – 6.0 (Laureys et al. 2020).

LAB can produce Lactic acid and are essential for fermentation. Some species are known to have probiotic properties, others are known for giving unique flavours to fermented foods and beverages (Zhang et al. 2023). The main LAB bacteria found in Kombucha are *Bifidobacterium* and *Lactobacillus*, often dominated by *Lactobacillus nagelii* and a high abundance of *Lactobaciullus mali*, which are mainly found in green tea Kombucha (Yang et al. 2022). Conversely, until now, it can't be demonstrated that it's an essential bacterium to produce Kombucha, although it has been shown to enhance the antioxidant activity (Wang et al. 2022).

2.2.4 Microbial interaction

Bacteria and yeast interactions are essential for Kombucha fermentation, and it turns out that it's a very complex relation. Studies have been done to pair combinations, which demonstrated that not all yeasts and bacteria consortia resulted in creating biofilms since not all yeasts stimulate the bacteria to produce cellulose. Hypotheses indicate that the relationship between yeast and bacteria is mutualistic. However, a study done by Landis et al. (2022) found that both parties involved are not always benefiting. It appeared that some yeasts are competing for nutrients or inhibiting the bacteria while being capable of growing in the absence of bacteria (Landis et al. 2022).

Yeast hydrolyses the sucrose into glucose and fructose (Figure 1). The yeast continues to convert the fructose to ethanol, while the AAB uses the glucose to convert into gluconic acid and the ethanol to convert into acetic acid (Chakravorty et al. 2019). This helps to keep the alcohol levels down while the acetic acid rises (Wang et al. 2022). The yeast production of ethanol assists the bacterial production of acetic acid, further, the acetic acid production may in return stimulate yeast production of ethanol. On the other hand, the conversion of glucose to high levels of gluconic acid, keeps the acetic acid bacteria levels down (Greenwalt 2000). Acetic acid bacteria use the glycerol produced by yeast and oxidize into dihydroxyacetone (DHA) and esters which contribute to the aroma of the Kombucha (Wang et al. 2022).

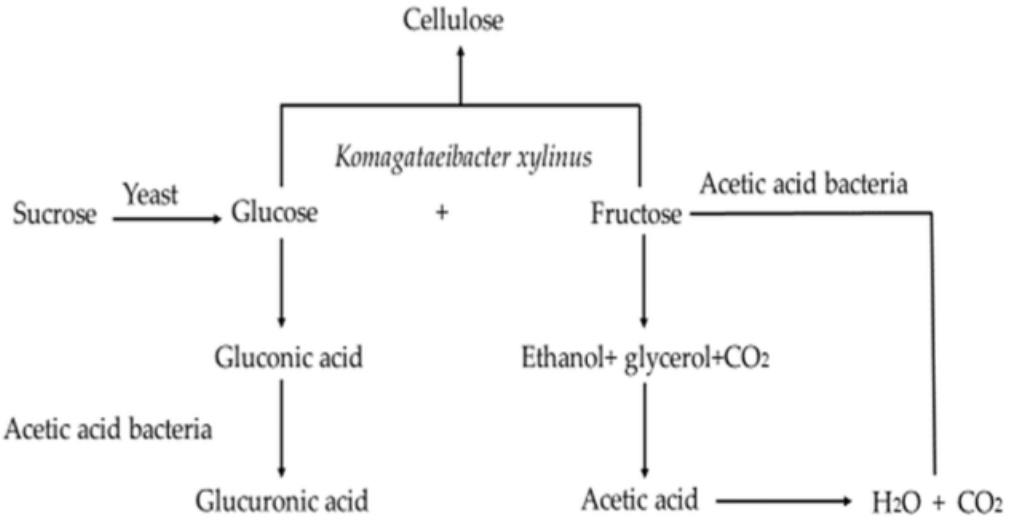

Figure 1. Metabolic activity of yeast and AAB during fermentation (Wang et al. 2022:4).

A common trend observed throughout the fermentation of Kombucha is that while sucrose decreased, fructose, glucose, ethanol, and acetic acid increased (Zhang et al. 2021). Another correlation that was captured in a study by Yang et al. (2022) showed how low bacterial diversity can be caused by the dominance of a single bacterium. Another reason of a low variety of microorganisms can be explained by yeasts performing the make-accumulate-consume strategy. Some yeasts can continue fermentation in aerobic conditions if carbohydrates are available, which entails they outcompete other microorganisms (Laureys et al. 2020). Finally, observations have been made in the trend of ethanol, where it is clear that high levels correlate with great concentrations of glycerol, in addition to low concentrations of sucrose, glucose, fructose, and organic acids (Harrisson et al. 2021). Fructose, being the preferred source of carbon, tends to decrease throughout fermentation, whereas glucose has been seen to rise during fermentation. In both cases, the significant change is usually observed during the first six days of the brewing (Guttapadu et al. 2000).

2.3 Effects of storage

The main carbon source during storage is sucrose, its availability will affect the viable cell counts of AAB, LAB, and yeasts. Throughout storage, fermentation progresses, which depletes the carbon source for the microorganism and lowers the pH. As a result, the viable cell count decreases with storage time. The temperature at which the Kombucha is stored in will affect the microbial activity. A study done with Kombucha stored at 4°C and 25°C, showed an increase in microbial activity

the first two weeks, reaching its peak on day 14. The viability gradually decreased with time in both cases, however, with a faster decrease at 4°C. Low temperatures inhibits the growth of microorganisms. Nevertheless, it also reduces the consumption of carbon sources. Further, the study showed that LAB was the microorganism most affected by storage, as it had the largest decline. Yeast was the least affected with AAB in second place (Li et al. 2023). Hence, to maintain microbial stability, according to Li et al. (2023) short-term storage is more suitable at 25°C, whereas storage for periods over one month is more suitable at 4°C.

Another effect that storage has is the increase in alcohol levels, specifically when not kept in a cold environment. However, there seems to be a gap that requires further research when it comes to shelf-life stability (Harrisson et al. 2023). A study on homemade Kombucha, has also recently shown that when stored for longer than four months in the fridge, the quantity of the polyphenol will decline (Yang et al. 2022).

2.4 Health effects

Kombucha is considered a functional food as it is perceived to contain live beneficial microorganisms at the point of consuming it (Harrison et al. 2023). Suggestions have been made that consuming Kombucha can help with numerous health issues, however, not enough studies have been done to prove the benefits.

Nevertheless, some of the correlations argued for are that it can lower blood pressure, mediating blood sugar levels and cholesterol levels. It can also help with weight loss due to lipase inhibition, which leads to controlling appetite (Chakravorty et al. 2019). In addition, Kombucha has been claimed to have anti-carcinogenic, anti-diabetic, anti-microbial, and anti-oxidative properties. These claims are linked to the acids and metabolites that are produced by the bacteria and yeast during fermentation. The antioxidant activity of Kombucha is also linked to its health benefits. This correlation comes from the green or black tea, which is used as the substrate (Zailani & Adnan 2022).

Lactobacillus species and related genera contain probiotic strains which are often found in Kombucha. Their probiotic reputation comes from its potential health benefits such as inhibition of pathogenic bacteria, promotion of gut health and activity, and modulation of signalling within the immune system. Hence, many commercial Kombucha products add probiotics to interest the consumers (Yang et al. 2022). Acetic acid bacteria also demonstrate health benefits such as as acid and bile resistance, activity against pathogens, and anti-cancer properties (Aghazadeh et al. 2015). Another health benefit comes from the oxidation of glucose into

glucuronic acid, which is the most significant detoxifier of the body as it can bind with toxic compounds in the liver (Bishop et al. 2022).

Furthermore, studies have identified large differences in the chemical composition across Kombucha products, which should be considered as Kombucha's health benefits are generalized (Yang et al. 2022). Tea is the component that provides the most antioxidants; therefore, which tea is used when producing Kombucha will affect the final composition and play a role in the potential health effects it might have (Martinez Leal et al. 2018).

Proven health effects have been seen from tea consumption; however, these studies have been done on consumers who drink four or more cups of tea a day (Stagga & Millin 2006). Kombucha, on the other hand, has a limited recommended daily consumption. Even though there are no clear recommendations, it is important to quantify the bioactive compounds to avoid chemical acidosis. (Oluranti Ojo & de Smidt 2023). Therefore, some of these health effects might not be associated with Kombucha.

3. Materials and Methods

3.1 Kombucha samples

Three different years of Sencha and Oolong Kombucha samples, together with the corresponding starter culture and one pasteurized sample, were picked up from the Källsjö Kombucha brewery in Ullared, the South of Sweden. Two commercialized bottles were purchased from the supermarket, Hemköp, in Stockholm; Humm and Renee Volatire. These two brands were selected due to their availability in the supermarket at the time. The home-brewed Kombucha samples were pipetted from the middle of the Kombucha liquid on the following days: 2, 4, 6, 8, 10, 12, and 14. Duplicates were taken for all 18 samples and put into falcon tubes, storing one in the freezer and one in the fridge.

3.2 Fermentation process

Källsjö

Tea was cooked with 5% sugar for 23 minutes at a temperature of 85°C -95°C, the remaining 70% of water was added cold together with the 10% starter culture. The Kombucha fermented in stainless steel tanks for 4-6 weeks, then was cooled down to 1°C for two weeks. This separated the yeast from other particles, which was rinsed from the bottom of the tank. Lastly, it was left to mature for up to two weeks, pressurized and extra carbonated before being bottled (Källsjö 2024).

Home-brewed

For the home-brewed Kombucha, 800g of tap water was boiled and mixed with 7.5% sugar and 0.6% black tea without any flavours. Once all sugar was dissolved and the tea mixture had cooled down, it was filtered into a glass jar removing the tea bags, and 100g of starter culture was added together with 10g of SCOBY. The Kombucha was left to ferment for 14 days, at room temperature covered with a clean cotton cloth, and stirred daily with a sterile spoon.

3.3 pH Measurements

The pH of each Kombucha sample was measured using an electronic pH meter. Each measurement was replicated three times.

3.4 Sensory evaluation

A one-person sensory evaluation was performed directly after opening the Kombucha bottles from Källsjö. The colour, odour, and taste were assessed and compared between the different vintages.

3.5 Isolation and identification of Bacteria and Yeasts

3.5.1 Culture specific media

The determination of lactobacilli bacteria and all types of bacteria was performed using selective media De Man-Ragosa Sharpe agar (MRS) and Tryptone glucose extract agar (TGEA), respectively. MRS agar was prepared by dissolving 62g/L in deionized water. After sterilization and cooling down, 10 ml/L of cycloheximide was added. The TGEA medium contained Peptone 5g, Beef extract 3g, Glucose 1g, agar 15g, and distilled water to a final volume of 1 litre (Remel, KS, USA).
To determine acetic acid bacteria, selective agar ABS, was prepared the following way: Glucose 50g, yeast extract 10g, bromophenol blue 20 mg, and bacteriological agar 20 g were dissolved in distilled water 1 litre and autoclaved. Once cooled down to 50˚C, 1 ml glacial acetic acid, 50 ml of pure ethanol, and 5000 U of penicillin dissolved in distilled water together with 5mg/L of cycloheximide were added to the medium and mixed thoroughly (Kim et al. 2019). YPD for the cultivation of yeast contained glucose 20g/L, peptone 20g/L, yeast extract 10g/L, agar 16g/L, and calcium carbonate 5g/L (Blomqvist et al. 2010). Post-autoclave and cooled down chloramphenicol 1ml/L was added. Malt extract agar (MEAC) for the cultivation of yeast and mould was prepared by dissolving 48g in 1 litre of distilled water. Chloramphenicol 1ml/L was added once autoclaved and cooled down. Dichloran Rose-Bengal Chloramphenicol (DRBC), a selective medium for moulds, was prepared according to the directions on the label. Chloramphenicol 1ml/L was added when it reached 50°C. All media were sterilized by autoclaving at 121°C for 15 min.

