

The effect of the fermented tea beverage kombucha on the gut microflora

A double-blind placebo-controlled study

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Popular science summary

The fermented tea drink kombucha has become an increasingly trendy drink during the past couple of years. Although it has historically been known for its health benefits in Asia, it has taken until recently for it to gain popularity in Western countries. Riding on the current health wave in society, kombucha is surrounded by numerous health claims, such as a high content of nutrients and beneficial fermentation products as well as being probiotic if consumed raw. Still little research has been done on the subject, meaning that few of these claims may be scientifically proven.

Tightening regulations have made it harder for food producers to place health claims on their product, including claims of probiotic effects. In order for a product to be classified as probiotic, it must contain a large amount of living bacteria that survive the digestive system and reach the colon. Even though some positive health effects can come from consumption of dead bacteria, living bacteria can in some cases colonize the colon or temporarily change the gut flora to the better. Probiotics have positive effects on the gut as well as the immune system, and has recently become a hot research topic as more and more interesting connections are found. Proving a probiotic effect of a food product is thus of high relevance for the producer.

This study investigated if the consumption of kombucha has an effect on the gut microbiota, and further if it depends on the content of living bacteria or is inherent even to a sterilized version of the drink. The study consisted of two parts. Firstly, the present species of bacteria and yeast in the kombucha culture were studied, as well as which are dominant and the content of living bacteria. Secondly a human study was performed to investigate the effect on the gut microflora. 42 participants were divided into three groups of which one consumed live kombucha; one sterilized kombucha with no living microorganisms; and one sterile water. The study took place over a period of three weeks. Fecal samples were taken before the start of consumption, the day after ended consumption and ten days after the ended consumption.

After studying the microbial communities of the fecal samples from the different groups, no significant effect of the kombucha could be found. There was no change in the composition of the gut flora for the group consuming either live or sterilized kombucha. It was also found that the kombucha used for the study was dominated by yeast, which has not been studied for its probiotic potential to the same extent that bacteria have. It also did not contain as many living microorganisms as are recommended as a probiotic dose.

A deeper analysis of the microbial community of the kombucha might give a better picture of it, and reveal a larger part of living bacteria. Many of the microbes that were found in the kombucha were hard to study in a laboratory environment, meaning the community may actually be different than what this study found. Previous studies have been able to identify a much larger number of bacteria. This may also increase the total number of microbes in the kombucha, although the results found in the study are consistent with the literature.

The data from the human trial was seemingly reliable as it showed clear correlations between the communities from one participant at different times, which was to be expected. The gut microbial community is relatively constant. This proves that the methods used were sensitive enough to provide good results. Also, there was a clear differentiation between the actual samples and the negative control samples, which

were used to verify the procedures. All in all, the methods for sample handling and data treatment seem to have been reliable enough to present the clear conclusion that there was no effect of treatment.

There is more work to be done in studying the community of kombucha as well as the drink's different health benefits. Despite the many, often positive, effects kombucha may have on the body; this study shows that altering the gut flora is not one of them. Nevertheless, it is a beverage that is enjoyed by many and many of the participants of the study experienced positive changes from drinking it such as decreased flatulence and improved stools. The many other health benefits of kombucha remain.

Abstract

Introduction

The fermented tea drink kombucha has gained large popularity over the past years. It has been praised for its nutritional content, organic acids and living community of beneficial bacteria. Multiple studies have been conducted on the effect of the nutrients and organic compounds on the body, but none on the effect of the microbial community on the human gut microbiota. That is thus the aim of this study.

Materials and methods

The study consisted of two parts. Firstly, the microbial community of kombucha was studied by culturing on an array of different media, with different pH and conditions. Colonies were then counted and large ones were randomly selected for sequencing.

The second part of the study consisted of a human trial, in which 42 participants consumed either living kombucha, sterilized kombucha or water during a period of three weeks. Fecal samples were collected after a washout period, prior to the trial, at the end of the three weeks and another ten days later. DNA from the samples was extracted, amplified and sequenced using Illumina sequencing.

Results

The kombucha used for the study was seemingly dominated by yeast, and contained 10^4 - 10^5 CFU/ml. No lactic acid bacteria or *Enterobacteriaceae* could be cultured. The data collected from the human study did not show an effect of treatment with living or sterilized kombucha. The results did show the composition of the microbial communities of the gut, even though they did not change significantly.

Discussion

The kombucha shows a low microbial content, below what is recommended as a probiotics dose. Yet the microbes present were hard to culture, and in many cases formed to small colonies to count. This implies that either the concentration is low, or that the result does not reflect the true microbial content due to a large part of the microbes being non-culturable, which has been the case in previous studies.

The data obtained from the human study seems sensitive and reliable enough to be able to draw conclusions from. There seem to have been some errors in the handling of the samples, although these are not believed to have affected the results.

Conclusion

The study found no effect of kombucha on the intestinal flora after three weeks of consumption. In order to improve the analysis of the kombucha itself, molecular methods should be used to analyze the community properly, and culturing conditions optimized to be able to get a better picture of the amount of living microbes.

Foreword and acknowledgements

This study was performed as a master thesis project at Lund University. The aim was to investigate the effect of kombucha on the human gut microbiota as well as how a study on probiotic effects of food is performed. The project intended to deepen the knowledge attained from five years of studies in the field of biotechnology and food, as well as provide practical laboratory practice.

Many people have contributed to the project, and aided in its success. Without their support it would not have been possible. A big thank you goes out to Roots of Malmö for producing such an interesting product to study, and also providing kombucha for the human trial. Special thanks to Nicholas Rosenstock at Roots, for all the knowledge, time and expertise you have contributed.

Another thank you goes to my supervisor Åsa Håkansson for inspiring the idea to the project and for your support during its progression. Also, thank you to Elisabeth Uhlig and Elin Oscarsson for your guidance in the lab.

Finally, thanks to all of the people who took time to participate in the study. Your contribution was deeply appreciated.

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Introduction

Aim

Over the past years the fermented tea beverage kombucha has gained large popularity. As the production includes the use of living microorganisms and the traditional version of the drink is never sterilized or pasteurized, it has been claimed that kombucha has a high content of bacteria (Marsh, O'Sullivan, Hill, Ross, & Cotter, 2014). Further more, it has been suggested that these microorganisms are beneficial to human health, thus being probiotic. Still, this has not been proven. The aim of the this study was to investigate if consumption of kombucha has an effect on the human gut microbiota, and further if the presumed effect depends on the content of living bacteria or is inherent even to sterilized kombucha. The hypothesis was that a difference should be seen in individuals consuming kombucha compared to a control group consuming water, and maybe even see a change in the group consuming sterilized kombucha due to the content of dead microbes. The goal is to gain deeper knowledge into the impact of this certain food product, containing living microorganisms, on the body as well as to see if it has probiotic potential.

This report gives a summarizing background of kombucha and previous studies of its microbial community, as well as a general overview of probiotics and methods for analyzing microorganisms in food. Following this, the method used during the project is described, both for the early examination of the kombucha culture and how the human study was performed. The main part of the report details the results of the study and discusses the conclusions.

Scope and limitation

The project was limited to the time span provided for a master thesis, being one term (January to June). This restricted the depth of the study, both for the culturing and isolation and the analysis of the human samples. Both fecal and saliva samples were collected, but only the fecal samples were analyzed due to time restriction. The fecal samples represent the gut microbiota, and since the gut flora is typically targeted in probiotic products and is a hot research topic this was deemed more interesting. During the project both bacterial and fungal communities from the samples were extracted, but only the bacterial were analyzed. This was because bacteria are commonly considered more interesting in terms of probiotic effect than yeast.

Background

Kombucha

The tradition of brewing kombucha goes far back in many Asian countries, especially China, where it has been made for more than 2000 years and is also known as tea fungus and Haipao (Marsh et al., 2014). Kombucha is made by fermenting sweetened black, and in some cases green, tea. The fermentation is performed by indigenous microorganisms entrapped in a natural biofilm that floats on the surface of the liquid. This biofilm is often referred to as a "pellicle". The pellicle houses multiple species of both yeast and bacteria living in symbiosis. It is made up of mostly cellulose produced by certain species of acetic acid bacteria (AAB) such as *Komagataeibacter xylinus* and can be considered an aerobic environment (Coton et al., 2017). The fermentation starts by different kinds of mainly osmotolerant fermentative species of yeast that hydrolyze the added sucrose into glucose and fructose. The yeast also

produces ethanol, carbon dioxide and organic acids. Acidotolerant species become more prominent later (Coton et al., 2017). Once the sucrose is broken down, species of mainly AAB start transforming the sugar into organic acids such as acetic acid and gluconic acid, as well as acetaldehyde. They also produce acetic acid from the ethanol (Coton et al., 2017; Marsh et al., 2014). At this stage there are also lactic acid bacteria (LAB) present and active, which produce lactic acid. This results in a final product that is rich in organic acids and carbon dioxide (Coton et al., 2017).

