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Characterisation of chemical, microbial and sensory profiles of commercial kombuchas

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ABSTRACT

The kombucha market is a fast-growing segment in the functional beverage category. The selection of kombuchas on the market varies between the traditional and flavoured kombuchas. Our research aimed to characterise the chemical, microbial, and sensory profiles of the commercial kombuchas. We analysed 16 kombuchas from 6 producers. The dominant metabolites were acetate, lactate, and ethanol, the last of which might put some kombuchas into the alcoholic beverage section in some countries. The metagenomic analyses demonstrated that LAB dominates in green tea, and AAB in black tea kombuchas. The main bacterial species were *Komagataeibacter rhaeticus* and *Lactobacillus ssp*, and yeast species *Dekkera anomala* and *Dekkera bruxellensis*. The sweet and sour balance correlated with acid concentrations. The free sorting task showed that commercial kombuchas clustered into three main categories "fruity and artificial flavour", herbal and tea notes", and "classical notes". Our research results showed the necessity of the definition of kombucha.

1. Introduction

Kombucha is a tea beverage fermented by symbiotic consortium of bacteria and yeast (Dufresne and Farnworth, 2000; Jayabalan et al., 2014). Up to now, the consortia of kombucha and kombucha biofilm in different environmental conditions have been investigated (Arıkan et al., 2020; Bharathiraja et al., 2016; Coton et al., 2017; Marsh et al., 2014). However, there is a lack of information about the chemical, microbial and sensory properties of commercial kombuchas.

In 2018, the kombucha market value was 1.8 billion USD, and by 2025, the expected value will be over 5 billion USD (Kim and Adhikari, 2020). The market is distributed into traditional and flavoured kombucha, where the last segment leads the market. The original brewing medium in flavoured kombucha has been replaced, e.g. by rooibos tea, coffee, juices, or some flavours have been added such as fruits and herbs (Kim and Adhikari, 2020). These untraditional components affect the sensory and microbial profiles of the kombucha causing huge diversity among composition and properties of commercial kombuchas (Dufresne and Farnworth, 2000; Jayabalan et al., 2014; Marsh et al., 2014; Reva et al., 2015).

Traditional kombucha is derived from black or green tea leaves by addition of 5–10% of sugar and inoculum of starter culture that consists of the previous batch of kombucha liquid and SCOBY (Symbiotic Culture of Bacteria and Yeast). The initial geographical origin, environmental conditions, and the growth medium of SCOBY determine the diversity of species in kombucha liquid and SCOBY (Marsh et al., 2014; May et al., 2019).

The dominant bacterial species in the kombucha consortium belong to acetic acid bacteria (AAB), mainly to the *Komagataeibacter* genus (earlier named as *Gluconacetobacter* and before as *Acetobacter* (Chakravorty et al., 2016; Marsh et al., 2014; Yamada et al., 2012)), such as *Komagataeibacter intermedius* (Reva et al., 2015), *Komagataeibacter xylinus* (De Filippis et al., 2018; Reva et al., 2015), *Komagataeibacter rhaeticus* (Semjonovs et al., 2017), *Komagataeibacter saccharivorans* (De Filippis et al., 2018; Reva et al., 2015) and *Komagataeibacter kombuchae* (Reva et al., 2015). Also, species from the genera of lactic acid bacteria (LAB) such as *Lactobacillus*, *Leuconostoc*, and *Bifidobacterium* have been identified in kombucha (Villarreal-Soto et al., 2018). The dominant

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yeast species in the kombucha belong to genera *Zygosaccharomyces (Zygosaccharomyces bailii)*, *Saccharomycodes* (*Saccharomycodes ludwigii*), *Candida*, *Torulaspora*, *Pichia*, *Brettanomyces/Dekkera (Brettanomyces bruxellensis*), *Schizosaccharomyces (Schizosaccharomyces pombe)* and *Saccharomyces (Saccharomyces cerevisiae)* (Coton et al., 2017; Dufresne and Farnworth, 2000; Jayabalan et al., 2014; Marsh et al., 2014; Reva et al., 2015).

The bacteria and yeasts in kombucha cooperate and compete. It leads to the cascades of reactions where different chemical components are formed, such as acetic acid, gluconic acid, glucuronic acid, and ethanol (May et al., 2019). All named components dynamically affect the sensory profile and microbial composition in the kombucha consortium (Dufresne and Farnworth, 2000; Jayabalan et al., 2014; Marsh et al., 2014; Reva et al., 2015). Although some information about the microbial and chemical composition of commercial kombuchas is available, the sophisticated sensory study that characterises kombucha properties has not been implemented (Kim and Adhikari, 2020). Therefore, current research aims to identify the microbial composition by using novel metagenomics methods for consortia analysis, which influences chemical and sensory characteristics of commercial kombuchas.

2. Materials and methods

2.1. Collection of kombuchas

The commercial kombuchas were obtained from the Estonian market in September–October 2020. Sixteen different kombuchas in two biological replicates were acquired for the metagenomic, sensory and chemical analyses. The kombuchas were from several countries and six brands were represented. The coding of kombuchas can be found in Supplementary Table A.1.

2.2. Determination of pH and titratable acidity

Five millilitres of the sample were suspended in 50 mL of distilled water to measure pH and titratable acidity (TA). The Food and Beverage Analyzer (Mettler-Toledo International Inc., Columbus, OH, USA) was used for pH and TA measurements. The acidity was determined through titration to pH 7 using 0.1 N of NaOH and expressed as 0.1 N of NaOH per 1 mL of kombucha. All measurements were performed in replicates.

2.3. Determination of metabolic products in kombucha

The liquid was filtered by using Millipore Millex-LG filters 13 mm Philic PTFE 0.2 μm Non-sterile (Germany). The Waters 2695 HPLC system (Waters Corporation, Milford, MA, USA) was used to analyse metabolic products (organic acids, sugars, and ethanol) in the kombuchas. The HPX-87H column (BioRad Hercules, CA) was used, at 35 °C and the system was eluted isocratically with 0.005 M of H_2SO_4 at 0.6 mL/min. The Waters 2487 dual absorbance detector (Waters Corporation, Milford, MA, USA) and the Waters 2414 refractive index detector (Waters Corporation, Milford, MA, USA) were used for detection and quantification of analytes. The data analysis was performed with Empower software (Waters Corporation, Milford, MA, USA).

