fermentation

Communication

Is It Possible to Brew Non-Alcoholic Kombucha? Brazilian Scenario after Restrictive Legislation

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Abstract: Kombucha is a traditional fermented beverage produced via the fermentation of a sweetened *Camellia sinensis* infusion added to a symbiotic culture of bacteria and yeast (SCOBY). During fermentation, a high level of ethanol can be produced as a yeast metabolite that can reach values above the legal limits for non-alcoholic beverages. In 2021, Brazil made the world's first kombucha-specific legislation to label beverages containing up to 0.50% ABV (alcohol by volume) as non-alcoholic. Headspace gas chromatography was used to quantify ethanol in 12 kombucha samples from different brands 12 months before and after the legislation was implemented. Before the legislation was implemented, 92% of the samples showed ethanol concentrations above 0.50% ABV, ranging from 0.47% to 3.56% ABV. One year later, an analysis of the same 12 kombucha brands showed that 67% of the samples were non-compliant with the new legislation, ranging from 0.10% to 2.40% ABV. The formation of ethanol during kombucha fermentation is a multivariate problem. Inoculum usually differs between sources, and the types and amounts of sugar and fruits, and the tea infusion percentage can also impact the final product. These parameters vary among producers. Some efforts to help kombucha producers achieve a more controlled and consistent production process are needed to ensure that commercially available kombuchas are properly non-alcoholic beverages and safe to consume.

Keywords: traditional fermented food; ethanol; alcoholic fermentation; *Camellia sinensis*

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1. Introduction

Kombucha is a traditional fermented beverage made via the fermentation of a sugared infusion of *Camellia Sinensis* by a cellulosic symbiotic consortium of yeasts and a bacterial biofilm under aerobic conditions [1]. It is a slightly acidic and carbonated beverage, and its fermentation can avoid the growth of pathogens and sustain a stable microbiological environment, producing a range of metabolites with potential health benefits [2].

Since 2016, the global market for kombucha has been consistently growing; in 2019, it was valued at around USD 1.84 billion [3]. By the end of 2021, Brazil was heading the artisanal kombucha market in Latin America [4]. The artisanal technics used to produce Brazilian commercial kombucha promote a wide variety of beverages in terms of composition because of the little control manufacturers hold over the processes [1,5–8].

The kombucha fermentation process creates bioactive peptides and biogenic amines. This process also converts phenolic compounds into biologically active compounds while reducing anti-nutrients [9]. Its composition varies depending on various components and concentrations; the ethanol concentration can reach values greater than the legal limits for non-alcoholic beverages. Regarding this concern, Talebi et al. (2017) analyzed the alcohol content of 18 kombuchas sold on the US market. They found that 100% of the samples had an ethanol content above the local limit of 0.50% ABV (alcohol by volume). Jang et al. (2021) analyzed 684 kombuchas in the Canadian market and found that 31.5% of the samples were non-compliant with the Canadian British Columbian regulatory ethanol concentration [8,10].

In July 2021, Brazil's MAPA (Ministerio da Agricultura, Agropecuaria e Abastecimento) released the Normative Instruction number 41 [11], which regulates the physical–chemical parameters of kombucha and set the alcohol levels for the kombucha to be sold as a non-alcoholic beverage. It is important to note that there are certain standards of identity and quality that the production of kombucha must adhere to. These standards include an alcoholic content under 0.5% for non-alcoholic beverages; an alcoholic content between 0.5 and 1.5% ABV for alcoholic beverage ratings; the use of drinking water, plant species used as infusions, sugars, and SCOBYs in production; and the allowance of food additives and technology aids authorized by MAPA, as well as juices, fruit pulp, plant extract, spices, honey, and natural aromatics [9,11]. Normative Instruction number 41 was the world's first specific kombucha legislation to guarantee that when labeled as a non-alcoholic beverage, kombucha does not exceed 0.50% ABV [11]. As the kombucha market is emerging, and due to the novelty of this legislation, the Ministry of Agriculture, Livestock and Supply provided 365 days for the market to comply [11]. This work evaluated the ethanol content in commercial kombuchas available in São Paulo City. We quantified the ethanol content of 12 kombucha bottles of different brands before the legislation was implemented via a headspace-GC-FID and found that 91.7% of the samples contained ethanol at a level above 0.50% ABV. One year later, we analyzed the same 12 kombucha brands after the legislation became valid to verify its effect on the market.

