

**MICROORGANISMS PRESENT IN SOUTHERN
BRAZILIAN HANDCRAFTED KOMBUCHA**

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Highlights: (1) The kombucha showed a high amount of yeast in original and flavored beverage. (2) The highest concentration of the evaluated microorganisms was found in the homogenized form. (3) It is suggested to lightly homogenize the beverage before consumption.

PRE-PROOF

(as accepted)

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ABSTRACT

Kombucha is a fermented beverage produced by a symbiotic culture of microbiologically active bacteria and yeast (SCOBY) with properties that are reportedly beneficial to health. A descriptive study was conducted with kombuchas produced on a small scale in Porto Alegre/RS. Samples were collected to isolate microorganisms present in the kombucha, as well as in the ingredients used (SCOBY and green tea). The beverages were analyzed in their original and flavored versions, with and without homogenization, in triplicate, totaling 12 samples. It was found that kombucha exhibited a high yeast count, with a significant difference in the beverage depending on homogenization, both in the homogenized original version (7.39 ± 6.46 log CFU/mL vs. non-homogenized 6.46 ± 5.39 log CFU/mL, $p < 0.0065$) and in the homogenized flavored version (7.54 ± 6.81 log CFU/mL vs. non-homogenized 7.20 ± 6.04 log CFU/mL, $p < 0.049$). The SCOBY presented a total yeast count of 6.78 ± 6.68 log CFU/mL, and no microbial growth was observed in the green tea. In conclusion, the analyzed kombucha exhibited a high yeast content in both the original and flavored versions, with a higher concentration of these microorganisms in the homogenized form, suggesting the importance of mild homogenization of the beverage before consumption.

Keywords: *Camellia sinensis*, Yeast, Fermented Beverages.

INTRODUCTION

Kombucha is a beverage obtained through the infusion of *Camellia Sinensis* leaves and fermentation from a symbiotic culture of bacteria and yeast (SCOBY)¹. Mildly sparkling and with a slightly sweet acidity, kombucha is the beverage with the highest estimated growth in the market². Recognized as a product containing bioactive compounds due to the presence of probiotic microorganisms and polyphenols, kombucha has also begun to be produced on an industrial scale, with various flavors available^{3,4}. It has been reported that the consumption of foods containing live microorganisms is directly related to several health benefits, such as gut health and strengthening of the immune system⁵. When the balance of the microbiota and immune system is disrupted, it can lead to health issues⁶. Among these health issues are the

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antagonistic action against pathogenic microorganisms, competitive adherence to the mucosa and intestinal epithelium, increased production of the intestinal mucus layer—reducing gut permeability—and modulation of the gastrointestinal immune system. These findings support the link between microbiota imbalance and the development of obesity and other metabolic diseases. Indeed, some diseases are accompanied by a shift in microbial composition, leading to an imbalance between beneficial and potentially pathogenic bacteria^{7,8}.

In this context, healthy and balanced eating habits from a nutritional point of view have a significant impact on the population's health, as well as on subsequent related health issues. In this regard, kombucha emerges as a healthy beverage, as it contains microorganisms, phenolic compounds, various vitamins, and minerals that may promote health benefits^{7,9}. However, studies on the quantification and identification of microorganisms present in kombucha are scarce. Thus, the present study aimed to determine the total amount of microorganisms in a commercially produced original and flavored kombucha from the state of Rio Grande do Sul, Brazil.

MATERIAL AND METHODS

A descriptive study was conducted with kombucha products produced on a small scale by a company in Porto Alegre/RS. Two types of kombucha (original and flavored) were included in the study, as well as some of the ingredients used in its preparation, including the SCOBY and green tea. The commercialized beverages' samples, as well as the ingredients, were acquired directly from the company.

INFORMATION ABOUT KOMBUCHA PRODUCTION

According to the data provided by the kombucha company, the beverage is prepared with filtered water, green tea (of the Japanese variety *Camellia Sinensis*), organic sugar, SCOBY (aprox. 50g/L), and 10% of the starter liquid, which were all weighed and measured precisely. The utensils used were made of Inox or glass, and there is ambient temperature control throughout the whole production process (between 25 and 29°C). After the first fermentation, the pH is measured (which cannot be higher than 4,5), and the sugar is measured with a refractometer. After the second fermentation, the beverage is kept under a pH of 3,5 with

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a Brix index of approximately 5. For the flavored beverages, fruit, spices or herbs are added. After bottling, the product is stored and kept between 1°C and 7°C.

For the present study, the products, as well as all ingredients, packaging, and caps used in the production process, were provided by the company for analysis control.