2.4. Sensory analysis

Sensory analysis was conducted in the Center of Food and Fermentation Technologies (TFTAK, Tallinn, Estonia) by an expert sensory panel accordingly with ISO 8586:2012. The panel consisted of eight trained assessors (seven females and one male), who were between 25 and 48 years old (average age 34). Panellists were subjected to previous training for kombucha sensory analysis. The evaluation was carried out with two different descriptive analysis methods. Quantitative Descriptive Analysis (QDA) was conducted to compare the sensory profile of different kombuchas. Free sorting was used to map kombuchas based on similarities perceived. Assessors evaluated samples in isolated booths in a sensory room which was in accordance with ISO 8589:2007. Planning and conducting sensory tests followed the ISO 6658:2017. Kombucha samples were stored refrigerated at 4 °C and were served at room temperature (22 °C). All samples were served in transparent 40 mL plastic cups that were coded with three-digit numbers. Palate cleansing was encouraged during evaluations with unsalted water crackers (Pladis LTD, London, UK) and available spring water (Eden Springs Estonia OÜ, Tallinn, Estonia). All sensory data was collected by using RedJade (RedJade Sensory Solutions LLC, Martinez, CA, USA).

QDA was performed as individual evaluations replicated in two sessions. A session lasted about 40 to 50 min. Assessors evaluated intensity of all attributes on a 10-point scale with word anchors (0 = "none", 1 = "very weak", 5 = "moderate", 9 = "very strong"). The initial attribute list was developed based on literature (Gramza-Michalowska et al., 2016; Neffe-Skocińska et al., 2017; Yavari et al., 2017) and was refined in panel discussions. The final attribute list is shown in Supplementary Table A.3. Four modalities were assessed in total: appearance, odour, texture, and taste. The additional comments of modality were possible to write in a voluntary text box.

To avoid loss of carbonization that can affect sensory results (texture), samples were served in groups consisting of 3–4 samples and each group was evaluated immediately after opening the bottle. Samples were grouped based on their flavouring characteristics to prevent the convergence effect where a very distinctive sample may reduce the perceived differences between other samples (Hollowood, 2018). The order of the groups was the same for all panellists, but the sample order within the group was balanced by Williams' Latin square design to avoid the effect of presentation (Macfie et al., 1989). There were two-minute breaks between each group assessment to prevent sensory fatigue. In addition, the first sample in both sessions was a reference kombucha that helped panellists to get ready for the evaluation and to compare it with the upcoming samples. Values on the scale for the reference sample were previously agreed by consensus.

The free sorting task was given in a session lasting about 15 min. All samples were presented simultaneously in a randomized order. Assessors were asked to sort each sample into a group and to arrange as many groups as necessary. Every formed group was characterised based on their differences. Data was cleaned similarly to Pétel et al. (2017) research, where synonyms were assembled, and descriptors used by less than 10% of participants were removed. Since there were 8 participants in total, then the 10% was rounded to one participant.

2.5. Amplicon-based metagenomic analysis

2.5.1. DNA extraction

A bottle with commercial kombucha was roiled and opened carefully in aseptic conditions. 100 mL of the kombucha beverage was centrifuged at 3950 ×*g* for 20 min at 4 °C. Then, the pellet was washed with 500 μL of sterile and cold 1 × PBS (*Phosphate-buffered saline*, BIO-RAD, CA, USA), centrifuged at 10,000 ×*g* for 10 min at 4 °C. For the gDNA extraction, the pellet was resuspended in 200 μL of 1 × PBS and added into the ZR BashingBead™ Lysis Tube (Zymo Research, Irvine, CA, USA). The gDNA isolation was performed by applying Quick-DNA™ Fungal/Bacterial Miniprep kit (Zymo Research) according to the instruction manual. Extracted gDNA was quantified by a Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using dsDNA BR Assay Kit (Thermo Fisher Scientific).

2.5.2. Amplicon sequencing

25 ng of extracted microbial gDNA was subjected to the amplicon library preparation. For the study of the bacterial composition, the V4 region of the 16S ribosomal RNA (rRNA) gene (Caporaso et al., 2011) was sequenced as described in Kazantseva et al. (2021) by Next Generation Sequencing (NGS) technology.

For the determination of fungi, the 5.8S-ITS2-LSU region of the rRNA

gene was sequenced using forward 5.8S-Fun 5′-**AACTTTYRRCAAYGGATCWCT**-3′ and reverse ITS4-Fun 5′-**AGCCTCCGCTTATTGATATGCTTAART**-3′ primers (Biomers, Germany); (Taylor et al., 2016). The library preparation and sequencing were carried out as mentioned before (Kazantseva et al., 2021) with some modifications. In the amplification stage of the library preparation, 25 cycles, annealing temperature of 58 °C and elongation time of 45 s were applied. The normalised library pool was sequenced on iSeq100 Sequencing System (Illumina, San Diego, CA, USA) using iSeq 100 i1 Reagent and 300 cycles single-end protocol.

2.6. Bioinformatic analysis

The sequencing data of the V4 region of the 16S rRNA gene were analysed by an open-source BION-meta package (Espinosa-Gongora et al., 2016; McDonald et al., 2016; www.box.com/bion, Danish Genome Institute, Denmark). Raw sequences (paired-end reads 2 × 150 bp) were cleaned at both ends applying a 99.5% minimum quality threshold for at least 18 of 20 bases for 5′-end and 28 of 30 bases for 3′-end, joined and contigs shorter than 150 bp were removed. Created sequences were cleaned from chimaeras and clustered by 95% oligonucleotide similarity (k-mer length of 8 bp, step size 2 bp). Obtained consensus reads were aligned to the SILVA reference 16S rRNA database (v123) using a word length of 8 and a similarity cut-off of 90%. The bacterial designation was analysed at different taxonomic levels down to species if applicable.

The sequencing data of the 5.8S-ITS2-LSU region of the rRNA genes were processed and analysed using custom pipeline of the Quantitative Insights Into Microbial Ecology [QIIME2, version 2019.10, (Bolyen et al., 2019)]. All following procedures in this section were conducted in the QIIME2 environment plug-ins. Demultiplexed single-end reads from iSeq100 (1 × 300 bp) were trimmed to remove primers and poor quality bases and then denoised with DADA2 (Callahan et al., 2016, R package, https://github.com/benjjneb/dada2). Taxonomies were assigned with a q2-feature-classifier plug-in and classify-sklearn (Pedregosa et al., 2011) method using the fungal ITS classifiers trained on UNITE reference database [version 2018.11.18 ("UNITE Community (2019) UNITE QIIME release for Fungi 2.," n.d.)].