2. Materials and Methods

2.1. Sampling

This study analyzed twelve different brands of Kombucha purchased at a local market. The samples were transported in thermally insulated boxes to the laboratory and stored under refrigeration at 4 °C until sample preparation. The samples were analyzed using a headspace-GC-FID analytical method that meets the AOAC INTERNATIONAL requirements for determining the ethanol content in kombucha. The samples were bought in March 2021 and April 2022. In brief, 10 mL of each sample was homogenized and centrifuged at 2790× *g* for 10 min before the supernatant was collected and frozen at −20 °C until the determination of the ethanol content [12].

2.2. Ethanol Determination

The kombucha samples were subjected to a thorough analysis of their ethanol concentrations utilizing gas chromatography. The process involved the addition of a 200 µL sample of the kombucha to a 10 mL headspace vial containing 1.8 mL of a 2-propanol aqueous solution (0.6 g/L), which served as an internal standard (>99% purity; Sigma-Aldrich, St. Louis, EUA). The mixture was then incubated, and a 500 µL headspace aliquot was manually sampled in which the vials were sealed with a rubber cap and an aluminum crimp seal and incubated for 30 min at 70 °C without agitation before they were injected into the gas chromatography system (Hewlett-Packard system model 6890, Little Falls, DE, USA), which was equipped with a flame ionization detector (FID) and a Poraplot-Q fused-silica capillary column (10 m × 0.32 mm i.d. × 0.5 µm film thickness) (Agilent Technologies, Santa Clara, CA, USA) for the quantitation of ethanol. Each sample was analyzed in triplicate to ensure the utmost accuracy. The GC instrument parameters were as follows: an injector temperature of 220 °C, a detector temperature of 280 °C, and an oven temperature held isothermally at 150 °C for the entire 5 min run. The injection mode was split with a split ratio of 50:1, and the flow rate of the hydrogen carrier gas was 1.8 mL/min. The injection was carried out manually using a 500 µL "gas-tight" siring (Hamilton, OH, USA). The acquired data were processed via Agilent's HPCHEM software (Hewlett Packard, Santa Clara, CA, USA).

To calculate the ethanol concentrations of the kombucha samples, a twofold serial dilution of the 10 g/L top standard was performed four times to prepare a standard curve. After the dilution, the ethanol concentrations were 10 g/L, 5 g/L, 2.5 g/L, and 1 g/L. The internal standard (IS) used was 2-propanol (0.6 g/L). Quality controls of 2 concentrations (5 and 10 g/L) were also employed to determine precision and bias.

The reagents were acquired from Sigma-Aldrich (>99% purity; St. Louis, MO, USA) [11].

This approach provided a comprehensive understanding of the results, which revealed the exact concentrations of ethanol in the Kombucha samples. The entire process was conducted with the highest level of precision and accuracy, ensuring that the final results were reliable and trustworthy.

2.3. Statistical Analysis

Statistical analyses were performed using Student's t-distribution with N = 3 to calculate the 95% confidence interval for the ethanol content obtained for each sample. The analyses were conducted using Prism GraphPad Software, Version 10 (Dotmatics, Boston, MA, USA).

3. Results

In the round of ethanol determination before the Brazilian legislation was implemented, the kombucha bottles presented ethanol contents ranging from 0.40% to 3.56% ABV, and 11 of the 12 bottles had an ethanol content above 0.50% ABV. Only one kombucha bottle presented with an ethanol content which did not contain an ABV value of 0.50% or above within a confidence interval of 95%, (Figure 1).