3.5.2 Enumeration

Serial dilutions were performed to generate 30-300 colonies on the medium, using 100 µL of Kombucha and 900 µL of peptone water. From each sample, 100µL of

dilution was spread-plated on each media. This was done in triplicates. The media was then incubated aerobically or anaerobically at the appropriate temperature and days (Table 1). The plates were observed and enumerated after incubation. Colonies were counted and the results expressed as colony-forming units per millilitre (Chen & Liu 2000). All samples that did not generate Colony-forming units (CFU) counts or were out of range were repeated to obtain counts within the target range.

Table 1. Media and incubation environment and duration.

Media / Incubation info	Temperature	Duration	Aerobic/ Anaerobic
MRS	30°C	±7	Anaerobic
ABS	30°C	±6	Aerobic
TGEA	30°C	±5	Aerobic
YPD	25°C	±5	Aerobic
DRBC	25°C	±5	Aerobic
MEAC	25°C	±5	Aerobic

3.5.3 Isolation

For each sample, one plate from each different medium was chosen. Four colonies per colony morphotype were inoculated on their corresponding media and incubated in the appropriate environment (Table 1). A second clean streak was done using the same procedure. Samples were then ready to be amplified.

3.6 Identification of bacteria and yeast

3.6.1 DNA extraction and PCR

Hypochlorite solution was used to wipe the workbench from any contaminations. For yeasts, 20uL of 0.02 M NaOH was pipetted into an Eppendorf tube, in the case of bacteria, 20uL of sterile water was used. Isolated colonies were taken with a sterile toothpick, suspended into the Eppendorf tube, and mixed well. It was put in a heating block at 95°C for 10 min. A Master mix was prepared containing 1 µL of each forward primer & reverse primer (Table 2), sterile Milli-Q water 8,5 µL, and DreamTaq Green 2x Master mix (buffer, Taq, dNTPs and loading dye) 12,5 µL for each reaction. PCR tubes were labelled and filled with 23 µL Master mix and 2µL of the boiled yeast or bacteria suspension, for the negative control, NaOH was added respectively Milli-Q water. They were mixed well and vortexed in the PCR machine with the appropriate program. Gel electrophoresis was performed to confirm that each sample contained either bacteria or yeast. A gel containing 1% agarose in 0.5 TBE buffer was prepared and loaded with samples. Once it had run

for about 30 minutes, the PCR products could be visualized under a UV light, and clear bands could be seen in the correct size.

Table 2. The primers used for PCR

Primer	Sequence	Region and length
NL1	5’ – GCA TAT CAA TAA GCG GAG GAA AAG-3’	D1/D2 region
NL 4	5’ – GGT CCG TGT TTC AAG ACG G -3’	ca 600bp
16Ss	5’ – AGA GTT TGA TCC TGG CTC – 3’	16S region
16Sr	5’ – CGG GAA CGT ATT CAC CG -3’	ca 1420

3.6.2 Sequencing

Amplified DNA samples obtained through PCR were sequenced according to the Sanger method by Macrogen Europe . The primers used were NL1 for yeast and 16 Ss for bacteria. Post-sequencing analysis was done by using the software SnapGene and the BLAST tool (basic alignment search).

3.7 High-performance liquid chromatography (HPLC) analysis

Standard curves had already been prepared by calculating 10g/L for pure ethanol, lactic acid, acetic acid, and glucose. The Kombucha samples were retrieved from the freezer and defrosted: 9 from the ready products, 2 from the starter cultures, and 7 from the Kombucha process. One sample at the time was pipetted into falcon tubes and diluted with Milli-Q (Table 3).

Table 3. Dilution of samples for HPLC analysis.

My data	ND	2 fold	5 fold	10 fold
Starter culture S & O	x	x	-	-
Sencha 2021, 2022	x	x	-	-
Sencha 2023	x	x	-	x
Oolong 2021, 2022	x	x	-	-
Oolong 2023	x	x	-	x
Pasteurized	x	-	x	-
Humm	x	x	-	-
HB day 1, 2, 3, 4	-	-	x	x
HB day 5, 6, 7	-	x	x	-

Each sample was centrifuged with Sorvall LYNX 4000 Centrifuge for 10 minutes at G-force 5000 xg. From the supernatant of each sample, 3 ml was pipetted into individual Eppendorf tubes and mixed with 300 uL of 5M H_2SO_4. Concentrations of glucose, lactic and acetic acid, and ethanol were quantified based on the standard curves of the pure compounds. Results were expressed as content in g/L.

4. Results

4.1 Samples

Different samples were used for this thesis. Information on the Kombucha products is shown in Table 4.

Table 4. Kombucha samples used.

	Origin	Ingredients	Year	Pasteurisation
Starter culture Sencha & Oolong	Sweden	Starter culture	2024	No
Källsjö Sencha	Sweden	Sencha (Guizho, Zhengan District China), Ecological sugar (4 %), Natural yeast- and bacteria culture.	2021 2022 2023	No
Källsjö Oolong	Sweden	Oolong (Anahui) China, ecological sugar (4%), natural yeast- and bacteria culture.	2021 2022 2023	No
Källsjö Rose	Sweden	Water, sugar, fermented green tea and carbon dioxide	-	Yes
Humm	Sweden	Vatten, rörsockerjuice, Svart te, levande Kombuchakultur, citronjuice, ingefärsjuice	2024	Nej
Renee Voltaire	England	Filtrerat vatten, rårörsocker*, Chun Mee (grönt te)* färskpressad ingefära (0,02%)*, Kombuchakultur.	2024	Nej
Home-brewed	Sweden	Black tea, sugar, water, bacteria culture, SCOBY	2024	No

4.2 Sensory evaluation

A sensory evaluation was performed on the three different vintages of Sencha and Oolong from Källsjö. The smell, taste and colour were evaluated, and results were noted in table 5.

Table 5. Sensory evaluation of Sencha and Oolong three vintages.

Samples	Taste	Color	Smell
Sencha 2021	Acidic, bitter, slight sting, refreshing, mild sweetness, very carbonated	Yellowish colour, not transparent	Acidic, musky
Sencha 2022	Less sting, fresh, slight acidic	Yellow tint, more transparent	Slightly acidic
Sencha 2023	Sweet, flowery, less bubbles than ‘21	transparent	Flowery, sweet
Oolong 2021	Slightly acidic but mild, slight sting, flowery flavors, very bubbly	Slight colour, not completely transparent	Flowers/Vanilla
Oolong 2022	Acidic Slightly flowery Clear and refreshing	Clearer than ’21 not completely transparent	Slightly more acidic and less flowery than ‘21
Oolong 2023	Strong of flower: vanilla, elderflowers Sweet, mild acidic, less bubbles than ‘21	Transparent	Slight acidic, tint of flowers, sweet

4.3 pH

The pH of each Kombucha sample was measured after opening each bottle, alternatively after sampling the brewing process. The values for the ready products are shown in Figure 2 and the for the fermentation process are seen in Figure 3. The Kombucha products show the lowest pH 3,2 and the highest 3,4. Observing the Oolong samples, year 2022 has the lowest pH and 2023 the highest, compared to the Sencha samples where there is a decrease in pH relative to age. During the fermentation process, the pH initiates at 3,9 decreasing with time to end on the 14th day at pH 2,8.

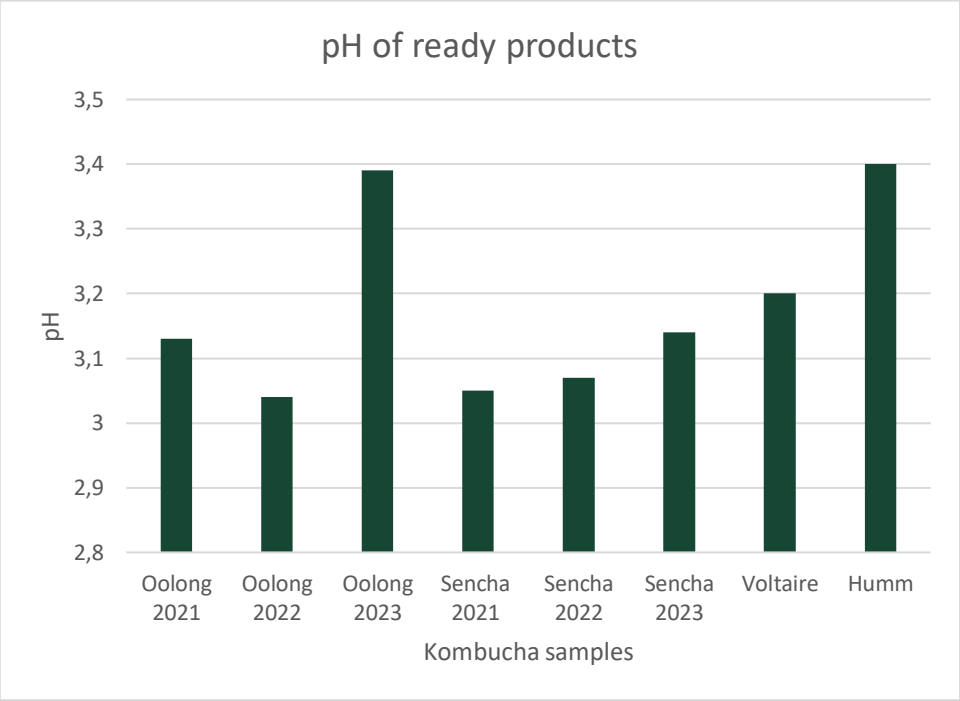

Figure 2. pH values from the final product of the different brands and years.