Kombucha, like many other fermented foods, have been found to contain complex communities of microorganisms. As the vessel remains open to the air during fermentation, the species composition is generally unstable, depending on the surrounding conditions, nutrition and environment. The kombucha community has been found to recruit other microorganisms, such as *Lactobacilli*, if present in the surrounding environment. The diversity of the community decreases over the time of the fermentation, and the finished drink is dominated by *Proteobacteria*, which often account for more than 90% of all bacteria present (Coton et al., 2017; Marsh et al., 2014). These are in turn dominated by the genera *Acetobacter* and *Gluconacetobacter*, the later greatly exceeding the first. A study using sequence-based analysis, rather than culture-based, found that *Gluconacetobacter* was the most prominent genus, accounting for >85% of all bacteria in most samples, whilst *Acetobacter* were <2% (Marsh et al., 2014). Among the AAB found in kombucha cultures the most dominant species are often *Acetobacter xylinum*, *Acetobacter aceti*, *Acetobacter pasteurianus*, *Komagataeibacter xylinus* and *Gluconobacter oxydans*. Lactic acid bacteria are present in much lower numbers, and sometimes not found at all (Coton et al., 2017). Newer studies have found more LAB being present, and that they increase later on in the fermentation (Marsh et al., 2014). The fore-mentioned study showed *Lactobacillus* in levels up to 30% of the total bacterial content. *Lactobacillus* has been found to increase the cellulose production of *Gluconacetobacter* as well as increase their growth (Marsh et al., 2014). One study found 84 different species of LAB in kombucha (Puspawati, 2016).

The yeast community in kombucha is complex and the species that dominate the culture change over time. The community generally includes species *Zygosaccharomyces*, *Dekkera*, *Saccharomyces*, *Candida*, *Hanseniaspora*, *Torulaspora*, *Torulopsis*, *Pichia*, *Saccharomycoides*, *Wallemia*, *Schizosaccharomyces* and *Lachancea* genera (Coton et al., 2017). The yeast species present seem to depend more on the fermentation environment and cultivation conditions than the bacterial community, which has been found to be more stable (Marsh et al., 2014; Reva et al., 2015). One study found that *Zygosaccharomyces* was the dominant genera at >95% of the liquid, with a greater diversity in the pellicle (Marsh et al., 2014).

The content of microorganisms in kombucha generally reaches 10^4 - 10^6 CFU/ml after 10 days of fermentation. At this point yeast is most commonly present in slightly higher numbers than the bacteria. Studies have also found that there are more microorganisms in the broth than in the pellicle. The diversity of microbes decreases during the fermentation, probably due to the very acidic conditions (pH 2.5) or lack of oxygen. The species present in the later stages of the fermentation are more acid tolerant (Marsh et al., 2014).

The gut microbiota

The human large intestine is colonized by a complex community of microorganisms, commonly referred to as the intestinal microflora. The gut contains approximately 10^{14} microbial cells and 400-500 different bacterial species (Plaza-Diaz, Gomez-Llorente, Fontana, & Gil, 2014). These microorganisms are essential for human health, as they contribute to the metabolism of food into nutrients and energy but are also important for the immune system.

The intestinal microflora can vary depending on age, gender and sickness or health (Marathe, Shetty, Lanjekar, Ranade, & Shouche, 2012).

The gut microbiota is dominated by strict anaerobes, and these outnumber the facultative anaerobes and aerobes by a magnitude of two to three. Among these, the phyla *Firmicutes* and *Bacteroidetes* dominate, although *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Fusobacteria*, and *Cyanobacteria* may also present but in much lower numbers (Marathe et al., 2012). Although this community can be composed of as many as hundreds of different bacterial species, it is believed that it is dominated to 99% by 30-40 species (Tannock, 2000). Among microorganisms present in the human gut are lactic acid bacteria, which despite being largely outnumbered by the obligate anaerobes are commonly found in fecal samples. The permanent content of *Lactobacillus* in the human intestinal tract varies between individuals, being always present in some and only transiently in some (Tannock, 2000). It has been found that the bacterial content of a fecal sample is representative of the intestinal flora, although it might not show the exact content.

Probiotics – a general overview

As people become more health-conscious, probiotics and symbiotic are becoming an increasingly important for the functional food market as a mean fill this new niche. This has given rise to an increased interest in probiotic foods. There is not a complete agreement as to what is considered a probiotic, but the commonly used definition is that of WHO stating that a probiotic is “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (WHO, 2002). There is increasing evidence showing that there is a positive correlation between the content of probiotics in functional food and other food products and good human health (Arévalo-Villena, Fernandez-Pacheco, Castillo, Bevilacqua, & Briones Pérez, 2018). The presence of probiotic strains in the human gut largely depends on environmental factors and they are usually only present during the period of consumption of a certain probiotic food (Tannock, 2000).

Lactic acid bacteria, primarily *Lactobacillus*, are commonly used in probiotic applications. Lactic acid bacteria also produce metabolites that can be beneficial to human health by for example inhibiting bacterial pathogens in the intestine, aiding digestion of lactose, lowering cholesterol levels, improving the immune system and by being anti-mutagenic and anti-carcinogenic (Puspawati, 2016).

Studies on probiotic properties of microorganisms have generally focused on lactic acid bacteria and bifidobacteria (Diosma, Romanin, Rey-Burusco, Londero, & Garrote, 2014). The probiotic characteristics of yeast have not been studied as thoroughly, although yeasts are commonly used in many foods and fermentation processes. The probiotic potential and properties of yeast are becoming increasingly relevant. Yeasts are commonly resistant to antibiotics, making it possible for them to persist in the gut even during antibiotic treatments (Arévalo-Villena et al., 2018). *Saccharomyces cerevisiae* var. *boulardii* is the most commonly used probiotic yeast strain, and is applied to treat gut disorders and to improve the state of the intestinal flora (Diosma et al., 2014). *S. cerevisiae* are more resistant to the conditions of the gastrointestinal system than non-*Saccharomyces* strains (Arévalo-Villena et al., 2018).

It has previously been believed that microorganisms must be alive in order to confer positive benefits on the host, which is required to be classified as probiotic. Recent studies have begun to prove this statement wrong by showing benefits from non-viable cells as well. Adams (Adams, 2010) presents what is called the probiotic paradox, that both dead and living cells can confer positive biological responses. Living bacteria can affect the gut flora and the immune response, whilst dead bacteria or components thereof can have an anti-inflammatory

effect on the intestinal system. Dead bacteria can stimulate proliferation of cells in the immune system and the secretion of multiple different substances from these cells, as well as switch on T-helper cells (Adams, 2010). In many cases, dead probiotic cells can lessen intestinal inflammation without the pro-inflammatory effect of some living cells.

Certain protective effects of probiotics are mediated by the bacterial DNA rather than by the cells adhering to the intestine or by their metabolites. Thus the effect can be seen from both living and dead cells, as long as the DNA is intact. Both living and dead cells can remove cholesterol, although living cells are able to remove more. This is because in addition to binding cholesterol to the cell surface, which can be done by dead and living cells alike, living cells can also assimilate the cholesterol (Adams, 2010).

Dead cells of different strains will have different effects on the body, just like different living cells do. For example, *Enterococcus faecalis*, which has in fact been developed into a commercial product in its non-viable form, claims to have immunostimulatory properties. It has been shown to stimulate IgA production as well as the production of certain antimicrobial peptides. Bifidobacteria, which are often used as probiotics in food products, can increase the production of TNF- α as well as IL-6. It is believed that this is due to interactions between the bacteria and the macrophages (Adams, 2010).

Kombucha as a probiotic

Commonly, probiotic foods are dairy products, but there is an growing demand for vegan options, as choosing a plant-based diet becomes increasingly common (Reva et al., 2015). Kombucha could comprise one such option. There is still much to be discovered about the symbiotic relationship between bacteria and yeast, as well as their effect on the human microbiota (Reva et al., 2015). Probiotic effects have not yet been proven to come from the microorganisms in kombucha (Marsh et al., 2014).

In order for a microorganism to have maximal impact on the gut flora they must survive the acidic conditions of the stomach, approximately pH 2, as well as the bile salts in the duodenum. One study found that out of 20 species of LAB isolated from kombucha, 15 species were resistant to low pH and 13 species were resistant to bile. The conditions under which the bacteria were grown (pH, bile salt content and time) were chosen to mimic the conditions in the body, being pH 2.0, a concentration of bile salt of 0.5% and 4 hours incubation. The acidic growth conditions decreased the all LAB populations tested, but without completely killing them, whilst the many isolates actually continued to grow in the presence of bile (Puspawati, 2016).

Culture based techniques

Culture-based analysis implies growing the microorganisms found in a sample externally in some kind of media, such as a liquid broth or agar plate. Once isolated, they are identified using morphological, physiological and cultural characteristics as well as metabolism (Nguyen, Nguyen, Nguyen, & Le, 2015). Culture-based techniques are labor intensive and time consuming, as there is a lot of manual work and time required for incubation. It also makes exact quantification difficult and favors species that are easily grown outside of their natural environment. The benefit of these methods is that they leave an isolate for further studies (Tedder et al., 1998).