2.7. Quantitative real-time PCR

To measure the bacterial and the fungal-specific gDNA concentration of the commercial kombucha, a quantitative real-time PCR (qPCR) was carried out using qTOWER^{3}G thermal cycler (Analytik Jena, Jena, Germany) by implementing a qPCRsoft 4.0 software. Standard curves for the quantification were performed applying DNA standards from Femto Bacterial or Fungal Quantification kits (Zymo Research, Irvine, CA, USA). Specific primer pairs 515F/806R (forward 5′-GTGYCAGCMGCCGCGGTAA-3′ and reverse 5′-GGACTACNVGGGTWTCTAAT-3′; Biomers, Germany) for bacterial V4 region of the 16S rRNA gene (Caporaso et al., 2011), and UNF1/UNF2 (forward 5′-GCATCGATGAAGAACGCAGC-3′ and reverse 5′-TTGATATGCTTAAGTTCAGCGG-3′) for fungal ITS2 region of the ribosomal gene (Fiedorová et al., 2019) were used. Each reaction in a total volume of 10 μL included 5× HOT FIREPol® SolisGreen qPCR Mix (Solis BioDyne, Tartu, Estonia), 5 pmol of each primer and 1 μL of extracted gDNA sample or commercial standard. The reaction conditions were as follows: initial denaturation at 95 °C for 12 min; 42 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s; final elongation at 72 °C for 7 min. To determine the specificity of amplification, an analysis of the product melting curve was performed after the last cycle of the amplification. All samples and standards were analysed in duplicates.

Based on the quantification data of the qPCR (Supplementary Table A.1) and according to the taxonomic proportional abundances of the sequencing results, the content of detected bacterial and yeasts species of each investigated kombucha was calculated. The number of the amplified PCR products (16S rRNA or ITS2 rRNA gene copies) per volume was calculated based on the equation: Copy number $= \frac{N_A \times m \times D}{AS \times M}$, where N_A is the Avogadro number 6.02 × 10^{23} $\left(\frac{\text{copies}}{\text{mol}}\right)$, m is the measured mass of DNA (g), D – dilution coefficient, AS is the amplicon size (bp) and M is the dsDNA molar mass 660 $\left(\frac{\text{g}}{\text{mol bp}}\right)$ (Duewer et al., 2018). To estimate the cell numbers, the average number of 16S rRNA genes in bacteria was considered as 4.2 (Větrovský and Baldrian, 2013), and 150 copies for ITS2 (Kobayashi, 2011).

2.8. Statistical analysis

Sensory results were statistically analysed and visualised with R version 4.1.0 (R Foundation for Statistical Computing, Vienna, Austria). For quantitative descriptive analysis, results were interpreted using principal component analysis (PCA), analysis of variance (ANOVA), and variable distributions presented as violin plots. Free sorting results were statistically analysed by multiple correspondence analysis (MCA) from R package "FactoMineR" 2.4. Dissimilarities between microbial consortia were assessed using non-metric multidimensional scaling (NMDS) function from R package "vegan" 2.5-7.

3. Results

3.1. Chemical composition of commercial kombuchas

All commercial kombuchas were evaluated for acidity and amounts of metabolites. The acidity was estimated by pH and TA measurements. The pH values of all products stayed below 4, and the average TA value was 3.1 ± 0.3 mL of 0.1 N NaOH (Supplementary Fig. A.1). Commercial kombucha K2 had the highest pH with the value of 3.7 ± 0.3, and the product H3 pH value was 2.8 ± 0.3, which was the lowest detected. This kombucha TA value was also the highest with the value of 6.0 ± 0.3 mL of 0.1 N NaOH. The pH values of fermented beverages of manufacturers H, M, and S changed between different products. However, the beverages of producer C showed rather equal results, the pH fluctuation was 0.1 units. The kombucha C3 showed the lowest TA value (0.9 mL of 0.1 N NaOH). The TA values also varied between different kombuchas of the same manufacturer and were negatively correlated to pH (Pearson's r − 0.56).

The concentration of acetic acid in analysed beverages was relatively high (Fig. 1A). Kombuchas H1–H3 showed elevated concentrations in the range of 3.54–6.40 g/L. The product V1 showed the lowest level of acetic acid (0.35 ± 0.002 g/L). In contrast, only C1, C3, M1, M2, M3, M5, S1, S2, and V1 beverages contained lactic acid. The highest concentration of lactic acid was in kombucha M5 and the lowest in C1 that corresponds to 3.01 ± 0.17 g/L and 0.21 ± 0.02 g/L, respectively.

Additionally, the commercial kombuchas contained residues of citric acid, malic acid, and succinic acid (Fig. 1B). The concentrations of these acids were 10-folds lower than that of acetic and lactic acid. Products H1 and M2 had the highest concentration of citric acid—0.36 and 0.34 g/L, and C1 had the lowest at 0.07 g/L. Malbaša et al., 2011 measured the citric acid concentration accounted for 2.5% of the total acidity, and Jayabalan et al., 2007 citric acid concentrations varied between 0.03 and 0.11 g/L. Our results showed this acid content in studied kombucha was insignificant, what was confirmed by the literature.

All products with except of the kombucha S1 contained gluconic acid (Fig. 1B). Manufacturer H beverages showed the highest concentrations of gluconic acid, other products contained relatively low amounts of this acid. Only four kombuchas contained glucuronic acid: C1, C2, C3 and S2, where the acid concentrations were up to 0.33 ± 0.02 g/L (data not shown).

The average concentration of detected ethanol was 7.35 ± 0.23 g/L. The highest concentration was in kombucha H1 at 14.77 ± 0.77 g/L,