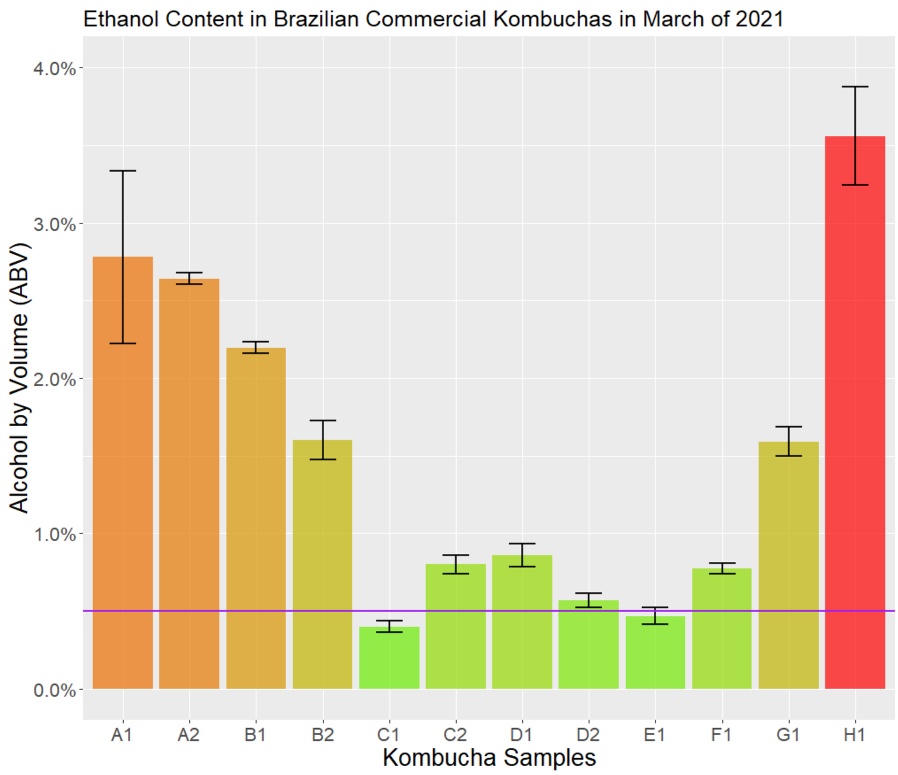

Figure 1. The ethanol content of commercial kombucha samples was analyzed in March of 2021 before legislation. The error bars indicate a confidence interval of 95% around each mean value. The horizontal purple line indicates 0.50% ABV (the current Brazilian legal limit). Kombucha samples from the same manufacturers are labeled with the same letter; the number indicates different flavors.

According to Figure 2, during the round of ethanol determination following the implementation of the Brazilian legislation, it was found that the kombucha bottles had ethanol contents that varied from 0.11% to 2.40% ABV. Out of the twelve bottles tested, eight had an ethanol content above 0.50% ABV.