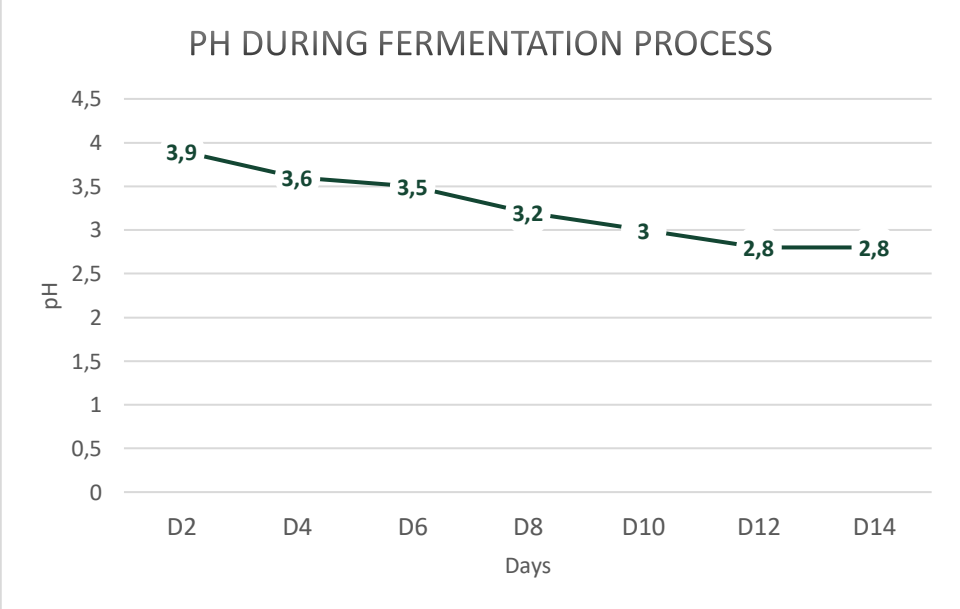

Figure 3. pH values taken on day 2, 4, 6, 8, 10, 12 and 14, throughout the fermentation process,

4.4 Enumeration

After incubation, the media plates were observed, and the colonies were counted to differentiate the plates that were within the range of 30-300 colonies. The plates outside this range were not used. The overview of colonies of colonies counted can be found in Appendix 1. The colony count of each plate was expressed in colony-forming units per millilitres. In Table 6, the CFU for the Kombucha samples are shown.

Table 6. CFU/ml from the different Kombucha samples. S= Sencha, O= Oolong, R.V = Renee Voltaire

Media	S 2021	S 2022	S 2023	O 2021	O 2022	O 2023	Pasteurise	Humm	R.V
MRS	$6{,}87\times10^{4}$	$1{,}19\times10^{5}$	$2{,}50\times10^{6}$	$1{,}82\times10^{6}$	$2{,}06\times10^{6}$	$2{,}77\times10^{5}$	$1{,}05\times10^{5}$	-	$3{,}67\times10^{5}$
ABS	$5{,}53\times10^{4}$	$2{,}37\times10^{5}$	$2{,}69\times10^{6}$	$1{,}60\times10^{6}$	$1{,}63\times10^{6}$	$3{,}30\times10^{5}$	$2{,}98\times10^{5}$	$3{,}33\times10^{2}$	$3{,}73\times10^{5}$
TGEA	$5{,}03\times10^{4}$	$1{,}62\times10^{5}$	$1{,}04\times10^{6}$	$1{,}62\times10^{6}$	$1{,}66\times10^{6}$	$3{,}33\times10^{5}$	$1{,}75\times10^{5}$	$1{,}15\times10^{6}$	$4{,}53\times10^{5}$
YPD	$1{,}22\times10^{5}$	$3{,}26\times10^{5}$	$2{,}72\times10^{6}$	$2{,}34\times10^{6}$	$2{,}81\times10^{6}$	$1{,}28\times10^{6}$	$1{,}95\times10^{5}$	$9{,}03\times10^{5}$	$4{,}00\times10^{5}$
DRBC	$5{,}13\times10^{4}$	$3{,}30\times10^{4}$	$3{,}50\times10^{5}$	$1{,}82\times10^{6}$	$2{,}06\times10^{6}$	$2{,}77\times10^{5}$	$2{,}94\times10^{5}$	$7{,}23\times10^{5}$	$2{,}57\times10^{5}$
MEAC	$8{,}77\times10^{4}$	$2{,}02\times10^{5}$	$1{,}79\times10^{5}$	$1{,}60\times10^{6}$	$1{,}63\times10^{6}$	$3{,}30\times10^{5}$	$3{,}25\times10^{5}$	$8{,}20\times10^{5}$	$3{,}90\times10^{5}$

The results in Figure 4 indicate a rise in the number of microbes from the Sencha sample from 2021 to 2023. Whereas, in the Oolong 2022 sample, there is a sudden peak in microbial activity. The pasteurized sample had low activity, similar to Sencha 2021 and 2022 as well as Oolong 2021 and 2023. Peaks of micro activity are found in Sencha 2023 and Oolong 2022. The commercialized Kombuchas lie in between the Källsjö samples lowest and highest points. The media that showed the highest amount of CFU, therefore the most microbial activity, are the bacterial media MRS, ABS, and yeast media YPD.

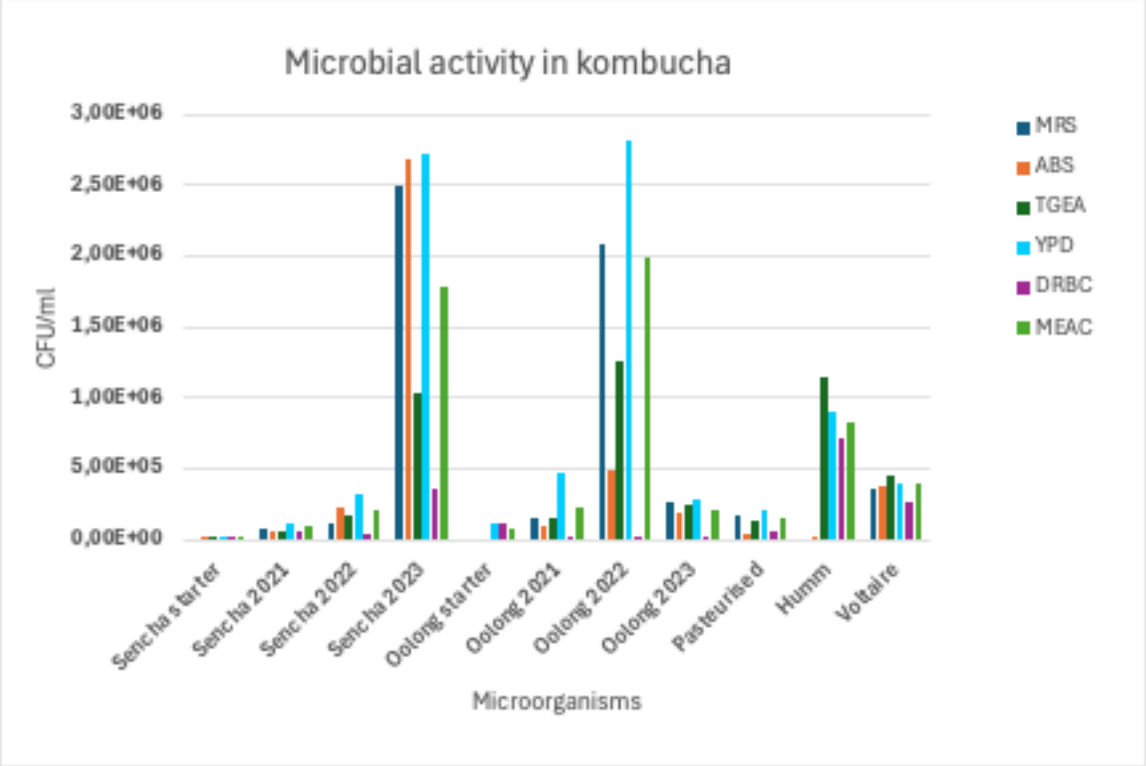

Figure 4. Shows CFU/ml on different selective media for different kombucha samples.

During the fermentation process of the home-brewed kombucha, the CFU/ml varied from $4{,}77 \times 10^4$ to the maximum amount of $2{,}81 \times 10^{6,}$ which is summarized in Table *7*. The plates that had the most growth were the bacterial selective media.

Table 7. CFU/ml from the fermentation process.

Media	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
MRS	$5{,}97 \times 10^4$	$3{,}23 \times 10^5$	$1{,}71 \times 10^5$	$1{,}82 \times 10^6$	$2{,}06 \times 10^6$	$2{,}77 \times 10^5$	$1{,}05 \times 10^5$
ABS	$2{,}57 \times 10^5$	$1{,}99 \times 10^6$	$1{,}18 \times 10^6$	$1{,}60 \times 10^6$	$1{,}63 \times 10^6$	$3{,}30 \times 10^5$	$2{,}98 \times 10^5$
TGEA	$4{,}77 \times 10^4$	$2{,}39 \times 10^5$	$1{,}57 \times 10^5$	$1{,}62 \times 10^6$	$1{,}66 \times 10^6$	$3{,}33 \times 10^5$	$1{,}75 \times 10^5$
YPD	$2{,}28 \times 10^5$	$4{,}50 \times 10^5$	$2{,}46 \times 10^5$	$2{,}34 \times 10^6$	$2{,}81 \times 10^6$	$1{,}28 \times 10^6$	$1{,}95 \times 10^5$
DRBC	$1{,}66 \times 10^5$	$3{,}23 \times 10^5$	$1{,}71 \times 10^5$	$1{,}82 \times 10^6$	$2{,}06 \times 10^6$	$2{,}77 \times 10^5$	$2{,}94 \times 10^5$
MEAC	$5{,}83 \times 10^5$	$1{,}99 \times 10^6$	$1{,}18 \times 10^6$	$1{,}60 \times 10^6$	$1{,}63 \times 10^6$	$3{,}30 \times 10^5$	$3{,}25 \times 10^5$

A trend of increased activity can be seen in Figure 5, where there is a peak between days 8 and 10 in both the MRS and TGEA media. The growth specifically on the ABS plates can be seen to rise already in the beginning of the fermentation process, reaching a peak on day 4. All media show a decline in activity on day 4 and day 12.

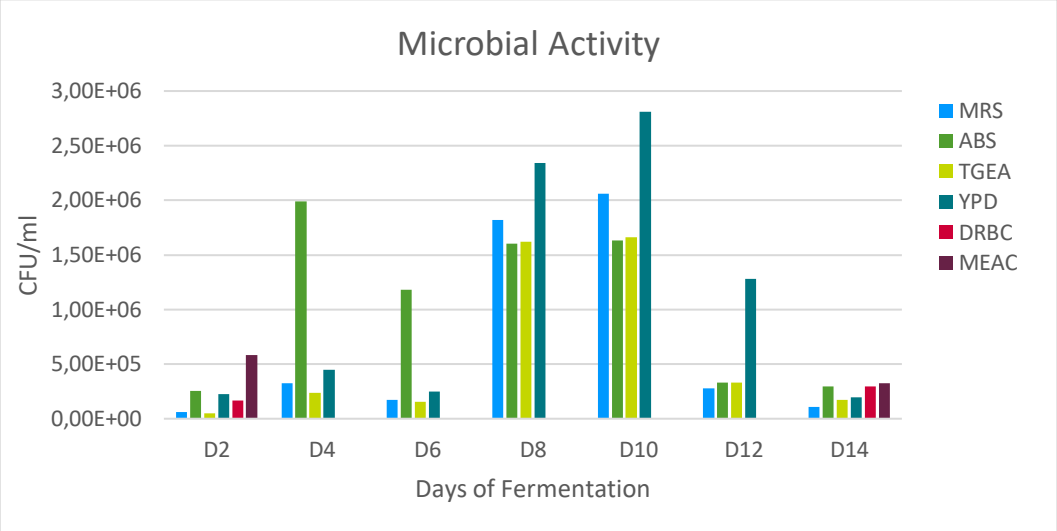

Figure 5. CFU/ml from the different selective media used during the fermentation.

4.5 Colony morphology

Each sample and corresponding media was observed, and four colonies from each morphotype were inoculated on the corresponding selective media. In Table 8, the colonies are described, and complementary images are presented, as shown below.

Table 8. Description of the morphology of colonies on the different media.