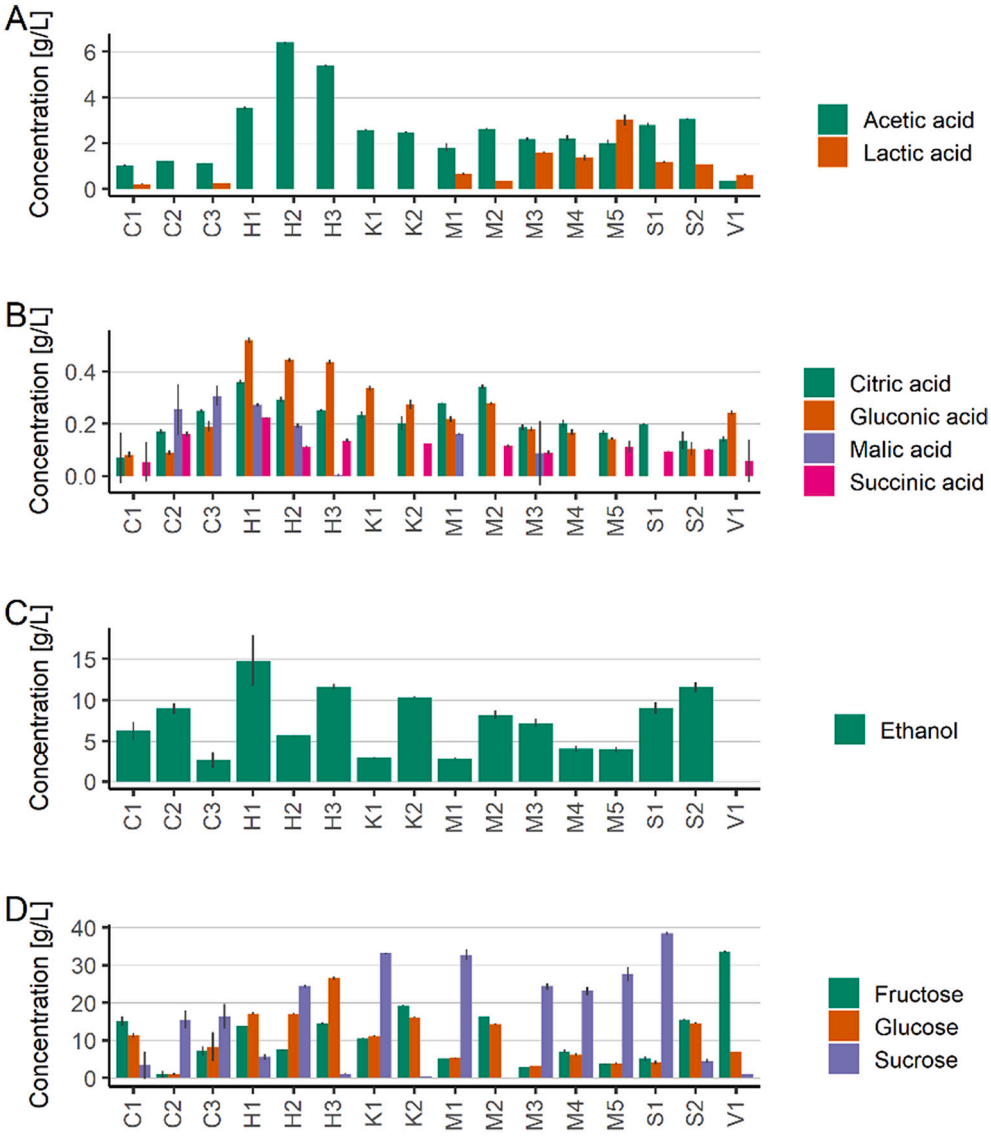

Fig. 1. Organic acids, ethanol, and sugar profiles of commercial kombuchas are shown as mean with standard deviations (n = 2). Metabolite profiles presented in acetic and lactic acids profiles (A); citric, gluconic, malic and succinic acids profiles (B); ethanol profiles (C); fructose, glucose, and sucrose profiles (D). The coding of kombuchas is explained in Supplementary Table A.1.

and the lowest in C3 with a value of 2.66 ± 0.75 g/L (Fig. 1C). The beverage V1 did not contain ethanol.

All products included sucrose; the concentrations varied between 0.21 ± 0.01 g/L to 38.49 ± 0.30 g/L (Fig. 1D). The highest sucrose concentration occurred in kombucha S1 and the lowest in M2. Also, relatively low concentrations of sucrose were in kombuchas V1 and H3 1.13 ± 0.01 g/L and 1.19 ± 0.03 g/L, respectively. Additionally, these two commercial kombuchas showed the highest concentrations of the monosaccharides' glucose and fructose.

3.2. Microbial composition of commercial kombuchas

Microbial gDNA were isolated from 15 out of 16 commercial kombuchas (Supplementary Table A.2). Repeated extraction of microbial DNA from V1 product failed because of the absence of any viable microbes which was confirmed by additional out-plating analysis (data not shown). The common universal origin of kombuchas from one starter culture among definite manufacturers was confirmed by NMDS clustering analysis (Supplementary Fig. A.2). Kombuchas from manufacturers S, M, and H formed distinct groups, but K1–K2 and C1–C3 did not, which is confirmed by their difference in bacterial composition (Fig. 2). However, K1 showed similarities with the H cluster but K2 could not be clustered.

The metagenomic 16S amplicon NGS results revealed that the most abundant genera among the kombuchas consortia refer to *Komagataeibacter*, *Lactobacillus* and *Bacillus* (Fig. 2A) that belong to the potential probiotic strains such as *Lactobacillus* and *Oenococcus*, and bacterium *Bacillus coagulans*. Also, some products contained *Acetobacter*, *Gluconobacter*, *Pseudomonas* and *Zymomonas* species.

The dominant species in kombuchas H1–H3 were related to *Komagataeibacter* and *Bacillus*, while *Lactobacillus* prevailed in M1–M5 beverages. The highest proportion of bacteria in S1and S2 beverages belonged to *Zymomonas mobilis*, but K1 and K2 kombuchas included mainly *Komagataeibacter* and *Gluconobacter* species. The most diverse bacterial composition across the one manufacturer was in C1–C3 products. Here, the common bacterial pattern was not detected, thus all three tested kombuchas had totally different major bacteria – *Pseudomonas putida* in C1, *Bacillus coagulans* in C2, and *Gluconobacter oxydans* in C3 beverage. Inclusively, the distinctive pattern in the bacterial composition of commercial kombuchas was shown - LAB dominated in green tea