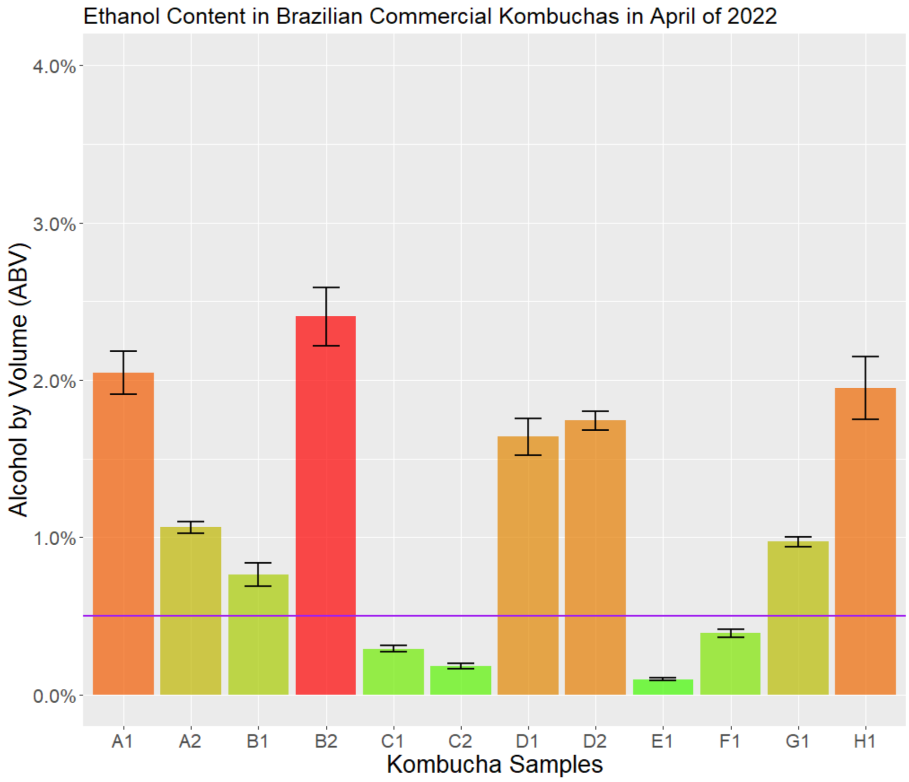

Figure 2. After legislation, the ethanol content in the commercial kombucha samples was analyzed in April 2022. The error bars indicate a confidence interval of 95% around each mean value. The horizontal purple line indicates 0.50% ABV (the current Brazilian legal limit). Kombucha samples from the same manufacturers are labeled with the same letter; the number indicates different flavors.

4. Discussion

In March 2021, before the Brazilian legislation was implemented, 97% of the kombucha bottles on the market presented an ethanol content greater than 0.50% ABV. Only one of the twelve kombucha bottles presented an ethanol content which was not equal to or greater than an ABV of 0.50%within a confidence interval of 95%. These results indicate the need for the proposed legislation, as most manufacturers produced kombuchas without ethanol concentration control. As can be seen, 91.7% of the samples were above 0.50% ABV, and one sample reached 3.56% ABV, with an ethanol content higher than a light beer (0.5% < ABV < 2.0%) [13]. Only sample C1 was below the 0.50% ABV value. Despite sample E1 having its mean value below the limit, the confidence interval contains values above 0.50% (Figure 1).

After the Brazilian legislation was implemented [10], in April 2022, 66.7% of the kombucha bottles on the market were noncompliant with the Brazilian legislation, presenting ethanol contents greater than 0.50% ABV.

As with any fermentation product, the composition of kombucha is affected by many parameters, such as the microbiological composition of the inoculum [6,14], tea type [15,16], carbon source [17], fermentation time [12,18,19], temperature [20,21], and the geometry of the fermentation vessel [22]. Controlling the concentration of the chemical components is a multivariate problem. Figure 3 shows a simplified scheme of sugar consumption and the formation of the primary metabolites in kombucha symbiosis. Ethanol is mainly formed via the action of yeasts. Then, acetic acid bacteria oxidize it into acetic acid, which will induce the production of ethanol by the yeasts [22]. The ethanol concentration is mediated by this symbiotic relation and all the factors impacting the microorganisms' metabolisms.

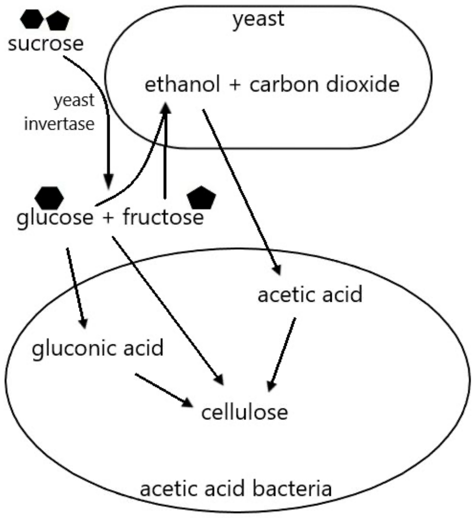

Figure 3. Sucrose's principal metabolic products via the action of the yeast and acetic acid bacteria present in kombucha.