DATA GATHERING

After the production and bottling of the beverage batch, the company sent the kombucha bottles, a fraction of the SCOBY, and the green tea used in the preparation to the Immunology and Microbiology Laboratory at PUCRS, all properly stored for transport. The analyses were conducted in triplicate, totaling 12 bottles (6 bottles of natural kombucha: 3 homogenized and 3 non-homogenized; 6 bottles of flavored kombucha: 3 homogenized and 3 non-homogenized). The study investigated the effect of homogenization to verify differences in microorganism quantification in the beverage when mixed with the usual residue at the bottom of the bottle (Figure 1).

Figure 1- Illustrative image of the residue deposited at the bottom of the bottle.



Source: authors

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Microbiological analysis

For microbiological analyses, 25mL of each bottle of kombucha, 25g of green tea and 25g of the SCOBY were diluted in 225mL of 0.1% peptone water, followed by serial dilutions (10^{-2} to 10^{-8}). It is worth noting that for the homogenized samples, the mixing of the residue with the beverage was performed using the same collection pipette.

The samples were subjected to total yeast count and total count of mesophilic aerobic microorganisms.

Total yeast count

For yeast counting, the surface plating method was used with successive decimal dilutions of the samples in YPD Agar (2% glucose, 2% peptone, and 1% yeast extract, supplemented with 2% agar) containing chloramphenicol at a concentration of 0.1 mg/mL. The plates were incubated at a temperature of $25 \pm 1^\circ\text{C}$ for 5 days for total yeast counting. All samples were plated in triplicate for each dilution.

Total Count of Mesophilic Aerobic Microorganisms

For the total mesophilic count, the pour plate method was used with successive decimal dilutions of the samples in Plate Count Agar (PCA). The plates were incubated at a temperature of $36 \pm 1^\circ\text{C}$ for 48 hours to determine the total count of mesophilic bacteria. The results were expressed as Colony-Forming Units per milliliter (CFU/mL). All samples were plated in triplicate for each dilution.

Statistical Analysis

The results were expressed as mean and standard deviation using Microsoft Office Excel®. For variance analysis, a paired t-test was performed between samples according to the homogenization of each type of beverage (original and flavored). The significance level adopted was 5% ($p < 0.05$).

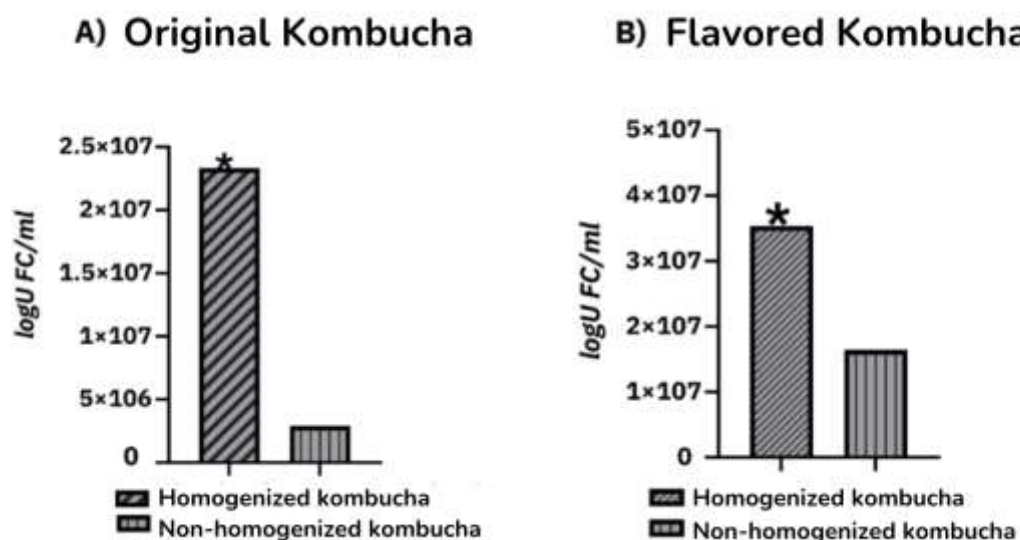
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RESULTS

Three samples of each beverage (original and flavored) were analyzed, totalling 6 samples. Each sample was examined in both homogenized and non-homogenized form. Figure 2 presents the analysis of yeast content according to the type of kombucha and its homogenization status.

Figure 2 - Total Yeast Count in Original and Flavored Kombucha, Homogenized or Not.

Note: Graphs A and B use a logarithmic scale for paired samples.