	Colony morphology
MRS	1. Big, white, round 2. Small, white, round 3. Wrinkly surrounding white dot
ABS	1. White, round 2. Light blue, round 3. Dark blue, round 4. Blue and white, round
TGEA	1. White, round 2. Off white, smudge
YPD	1. White, round 2. White, wrinkly surrounding 3. White, circle surrounding dot
DRBC	1. Big, white, round 2. Big, pink/white 3. Small, pink
MEAC	1. Big, white, round 2. Small, white, round 3. White, smudge

In figure 6 different coloured colonies can be seen on the ABS media, studying them under incubation they showed to change colour over time from white to light blue at first, to dark blue in the end. On the MRS media (figure 7) the colonies were mainly white dots, some grew over time, and others ended up with a round wrinkly circle surrounding them.

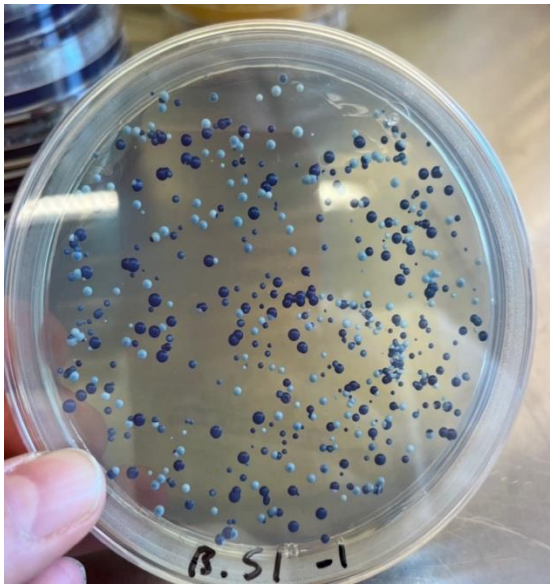

Figure 6. ABS media – 7 days of incubation.

Figure 7. MRS media - 7 days incubation.

TGEA media (figure 8) didn't have big variations in the morphology of its colonies. YPD, being the selective media for yeast, was the one that had the most growth of colonies mostly white dots with a wrinkly surrounding circle, which can be clearly seen in figure 9.

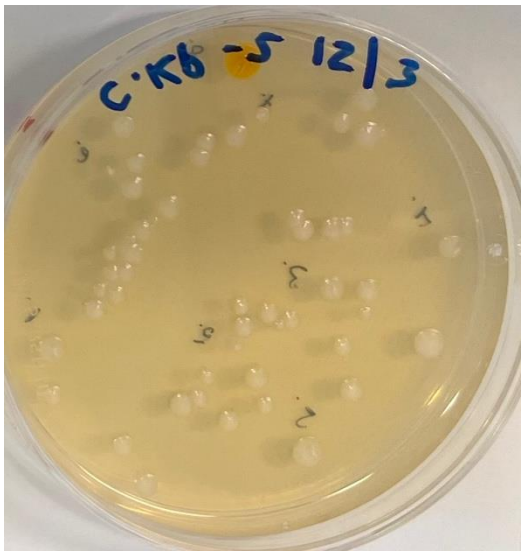

Figure 8. *TGEA media – 5 days of incubation.*

Figure 9. *YPD media - 6 days incubation.*

DRBC media (figure 10) grew pink and white colonies; observing the incubation it was also clear that the colour changed over time from a dark pink to a lighter tone, with some becoming completely white. Lastly, on MEAC media grew white colonies, frequently starting as a white round colony and morphing into a colony resembling a fried egg which is represented in figure 11.

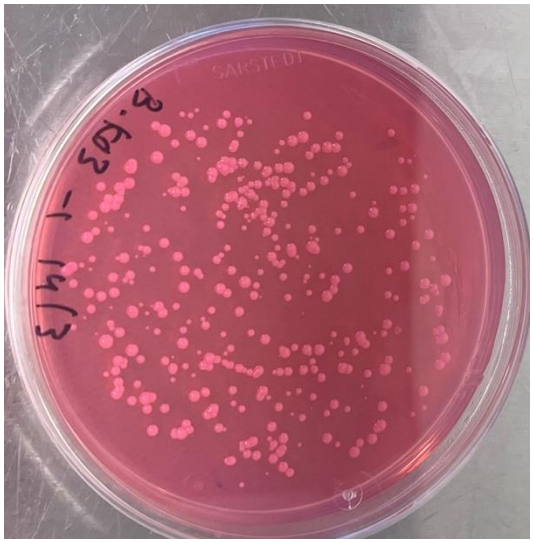

Figure 10. DRBC media – 6 days of incubation.

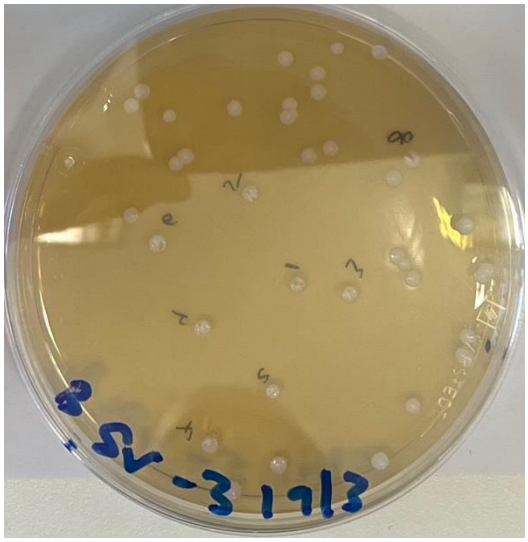

Figure 11. MEAC media - 8 days incubation.

4.6 Identification of yeasts and bacteria

The results from isolating single colonies by inoculation on media showed to be difficult as many colonies didn't grow, the ones that did were used for PCR and sequencing for identification. Table 9 below presents the results of the identified yeasts. In the ready-made Kombucha products and during the fermentation there was an abundance of *Brettanomyces bruxellensis* and *Brettanomyces anomalus.* On the contrast, in the Kombucha products there was a low abundance of *Pichia sp., Hanseniaspora sp. Candida boidinii and Prototheca zopfii.* In most products the diversity was low, interestingly Sencha 2023 and Humm had the largest variation composition of yeasts, containing three different species. During the fermentation there was a higher variation of species in total, but also within the samples.

Table 9. Species grown on medium selective for yeasts.

	YPD	DRBC	MEAC	MRS	ABS	TGEA
Starter cultures	-	-	-	-	-	-
Sencha 2021	B. bruxellensis	B. anomalus	B. anomalus	B. anomalus	B. bruxellensis	B. bruxellensis
Sencha 2022	B. anomalus	B. anomalus	B. anomalus	B. anomalus	B. bruxellensis	B. bruxellensis
Sencha 2023	- B. anomalus - B. bruxellensis	- B. anomalus - Hanseniaspara sp. - Candida boidinii	- Hanseniaspara sp. - B. anomalus	B. anomalus	B. bruxellensis	B. bruxellensis
Oolong 2021	B. anomalus	B. anomalus	B. anomalus	- B. anomalus - B. bruxellensis	B. bruxellensis	B. bruxellensis
Oolong 2022	B. anomalus	B. anomalus	B. anomalus	B. anomalus	B. bruxellensis	B. bruxellensis
Oolong 2023	B. anomalus	B. anomalus	B. anomalus	B. anomalus	B. bruxellensis	B. bruxellensis
Pastuerised	- B. anomalus - B. bruxellensis	- B. anomalus - B. bruxellensis	- B. anomalus - B. bruxellensis	B. bruxellensis	-	-
Humm	- Pichia sp. - B. Anomalus - B. Bruxellensi	-	Prototheca zopfii	-	-	-
R. Voltaire	- B. anomalus - B. bruxellensis	-	- B. anomalus - B. bruxellensis	-	-	-
Fermentation process						
D2	-Zygosaccharomyces lentus. -Zygosaccharomyces kombuchaensis	Zygosaccharomyces lentus	- Zygosaccharomyces kombuchaensis. - Zygosaccharomyces lentus	B. anomalus	- B. anomalus - B. bruxellensis	- B. anomalus - B. bruxellensis
D4	- Zygosaccharomyces kombuchaensis. - B. Anomalus - B. Bruxellensi	-	-	B. anomalus	- B. anomalus - B. bruxellensis	- B. anomalus - B. bruxellensis
D6	- Zygosaccharomyces kombuchaensis. - B. Anomalus - B. Bruxellensi	-	-	B. anomalus	- B. anomalus - B. bruxellensis	- Saccharomyces cerevisiae. - B. Anomalus. - B. Bruxellensi
D8	- Brettanomyces anomalus - Zygosaccharomyces lentus	-	-	B. anomalus	- B. anomalus - B. bruxellensis	- Saccharomyces cerevisiae. - B. Anomalus - B. Bruxellensi
D10	- Zygosaccharomyces kombuchaensis. - Zygosaccharomyces lentus	-	-	- B. anomalus - B. bruxellensis	- B. anomalus - B. bruxellensis	- B. anomalus - B. bruxellensis
D12	Zygosaccharomyces lentus	- B. anomalus - B. bruxellensis	Zygosaccharomyces lentus	- B. anomalus - B. bruxellensis	- B. anomalus - B. bruxellensis	- B. anomalus - B. bruxellensis
D14	Zygosaccharomyces lentus	-	Zygosaccharomyces lentus	- B. anomalus - B. bruxellensis	- B. anomalus - B. bruxellensis	- B. anomalus - B. bruxellensis

Bacteria were difficult to cultivate; after PCR and gel electrophoresis, there were only 24 samples that identified as bacteria. From the sequencing, only four samples were pure enough to show results, which are shown in table 10. Four different species were found.

Table 10. Bacteria identified through sequencing.

	MRS	**TGEA**	**MEAC**
Sencha 2022	Staphylococcus sp.	-	-
Oonlog 2023	-	Falsarthrobacter nasiphocae	Staphylococcus sp.
Humm	-	-Staphylococcus warneri - Asaia sp.	-

4.7 HPLC

Concentrations of sugars, alcohols, and organic acids from the different ready Kombucha products are shown on the left of Table 11, and on the right the results from the fermentation. Lactic acid was not found in any samples. Sucrose could not be quantified due to the inability of the machine available.

Table 11. Acetic acid, glucose and ethanol values in g/L. On the left side are the results from the ready Kombucha products. On the right are the results from the samples of the fermentation process. O= Oolong S= Sencha, R-V = Renee Voltaire

Kombucha ready products	**Acetic Acid**	**Glucose**	**Ethanol**	Fermentation samples	**Acetic Acid**	**Glucose**	**Ethanol**
S ST	6,05	6,02	14,19	**D2**	0,99	33,90	1,16
S 2021	4,18	25,39	9,88	**D4**	1,32	33,14	2,99
S 2022	2,68	24,63	6,54	**D6**	1,58	30,36	5,09
S 2023	1,32	26,61	3,63	**D8**	2,86	39,05	9,93
O ST	20,32	13,66	7,24	**D10**	3,41	38,76	13,12
O 2021	2,31	17,63	13,65	**D12**	4,38	37,42	16,37
O 2022	3,84	16,45	18,14	**D14**	5,65	34,41	18,39
O 2023	1,89	21,88	6,29				
Past.	2,67	21,43	7,79				
Humm	2,5	17,93	2,55				
R. V	2,31	2,31	1,82				

Sencha samples showed the highest glucose values, as shown in Figure 12. Sencha and Oolong show an increasing trend as the vintages get younger, with a slight decline in glucose in the year 2022. Ethanol is found to increase with time in both Sencha and Oolong. However, Oolong sample year 2022 shows the highest quantity of ethanol. The lowest result of ethanol was found in the commercialized samples: Humm and R.V. Acetic acid, steadily rises with age in Sencha Kombucha. Oolong starter culture shows the highest acetic acid concentration, while the other Oolong samples are low. The year 2022 in Oolong Kombucha shows the highest concentration, whereas the year 2021 is similar in concentrations to the pasteurized and commercialized samples.