Due to the microbiological variability among the inocula used by manufacturers [6,23], the kombucha producers should screen most of the variables in the production of ethanol for each SCOBY and substrate used to develop optimized models for producing kombucha with an ABV value under 0.5%. Most kombucha producers use the backslopping technique to introduce the inoculum into the fermentation process. This technique consists of adding part of the previously fermented batch as the starter for the new batch to be fermented. The kinetics of the microorganisms can change from batch to batch; even when maintaining the same fermentation parameters, differences in the ethanol content can be observed [24]. Therefore, monitoring ethanol during fermentation should be considered a quality control procedure.

Some attempts to control the microbial population in kombucha and then control the production of metabolites were made by Li et al. (2022) with a synthetic microbial community made from screened yeasts and bacteria from traditional kombucha with the desired characteristics to improve the sensorial attributes of the beverage [24]. Nguyen et al. (2015) also studied the ratio of co-cultured yeast and bacteria isolated from kombucha to optimize the production of glucuronic acid [25]; these approaches could be used to find optimal combinations of yeast and acetic acid bacteria to minimize ethanol production while maintaining a high content of the target metabolites that are of sensorial and health interest.

Our results showed no pattern in the ethanol content among the producers, probably because of the use of particular conditions for the above-listed parameters in the production process. Regarding producers, the results showed that only three (C, E, and F) of the eight brands complied with the ethanol legal limit. It can be noted that for some producers, such as "A", "B", and "C", there was a significant difference ($p < 0.05$) between their flavors analyzed, but for "D", the different flavors did not show a significant difference in ethanol content ($p < 0.05$). The variations observed in the flavors from a particular brand can be attributed to the fact that several manufacturers offer an unfiltered and unpasteurized type of kombucha that contains live yeast cells. Consequently, the ethanol composition can alter during storage depending on the type of flavoring agent used. Talebi et al. (2017) verified an increase in ethanol content even in refrigerated bottles [8]. The alcoholic fermentation of residual sugars from the initial fermentation or flavoring ingredients like fruit juices between kombucha flavors of the same producer seems challenging to control. Furthermore, it is plausible that the resemblances in the ethanol concentrations among the kombucha flavors of the "C" and "D" brands can be attributed to the extraction of viable yeast cells from the beverage or the utilization of flavoring agents that lack fermentable sugars.

This scenario must concern both kombucha manufacturers and regulators as we can see the difficulty kombucha producers have in conforming the products already on the market to the new legislation. More knowledge about kombucha fermentation is needed. However, also it is crucial to recognize that each producer works with different consortiums of yeast and bacteria. Sometimes, these producers do not know the specific content of microorganisms in their SCOBY, challenging the ethanol control content among them. The initiative of the Natural Health and Food Products Research Group from the Canadian BCIT to test a significant number of commercially available kombuchas and produce public documents about controlling ethanol in kombucha production [26] and the proper legislation in Brazil were pioneering approaches that could be excellent strategies for other countries to follow, improving the diagnostic scenario and helping kombucha manufactures achieve the demanded legal limits to support a growing market, providing safe products to the consumer.

5. Conclusions

These findings highlight the need for the improved regulation and monitoring of kombucha production to ensure compliance with the legal ethanol content limits. Producers should consider implementing quality control procedures to monitor ethanol levels during fermentation and storage. Additionally, optimizing the microbial community used for fermentation could lead to improved control over the production of metabolites and reduce the ethanol content. Further research on the impacts of different parameters on the composition of kombucha and strategies for controlling ethanol production would be beneficial for the industry and consumers alike.