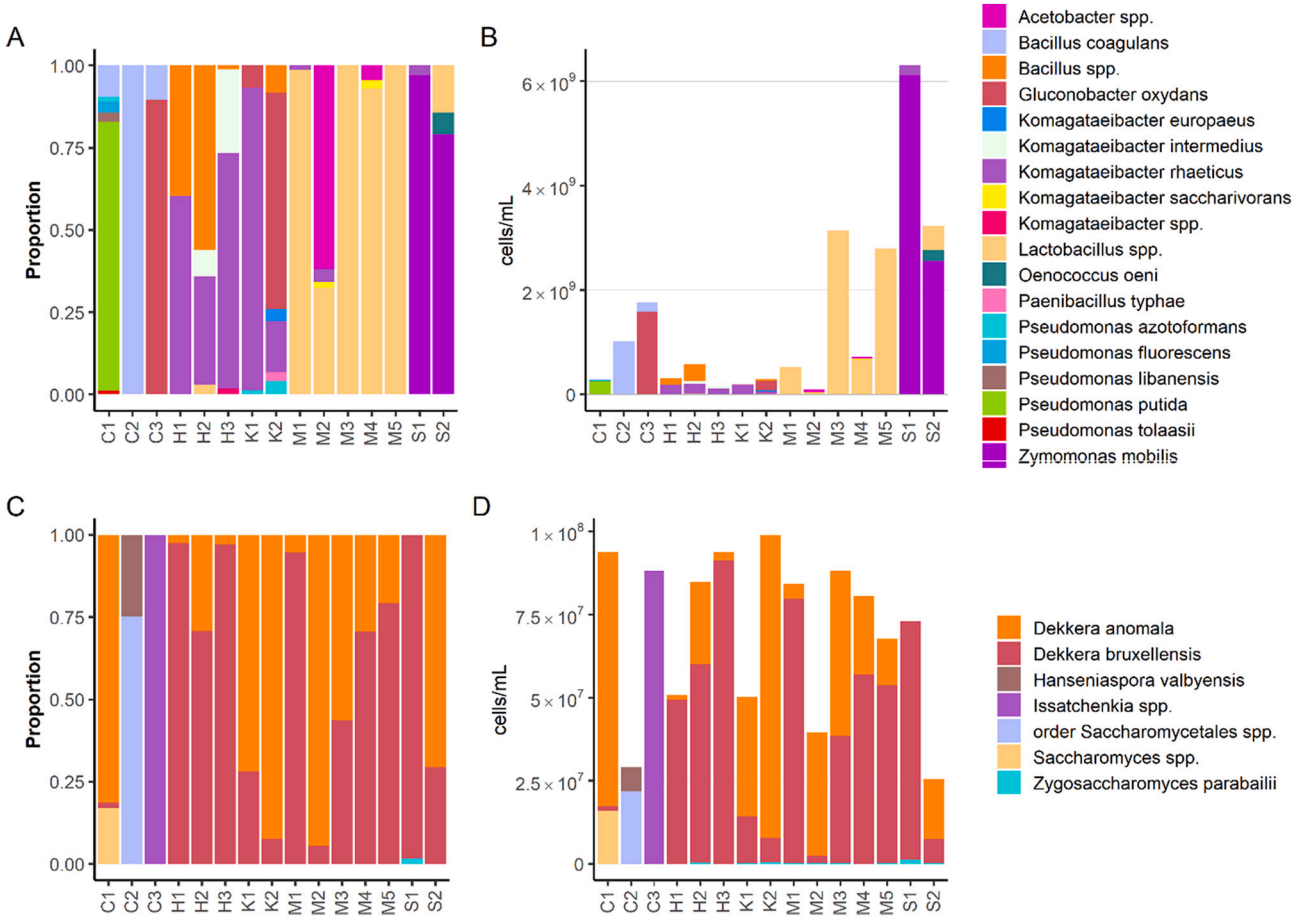

Fig. 2. Microbial composition of kombuchas according to 16S NGS for bacteria (A, B) and ITS2 NGS for yeasts (C, D) graphed as relative (A, C) and normalised abundances (B, D). The coding of kombuchas can be found in Supplementary Table A.1.

and AAB in black tea beverages.

In general, most kombuchas had 2–3 dominant bacteria in their composition. However, beverages H2, H3, and M2 contained mixture of four dominant bacterial cultures, and K2 and C1 were even richer. Products H2, H3 and M2 consisted mainly of common bacterial species, while the richness of C1 was due to the presence of various *Pseudomonas* species along with *B. coagulans*. The diversity of K2 drink was also caused by additional atypical bacteria for kombuchas like *Pseudomonas azotoformans* and *Paenibacillus typhae*.

The proportion of different bacterial species in fermented beverage is a valuable parameter to characterise and compare commercial products. The results of normalised absolute data based on bacteria-specific qPCR are represented on Fig. 2B. The products of S and M manufacturers have the highest number of bacterial cells in their composition, up to 6.4×10^9 cells/mL. At the same time, manufacturers H and K have a lower bacterial load in their drinks (~5.0×10^8 cells/mL), while C manufacturer shows intermediate values (~0.5×10^8–1.8×10^9 cells/mL). We also detected the difference in the bacterial load of the manufacturers M and C kombuchas, which points to the fact that diversity is not always connected with the abundance of bacteria. The prevalent bacterial genera for S and M were *Zymomonas* and *Lactobacillus*, correspondently, while the C manufacturers' drinks were rich in *B. coagulans* and *G. oxydans* species.

The diversity of yeasts in analysed kombuchas detected by metagenomic ITS2 amplicon NGS was more modest. All identified yeasts belonged to order *Saccharomycetales*, mainly to genus *Dekkera/Brettanomyces* (Fig. 2C). Kombuchas H, K, M and S had different proportions of *Dekkera anomala* and *Dekkera bruxellensis* in their composition. The S1 beverage along with dominant *D. bruxellensis* contained low portion of *Zygosaccharomyces parabailii*. Similarly to the bacterial composition, C group products stayed apart and were more unique regarding the yeasts presented. Moreover, they differed even inside the manufacturing group. Together with *Dekkera* species, C1 product contained *Saccharomyces* spp. Despite detected *Dekkera* genus, C2 included *Hanseniaspora valbyensis* and other *Saccharomycetales spp*, while C3 was dominantly represented by *Issatchenkia* spp.

For absolute quantification, we evaluated the fungal cell number based on DNA concentration measured by ITS2-specific qPCR and considering the average *Saccharomycetales* amplicon size. This normalisation did not affect the pattern of the cell amount so drastically as in the case of bacteria (Fig. 2). So, the pattern of yeasts determined by ITS2 sequencing remained more uniformed and without manufacturers distinctness. To compare, the difference in cell numbers between different kombuchas was only fourfold: the lowest count of fungal cells we found in beverages S2 and C2 (~2.5–3×10^7 cells/mL), and the highest number in H3, K2 and C1 (~1.0×10^8 cells/mL).

3.3. Sensory analysis

According to the ANOVA, all sensory attributes were significant. The kombuchas were mostly distinguishable by their sweet odour and taste based on the QDA results presented as a PCA plot (Fig. 3). In general, kombuchas from the same manufactures were sensorially similar. Overall additives intensities varied in taste and odour due to the different content of flavours. Tea, sourness, and vinegar notes are rather characteristic attributes for kombuchas, which was also noticeable in various products such as H1-H3. Products C1–C3 and V1 were perceived as the sweetest. Each manufacturer's (C, H, K, M) own products were