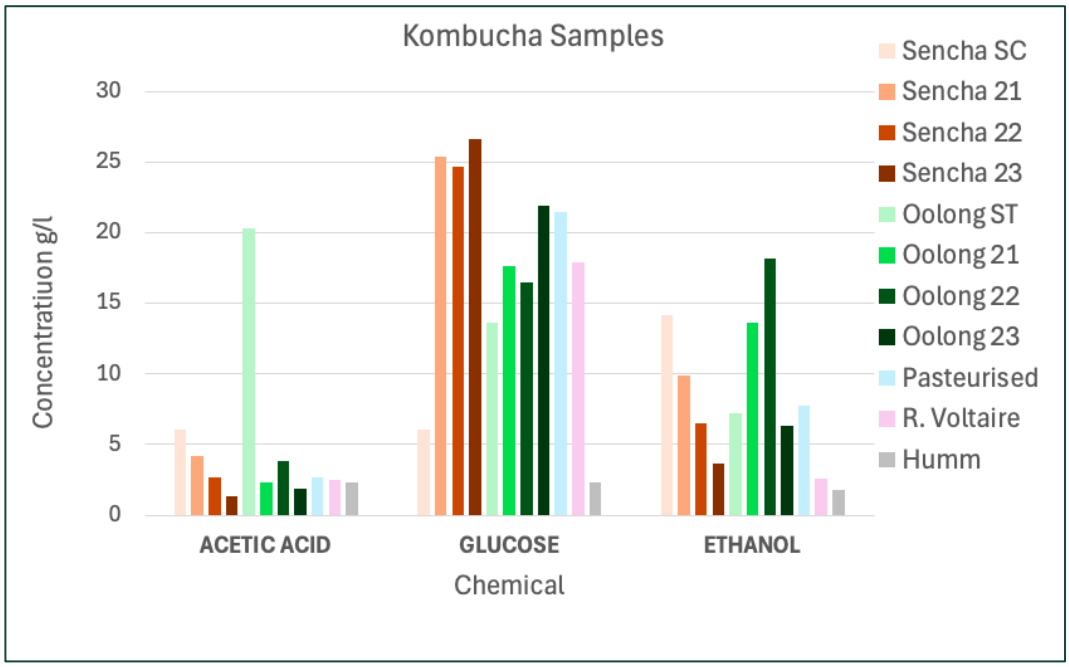

Figure 12. Acetic acid, glucose and ethanol levels for all ready-made Kombucha samples.

During the fermentation there is a clear increasing trend in both the results for acetic acid and ethanol (figure 12). The glucose concentrations show a peak in day 8 and 10, however the levels stay at a level over 30g/L throughout the whole fermentation process.

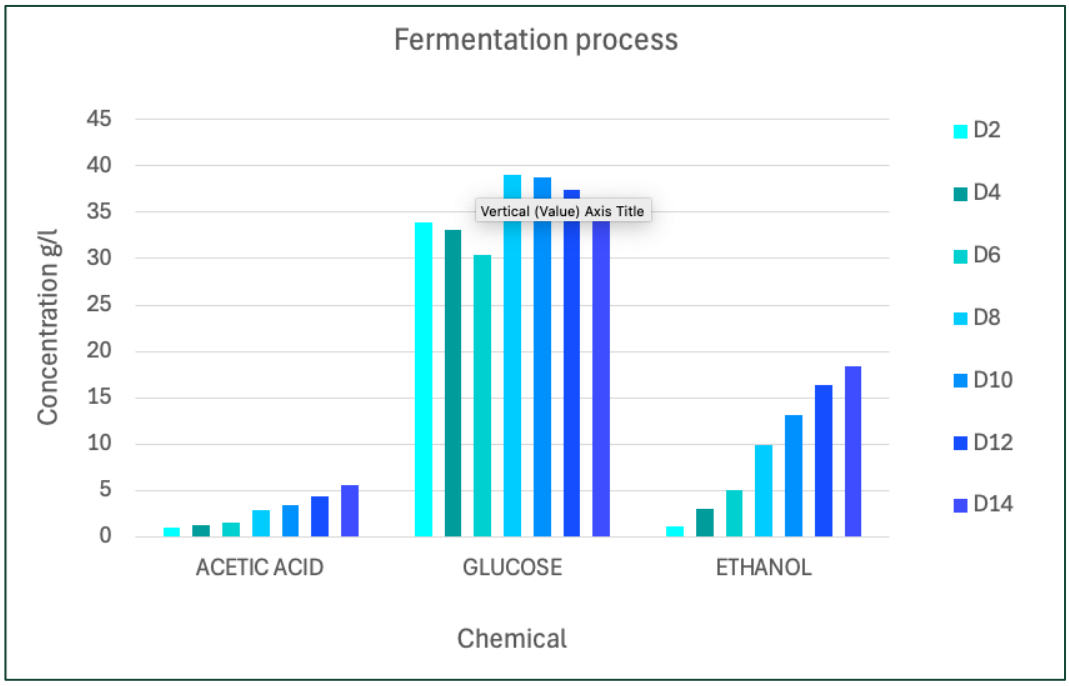

Figure 13. Acetic acid, glucose and ethanol levels during the fermentation process.

5. Discussion

With the rise of popularity of Kombucha, the interest in its characterisation and chemical composition has increased. The interaction between AAB and yeast is frequently speculated on, however, a better comprehension of the microbial diversity and how they interact would be useful for managing and engineering Kombucha production (Landis et al. 2022). This study aimed to contribute to this knowledge, specifically intended for Källsjö Kombucha brewery.

5.1 Microbial activity

The concentration of components in Kombucha is influenced by the initial brew of substrate and sucrose but also by the length of fermentation and the nature of the Kombucha culture (Zailani & Adnan 2022). This was clearly portrayed in the results (Fig. 4) as some were very different from each other compared to others that turned out to be quite similar. For Sencha and Oolong there were three different vintages of each kind to compare, which showed that the lowest microbial activity was in the oldest bottles from the year 2021. In the Sencha samples, there is an increasing trend with time, indicating the highest activity in the year 2023, which is to be expected due to the decline with time in carbon sources for the microorganisms. Nevertheless, the Oolong Kombucha shows a peak in the year 2022, which decreases in the year 2023. The combination of microbes could have affected this peak, such as a different yeast that created different activity or storing conditions. Another reason for this unexpected peak could have been due to any stressful events, such as drastic temperature changes, which can disturb the proportions and diversities within the Kombucha consortium, especially in the yeast community (Maas et al. 2022).

For a better understanding of these types of unexpected results, the study should have included several bottles from the same batch. We were limited to one bottle from each year and type of Kombucha. With more samples, the results would have been more reliable, and a wider perspective would have been reached, as we could have observed if the same results were presented in other samples. The colony forming units of the pasteurized sample was low, at a similar level as the vintages of 2021 and 2022, which is expected due to heat treatment destroying much of the microbes. The commercialised samples indicated results in between the highest and lowest values of the Källsjö samples, Humm showing the higher values of the two.

During the fermentation process (figure 5) of the homebrewed Kombucha the total number of cultivable microbes is highest during days 8 and 10. In the beginning of the fermentation, especially on day four, the CFU on the AAB media plate was high, which after day 10 did a significant decline. As fermentation progresses, yeast, AAB and LAB metabolise, producing organic acids that bring the pH value down, meanwhile the SCOBY is growing, creating a more anaerobic environment. These conditions are not optimal for all microbes, which means new ones evolve, and many don't survive. AAB is one microbe that is determined by oxygen availability; at the start of the fermentation where there is abundance of oxygen and sugar there will be more AAB activity (Laureys et al.). This also justifies the results from the homebrewed Kombucha fermentation.

5.2 Identification

The morphology of the colonies on the same selective media was similar across most Kombucha samples. There were also similarities across different media. This was reflected in the sequenced samples, as the results show a dominance of *Brettanomyces bruxellensis* and *Brettanomyces anomalus* (Table 9). There was little diversity, which is common since one species often becomes predominant. This also confirms the similarities of the colonies growing in the different media. *Brettanomyces* is one of the most common yeast members in Kombucha, which reflects on the results received (Yang et al. 2022). There was also a low abundance of yeast species, such as *Hanseniaspara sp., Candida boidinii, Pichia sp.* and, the a unicellular yeast-like aerobic algae *Prototheca zopfii*. The latter two were found in Humm Kombucha, whereas the two first mentioned were in Källsjö sample Sencha 2023. This was also the sample with the most diversity, containing four different species. *Candida boidinii,* which metabolizes glucose and fructose, is often found in Kombucha. *C. boidinii is* often discovered in alcoholic fermentations, which can explain why it thrives in the Kombucha environment. It's known to give specific desired flavours in fermented foods, although it's not always a positive yeast, as it can also cause spoilage in the aroma (Robinson 1999). *Haseniaspara* sp. has shown to be a yeast that makes a good pair with other yeasts such as *B. bruxellensis* and acidic bacteria. (Tran et al. 2022). Consequently, Kombucha tends to have an abundance of these microbes, which explains its presence. In Humm the yeast *Pichia* and *Prototheca zopfiihas* was found; the first one is commonly used in the starter culture for fermented foods such as wine and chocolate. It proved to produce high amounts of esters, which are important for the aroma in beverages (Wyk et al. 2023). *Prototheca zopfii,* the heterotrophic alga lacks chlorophyll and reproduces by endosporulation. It's commonly found in various environments, including food, and it plays a weak infectious role amongst immunocompromised patients (Abu Alo et al. 2021). The presence of this algae

must be due to some contamination during the experiment, as it was only found in one of the MEAC media plates, it didn't show in any yeast-specific media.

During fermentation, there was more activity to be seen, including a more varied microbial diversity. This is usually the case in homebrewed Kombucha compared to industrial brewed, since the industrial environment is much more sterile. The homemade fermentation illustrated *Zygosaccharomyces* and *Brettanomyces* yeast genera during the whole fermentation process. *Z. lentus* and *Z. kombuchaensis,* two very similar species were found throughout the fermentation. These are yeast strains in Kombucha that produce alcohol, undergo carbonation, and contribute to the formation of the SCOBY (Zubaidah et al. 2023). *Zygosaccharomyces* has a long history of spoilage in food and beverages. *Z. lentus* being one that can create the most spoilage to processed foods, due to its resistance to weak acids, high sugar or salt concentrations, and capacity to ferment fructose as well as having a high tolerance to ethanol. However, in Kombucha production, these species play an important role (Sa-Correia et al. 2014). *Saccharomyces cerevisiae,* the most common yeast in Kombucha, is essential in the symbiosis between yeast and AAB, keeping foreign microbes away (Zubaidah et al. 2023). To find this in the Kombucha is a positive find, although it was only found on days 6 and 8.