The majority of all studies made on kombucha have used culture-based techniques, which propose a limit to which species can actually be discovered. These techniques exclude species that are hard to isolate and there is a large risk of misidentification based on phenotypic traits. These methods are also low-throughput, meaning the number of isolates that can be

investigated are limited (Marsh et al., 2014). Some sources propose that 88% of all species found in fecal samples are culturable, whilst others give much lower numbers. Whatever the exact number is there is a large portion of the intestinal community that cannot be cultured (Tannock, 2000). The same is true for the microorganisms in kombucha. Many of them are not culturable outside of the complex community from which they originate. Therefore, molecular methods such as next-generation sequencing are better suited to study the community (Reva et al., 2015).

Sequencing

Non-culture based technologies are referred to as molecular methods such as next-generation sequencing (NSG) are useful when studying the microbial communities in fermented foods, which are generally quite complex, as well as the phylogenetic diversity, composition and dynamic structural changes of these communities. It makes it possible to identify unculturable species. NSG can be used to investigate and predict the relationships between species in complex communities (Reva et al., 2015). Molecular methods have high sensitivity and high throughput (Tedder et al., 1998).

Materials and methods

Content of microorganisms

Culturing and microbial enumeration

To enumerate and then further isolate microorganisms present in the kombucha from the brand “Roots of Malmö”, serial dilutions were plated on different kinds of agar to promote growth of different microorganisms. The pH of the kombucha was measured using a pH electrode to be able to make agars with a similar acidity. Dilutions of the kombucha broth were cultured on tryptic soy agar (TSA) (Merck, Germany), both with its normal pH (approximately 5) and with the pH lowered to 3.6-4.1 using acetic acid, and incubated aerobically as well as anaerobically in 30°C for 3 days. Dilutions were also cultured on glucose agar (20 g/L glucose, 5 g/L peptone, 5 g/L yeast extract, 15 g/L agar, 1.15 g/L citric acid, 2.7 g/L disodium phosphate), with the pH lowered to 3.6-4.1 using acetic acid, and incubated aerobically in 30°C for 3 days. For the total count of LAB, dilutions were cultured on MRS agar (Merck, Germany), with pH 5.5, and Rogosa agar (Merck, Germany), and incubated anaerobically at 37°C for 3 days. For the total count of yeast, dilutions were cultured on malt agar (Merck, Germany), both with its normal pH and with the pH lowered to 3.6-4.1 using acetic acid, and incubated aerobically for 7 days at room temperature. To see if any *Enterobacteriaceae* were present in the kombucha, dilutions were cultured on Violet Red Bile Dextrose (VRBD) agar Merck, Germany) and incubated aerobically in 37°C for 24 hours. Dilutions were made in peptone water. Duplicates were made for each dilution and media type.

After incubation, plates with 30-300 colonies were counted.

Isolation

Sixteen colonies from each kind of agar were picked and cultured for isolation on the same type of agar, and incubated under the same conditions and time as earlier. After incubation one colony from each isolate was picked and added to 1 ml freezing medium. The samples were then frozen in -80°C until used for sequencing.

Extraction and PCR amplification of isolates

The frozen isolates were cultured on their corresponding agar or in broth and incubated for the same time as during previous culturing, as a final mean of isolation before PCR. Colonies from MRS and malt were cultured in broth and colonies from TSA and glucose agar on agar. After incubation, DNA-extraction was performed. For the plate-grown colonies, approximately half of a 10 µl loop of cell mass was added to 1 ml of MilliQ water together with extraction beads. For the microorganisms grown in broth, the broth was centrifuged and the supernatant discarded. Any remaining liquid was mixed with the pellet, and 100 µl of this was added to 1 ml of MilliQ water together with extraction beads. These were shaken for 30 minutes to lyse the cells before centrifuging for one minute.

As it was not known if the isolates were bacteria or yeast all samples were first run through a PCR using fungal primers and for the samples that did not give results, bacterial primers were used. For the identification of yeast isolates, the D1/D2 region of the 26S rRNA gene was amplified using the primers NL1 (5'- GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'- GGT CCG TGT TTC AAG ACG G-3') (Coton et al., 2017; Reva et al., 2015). 2,5 µl of supernatant from the extraction was added to 22,5 µl of master mix, containing 18,375 µl nuclease-free water, 2,5 µl TopTaq buffer, 0,5 µl of each primer, 0,5 µl dDNTP and 0,125 µl TopTaq polymerase.

For the identification of bacterial isolates S, 16S rRNA gene amplification was performed using the primers BSF8 (5'-AGA GTT TGA TCC TGG CTC AG-3') and BRR1541 (5'-AAG GAG GTG ATC CAG CCG CA-3') (Coton et al., 2017). 2,5 µl of supernatant from the extraction was added to 22,5 µl of master mix, containing 18,375 µl nuclease-free water, 2,5 µl TopTaq buffer, 0,5 µl of each primer, 0,5 µl dDNTP and 0,125 µl TopTaq polymerase.

All PCR amplification was run using the following conditions: 3 minutes initial denaturation at 94°C, 30 cycles of 1 minute denaturation at 94°C, 45 seconds annealing at 50°C, 2 minutes extension at 72°C, ending by 10 minutes final extension at 72°C. The samples were then chilled to 4°C. The amplifications were performed in an Eppendorf Mastercycler gradient.

The PCR samples were examined by gel electrophoresis on agarose gels to guarantee the quality of the PCR product. The gels were stained using GelRed for visualization of the result.

Sanger sequencing

All samples that produced clear bands after gel staining were sent away for Sanger sequencing. The resulting sequences were then analyzed and identified using the online resource BLAST.

Human trial

Study design and participants

This double blind, placebo-controlled study was designed to assess the effect of kombucha on the human gut flora (study registered at ClinicalTrials.gov). The study was carried out between March 2018 and May 2018.

For the study, 42 healthy individuals were chosen according to the following inclusion and exclusion criteria:

- The participants should be 18 years of age or older.
- The participants should not have been diagnosed with any gastrointestinal problems, such as IBD or IBS, or have any other known illnesses.
- The gender of the participants was not taken into consideration.
- All participants should live in the region of Skåne and have access to Malmö, Lund or Helsingborg.

The overall study protocol, informed consent form and information to participants were approved by the Ethics Board in Lund, Sweden. All subjects provided a written consent before commencing any part of the study.

The enrolled test subjects were randomly assigned to receive one of two kinds of kombucha (live or sterilized) or a control drink (water). Randomization was performed by three-treatment computer software. All randomization codes were kept secure until all data was analyzed. All participants as well as scientific personnel working with the study were blind to the group assignment. Independent persons performed the randomization and prepared the packaging and labeling of the samples.

All participants received a box with 24 identical bottles that were unlabeled, but of the same style as regular kombucha from the brand “Roots of Malmö”, who provided the kombucha for the study. All kombucha was brewed and prepared at “Roots of Malmö” three months prior to the study start, and stored cold. The live kombucha was unflavored and unpasteurized. The sterilized kombucha was treated by boiling by “Roots of Malmö” and contained no living organism according to lab tests (culturing on malt and glucose agar). This functioned as a placebo, and the participants assigned to this group did not know that they were not drinking living kombucha. This group was also used to study the effect of dead microbes. The third group was the control group, who were given colored water in bottles that were identical to the kombucha bottles. This group was told that they had received a probiotic enriched drink with the same bacterial content as kombucha but none of the flavoring byproducts.

All the participants began the study within a time interval of 2 days in order to minimize variation due to outside factors. A questionnaire and overview description of the study protocol was handed out at the start of the study. Before the intervention, all participants went through a two week washout period, during which they were not allowed to consume any foods with a high content of living organisms, such as yoghurt, probiotic drinks, fermented vegetables or other kombucha. On the final day of this control period fecal samples were collected.

During the test period that followed, the participants consumed 330 ml daily of their designated drink. The dietary restrictions from the washout period continued. The day after the 3-week test period, new saliva and fecal samples were collected. Each participant was asked to fill in a survey concerning his or her experiences during the trial. The final stage of the study was the posttest period of 10 days, where the participants continued to follow the same diet as during the washout period. On day 11 after the trial, final saliva and fecal

samples were collected. 10 days were chosen as this is the approximate turnover time for the cells in the intestine, meaning anything that had not colonized the colon would no longer remain.

All samples collected during the study were taken by the participants in their homes. These were then frozen at approximately -18°C and transported to a collection location. The samples were kept frozen until analysis.

Extraction

The extraction was performed using the Extract-N-Amp™ Plant PCR Kit (Sigma-Aldrich catalog no. XNAP2) and a protocol modified from the product information and that used by Videvall et al. (Videvall, Strandh, Engelbrecht, Cloete, & Cornwallis, 2017).