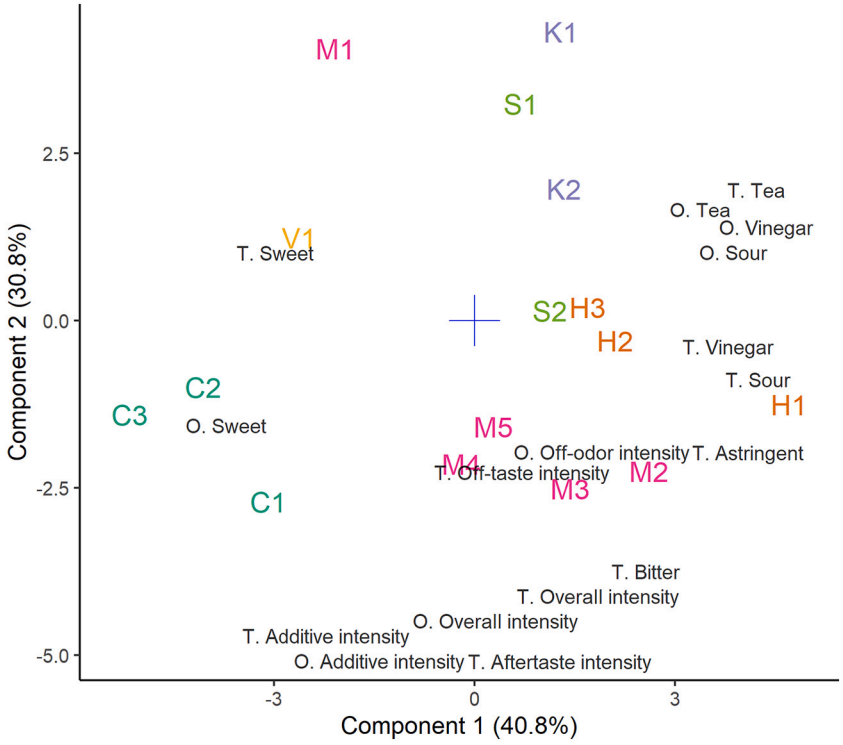

Fig. 3. Principal component analysis (PCA) of quantitative sensory analysis of kombuchas. Abbreviations: O – odour; T – taste. The coding of kombuchas is explained in Supplementary Table A.1.

clustered together on the plot. Product M1 was the only one that differed from other M kombuchas.

The sensory results were in concurrence with the chemical analyses (Fig. 4), which indicate good sensory panel stability. However, the sourness and sweetness may be influenced by other properties, e.g. ingredients used. In general, products with higher TA were perceived as sourer in taste (Fig. 4A). This also corresponds to the sensory results that beverages C1–C3 and V1 were the sweetest and the least sour. The kombuchas with higher total sugar content were mostly perceived as having a sweeter taste (Fig. 4B). However, based on the chemical analyses, the beverages C1–C3 had lower sugar content compared to others. It could be explained with the unbalanced taste profile between sourness and sweetness or the most intense additional flavours. According to the comments from the sensory panel, all C beverages were perceived as fruity and floral, that in turn may be sensorially associated with sweeter nuances.

Free sorting task produced 36 different descriptors, that were categorized into 10 groups: herbal (6 assessors), classical kombucha (5), fruity (4), artificial flavour (4), tea (4), vinegar (3), sweet (3), sweet and sour (2), berry (2), off-flavour (2). Based on the sorting task, the commercial kombuchas can be grouped into three clusters (Fig. 5). The first cluster includes kombuchas with “fruity” and “artificial flavours”,

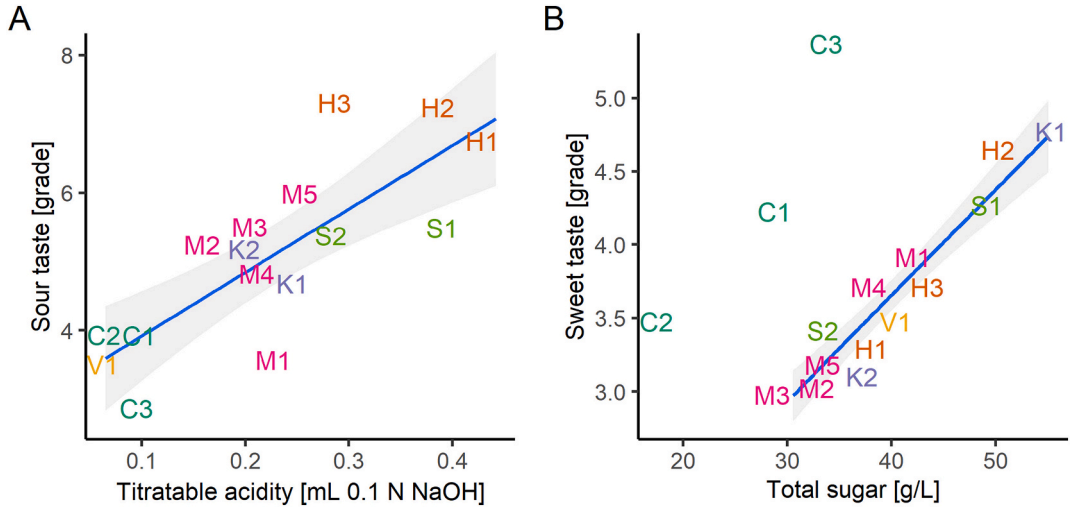

Fig. 4. Average sensory results in ascending order compared with the trendline for chemical analysis average results. A represents comparison for sour taste (sensory analysis) and TA (chemical analysis); B represents comparison for sweet taste (sensory analysis) and total sugar (chemical analysis). The blue lines are linear fit, and the grey areas show their confidence intervals. The coding of kombuchas can be found in Supplementary Table A.1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