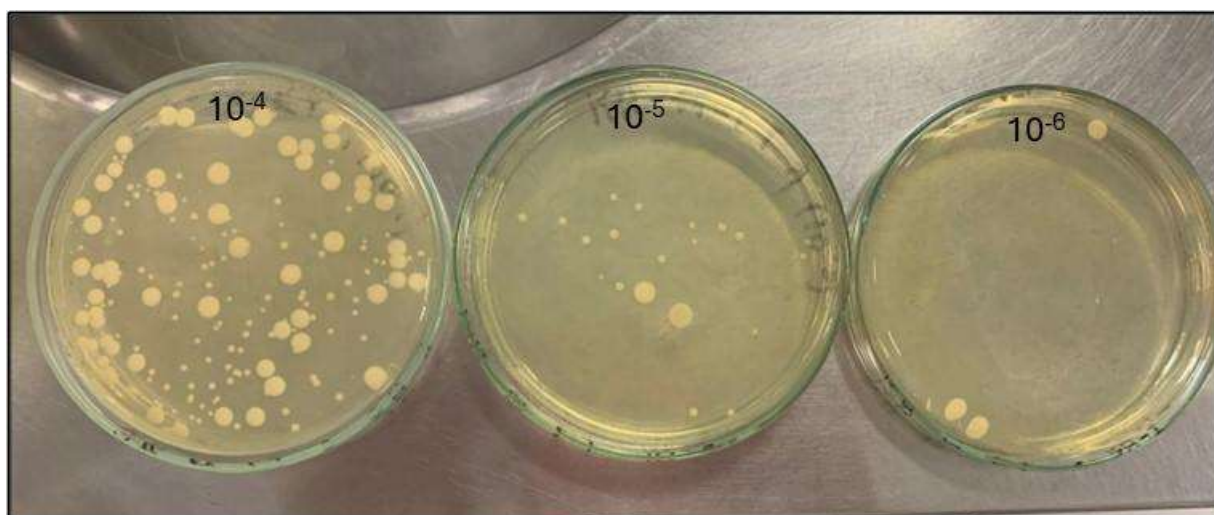
Source: authors

A significant difference in total yeast content was observed with the homogenization of the beverage in both original and flavored kombucha. Furthermore, when comparing the original and flavored beverages, the flavored kombucha exhibited a higher yeast concentration than the original, in both homogenized and non-homogenized forms.

Figure 3 illustrates the yeast quantification using the surface plating method from successive decimal dilutions of kombucha samples in the Laboratory of Immunology and Microbiology at the Pontifical Catholic University of Rio Grande do Sul (PUCRS).

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Figure 3 - Yeast Growth Using the Surface Plating Method from Successive Decimal Dilutions of Kombucha Samples.



Source: authors

The SCOBY analysis showed an average yeast count of 6.78 ± 6.68 log CFU/mL. On the other hand, no microbial growth was observed in the analyses of green tea.

Regarding the total count of mesophilic aerobic microorganisms, it was not possible to quantify the bacteria present in the beverage due to the analysis methodology used, which revealed a predominance of yeasts in the chosen dilutions for counting. However, bacteria were observed in the lower dilutions, though their colonies, along with the yeast colonies, were too numerous to count.

DISCUSSION

The study of microorganisms in food is widely recognized in scientific literature due to its impact on microbiota. In this context, kombucha has attracted research interest due to its classification as a "living" beverage. According to Normative Instruction No. 41/2019 from the Ministry of Agriculture, Livestock, and Supply (MAPA), kombucha is defined as a "beverage

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obtained through aerobic respiration and anaerobic fermentation of a must derived from the infusion or extract of *Camellia sinensis* and sugars by a symbiotic culture of microbiologically active bacteria and yeast (SCOBY),¹ which forms a floating cellulose biofilm¹¹. Currently, kombucha is one of the most prominent fermented beverages, available in a wide variety of homemade and industrially produced options. Additionally, these products offer an extensive range of flavors, often incorporating fruits, herbs, and/or spices^{3,2}.

The present study investigated yeast levels and mesophilic aerobic microorganisms present in the beverage, revealing high yeast concentrations in both the original and flavored versions, as expected. Yeasts are classified as fungi and are known to ferment sugars, producing alcohol and carbon dioxide. They are also rich in proteins, carbohydrates, B vitamins, and minerals^{1,3}. These microorganisms are considered beneficial for gut health and may positively impact the immune system^{5,14}, highlighting the potential of this beverage.

In this study, kombucha was homogenized due to the observation of residue at the bottom of the containers, a factor not yet analyzed in prior research. A significant difference in yeast concentration was observed with liquid homogenization in both types of kombucha, supporting the hypothesis that this residue could enhance the beverage's beneficial microorganisms when homogenized. This finding suggests that a slight homogenization of the beverage before consumption may be advisable.