The fecal samples were thawed on ice. From these a 30-80 mg feces from each sample was collected using the tip of a sterile, disposable loop and added to 2 ml of MilliQ water. These were vortexed for 30 seconds, before 1 ml of the slurry from each was transferred to a new tube. 8-10 sterile glass beads (approximately 0,5-1 mm in diameter) were added to each sample and they were then shaken for 30 minutes. The samples were then centrifuged at $200\times g$ for 1 minute.

5 μl of the slurry was added to 20 μl of Extract-N-Amp™ Plant PCR Kit extraction buffer in a sterile tube. The tubes were vortexed and heated in a heating block at 95°C for 10 minutes and then centrifuged at $2500\times g$ for 5 minutes. 20 μl of Extract-N-Amp™ Plant PCR Kit Dilution buffer was added to each sample and they were then vortexed. The samples were frozen at -20° until direct PCR.

Amplification

For the amplification of DNA from the collected fecal samples 16S rRNA gene amplification was performed using the primers 341F (5'-CCT ACG GGN GGC WGC AG-3') and 805R (5'-GAC TAC HVG GGT ATC TAA TCC-3')(Herlemann et al., 2011) with Illumina specific barcodes attached (i5 and i7). Thermo Scientific Phire Hot Start II was used for the amplification. 1,5 μl of supernatant from the extraction was added to 23,5 μl of master mix, containing 12,5 μl nuclease-free water, 5 μl 5X Phire Reaction buffer (containing MgCl_2), 2 μl of each primer (10 μM), 1 μl Ultrapure™ BSA (20 mg/ml), 0,5 μl dNTP (10 mM) and 0,5 μl Phire Hot Start II polymerase, giving a final volume of 20 μl .

The reaction with bacterial primers was run using the following conditions: 30 seconds initial denaturation at 98°C , 33 cycles of 5 seconds denaturation at 98°C , 6 seconds annealing at 55°C , 10 seconds extension at 72°C , ending by 90 seconds final extension at 72°C . The samples were then chilled to 4°C . The amplifications were performed in an Eppendorf Mastercycler gradient.

For the amplification of DNA from the collected fecal samples 26S rRNA gene amplification was performed using the primers ITS7g (5'-GTG ART CAT CGA RTC TTT G-3')(Ihrmark et al., 2012) and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-3')(White, 1990) with Illumina specific barcodes attached (i5 and i7). 1,5 μl of supernatant from the extraction was added to 23,5 μl of master mix (same as for bacterial primers), giving a final volume of 20 μl .

The reaction with bacterial primers was run using the following conditions: 30 seconds initial denaturation at 98°C , 34 cycles of 5 seconds denaturation at 98°C , 6 seconds annealing at 55°C , 10 seconds extension at 72°C , ending by 90 seconds final extension at 72°C . The

samples were then chilled to 4°C. The amplifications were performed in an Eppendorf Mastercycler gradient.

The PCR samples were examined by gel electrophoresis on agarose gels to guarantee the quality of the PCR product. The gels were stained using GelRed for visualization of the result. The samples were kept frozen until further analysis.

One negative control consisting of purified water was analyzed together with each set of samples.

Illumina sequencing

Illumina sequencing and the following data analysis were not performed in person, but left to be done at another institution at the University. These steps will therefore not be described in the same depth as the previous.

Before sequencing, the PCR products from the amplification were taken through multiple purification and quality control steps. They were first purified to remove any remaining primers, primer dimers and salts using the Agencourt AMPure kit (Agencourt Bioscience Corporation, Beverly, MA, USA), which purifies using a magnetic bead-based separation. The purified samples were then quantified using PicoGreen ds DNA Quantification Kit (Molecular Probes, Eugene, OR, USA) on a FLUOstar OPTIMA (BMG LABTECH GmbH, Ortenberg, Germany). A second amplification in 8 cycles was then used to add Nextera (Illumina Inc., San Diego, CA, USA) sample barcodes for the Illumina. The purification and quantification steps were repeated before adding equal molar amounts of the samples to a final pool. Size and proper annealing of the samples was controlled before being submitted for sequencing on a MiSeq sequencer using the MiSeq Reagent Kit v3 chemistry (Illumina Inc., San Diego, CA, USA) at the Next-generation sequencing facility at Lund University, Lund, Sweden.

Analysis of sequencing data

The bacterial and fungal gene sequences obtained from the Illumina sequencing were clustered using USEARCH UCLUST fast algorithms at a 97% sequence similarity. This provided a set of operational taxonomic units (OTUs) based of the reads from the sequencing. All OTUs with less than one read in total from all samples (singletons) were removed. The reads were analyzed using a chimera checker to remove any bad sequences. To further reduce the dataset and make it easier to handle, all sequences that occurred in only one PCR-product were removed, as well as any OTUs that had less than ten reads.

The taxonomic identities of the OTUs were determined by the use of the Basic Local Alignment Tool (BLASTN program 2.2.25, blast.ncbi.nlm.nih.gov) against the HIT database. The database is composed of 2473 unique sequences from prokaryotic species-like groups obtained from human gut microbiome samples, which have been annotated and quality-checked (Ritari, Salojarvi, Lahti, & de Vos, 2015).

Any sequence with a 97% similarity to the top hit sequence in the database and at least 80% coverage of the sequence length were assigned to the matched genus and species. If the similarity was 94-96% only the found genus was assigned to the sequence. In the case that the similarity was 91-93% compared to the database, the family name was assigned to the sequence. If the similarity was 90% or below, the sequence was discarded. Rarefaction was performed to the median number of reads across all samples.

The abundance of the OTUs for each sample were summarized and presented in a matrix showing the rarefied relative abundance of species per sample. This was then used to compare

the community compositions between treatments. A non-metric multidimensional scaling (NMDS) was performed on the data set, creating a distance matrix based on the Bray Curtis similarity.

All statistics were run in the programming software R using the Vegan Stats package to perform a Permutational Multivariate Analysis of Variance Using Distance Matrices (PerMANOVA) analysis.

Symptoms questionnaire

At the end of the study, participants were asked to fill in a questionnaire consisting of 5 items, apart from when they had started and ended the trial and if they had deviated from the study protocol. These items were as follows: (i) change in fecal character, (ii) flatulence, (iii) abdominal pain, (iv) other changes, (v) smoking or use of snuff. For each item the participants answered yes or no, and were allowed to elaborate in text if necessary. This gave both quantitative data as well, as qualitative. The qualitative data was used only as a complement to the quantitative, in the cases where participants seemed to have experienced something particular.

The questionnaires were analyzed using a 1-way ANOVA-analysis to see if there was a significant difference in between the different study groups.

Results

Viable count

Table 1 shows the results from culturing dilutions of kombucha on an array of different kinds of agar.

No microorganisms grew on the VRBD-plates, implying that there were no *Enterobacteriaceae* present.

There was no growth on the TSA-plates with normal pH that were incubated aerobically. On the plates incubated anaerobically, there was growth, but the colonies were too small to count, giving the agar a misty look on the least diluted plates.

The bacteria grown on the Rogosa and MRS-plates did not become large or distinct enough to count after the first incubation. Thus there is no result for the number of lactic acid bacteria present in the kombucha. Still, bacteria from these plates were spread again on new plates twice, after which distinct colonies could be used for sequencing.

Table 1 shows that glucose-agar and TSA gave similar counts of microorganisms. There were approximately five times more microorganisms growing on the malt agar with pH 3.6-4.1, which was used to culture yeast, than on the glucose-agar and TSA. The count on the malt agar with normal pH was approximately ten times higher than that on the malt with lower pH. Based on these counts, the total amount of microorganisms can be approximated to 10^6 - 10^7 , although this does not take into account the anaerobic bacteria, which could not be counted. This number is based on the count from the malt agar, as it is possible that the same organisms grew on the other agars and these should not be counted double. With this found concentration of microorganisms, the number of bacteria in one bottle (330 ml) of kombucha can be approximated to 10^7 - 10^8 .

Table 1. Microbial count after culturing kombucha on a variety of agar types. CFU is given as the mean of two duplicates.

Agar type	pH	Conditions	Result	CFU/ml
VRBD	Normal	Aerobic	No growth	-
TSA	Normal	Aerobic	No growth	-
Rogosa	Normal	Anaerobic	Growth	Not enumerable
MRS	Normal	Anaerobic	Growth	Not enumerable
Glucose	Low, 3,6-4,1	Aerobic	Growth	19 500
TSA	Low, 3,6-4,1	Aerobic	Growth	16 000
Malt	Normal	Aerobic	Growth	7 100 00
Malt	Low, 3,6-4,1	Aerobic	Growth	91 000

Dominant species

The kombucha is assumed to be dominated by yeast rather than bacteria, as a majority of the samples were able to be amplified using fungal primers but not bacterial primers. Results from the sequencing of the isolates have not yet arrived.

Human study

There was not enough time within the scope of the study to analyze the data on the fungal communities. The results and the discussion on the data from the human study will therefore focus on the bacterial communities.

Out of the 42 participants who signed up for the study not all completed it. *Table 2* shows the number of participants in the different treatment groups who have handed in each sample.

Table 2. Number of participants handing in each of the three samples.