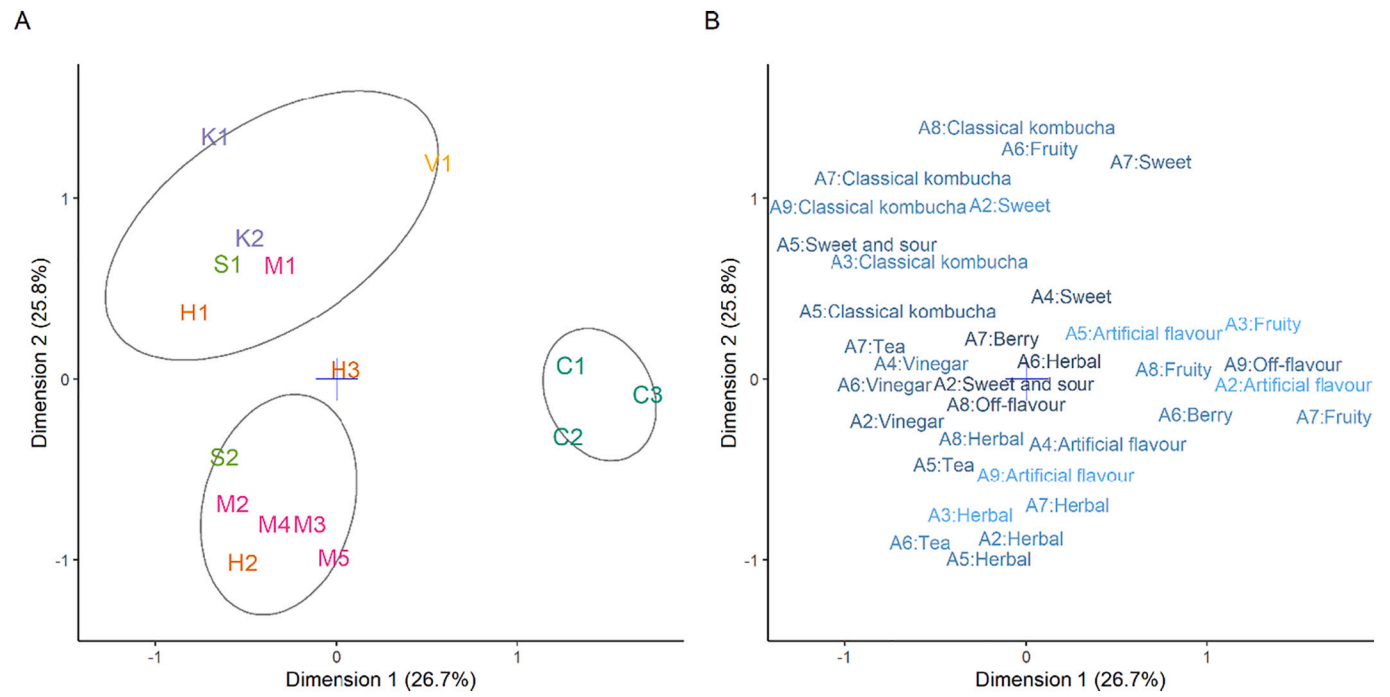

Fig. 5. Multiple correspondence analysis (MCA) for free sorting task, where A demonstrates clustering of different samples and B characterises the associating descriptors. Colour of variable categories is according to $\cos^2$; the lighter, the better this category is represented. Ellipses are drawn at 80% confidence level for three groups identified with hierarchical clustering. The coding of kombuchas is explained in Supplementary Table A.1.

mostly from manufacturer C. As the QDA confirmed, these kombuchas were distinctive by their additive intensity, highest perceived sweetness, and lower sourness. The second cluster (M2–M5, H2, S2) can be described as kombuchas with "herbal" and "tea" notes. These products had herbal ingredients like sage, hop, ginger, turmeric, mint. However, some kombuchas among that group had "vinegar" nuances. The third cluster (K1, K2, S1, M1, H1, V1) was often described as "classical kombuchas" with some "sweet" nuances. All unflavoured versions of kombuchas (coded with 1) and all kombuchas produced by K were included into this group. Another interesting tendency was that all kombuchas produced by H were clustered differently. Kombucha H1 was made with white tea and was grouped into classical kombucha cluster, whereas H2 was made with green tea and grouped into the herbal cluster. H3 (based on red tea) was the only sample that was not clustered into any group as it had similarities to both previously mentioned clusters.

3.4. Finding relations between microbial composition, sensory attributes, and chemical components

The abundance of bacteria and yeast cells, sensory attributes, and chemical composition were compared using Spearman's rank correlation analysis (Supplementary Figs. A.3, A.4). This revealed positive correlations between acetic acid concentration and *Komagataeibacter rhaeticus*, *Komagataeibacter intermedius*, and *Bacillus* spp., and a negative correlation with *Bacillus coagulans*. As expected, vinegar taste had a strong positive correlation with acetic acid. Astringent taste correlated negatively with sucrose concentration and positively with ethanol. The off-taste intensity correlated positively with *Pseudomonas* spp., while *Lactobacillus* spp. showed positive relation to lactic acid concentration.

We must emphasize that we had no information about the fermentation process and its stages, any possible attempts to preserve the product, the time on the shelf, or the content of other additives (Supplementary Table A.1), which could be mixed into the drinks either before or after fermentation. All these factors are expected to strongly influence the sensory profile and microbial and chemical composition of the final product. This in turn could cause spurious correlations that are difficult to refute or justify. For all these reasons, Supplementary Figs. A.3 and A.4 should be interpreted with caution.

4. Discussion

Few publications connected with kombucha manufacturing have explored the marketing (Kim and Adhikari, 2020), the SCOBY composition (Harrison and Curtin, 2021), the diversity of the microbial community and its dynamics on an industrial scale (Coton et al., 2017). To the best of our knowledge, this study is the first that identifies the microbial composition of commercially available kombuchas by using metagenomic methods for consortia analysis and characterises their chemical and sensory profiles.

All studied kombuchas differed by their microbial composition, chemical parameters, and thereby sensory characteristics. The presence of AAB and LAB in kombucha and their production of organic acids determine the level of pH in the beverage. According to the Food and Drug Administration (FDA) Food Code model, the pH of commercial kombuchas must be in the range of $2.5 \leq \text{pH} < 4$ (Kim and Adhikari, 2020; Nummer, 2013). Low pH values are required to decrease the contamination risk. Also, the pH must be $\geq$2.5 to prevent damages caused by drinking acidic drink (Greenwalt et al., 2000; Kim and Adhikari, 2020; Nummer, 2013). The kombuchas in this study were in the required range (pH 2.8–3.7) and therefore according to this criterion were safe to consume.

All beverages contained acetic acid produced primarily by the AAB. This acid has been shown to inhibit the growth of 10 of the 14 pathogenic bacteria such as *Escherichia coli* and *Salmonella enteritis* (Sreeramulu et al., 2000). Our results also confirmed AAB dominance in market kombuchas with a high concentration of acetic acid and lack of pathogenic strains.

Another organic acid frequently found in kombuchas is lactic acid (Coton et al., 2017; Dufresne and Farnworth, 2000; Reiss, 1994) that is produced by LAB after fermentation from added sucrose (Dufresne and Farnworth, 2000; Reiss, 1994). It was detected in ten studied

commercial kombuchas at relatively high concentrations. The metagenomic analysis revealed the appearance of some LAB species, such as *Lactobacillus* and *Oenococcus*. LAB species were dominant in the kombuchas from manufacturer M and, correspondingly, the concentration of lactic acid was also the highest.

Others distinctive acids in kombucha are gluconic and glucuronic acid, whose central producers are AAB (Chen and Liu, 2000; Dufresne and Farnworth, 2000; Jayabalan et al., 2014; May et al., 2019). Our results showed that the amount of these acids was insignificant. Controversially, these concentrations vary in the literature from 2.3 g/L (Jayabalan et al., 2007) to 7.36 g/L (Chakravorty et al., 2016). Moreover, the metabolic pathways of gluconic and glucuronic acids showed that these acids could be metabolised to other chemical components, such as ascorbate, amino sugars, cofactors, and inositol ("KEGG COMPOUND C00191," n.d.).