Table 10 shows the bacteria that *Falsarthrobacter nasiphocae* known for inhabiting the air, probably came from contamination during the study. S*taphylococcus warneri* found in hair and skin, can also be explained as a contamination from lab errors. Finally, in the TGEA media *Asaia sp.* was found in the Humm sample, one of the commercialized Kombuchas. This is a common species found in Kombucha, coming from the Acetobacteraceae family (Kaashyap 2021). It originates from tropical climates where its natural habitats have been reported to be flowers of the orchid, plumbago, and fermented glutinous rice. However, there is no risk for human health (Moore et al. 2002).

A limitation of this research was the possibility of contamination during the extraction and/ or sequencing steps, which resulted in only a few identifications of bacteria. Another possibility for the lack of acetic acid bacteria could be that they are difficult to isolate due to its high nutritional demand, to such an extent that some species are not culturable. Different techniques need to be used for these samples (Wang et al. 2022). Another reason for using different identification techniques, is the possibility for the AAB to be in a VBNC state, when culture dependent methods won't give the accurate results. Some studies have shown that there is a higher content of acetic and lactic acid bacteria in the biofilm compared to the liquid, where more of the yeast is found (Maas et al. 2022). Therefore, it would be interesting for further studies to take samples from different parts of the kombucha

tank to see the difference and confirm if bacteria are found closer to the SCOBY. In this study, samples were only taken from the middle of the Kombucha tank during fermentation. In regards to the ready-made products, it would be interesting to compare bottles of the same batch and year and what can be identified.

Even though specific media was used for different microbes such as yeast and LAB and AAB, and agents were used to hinder the growth of unwanted microorganisms, yeast still grew on the bacteria selective media where no bacteria was found. One explanation could be that yeast inhibited the bacteria from growing, as it is common that a high fungal diversity results in low bacteria diversity (Harrisson et al. 2021). This also validates that some yeasts, such as *Brettanomyces*, *Candida*, *Pichia* can grow on agar with cycloheximide if levels are low (Butzke 2010).

5.3 HPLC

The concentrations of sugars, alcohol, and organic acids were measured and monitored throughout the fermentation. Lower acetic acid concentrations were observed in younger kombucha samples compared to the older variants, which is expected as the more time passes the more acetic acid is produced (Figure 11). This also explains the reason why the older vintages have a more of an acidic flavour (Table 5). The pH values are expected to decline with time as the organic acids produced accumulate. Observing Sencha, the pH values decline (Figure 2) with the years while the acetic acid increases. Acetic acid concentrations are commonly around 10g/L in Kombucha (Greenwalt et al. 1998). Observing the ready-made products, the levels are around 2-3 g/L in most cases, which would be low if compared to 10 g/L. During the fermentation, the amounts of the acetic acid are generally higher, reaching a level of 5,65 g/L on the last day (Table 11). Only the Sencha vintage from 2021 is close to this quantity. Throughout the fermentation process (Figure 12) an increasing trend is seen in both the acetic acid and ethanol. One difference to keep in mind, is the length of fermentation, as the one done in the study was 14 days compared to Källsjö, which was 30 days. Fermenting for a longer period, such as 30 to 60 days, will result in a decrease in acetic acid towards the end. The decline is created when acetic acid is used as a carbon when sugars in the tea have been used up. Alternatively, because of a decrease in ethanol metabolism by yeast due to low pH (Martinez Leal et al. 2018).

Ethanol levels are expected to increase throughout the fermentation process and the longer the Kombucha has been stored. When the yeast runs out of glucose, it will turn to fructose as a carbon source, which will be converted into ethanol. In return AAB use the ethanol to produce acetic acid, keeping the alcohol levels down. This trend can be observed in the Oolong starter culture, which shows high acetic acid

and low ethanol content. Conversely, some of the Kombucha samples with low acidic acid show high ethanol, such as Oolong 2022. AAB are aerobic bacteria, which means that in stored anaerobic conditions, they won't grow, limiting the conversion of ethanol and glucose into acetic acid and gluconic acid (Chakravorty et al. 2019). Sencha vintages show the expected increase in ethanol as the samples get older. A known and continuous challenge for the Kombucha producers is to keep alcohol levels down. Pasteurization could be one solution; however, it means the composition and aromas will be affected (Harrisson et al. 2023). Conversely, the pasteurized sample from Källsjö, shows similar ethanol and acetic acid results to some of the unpasteurized samples, such as Oolong 2023 and Sencha 2022. Humm is the only example where acetic acid, ethanol, and glucose have lower levels compared to the rest of the samples. The manufacturers might use some techniques to keep the microbial activity and synthesis of organic acids and ethanol down, such as adding additional sucrose.

Glucose levels are highest in Sencha samples, without much change throughout the vintages, although it's a little higher in the year 2023. Glucose is expected to be higher at younger ages and to decrease with time as it is converted into organic acids. Nonetheless, high amounts of glucose were found in all Kombucha samples except in Humm, which could mean that sucrose was broken down at a faster rate than what bacteria could be used up. In the observed fermentation, glucose levels are declining the first three days, which is expected as yeast is converting sucrose to glucose and fructose; furthermore, glucose is used by the acetic acid bacteria. Meanwhile, the SCOBY grows and creates a more unpleasant environment for the AAB bacteria, which means the glucose created by the yeasts is not being used up and therefore increases. The dominance of yeast in all kombucha samples could also explain the lack of use of glucose, as yeast uses fructose to produce ethanol. Unfortunately, due to the inability to check fructose levels with HPLC, the results are insufficient to analyse.

No lactic acid was found, indicating that no LAB were present in the Kombucha samples. The reason for this could be the pH of the Kombucha samples, as it reached levels under 3.5, which is an environment that many LAB cannot survive in (Laureys et al. 2020).

6. Conclusion

This study has served the purpose of getting a better understanding of the composition of Källsjö Kombucha and how storage has affected the beverage. As the kombucha gets older, the pH declines and so does the microbial activity. Ethanol and Acetic acid increase with time, which can also explain the decrease in the pH value. Even though this trend is expected, it is not always the outcome, which shows in Oolong year 2022. The reason for these results can be due to a variety of factors such as temperature during fermentation or storage conditions. The results of the identification indicated that yeast dominated the Kombucha, both the commercialised and the home-brewed Kombucha. However, there is reason to believe that the shortage of bacteria identified is not due to their absence in the Kombucha, but rather to errors during the lab work or the methods used, which might not have been suitable for all microorganisms. Alternatively, due to VBNC state of the ABS.

For future studies, more samples from the same batches would be interesting to study as well as trying different methods which can make it easier to identify ABS which is difficult to isolate.

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Popular science summary

Kombucha is a fermented drink that has been consumed since 220 BC, it gained popularity then and today due to its health benefits (Greenwalt et al. 2000). The composition of kombucha contains microorganisms that seem to help in different ways such as lower blood pressure and cholesterol, as well as improve gut health and activity. Studies show that the composition of Kombucha varies not only among different countries but also between batches. The fermentation parameters will influence these changes, which also means that one cannot generalize the potential health effects (Yang et al. 2022). As the market grows, both consumers and manufacturers are showing more interest in what the beverages contain.

One brewery in the South of Sweden, Källsjö, reached out and wanted to understand the composition of their Kombucha. They were also interested in finding out how storage and pasteurization affect the composition. This study merely observed samples from Källsjö, however, some commercialised samples and samples from a homebrewed fermentation were included for comparison.

The main components of Kombucha are yeasts, acetic acid, and lactic acid, which also play important roles in the health benefits. Yeast is often found to dominate the ecosystem, which is also what we observed in the different Kombucha samples. In the Källsjö Oolong and pasteurized sample only *Brettanomyces bruxellensis* and *Brettanomyces anomalus* was found. Sencha had a bigger variety including *Hanseniaspara sp., Candida boidinii*, *Pichia sp.* and, *Prototheca zopfii*. The only AAB identified, *Asaia sp.* was in the commercialized Kombucha HUMM. In the fermentation process, more diversity in the microorganisms was found, which was expected as it was a less controlled environment. Additionally to the yeasts already mentioned *Zygosaccharomyces lentus* and Z. *Kombuchaensis* and *Saccharomyces cerevisiae* were identified. All yeasts are common in Kombucha and fermented foods.

Other important components affecting the taste and composition are the organic acids produced by the microorganisms. Acetic acid and ethanol are byproducts produced by the breakdown of the sucrose in the tea. The microbial interaction is essential, and therefore learning how to control the fermentation and achieve the ideal end product is of great importance to Kombucha manufacturers.

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Appendix 1

		Control - C	No Dilution - ND	1×10^+01	1×10^+02	1×10^+03	1×10^+04
SENCHA							
2021 - A							
1w 1d	**MRS**	0	-	>300	70	12	2
1w	**ABS**	0	-	>300	53	8	2
1w	**TGEA**	0	-	287	54	9	-
1w	**YPD**	0	-	>300	176	16	4
1w 1d	**DRBC**	0	>300	>300	45	4	-
1w	**MEAC**	0	>300	>300	139	23	-
2021 - B							
1w 1d	**MRS**	0	-	>300	79	11	2
1w	**ABS**	0	-	>300	77	7	0
1w	**TGEA**	0	-	321	39	5	-
1w	**YPD**	0	-	>300	103	3	0
1w 1d	**DRBC**	0	>300	>300	66	4	-
1w	**MEAC**	0	>300	>400	66	9	-
2021 - C							
1w 1d	**MRS**	0	-	>300	57	9	0
1w	**ABS**	0	-	>300	36	8	0
1w	**TGEA**	0	-	238	58	8	-
1w	**YPD**	0	-	>300	86	4	0
1w 1d	**DRBC**	0	>300	197	43	6	-
1w	**MEAC**	0	>300	344	58	10	-
SENCHA 2021							
1w 1d	**MRS**	0	-	>300	68,67	10,67	1,33
6d	**ABS**	0	-	>300	55,33	7,67	0,67

6d	**TGEA**	0	>300	282,00	50,33	7,33	-
6d	**YPD**	0	>300	>300	121,67	139,67	1,33
6d	**DRBC**	0	>300	>300	51,33	4,67	-
6d	**MEAC**	0	>300	>300	87,67	14,00	-
2022 - A							
1w	**MRS**	0	>300	>300	309	42	1
5d	**ABS**	0	>300	>300	246	45	1
1w	**TGEA**	0	>300	>400	146	10	0
1w	**YPD**	0	>300	>300	318	28	5
1w	**DRBC**	0	223	74	26	0	0
1w	**MEAC**	0	>300	>300	221	20	1
2022 - B							
1w	**MRS**	0	>300	>300	0	31	0
5d	**ABS**	0	>300	>300	188	31	1
1w	**TGEA**	0	>300	>400	129	14	0
1w	**YPD**	0	>300	>300	280	19	0
1w	**DRBC**	0	0	0	1	1	0
1w	**MEAC**	0	>300	>300	175	8	1
2022 - C							
1w	**MRS**	0	>300	>300	48	24	0
5d	**ABS**	0	>300	>300	278	29	3
1w	**TGEA**	0	>300	>400	212	14	2
1w	**YPD**	0	>300	>300	380	29	6
1w	**DRBC**	0	>400	305	72	9	0
1w	**MEAC**	0	>300	>300	210	29	2
SENCHA 2022							
1w	**MRS**	0	>300	>300	119,00	32,33	0,33
5d	**ABS**	0	>300	>300	237,33	35,00	1,67
1w	**TGEA**	0	>300	>400	162,33	12,67	0,67
1w	**YPD**	0	>300	>300	326,00	25,33	3,67
1w	**DRBC**	0	>300	189,50	33,00	3,33	0,00
1w	**MEAC**	0	>300	>300	202,00	19,00	1,33
2023 - A							
1w 5d	**MRS**	0	>300	>300	>300	311	13
5d	**ABS**	0	-	>300	>300	213	-