Group	Sample 1	Sample 2	Sample 3
Kombucha	14	14	13
Sterile kombucha	14	13	12
Water	10	10	10
Total	38	37	35

Overall community

The samples from the participants contained varying amounts of sequences, between 15 000 and 60 000 per sample. An analysis of the communities found within the different treatment groups and at different time points show that Clostridia and Bacteroida were the dominating classes in all communities. This is visualized in *Figure 1*. The five most abundant taxa were found to be *Faecalibacterium prausnitzii*, *Bacterioides vulgatus*, *Gemmiger formicilis*, *Oscillibacter valericigenes* and *Clostridium clariflavum*. These were present in 109-111 of the total 112 samples. The top taxon represents 5,4% of the total community (all samples). The eighteen most abundant taxa represent 50% of the total community. For more details on the dominant taxa, see *Appendix 1*.

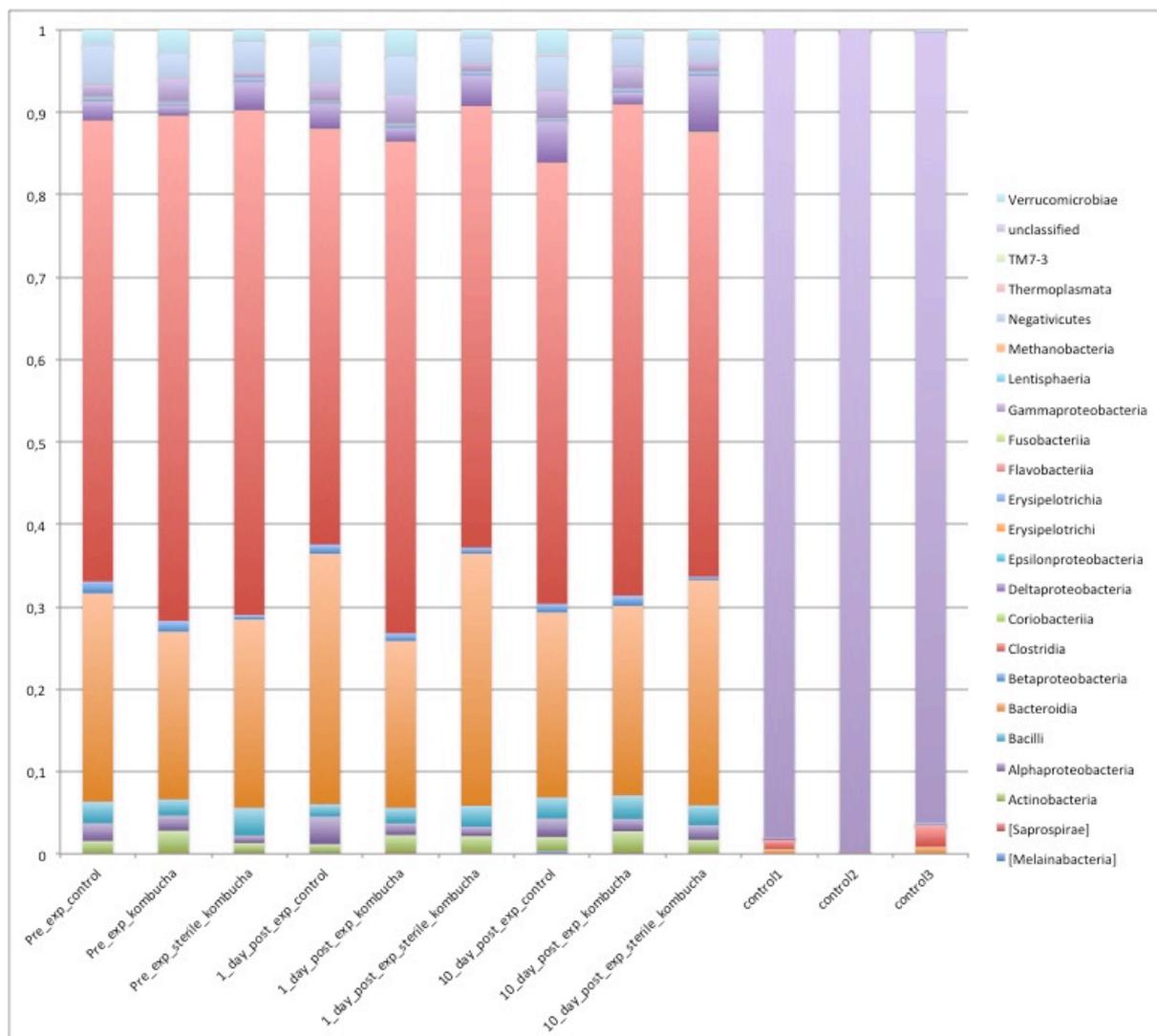


Figure 1. Community distribution between different classes of bacteria in all three treatments and all three time points, and additionally the content of the negative controls.

Data treatment

There was a reduction of reads and clusters/OTUs in the data set during the treatment of the data. This reduction is shown in Table 3.

Table 3. Sequencing metadata showing the number of clusters and reads and the reduction of data in the simplification steps performed.

Reducing step	Number of clusters/OTUs	Number of reads	Reduction of reads from previous step [%]	Reduction of reads from start [%]
Raw data	141 102 clusters	3 872 991	-	-
Elimination of singletons	50 052 clusters	3 781 941	2,4%	2,4%
Elimination of OTUs occurring in 1 sample or with less than 10 reads	11 645 clusters	3 649 865	3,5%	5,8%
Rarification	No change	3 082 770	15,5%	20,4%
Elimination of reads with less than 91% similarity to database	10 885 clusters	-	-	-
Elimination of OTUs corresponding to the same taxon	3855 OTUs	-	-	-

Change in communities

Figures 2-6 show the result of the human study after data treatment. The figures show the clustering of the intestinal communities from the participants, where the distance between points correlate to how similar or dissimilar they are. As can be seen in *Figure 2a*, all negative controls cluster separately from the sample data points. In order to see the separation between the community samples, the negatives had to be removed from the plot for greater separation. This is shown in *Figure 2b*, which reports that centroid ellipses representing a 95% confidence interval of the mean of the points within one treatment overlap, meaning there is no significant difference between treatment groups if looking over all time points.

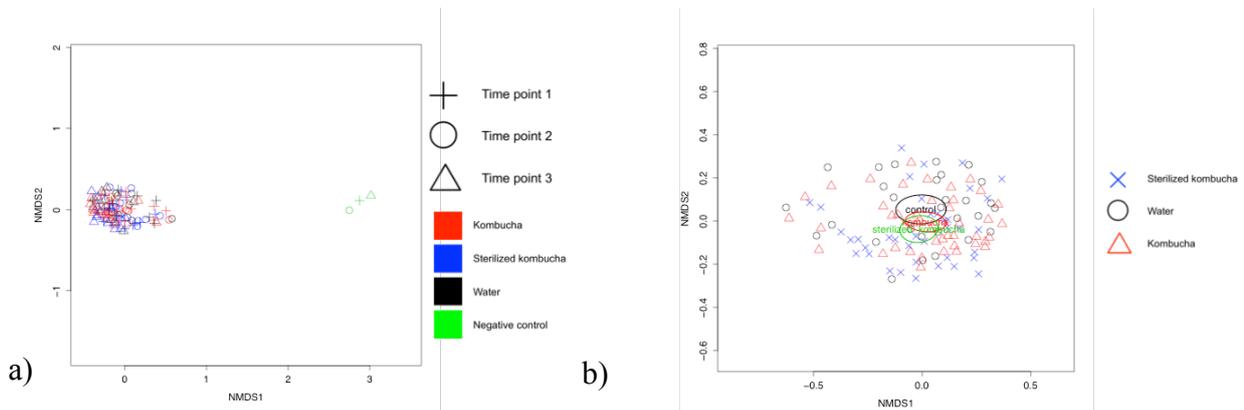


Figure 2. NMDS-plot based on the 3854 OTU data set, and the negative controls from each data set. Data points are grouped after treatment. Each point represents one participant.

a) All time points and all treatments together, including negative controls (marked in green).

b) All time points and all treatments together. The centroid ellipses show the 95% confidence interval of the mean of the data points in each treatment group. Ellipses for control (black), sterilized kombucha (green) and live kombucha (red) are marked.

A closer look at the communities at the separate time points reveals no significant change between the treatments during time the study. In the samples from before the start of treatment (time point 1) the communities in the sterile kombucha treatment group differ slightly from the other groups, as shown in *Figure 3a*. At time point 2 no significant difference could be observed, as seen in *Figure 3b* where the centroid ellipses of the means within each group overlap. Finally, *Figure 3c* shows time point 3 where the results are similar to time point 2 and no difference between the communities from different treatment groups could be observed.