The chemical analysis demonstrated significantly elevated concentrations of ethanol. AABs use ethanol to produce acetic acid (De Filippis et al., 2018; Jayabalan et al., 2014; Matsushita et al., 2016; Nguyen et al., 2015; Yavari et al., 2017). In some cases, high concentrations of ethanol can decrease the population of LAB due to toxicity (May et al., 2019). This could happen in the case of H and C kombucha products, as the increased ethanol concentration was accompanied by a low number of bacterial cells. Moreover, the level of acetic acid for manufacturer H was high, which was confirmed both by the chemical analysis results as well as the highest sourness in taste. However, not only yeasts have the capability to produce ethanol. It was shown that *Z. mobilis* possesses this feature (Cao et al., 2019; Doelle et al., 1993; Marsh et al., 2013; Weir, 2016), which could be the reason of the heightened level of ethanol in the products of manufacturer S. Additionally, the incorrect storage temperature of products could cause overproduction of ethanol. Only kombucha V1 from the investigated kombuchas did not contain ethanol. Also, it was very distinct from other beverages and did not have any residues of microbial DNA or alive microbes, which might indicate that it was somehow processed (Kim and Adhikari, 2020; Nummer, 2013). The accepted concept of kombucha is that it should be a "living organism". Therefore, the sample V1 should not be categorized as kombucha but might be named as a carbonated soft drink. Sensory results also confirmed that V1 was the most different sample.

In our work, the sensorial free sorting task and QDA showed that the commercial kombuchas can be grouped into three clusters. The "fruity and artificial flavour" cluster included products C1–C3 that distinguished by the higher sweetness, additive intensity, and lower sourness. It could be associated with the processed product, where the bacteria are added after fermentation to preserve the title of kombucha (Kim and Adhikari, 2020). It can also be confirmed by the detection of bacteria that are atypical in kombucha, and the information on the bottle label (*Bacillus coagulans* as an ingredient). Market kombuchas M2–M5, H2, and S2 had "herbal" and "tea" notes. All these products were made from green tea, contained *Lactobacillus* species, and some herbal additives. Coton et al., 2017 also showed that LAB dominated in green tea kombuchas. The commercial kombuchas K1, K2, S1, M1, H1, and V1 were clustered as "classical kombuchas" without distinctive non-characteristic attributes. Samples K1, K2, S1, M1 and H1 contained *Komagataeibacter rhaeticus* and yeast *Dekkera* species, and all of them except M1 were made of black tea. The same observation was made by Villarreal-Soto et al., 2020 and Coton et al., 2017, who demonstrated the same *K. rhaeticus* dominance in the black tea beverage.

According to the sensory analysis, the products of manufacturer H demonstrated the most "vinegar" notes in odour and taste. It can be caused by the presence of *K. rhaeticus* and other species of the *Komagataeibacter* genus, due to their ability to withstand high acetic acid and ethanol content (Gaggìa et al., 2019; Nguyen et al., 2015; Semjonovs et al., 2017). Interestingly, the bacterial cell numbers of H2 kombucha were higher than in the case of H1 and H3, while the yeast cell numbers were the medium ones. These fluctuations in bacterial and fungal cell numbers could be explained by dynamic and symbiotic processes in kombuchas (Jayabalan et al., 2014; Reva et al., 2015; Villarreal-Soto et al., 2020). Furthermore, Kombucha H2 had the highest concentration of acetic acid and the lowest concentration of ethanol from all H manufacturer products. Additionally, the relative population of the H2 yeasts was decreased probably due to the increase of *Komagataeibacteria* species amount. These findings may indicate that the kombuchas of the same producer H tended to be in different fermentation phases (Coton et al., 2017; Jayabalan et al., 2014; Villarreal-Soto et al., 2018). Moreover, all three kombuchas were produced with different substrates (H1 – white tea, H2 – green tea, H3 – hibiscus), which might also affect the consortia composition (Villarreal-Soto et al., 2018).

The absence of a commonly recognised kombucha definition and uncertain technological characteristics allow high diversity in market kombuchas. This permits the appearance of a beverage with a kombucha label but omits its possible beneficial qualities, as in the case of several drinks we studied. The FDA is elaborating to set the variance limits for kombucha safety plan (Nummer, 2013), and the Kombucha Code of Practice has stated the product standards and safety requirements ("Kombucha Code of Practice – Kombucha Brewers International," n.d.). The Kombucha Code of Practice has defined that kombucha is made from tea leaves and fermented with symbiotic consortia of bacteria and yeasts. Despite that, no official definition of kombucha has been declared yet by European Food Safety Authority or FDA.

5. Conclusion

In this paper, we analysed 16 kombuchas from 6 manufacturers. Their chemical and microbiological composition differed between production companies and inside the same manufacturer. The prevailing acid in the majority of kombuchas was acetic acid, however, lactic acid dominated in one beverage. Due to the fact the ethanol concentrations in analysed kombuchas were relatively high (2.66 to 14.77 g/L), some of the commercial kombuchas depending on the local legislation could be considered as alcoholic beverages. Based on the sensory analysis, the commercial kombuchas were sorted into three clusters "fruity and artificial flavour", "herbal and tea notes", and "classical". The analyses of the kombucha consortia showed that LAB is dominant in green tea and AAB in black tea kombuchas. Also, sensory and metagenomic analyses confirmed that the substrate and microbial composition of starter culture are the main influencers of the final product properties.

We were the first ones who compared several products from different brewers, analysed the chemical composition and microbiological consortia by metagenomic amplicon NGS, and established the connections with sensory profiles. Further research should investigate the substrate effect on temporal microbial composition in more detail. Definite and accepted industry standards have to be set to ensure product quality in the fast-growing market of kombuchas.

CRediT authorship contribution statement

Maret Andreson, Rain Kuldjärv, Mary-Liis Kütt: Conceptualization. Maret Andreson, Jekaterina Kazantseva: Data curation. Maret Andreson, Esther Malv, Aleksei Kaleda: Formal analysis. Rain Kuldjärv: Funding acquisition. Maret Andreson, Rain Kuldjärv, Esther Malv, Jekaterina Kazantseva, Helen Vaikma: Investigation, Methodology. Rain Kuldjärv, Maret Andreson: Project administration, Resources. Aleksei Kaleda: Software, Mary-Liis Kütt, Raivo Vilu: Supervision. Maret Andreson, Rain Kuldjärv, Jekaterina Kazantseva, Aleksei Kaleda: Validation. Maret Andreson, Esther Malv, Jekaterina Kazantseva, Helen Vaikma: writing-original draft. Maret Andreson, Jekaterina Kazantseva, Esther Malv, Helen Vaikma, Rain Kuldjärv, Mary-Liis Kütt, Aleksei Kaleda, Raivo Vilu: writing-review and editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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