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References

1. Laureys, D.; Britton, S.J.; De Clippeleer, J. Kombucha Tea Fermentation: A Review. *J. Am. Soc. Brew. Chem.* **2020**, *78*, 165–174. [CrossRef]
2. Martínez Leal, J.; Valenzuela Suárez, L.; Jayabalan, R.; Huerta Oros, J.; Escalante-Aburto, A. A review on health benefits of kombucha nutritional compounds and metabolites. *CyTA-J. Food* **2018**, *16*, 390–399. [CrossRef]
3. Kombucha Market Size, Share & COVID-19 Impact Analysis, By Type (Natural and Flavored), Distribution Channel (Supermarkets/Hypermarkets, Convenience Stores, Health Stores, and Online Retail), and Regional Forecast, 2020–2027. Available online: https://www.fortunebusinessinsights.com/industry-reports/kombucha-market-100230 (accessed on 1 May 2022).
4. Latin America Kombucha Market Analysis by Types (Bacteria, Yeast, Mould, Others), by Flavours (Herbs & Spices, Citrus, Berries, Apple, Coconut & Mangoes, Flowers, Others) and by Region Latin America (Brazil, Argentina, Mexico)–Regional Industry Size, Share, Growth, Trends, Competitive Analysis and Forecast Report 2022 to 2027. Available online: https://www.marketdataforecast.com/market-reports/la-kombucha-market (accessed on 1 May 2022).
5. Chakravorty, S.; Bhattacharya, S.; Chatzinotas, A.; Chakraborty, W.; Bhattacharya, D.; Gachhui, R. Kombucha tea fermentation: Microbial and biochemical dynamics. *Int. J. Food Microbiol.* **2016**, *220*, 63–72. [CrossRef] [PubMed]
6. Chen, C.; Liu, B. Changes in major component of tea fungus metabolites during prolonged fermentation. *J. Appl. Microbiol.* **2000**, *89*, 834–839. [CrossRef] [PubMed]