NMDS-tables at 3854 OTU-level – Time point 1, 2 and 3

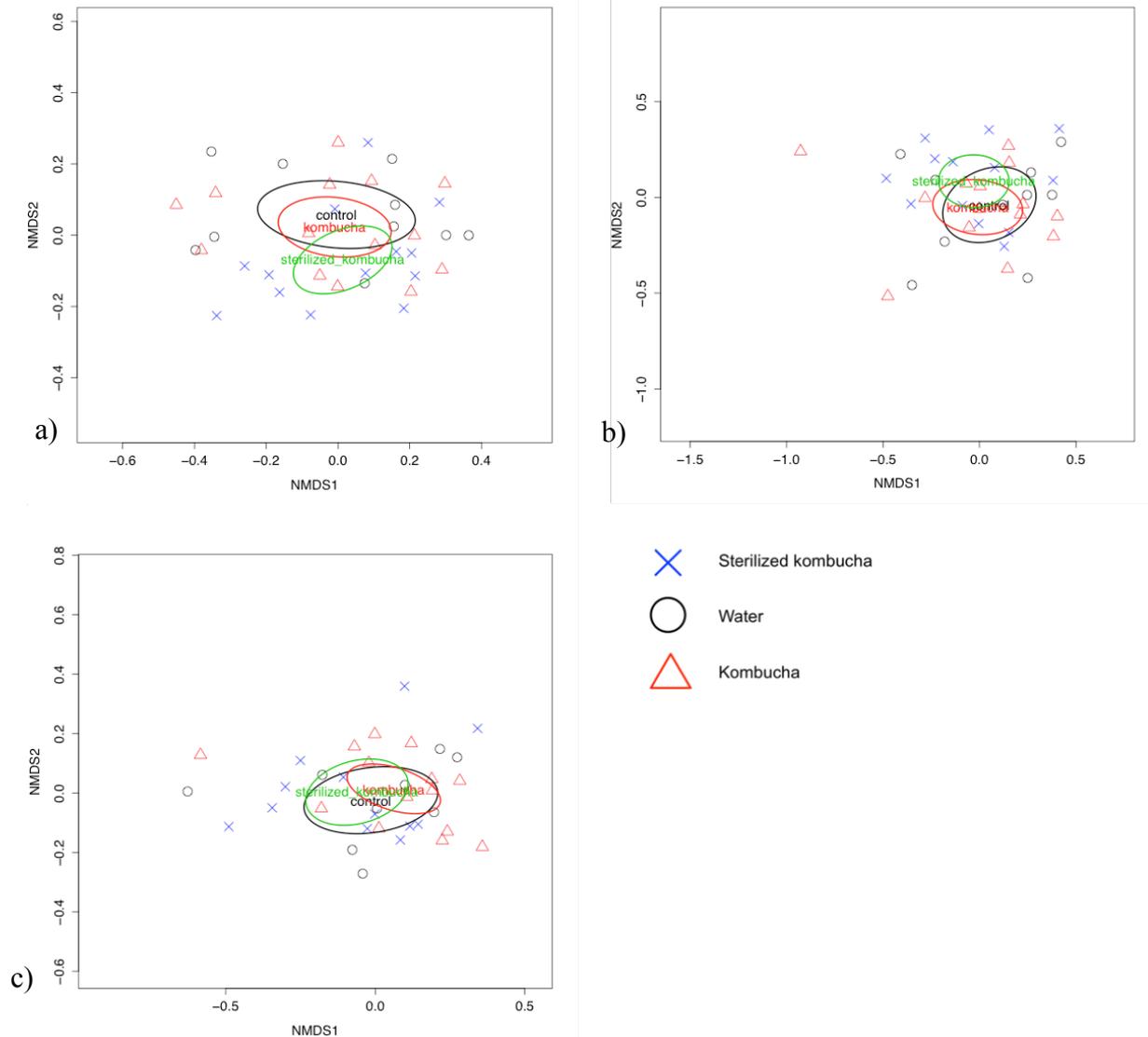


Figure 3. NMDS-plot showing all treatments at a given time point, based on the 3854 OTU data set. Data points are grouped after treatment. Each point represents one participant. The centroid ellipses show the 95% confidence interval of the mean of the data points in each treatment group. Ellipses for control (black), sterilized kombucha (green) and live kombucha (red) are marked.

a) Time point 1

b) Time point 2

c) Time point 3

By combining the data from the groups given live and sterilized kombucha and comparing them to the control group given water the effects of the kombucha itself could be examined. *Figure 4a* shows this comparison, but no significant difference can be observed between the communities from the two groups. A similar comparison was made between the participants consuming living microorganisms (live kombucha group) and the participants consuming drinks without microorganisms (sterile kombucha and water groups). The result, shown in *Figure 4b*, does not indicate a difference between the treatments.

NMDS-tables at 3854 OTU-level, time point 2

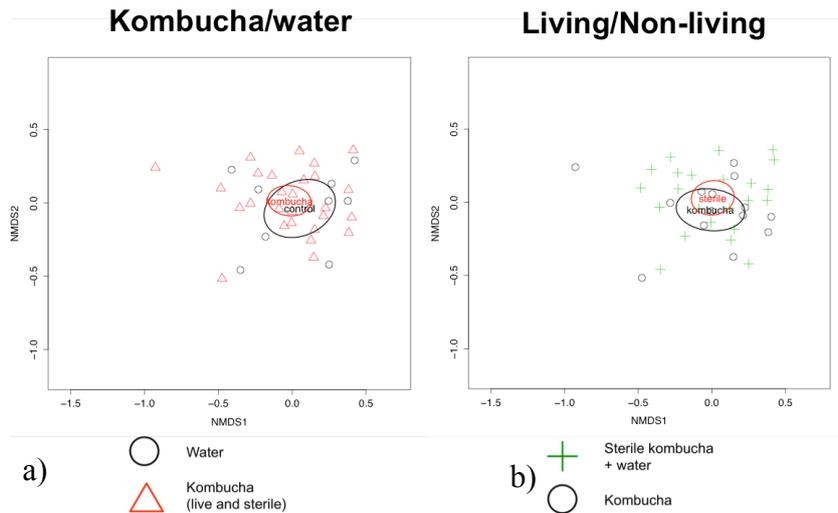


Figure 4. NMDS-plot based on the 3854 OTU data set. Each point represents one participant. The centroid ellipses show the 95% confidence interval of the mean of the data points in each treatment group.

a) Comparing kombucha treatments, live and sterilized kombucha (red ellipse), to the control group, water (black ellipse), at time point 2.

b) Comparing treatment with living microorganisms, live kombucha (black ellipse), to treatments with no living microorganisms, sterilized kombucha and water (red ellipse), at time point 2.

If considering the difference in communities among all participants over time, an overall difference can be seen if comparing time point 2 to time point 1 and 3. This is visualized in *Figure 5*, where communities from all treatments are clustered together based on time point.

NMDS-tables at 3854 OTU-level Comparing time points

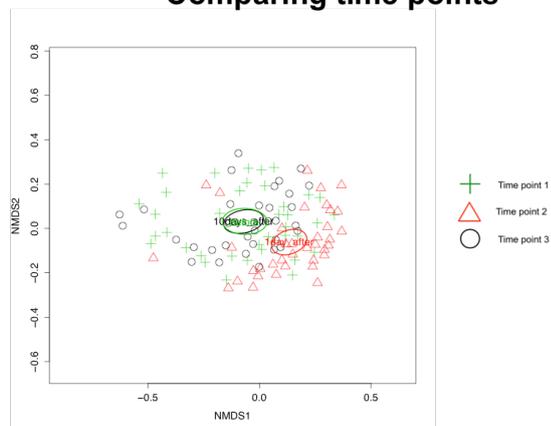


Figure 5. NMDS-plot comparing all treatments together at all three time points, based on the 3854 OTU data set. Data points are grouped after time point. Each point represents one participant. The centroid ellipses show the 95% confidence interval of the mean of the data points in each treatment group.

There was a consistent clustering within the samples from each participant in the study. Even though there was a slight change for most participants, the communities remained relatively constant, as seen in *Figure 6* where each participant is represented by a symbol. The symbols cluster together relatively close.

NMDS-table at 3854 OTU-level Comparing individuals

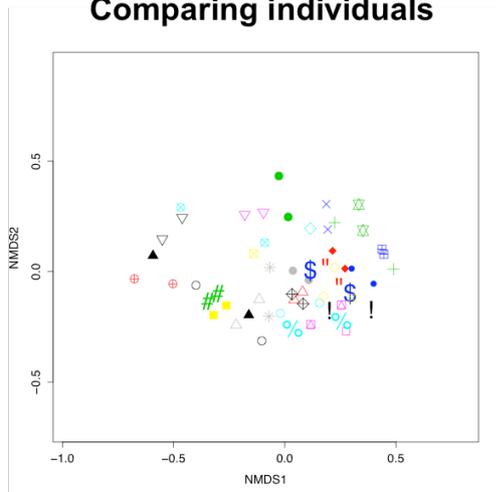


Figure 6. NMDS-plot showing all individual participants at each time point, based on the 3854 OTU data set. Each symbol represents one participant.

Dissimilarity testing

By comparing reads from within one treatment to the reads from another it was possible to find the mean differences between treatment groups and time point. The dissimilarity between time point 1 and time point 2 was found to be the same for all treatment groups, as shown in Figure 7.