The scientific literature on kombucha is expanding, yet most studies focus on the beverage or the SCOBY in isolation, neglecting the contribution of the ingredients used in its fermentation process. This study aimed to assess the yeast and mesophilic aerobic microorganism populations present in both the kombucha beverage and its ingredients, particularly the SCOBY and green tea, as these may influence microbial growth dynamics and the subsequent results. These factors are critical for a comprehensive understanding of kombucha fermentation. However, the analysis did not include the evaluation of coliforms in the water used for production or the presence of *Salmonella* and *Escherichia coli* in the final beverage, as the production facility adheres to the standards set by Ordinance No. 888/2021¹⁵ and Normative Instruction No. 161/2022 from the Ministry of Health^{1,6}, ensuring compliance with established safety regulations.

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The term SCOBY, coined by Len Porzio in the 1990s, refers to a biofilm resulting from the symbiotic association of yeasts and acetic acid bacteria responsible for fermenting kombucha¹. This microbiological diversity influences the nutritional properties of the beverage, as it can vary depending on geographical location and production factors such as tea type, sugar concentration, temperature, and fermentation time. These variables quantitatively and qualitatively affect SCOBY development and composition, given that it is a living organism^{10,7}. As a result, each SCOBY—and consequently each kombucha—will have distinct characteristics, potentially leading to different microorganism concentrations and nutritional quality.

In this study, the analysis of ingredients, including the SCOBY and green tea, confirmed the presence of yeasts only in the SCOBY, as expected. The traditional culture method used in this study did not allow for the quantification of mesophilic aerobic microorganisms in the beverages or the SCOBY due to yeast dominance, preventing bacterial measurement. In contrast, the analyzed green tea did not exhibit microbial growth, demonstrating the quality and safety of the material. While recent studies have employed genetic mapping and DNA characterization methodologies^{2,18,19}, these techniques remain a high-cost alternative for microorganism quantification and identification.

Kombucha has gained popularity amongst researchers and consumers due to its probiotic characteristics²⁰ and potential health benefits²¹. Brazilian legislation defines a probiotic as any live microorganism that, when administered in adequate amounts, confers health benefits to the individual. The Collegiate Board Resolution No. 241, issued on July 26, 2018²², specifies that the health benefit associated with probiotic use must be clearly identified and supported by evidence. Criteria for classifying a microorganism as probiotic include non-pathogenicity, survival through the gastrointestinal tract, demonstrated positive effects in human studies, cholesterol reduction capability, host specificity, acid production, and acid resistance, among others^{1,18,23}.

Kaashyap et al.¹⁹ identified seven genera of commercially recognized probiotics: *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, *Streptococcus*, *Enterococcus*, *Escherichia*, and *Bacillus*. In a study by Suhre et al.², taxonomic characterization of kombucha from six

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different Brazilian brands identified *Komagataeibacter*, *Gluconobacter*, *Acetobacter peroxydans*, *Liquorilactobacillus ghanensis*, *Oenococcus oeni*, and the predominant fungal class *Saccharomycetales*. A 2019 review²⁴ highlighted that high-throughput sequencing analyses demonstrated that, after fermentation, the predominant yeasts in kombucha belong to the *Candida* and *Zygosaccharomyces* genera, while the most abundant bacterial genera include *Komagataeibacter*, *Lyngbya*, *Gluconobacter*, *Lactobacillus*, and *Bifidobacteria*.

In Brazil, there is no official regulation regarding the necessary microorganism concentration for a food or beverage to be considered probiotic. The concentration and diversity of microorganisms in some products vary, and little is known about specific strains, emphasizing the need for further research. Although kombucha is often marketed as a health-promoting beverage, human clinical studies remain limited^{2,3,4}. Despite its growing popularity due to its supposed nutritional benefits, its effects on the microbiome and overall health remain unclear due to a lack of human studies.

FINAL CONSIDERATIONS

Based on the findings, the analyzed kombucha sample exhibited a high yeast concentration in both the original and flavored version, with an even higher concentration when homogenized. This suggests the importance of lightly homogenizing the beverage before consumption.

Additionally, there is a clear need for further studies on kombucha's microbiological properties and its functional effects on human health.

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Giulia Bacim de Araujo e Silva: Curadoria de dados, Investigação, Metodologia, Validação de dados e experimentos, Design da apresentação de dados, Redação do manuscrito original, Redação - revisão e edição.

Marina Borba Tósca: Curadoria de dados, Investigação, Metodologia, Validação de dados e experimentos, Design da apresentação de dados, Redação do manuscrito original e Redação - revisão e edição.

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