7. Coton, M.; Pawtowski, A.; Taminiau, B.; Burgaud, G.; Deniel, F.; Coulloumme-Labarthe, L.; Fall, A.; Daube, G.; Coton, E. Unraveling microbial ecology of industrial-scale Kombucha fermentations by metabarcoding and culture-based methods. *FEMS Microbiol. Ecol.* **2017**, *93*, fix048. [CrossRef] [PubMed]
8. Talebi, M.; Frink, L.A.; Patil, R.A.; Armstrong, D.W. Examination of the Varied and Changing Ethanol Content of Commercial Kombucha Products. *Food Anal. Methods* **2017**, *10*, 4062–4067. [CrossRef]
9. Oliveira, Í.A.C.L.d.; Rolim, V.A.d.O.; Gaspar, R.P.L.; Rossini, D.Q.; de Souza, R.; Bogsan, C.S.B. The Technological Perspectives of Kombucha and Its Implications for Production. *Fermentation* **2022**, *8*, 185. [CrossRef]
10. Jang, S.S.; McINTYRE, L.; Chan, M.; Brown, P.N.; Finley, J.; Chen, S.X. Ethanol concentration of kombucha teas in British Columbia, Canada. *J. Food Prot.* **2021**, *84*, 1878–1883. [CrossRef]
11. BRASIL. Ministério da Agricultura, Pecuária e Abastecimento/Gabinete da Ministra. Instrução Normativa n° 41, de 17 de Setembro de 2019. Estabelece o Padrão de Identidade e Qualidade da Kombucha em todo o território Nacional. Diário Oficial da União, Brasília, 18 de Setembro de 2019. Seção 1. Available online: https://www.in.gov.br/en/web/dou/-/instrucao-normativa-n-41-de-17-de-setembro-de-2019-216803534 (accessed on 24 August 2023).
12. Ebersole, B.; Liu, Y.; Schmidt, R.; Eckert, M.; Brown, P.N. Determination of ethanol in kombucha products: Single-laboratory validation, first action 2016.12. *J. AOAC Int.* **2017**, *100*, 732–736. [CrossRef]
13. BRASIL, Ministério da Agricultura, Pecuária e Abastecimento/Gabinete da Ministra. Instrução Normativa n° 65, de DE 10 DE DEZEMBRO DE 2019. Estabelece os Padrões de Identidade e Qualidade Para os Produtos de Cervejaria. Diário Oficial da União, Brasília, DE 10 DE DEZEMBRO DE 2019. Seção 1. Available online: https://www.in.gov.br/en/web/dou/-/instrucao-normativa-n-65-de-10-de-dezembro-de-2019-232666262 (accessed on 24 August 2023).
14. Chu, S.C.; Chen, C. Effects of origins and fermentation time on the antioxidant activities of kombucha. *Food Chem.* **2006**, *98*, 502–507. [CrossRef]
15. Jakubczyk, K.; Kałduńska, J.; Kochman, J.; Janda, K. Chemical profile and antioxidant activity of the kombucha beverage derived from white, green, black and red tea. *Antioxidants* **2020**, *9*, 447. [CrossRef]
16. Kaewkod, T.; Bovonsombut, S.; Tragoolpua, Y. Efficacy of kombucha obtained from green, oolong, and black teas on inhibition of pathogenic bacteria, antioxidation, and toxicity on colorectal cancer cell line. *Microorganisms* **2019**, *7*, 700. [CrossRef] [PubMed]
17. Malbaša, R.; Lončar, E.; Djurić, M. Comparison of the products of Kombucha fermentation on sucrose and molasses. *Food Chem.* **2008**, *106*, 1039–1045. [CrossRef]
18. Gramza-Michałowska, A.; Kulczyński, B.; Xindi, Y.; Gumienna, M. Research on the effect of culture time on the kombucha tea beverage? s antiradical capacity and sensory value. *Acta Sci. Pol. Technol. Aliment.* **2016**, *15*, 447–457. [CrossRef]
19. Lee, S.P.; Kim, C.S. Characterization of Kombucha beverages fermented with various teas and tea fungus. *Prev. Nutr. Food Sci.* **2000**, *5*, 165–169.
20. De Filippis, F.; Troise, A.D.; Vitaglione, P.; Ercolini, D. Different temperatures select distinctive acetic acid bacteria species and promotes organic acids production during Kombucha tea fermentation. *Food Microbiol.* **2018**, *73*, 11–16. [CrossRef]
21. Lončar, E.; Djurić, M.; Malbaša, R.; Kolarov, L.J.; Klašnja, M. Influence of working conditions upon kombucha conducted fermentation of black tea. *Food Bioprod. Process.* **2006**, *84*, 186–192. [CrossRef]
22. Cvetković, D.; Markov, S.; Djurić, M.; Savić, D.; Velićanski, A. Specific interfacial area as a key variable in scaling-up Kombucha fermentation. *J. Food Eng.* **2008**, *85*, 387–392. [CrossRef]
23. Harrison, K.; Curtin, C. Microbial composition of SCOBY starter cultures used by commercial kombucha brewers in North America. *Microorganisms* **2021**, *9*, 1060. [CrossRef]
24. Li, R.; Xu, Y.; Chen, J.; Wang, F.; Zou, C.; Yin, J. Enhancing the proportion of gluconic acid with a microbial community reconstruction method to improve the taste quality of kombucha. *LWT* **2022**, *155*, 112937. [CrossRef]
25. Nguyen, N.K.; Nguyen, P.B.; Nguyen, H.T.; Le, P.H. Screening the optimal ratio of symbiosis between isolated yeast and acetic acid bacteria strain from traditional kombucha for high-level production of glucuronic acid. *LWT-Food Sci. Technol.* **2015**, *64*, 1149–1155. [CrossRef]
26. Kombucha–A Reference Page for Manufactures and Stakeholders. Available online: https://www.bcit.ca/applied-research/natural-health-food-products/our-projects/kombucha/ (accessed on 7 June 2022).