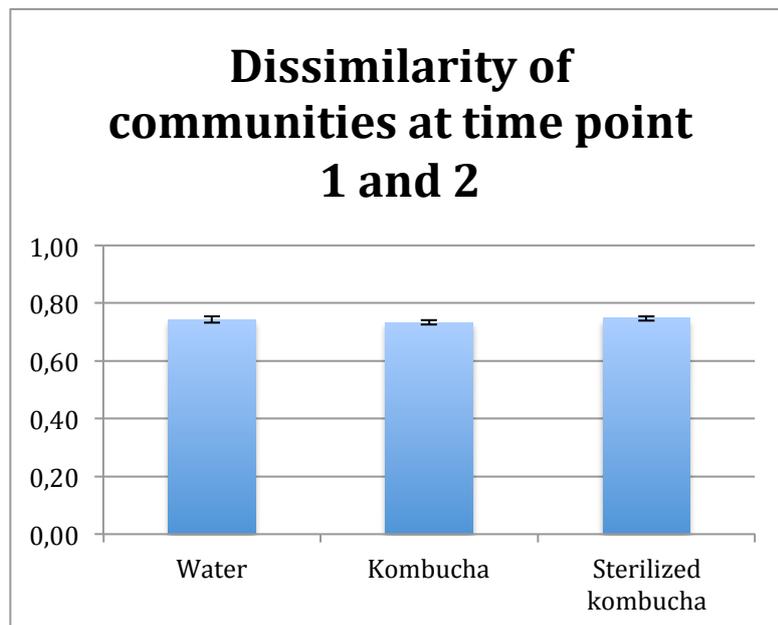


Figure 7. Dissimilarities in communities between time point 1 and 2 for all treatment groups, measured by comparing distance between data using Bray Curtis dissimilarities.

Statistical analysis

The PerMANOVA analysis showed no significant difference between treatments at any of the time points. A significant difference was found within the community when combining all three treatment groups and considering all time points. There was also a significant difference between the total community (all treatments combined) at time point 2 compared to the other time points. The results are consistent both for the 10 877 clusters level and the 3855 OTUs level. The results from the PerMANOVA analysis are shown in Table 4.

Table 4. Results for PerMANOVA analysis at different levels,

Level	Comparison	p-value	Significant difference within group	Significance level
10 887 clusters	All treatment groups, all time points	0,0003332	Yes	0
	Kombucha/control, all time points	0,0006664	Yes	0
	Living microbes/No living microbes, all time points	0,0006664	Yes	0
	All treatment groups, first time point	0,1123	No	-
	All treatment groups, second time point	0,4015	No	-
	All treatment groups, third time point	0,3715	No	-
3855 OTUs	All treatment groups, all time points	0,0006664	Yes	0
	Kombucha/control, all time points	0,02566	Yes	0,01
	Living microbes/No living microbes, all time points	0,001666	Yes	0,001
	All treatment groups, first time point	0,6011	No	-
	All treatment groups, second time point	0,6598	No	-
	All treatment groups, third time point	0,6448	No	-

Participant questionnaire

Figure 8 shows a summary of the results from the participant questionnaire. It should be noted that not all participants filled out the questionnaire. In the different groups, kombucha, placebo and control, 8/16, 8/15 and 6/11 participants filled out the survey.

The results do not show any statistically verifiable difference between the different study groups for any of the questions. ANOVA-analysis confirmed that there was no significant different.

Three out of the eight answering participants in the kombucha group reported that they experienced improved consistency of the feces/easier defecation. In the placebo group (sterile kombucha) one person out of eight reported firmer feces, one softer, one diarrhea and one softer that later normalized. In the control group (water) one person out of six reported firmer feces.

Other symptoms reported by the group consuming kombucha were decreased bloating, more energy, dizziness and a feeling of increased gastric acid, each by one participant.

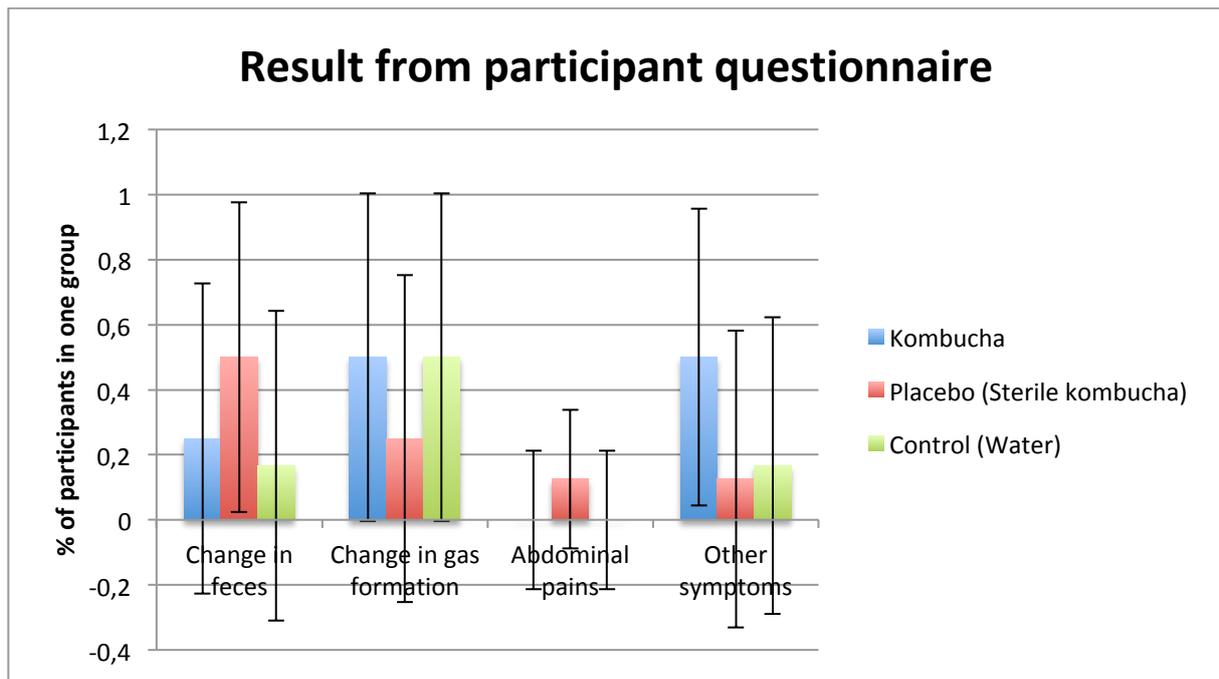


Figure 8. Result from participant questionnaire detailing how the participants felt during the study period. Not all participants handed in the questionnaire. Error bars are based on the pooled standard deviation.

Discussion

Viable count

The content of microorganisms in the kombucha is approximated to 10^6 - 10^7 CFU/ml based on the results from culturing on malt agar, which is higher than the bacterial counts presented in other studies (Marsh et al., 2014). That is probably explained by the fact that the content found in this study is largely based on the yeast content. The results show that there is much more yeast than bacteria present, which also corresponds to the literature which states that yeast is generally dominant in kombucha (Marsh et al., 2014). As the malt agar, which is selective for yeast, gave the highest counts one could assume that either the yeast could not grow properly on glucose agar and TSA or that there was higher competition on these plates, implying that some organisms outcompeted others leading to a lower count. Both yeast and bacteria should be able to grow on these plates, and the sequencing results show that they did.

The results show better growth on media with lower pH, in all cases but for malt agar. This is to be expected, as the microorganisms present at the end of the kombucha fermentation should be more acid tolerant (Coton et al., 2017). As for the malt agar, it seems that the yeast growing on this media prefer higher pH.

As no growth media seemed to enable growth of all microorganisms, no total count could be made. Since no media was found to be selective for only bacteria, no total bacterial count could be performed either. According to previous studies and literature, a large part of the bacteria present in the drink are not culturable (Marsh et al., 2014), implying that the number of living bacteria may actually be higher. A larger number of lactic acid bacteria have previously been found when using molecular methods instead of culturing (Marsh et al., 2014; Puspawati, 2016). The conclusion can be drawn that the lactic acid bacteria present in kombucha are hard to culture, which could explain the problems encountered with growing them for the study. Small colonies appeared on the Rogosa and MRS agars (selective for

lactobacilli), but they were not large enough to count. This might imply that the bacteria were not adapted to the culturing environment. Molecular methods might have given better results as to the content of lactic acid bacteria.

Since the pH of kombucha is low (approximately 3,3), no growth of *Enterobacteriaceae* was expected. These bacteria generally do not proliferate in such acidic environments. As there were no colonies on the selective VRBD agar plates, the results stand in line with the theory and it can be assumed that there are no harmful *Enterobacteriaceae* present in the kombucha. Kombucha is generally considered a safe product, due to the environment it provides, in which good microbes outcompete harmful ones.