5d	**TGEA**	0	>300	>300	>300	93	11
5d	**YPD**	0	>300	>300	>300	273	17
5d	**DRBC**	0	>300	>300	141	12	0
5d	**MEAC**	0	>300	>300	>300	128	31
2023 - B							
1w 5d	**MRS**	0	>300	>300	>300	291	21
5d	**ABS**	0	-	>300	>300	213	-
5d	**TGEA**	0	>300	>300	>300	102	13
5d	**YPD**	0	>300	>300	>300	283	19
5d	**DRBC**	0	>300	>300	362	40	5
5d	**MEAC**	0	>300	>300	>300	210	33
2023 - C							
1w 5d	**MRS**	0	>300	>300	>300	149	27
5d	**ABS**	0	-	>300	>300	380	-
5d	**TGEA**	0	>300	>300	>300	118	14
5d	**YPD**	0	>300	>300	>300	260	36
5d	**DRBC**	0	>300	>300	347	53	2
5d	**MEAC**	0	>300	>300	>300	200	43
SENCHA 2023							
1w 5d	**MRS**	0	>300	>300	>300	250,33	20,33
5d	**ABS**	0	-	>300	>300	268,67	-
5d	**TGEA**	0	>300	>300	>300	104,33	12,67
5d	**YPD**	0	>300	>300	>300	272,00	24,00
5d	**DRBC**	0	>300	>300	283,33	35,00	2,33
5d	**MEAC**	0	>300	>300	>300	179,33	35,67
OONLOG							
2021 - A							
1w	**MRS**	0	>300	>300	177	19	13
1w	**ABS**	0	>300	>300	97	18	43
1w	**TGEA**	0	>300	>300	207	17	11
1w	**YPD**	0	>300	>300	416	60	17
1w	**DRBC**	0	34	21	0	0	0
1w	**MEAC**	0	>300	>300	317	4	31
2021 - B							
1w	**MRS**	0	-	>300	182	20	2

1w	**ABS**	0	>300	>300	152	22	-
1w	**TGEA**	0	-	>300	177	18	1
1w	**YPD**	0	-	>300	>400	61	2
1w	**DRBC**	0	0	24	1	0	0
1w	**MEAC**	0	-	>300	255	38	8
2021 - C							
1w	**MRS**	0	>300	>300	96	4	0
1w	**ABS**	0	>300	>300	61	6	-
1w	**TGEA**	0	>300	>300	99	12	0
1w	**YPD**	0	>300	>300	138	18	1
1w	**DRBC**	0	106	10	0	0	0
1w	**MEAC**	0	>300	>300	124	35	1
OONLOG 2021							
1w	**MRS**	0	>300	>300	151,67	14,33	5,00
6d	**ABS**	0	>300	>300	103,33	15,33	-
6d	**TGEA**	0	>300	>300	161,00	15,67	4,00
6d	**YPD**	0	>300	>300	>300	46,33	6,67
6d	**DRBC**	0	46,67	18,33	0,33	0,00	0,00
6d	**MEAC**	0	>300	>300	232,00	25,67	13,33
2022 - A							
1w	**MRS**	0	>300	>300	>300	233	39
5d	**ABS**	0	-	>300	261	47	-
1w	**TGEA**	0	>300	>300	>300	128	14
1w	**YPD**	0	>300	>300	>300	250	49
1w	**DRBC**	0	>300	226	27	3	0
1w	**MEAC**	0	>300	>300	>300	228	17
2022 - B							
1w	**MRS**	0	>300	>300	>300	208	25
5d	**ABS**	0	-	>300	323	56	-
1w	**TGEA**	0	>300	>300	>300	106	23
1w	**YPD**	0	>300	>300	>300	325	33
1w	**DRBC**	0	>300	320	46	5	0
1w	**MEAC**	0	>300	>300	>300	204	29
2022 - C							
1w	**MRS**	0	>300	>300	>300	187	32

5d	**ABS**	0	>300	>300	185	44	-
1w	**TGEA**	0	>300	>300	>300	143	14
1w	**YPD**	0	>300	>300	>300	269	37
1w	**DRBC**	0	>300	219	39	1	0
1w	**MEAC**	0	>300	>300	>300	168	18
OONLOG 2022							
6d	**MRS**	0	>300	>300	>300	209,33	32,00
5d	**ABS**	0	-	>300	256,33	49,00	-
5d	**TGEA**	0	>300	>300	>300	125,67	17,00
5d	**YPD**	0	>300	>300	>300	281,33	39,67
5d	**DRBC**	0	>300	255,00	37,33	3,00	0,00
5d	**MEAC**	0	>300	>300	>300	200,00	21,33
2023 - A							
1w 6d	**MRS**	0	>300	>300	288	34	1
5d	**ABS**	0	>300	>300	213	-	-
5d	**TGEA**	0	>300	>300	301	29	6
5d	**YPD**	0	>300	>300	249	37	2
5d	**DRBC**	0	>300	172	17	1	0
5d	**MEAC**	0	>300	>300	193	28	1
2023 - B							
1w	**MRS**	0	>300	>300	333	49	4
6d	**ABS**	0	>300	>300	0	-	-
6d	**TGEA**	0	>300	>300	284	41	3
6d	**YPD**	0	>300	>300	393	42	3
6d	**DRBC**	0	>300	348	51	1	0
6d	**MEAC**	0	>300	>300	315	40	5
2023 - C							
1w 4d	**MRS**	0	>300	>300	155	20	4
6d	**ABS**	0	>300	>300	380	-	-
6d	**TGEA**	0	>300	>300	151	43	0
6d	**YPD**	0	>300	>300	235	24	2
6d	**DRBC**	0	>300	252	42	1	0
6d	**MEAC**	0	>300	>300	118	12	2
OONLOG 2023							
1w	**MRS**	0	>300	>300	258,67	34,33	3,00

6d	**ABS**	0	>300	>300	197,67	-	-
6d	**TGEA**	0	>300	>300	245,33	37,67	3,00
6d	**YPD**	0	>300	>300	292,33	34,33	2,33
6d	**DRBC**	0	>300	257,33	36,67	1,00	0,00
6d	**MEAC**	0	>300	>300	208,67	26,67	2,67
Pasteurised							
2023 - A							
1w 5d	**MRS**	0	>300	>300	114	14	3
1w 5d	**ABS**	0	-	-	-	-	-
1w 5d	**TGEA**	0	>300	>300	89	32	1
1w 5d	**YPD**	0	>300	>300	119	15	1
1w 5d	**DRBC**	0	>300	>300	75	8	0
1w 5d	**MEAC**	0	>300	>300	70	10	0
2023 - B							
1w 5d	**MRS**	0	>300	>300	333	49	4
1w 5d	**ABS**	0	>300	>300	1	0	0
1w 5d	**TGEA**	0	>300	>300	284	41	3
1w 5d	**YPD**	0	>300	>300	393	42	3
1w 5d	**DRBC**	0	>300	>300	51	1	0
1w 5d	**MEAC**	0	>300	>300	315	40	5
2023 - C							
1w 5d	**MRS**	0	>300	>300	86	6	9
1w 5d	**ABS**	0	>300	>300	72	9	0
1w 5d	**TGEA**	0	>300	>300	54	10	1
1w 5d	**YPD**	0	>300	>300	142	14	1
1w 5d	**DRBC**	0	>300	>300	53	6	1
1w 5d	**MEAC**	0	>300	>300	74	13	0
Pasteurised							
1w 5d	**MRS**	0	>300	>300	177,67	23,00	5,33
1w 5d	**ABS**	0	>300	>300	36,50	4,50	0,00
1w 5d	**TGEA**	0	>300	>300	142,33	27,67	1,67
1w 5d	**YPD**	0	>300	>300	218,00	23,67	1,67
1w 5d	**DRBC**	0	>300	>300	59,67	5,00	0,33
1w 5d	**MEAC**	0	>300	>300	153,00	21,00	1,67
HUMM							
2023 - A							

1w 5d	**MRS**	0	>300	>300	>300	>300	135
1w 5d	**ABS**	0	11	5	2	0	0
1w 5d	**TGEA**	0	>300	>300	>300	165	7
1w 5d	**YPD**	0	>300	>300	>300	122	12
1w 5d	**DRBC**	0	>300	>300	>300	83	11
1w 5d	**MEAC**	0	>300	>300	>300	107	12
2023 - B							
1w 5d	**MRS**	0	>300	>300	>300	>300	116
1w 5d	**ABS**	0	68	5	0	0	0
1w 5d	**TGEA**	0	>300	>300	>300	94	17
1w 5d	**YPD**	0	>300	>300	>300	90	10
1w 5d	**DRBC**	0	>300	>300	>300	52	13
1w 5d	**MEAC**	0	>300	>300	>300	63	12
2023 - C							
1w 5d	**MRS**	0	>300	>300	>300	>300	136
1w 5d	**ABS**	0	21	2	0	1	0
1w 5d	**TGEA**	0	>300	>300	>300	87	6
1w 5d	**YPD**	0	>300	>300	>300	59	2
1w 5d	**DRBC**	0	>300	>300	>300	82	6
1w 5d	**MEAC**	0	>300	>300	>300	76	9
HUMM							
1w 5d	**MRS**	0	>300	>300	>300	>300	129,00
1w 5d	**ABS**	0	33,33	4	0,67	0,33	0,00
1w 5d	**TGEA**	0	>300	>300	>300	115,33	10,00
1w 5d	**YPD**	0	>300	>300	>300	90,33	8,00
1w 5d	**DRBC**	0	>300	>300	>300	72,33	10,00
1w 5d	**MEAC**	0	>300	>300	>300	82,00	11,00
VOLTAIRE							
2023 - A							
1w 1d	**MRS**	0	-	>300	>300	33	0
1w 1d	**ABS**	0	-	>300	210	39	3
1w 1d	**TGEA**	0	-	>300	>300	32	2
1w 1d	**YPD**	0	-	>300	>300	38	5
1w 1d	**DRBC**	0	-	116	110	13	2
1w 1d	**MEAC**	0	-	>300	>300	42	6
2023 - B							