In order to achieve more reliable results on the microbial content of kombucha, the method for culturing should have been further optimized to enable growth of more kinds of organisms. A suitable media for a total bacterial and fungal count should be developed as well as a protocol for culturing anaerobic bacteria. This was not successful in the study. No media were found to exclude yeast, and give a pure bacterial count. To get reliable data on the actual microbial content, and not only the culturable species, molecular methods would have to be used. There was not room for this within the scope of this study. Still, the problem with molecular methods is that they enumerate both living and dead microorganisms. Even though both of these can have positive health benefits, it would be preferable to know both the total amount and number of living microorganisms present.

Compared to the recommended daily dose of probiotics, which is 10^9 CFU, the amount of living microorganisms in one bottle of kombucha is too low, based on the culturing results. One bottle, which was the daily dose during the study, would only contain 10^7 - 10^8 living microorganisms, yeast included. The consumer would therefore have to drink a very large amount of kombucha each day in order to receive the suggested amount of living organisms, and even then not all microorganisms will be of the beneficial kind. Yet since not all organisms in kombucha are culturable, the microbial counts should not be trusted. Instead it is more interesting to look at the results from the human study to see if kombucha could be a suitable probiotic. As stated previously, the definition of probiotics only demands that there is a positive benefit from the product and not that there is a certain concentration of microbes.

Sequencing of isolates

The sequencing results were not done when this report was written. But based on the primers that annealed to the DNA from the isolates, yeast seems to be dominant and only one isolate used the bacterial primers. This is generally the case with kombucha, although there are probably more bacteria present than what was found in this study.

Sequencing of human intestinal communities

Regardless if there was a treatment effect or not it was expected that there should be a similarity in the community from one individual at different time points. The intestinal flora is relatively constant. The data points from different individuals tend to segregate and group together, as was shown in *Figure 6*, which confirms the previous expectations. This also indicates that the measures used to analyze the data were sensitive and accurate enough to show a representative picture of the microbial community.

The aim of the study was to examine if kombucha, living or dead, had an effect on the gut microbiota. For this to be the case a significant change in community should be visible from time point 1 (before start of consumption) to time point 2 (1 day after ended consumption). If there were a remaining effect from the treatment this change would persist even in the

samples from time point 3. In order to draw a conclusion on the effect, no change should be observed in the control group, which only consumed water.

All tests performed to analyze the data show a conclusive result; that there is no effect from either live or sterilized kombucha on the intestinal microbiota. Neither the clustering, the statistical analysis nor the table of differences, show a difference due to treatment between the three time points. The samples from the kombucha group should have more altered communities than the other, but there is no such structure to the data.

A significant difference was found between time point 2 and time point 1 and 3 for all treatments. As the change was present in both the control group and the two kombucha groups this does not prove an effect of the kombucha. Instead this overall change could be attributed to errors during the analysis of the set of samples from time point 2. The negative control from this time point contained a significant number of reads, but as shown in *Figure 1*, but was dominated by one single class. This indicates a contamination of the samples. Another theory could be that there was an effect from some regional change affecting all participants, but no such change is known of. Also, if that was the case, the change would probably be present in time point 3 as well, which it is not. The fact that the communities are so similar for most participants between time point 1 and 3, strengthens the belief that was an error in the samples from time point 2.

An error in the handling of the second set of samples, such as a contamination, could possibly have resulted in a change large enough to mask any possible results. Yet, as seen in *Figure 1*, bacteria that were barely present in the samples from this time point dominated the possibly contaminated negative control. Also, this negative control is completely separated from the samples in the NMDS tables, implying it has in fact not affected the samples. If this was the case, this negative control should have clustered closer to the samples than the other, clean, negative controls. Thus it is deemed that even though an error may have occurred in the second set of samples, it has not affect the ability to see any possible results.

As the participants' communities were different from the start, it is not surprising that some variation between the treatment groups. Without a difference between the different time points this does not prove anything. The PerMANOVA analysis strengthens the belief that there was an error in samples from time point 2, as it shows a statistical difference between time point 2 and the other time points for all treatments. There should not be a significant difference in the control group, as they have not changed anything.

Possible improvements and possible extensions of the study

It is believed that the database used for taxonomic assignment was reliable and suitable to use for the means of this study. There were less than 100 reads that had 90% or lower similarity to a match in the database. For further analysis the sequences could be BLASTed against a global 16S data set to see if the number of sequences not matched could be decreased.

One possible area for improvement of the data treatment is the clustering. As the analysis of the data is based on the clustering of the communities being reliable this should be done as robustly as possible. One way to improve clustering is to first align the sequences, which shows how different they are depending on how well they align. This knowledge can be used to cluster within phylogenetic groups, making the clustering more robust. Due to the time limit of this project and the size of the data set it was not possible to perform alignment.

The study could have been improved by having more replications, to minimize the effect of any errors in handling single samples or deviations from procedures by individual participants. Yet, even though some participants dropped out of the study or failed to hand in

samples, there was still a minimum of ten participants in each treatment group which should be enough to draw statistically sound conclusions.

An interesting extension of the study would have been to perform an indicator species analysis. Even if no overall difference could be found between the treatment groups there might have been a significant difference in certain species or genera. Such a small difference might not be enough to trigger a move in the clustering, but may still have an impact on the individual.

Participant questionnaire

Unfortunately, only approximately half of the participants in each group filled out the questionnaire detailing any observed symptoms during the study. As the total number of participants was low to start with this drastically lowers the reliability of the results from the survey. Although the results indicate that the kombucha group and the placebo group have experienced more symptoms during the study, there were too few participants to validate it statistically. It can be said that there were less comments on observed symptoms and changes from the control group, which was to be expected, although it cannot be statistically proven. Thus, no real conclusions can be drawn from the questionnaire, although the comments were interesting. This could have been improved if all participants would have completed the survey, which would have close to double the sample size. If the results from the entire group had followed the same trend as the ones that were received did, the increase in answers would probably have been enough to give a significant difference. Another improvement would be to have had more participants in total, thus increasing the sample size. If all of the participants would have handed in the questionnaire, conclusions might have been able to be drawn.

Conclusions

The study found no effect of kombucha on the intestinal flora after three weeks of consumption. The data obtained during the study is believed to be reliable, and the study design and data handling shows no immediate weaknesses. Despite that there seem to have been some errors in the handling of some samples, this does not seem to have had a significant effect on the results. The data is good enough to believe that kombucha, living or dead, does not change the gut microbial community over a period of three weeks.

The lack of effect could possibly be explained by the fact that the kombucha used in the study was found to have a much lower content of living microorganisms than is recommended as a probiotic dose. Still, there may have been a large part of the microbes in the kombucha that could not be cultured. There was found to be a large majority of yeast in the kombucha, and although some yeast has been proven to have an effect in the gut microbiota it has not been studied as deeply as bacteria. It is not known if the high content of yeast was representative of the kombucha community or if the results were skewed due to not being able to culture all bacteria properly.

No conclusion could be drawn concerning the dominant species in the kombucha the participants were given. This would require optimizing of the culturing methods and perhaps the complement of molecular methods to study the community.

Despite the fact that it is not statistically verifiable, several participants observed positive changes during the time of the study. Even without an effect on the microbial community of the gut, the other health benefits of kombucha remain.

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Appendix

Appendix 1. Dominant taxa in total community from all collected samples

Accumulated percentage of reads	Percentage of reads	Read abundance	Samples observed	Phylum	Class	Order	Family	Genus	Species
5,4%	5,4%	167268	110	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Faecalibacterium	Faecalibacterium prausnitzii
10,1%	4,7%	143412	109	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	Bacteroides vulgatus
14,3%	4,1%	127254	111	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Gemmiger	Gemmiger formicilis
18,0%	3,8%	115552	111	Firmicutes	Clostridia	Clostridiales	Oscillospiraceae	Oscillibacter	Oscillibacter valericigenes
21,5%	3,5%	107013	110	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	Clostridium clariflavum
24,8%	3,3%	101743	110	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified	Gemmiger formicilis
27,7%	2,8%	87342	54	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	Prevotella copri
30,3%	2,6%	79769	52	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio	Desulfovibrio fairfieldensis
32,9%	2,6%	79218	108	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclassified	Fusicatenibacter saccharivorans
35,4%	2,5%	78274	99	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclassified	Soleaferrea massiliensis
37,8%	2,4%	72295	110	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	Bacteroides xyliamisolvens
39,8%	2,0%	61974	102	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Alistipes	Alistipes putredinis
41,8%	2,0%	61276	110	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Roseburia	Roseburia hominis
43,7%	1,9%	59719	109	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	Bacteroides timonensis
45,5%	1,7%	53605	81	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia	Akkermansia muciniphila
47,2%	1,7%	53495	87	Firmicutes	Clostridia	Clostridiales	unclassified	unclassified	Christensenella minuta
48,9%	1,7%	52512	109	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Eubacterium	Eubacterium rectale
50,5%	1,5%	47361	45	Firmicutes	Negativicutes	Selenomonadales	Veillonellaceae	Dialister	Dialister succinatiphilus