1w 1d	**MRS**	0	-	>300	>300	42	5
1w 1d	**ABS**	0	-	>300	311	42	3
1w 1d	**TGEA**	0	-	>300	>300	47	3
1w 1d	**YPD**	0	-	>300	>300	45	5
1w 1d	**DRBC**	0	-	>300	>300	28	3
1w 1d	**MEAC**	0	-	>300	>300	39	4
2023 - C							
1w 5d	**MRS**	0	-	>300	>300	35	2
1w 5d	**ABS**	0	-	>300	268	31	3
1w 5d	**TGEA**	0	-	>300	>300	57	8
1w 5d	**YPD**	0	-	>300	>300	37	0
1w 5d	**DRBC**	0	-	>300	>300	36	2
1w 5d	**MEAC**	0	-	>300	>300	36	5
VOLTAIRE							
1w 5d	**MRS**	0	-	>300	>300	36,67	2,33
1w 5d	**ABS**	0	-	>300	263,00	37,33	3,00
1w 5d	**TGEA**	0	-	>300	>300	45,33	4,33
1w 5d	**YPD**	0	-	>300	>300	40,00	3,33
1w 5d	**DRBC**	0	-	>300	>300	25,67	2,33
1w 5d	**MEAC**	0	-	>300	>300	39,00	5,00
Sencha starter							
2021 - A							
1w 1d	**MRS**	0	-	-	-	-	-
1w	**ABS**	0	-	-	-	-	-
1w	**TGEA**	0	-	-	-	-	-
1w	**YPD**	0	>300	>300	100	14	
1w 1d	**DRBC**	0	>300	>300	121	3	
1w	**MEAC**	0	>300	>300	69	8	
SENCHE St							
1w 1d	**MRS**	0	-				
	ABS	0	-				
6d	**TGEA**	0	>300				
6d	**YPD**	0	>300	>300	110,00	14,00	
6d	**DRBC**	0	>300	>300	121,00	3,00	
6d	**MEAC**	0	>300	>300	69,00	8,00	

Oonlog st							
1w 1d	**MRS**	0	-				
1w	**ABS**	0	-	232	24	3	
1w	**TGEA**	0	-	82	7	0	
1w	**YPD**	0	-	216	21	2	
1w 1d	**DRBC**	0	-	203	23	2	
1w	**MEAC**	0	-	192	18	3	
SENCHE 2021							
1w 1d	**MRS**	0	-				
6d	**ABS**	0	-	232	24,00	3,00	0,00
6d	**TGEA**	0	>300	82,00	7,00	0,00	0,00
6d	**YPD**	0	>300	216	21,00	2,00	0,00
6d	**DRBC**	0	>300	203	23,00	2,00	0,00
6d	**MEAC**	0	>300	192	18,00	3,00	0,00

STARTSVÄTSKA OONLOG		**Control - C**	**No Dilution - ND**	**1×10^+01**	**1×10^+02**	**1×10^+03**	**1×10^+04**
STARTVÄTSKA PIA							
	MRS	0	-	-	-	-	-
1w 1d	**ABS**	0	>300	>300	>300	283	-
6d	**TGEA**	0	>300	>300	183	82	3
6d	**YPD**	0	>300	>300	521	101	6
6d	**DRBC**	0	>300	>300	366	71	5
6d	**MEAC**	0	>300	>300	425	105	8
PIA SAMPLE 1							
A. 6d	**MRS**	0	>300	341	63	7	0
1w	**ABS**	0	-	>300	239	21	2
1w	**TGEA**	0	>300	141	46	3	0
1w	**YPD**	0	>300	406	117	4	3
1w	**DRBC**	0	>300	>300	93	22	7

1w	**MEAC**	0	>300	>300	346	76	9
B. 9d	**MRS**	0	>300	>400	73	13	2
1w	**ABS**	-	-	>300	256	26	0
1w	**TGEA**	0	>300	357	48	6	0
4d	**YPD**	0	>300	>400	66	7	1
4d	**DRBC**	0	>300	>400	77	136	12
1w	**MEAC**	0	>300	>400	>400	5	1
C. 9d	**MRS**	0	>300	254	43	5	0
1w	**ABS**	-	-	>300	275	25	2
1w	**TGEA**	1	>300	179	49	3	0
1w	**YPD**	0	>300	>400	>400	76	12
1w	**DRBC**	0	>300	>400	327	119	11
1w	**MEAC**	0	>300	>400	>400	94	6
PIA SAMPLE 1 - AVERAGE							
1w	**MRS**	0	>300	331,67	59,67	8,33	0,67
1w	**ABS**	0	>300	>300	256,67	24,00	1,33
1w	**TGEA**	0	>300	225,67	47,67	4,00	0,67
1w	**YPD**	0	>300	>400	227,67	29,00	5,33
1w	**DRBC**	0	>300	>300	165,67	92,33	10,00
1w	**MEAC**	0	>300	>300	>400	58,33	5,33
PIA SAMPLE 2							
A. 6d	**MRS**	0	>300	>400	278	36	3
1w	**ABS**	0	-	>300	>300	201	30
5d	**TGEA**	0	>300	>400	203	40	2
5d	**YPD**	0	>300	>400	413	57	6
B. 6d	**MRS**	0	>300	>400	374	41	3
1w	**ABS**	-	-	>300	>300	150	20

5d	**TGEA**	0	>300	>400	248	45	4
5d	**YPD**	0	>300	>400	436	66	6
C. 6d	**MRS**	0	>300	>400	316	28	1
1w	**ABS**	-	-	>300	>300	246	17
5d	**TGEA**	0	>300	>400	267	15	2
5d	**YPD**	0	>300	>400	356	57	7
PIA SAMPLE 2 - AVERAGE							
6d	**MRS**	0	>300	>400	322,67	35,00	2,33
1w	**ABS**	0	>300	>300	>300	199,00	22,33
5d	**TGEA**	0	>300	>400	239,33	33,33	2,67
5d	**YPD**	0	>300	>400	449,67	60,00	6,33
PIA SAMPLE 3							
A. 6d	**MRS**	0	>300	>400	119	19	0
1w	**ABS**	0	-	>300	>300	171	31
5d	**TGEA**	0	>300	>400	127	3	2
5d	**YPD**	0	>300	>400	173	14	2
B. 6d	**MRS**	0	>300	>400	79	7	3
1w	**ABS**	-	-	>300	>300	164	20
5d	**TGEA**	0	>300	>400	77	6	4
5d	**YPD**	0	>300	>400	65	6	6
C. 6d	**MRS**	0	>300	>400	316	0	0
1w	**ABS**	-	-	>300	>300	19	0
5d	**TGEA**	0	>300	>400	267	1	0
5d	**YPD**	0	>300	>400	356	2	6
PIA SAMPLE 3 - AVERAGE							
6d	**MRS**	0	>300	>400	171,33	8,67	1,00
1w	**ABS**	0	>300	>300	>300	118,00	17,00
5d	**TGEA**	0	>300	>400	157,00	3,33	2,00
5d	**YPD**	0	>300	>400	246,00	7,33	4,67

PIA SAMPLE 4							
A. 9d	**MRS**	0	>300	>300	>300	118	9
6d	**ABS**	0	>300	>300	>400	144	10
6d	**TGEA**	0	>300	>300	358	112	17
6d	**YPD**	0	>300	>300	>400	186	28
B. 9d	**MRS**	0	>300	>300	>300	236	31
6d	**ABS**	0	>300	>300	>400	182	27
6d	**TGEA**	0	>300	>300	>400	215	20
6d	**YPD**	0	>300	>300	>400	242	47
C. 9d	**MRS**	0	>300	>300	>300	193	16
6d	**ABS**	0	>300	>300	>400	153	17
6d	**TGEA**	0	>300	>300	>400	158	19
6d	**YPD**	0	>300	>300	>400	275	38
PIA SAMPLE 4 - AVERAGE							
1w 2d	**MRS**	0	>300	>300	>300	182,33	18,67
6d	**ABS**	0	>300	>300	>400	159,67	18,00
6d	**TGEA**	0	>300	>300	>400	161,67	18,67
6d	**YPD**	0	>300	>300	>400	234,33	37,67
PIA SAMPLE 5							
A. 9d	**MRS**	0	>300	>300	>300	214	25
6d	**ABS**	0	>300	>300	>400	170	22
6d	**TGEA**	0	>300	>300	>400	182	22
6d	**YPD**	0	>300	>300	>400	323	22
B. 9d	**MRS**	0	>300	>300	>300	205	24
6d	**ABS**	0	>300	>300	>400	179	14
6d	**TGEA**	0	>300	>300	>400	152	21
6d	**YPD**	0	>300	>300	>400	313	28
C. 9d	**MRS**	0	>300	>300	>300	200	20
6d	**ABS**	0	>300	>300	>400	139	16
6d	**TGEA**	0	>300	>300	>400	165	18

6d	**YPD**	0	>300	>300	>400	206	44
PIA SAMPLE 5 - AVERAGE							
1w 2d	**MRS**	0	>300	>300	>300	206,33	23,00
6d	**ABS**	0	>300	>300	>400	162,67	17,33
6d	**TGEA**	0	>300	>300	>400	166,33	20,33
6d	**YPD**	0	>300	>300	>400	280,67	31,33
PIA SAMPLE 6							
A. 8d	**MRS**	0	>300	>300	234	56	3
5d	**ABS**	0	>300	>400	55	32	5
5d	**TGEA**	0	>300	>300	202	51	1
5d	**YPD**	0	>300	>400	330	152	5
B. 8d	**MRS**	0	>300	>300	219	7	4
5d	**ABS**	0	>300	>400	109	47	6
5d	**TGEA**	0	>300	469	258	39	4
5d	**YPD**	0	>300	>400	254	207	16
C. 8d	**MRS**	0	>300	>300	62	20	2
5d	**ABS**	0	>300	>400	249	20	0
5d	**TGEA**	0	>300	353	45	10	0
5d	**YPD**	0	>300	>400	271	25	8
PIA SAMPLE 6 - AVERAGE							
1w 1d	**MRS**	0	>300	>300	>300	27,67	3,00
5d	**ABS**	0	>300	>300	>400	33,00	3,67
5d	**TGEA**	0	>300	>300	>400	33,33	1,67
5d	**YPD**	0	>300	>300	>400	128,00	9,67
PIA SAMPLE 7							
A. 6d	**MRS**	0	>300	>300	62	30	1
1w	**ABS**	0	>300	>300	157	22	13
6d	**TGEA**	0	>300	>400	242	10	7
6d	**YPD**	0	>300	>400	338	28	16

6d	**DRBC**	0	>300	>400	126	37	12
6d	**MEAC**	0	>300	>400	299	18	13
B. 6d	**MRS**	0	>300	>300	67	21	3
1w 1d	**ABS**	0	>300	>300	346	15	9
6d	**TGEA**	0	>300	>400	75	8	5
6d	**YPD**	0	>300	>400	161	85	9
6d	**DRBC**	0	>300	>400	396	144	14
6d	**MEAC**	0	>300	>400	403	39	7
C. 6d	**MRS**	0	>300	>300	187	17	7
1w 1d	**ABS**	0	>300	>300	390	92	68
6d	**TGEA**	1	>300	>300	207	8	3
6d	**YPD**	0	>300	>400	87	127	3
6d	**DRBC**	0	>300	>300	360	23	2
6d	**MEAC**	0	>300	>400	272	38	2
PIA SAMPLE 7 - AVERAGE							
6d	**MRS**	0	>300	>300	105,33	22,67	3,67
1w 1d	**ABS**	0	>300	>300	297,67	43,00	30,00
6d	**TGEA**	0	>300	>400	174,67	8,67	5,00
6d	**YPD**	0	>300	>400	195,33	80,00	9,33
6d	**DRBC**	0	>300	>400	294,00	68,00	9,33
6d	**MEAC**	0	>300	>400	324,67	31,67	7,33

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