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Microbial–physicochemical integrated analysis of kombucha fermentation

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ABSTRACT

Kombucha is a drink produced by spontaneous fermentation, and several studies have been conducted to unveil its microbiological and physicochemical aspects with numerous human health claims. The integration of these results is fundamental to understand and discuss the biological activities attributed to kombucha. In the present study, we isolated bacteria and yeasts involved in the fermentation of kombucha produced with green (GK) and black (BK) teas, as well as the amplicon metagenomic of the microbial communities (16S and ITS) during 0, 3, 5, 10, and 15 days of fermentation, at 28 °C. Microbial communities were linked to key biochemical parameters monitored during fermentation such as pH, total titratable acidity, total reducing sugars, polyphenols, acetic acid, and ethanol production. Moreover, ordination analysis (principal component analysis, PCA) revealed clear GK and BK separation groups during the fermentation process. Caffein, gallic acid, and chlorogenic acids majorly influenced the separation of GK and BK. Furthermore, the presence of *Komagataeibacter* spp. and catechins exerted selective pressure against microbial contamination. This study essentially contributes to the knowledge about the effects of integrated microbiota to the chemical results of the kombucha fermented in GK and BK teas.

1. Introduction

Kombucha is a sweetened beverage prepared from green (GK) or black (BK) tea (*Camellia sinensis*) by fermenting a complex culture of yeast and acetic acid bacteria. Kombucha has several health benefits, and studies have reported that fermentation plays a pivotal role in producing bioactive compounds. The starter culture used for kombucha fermentation reveals remarkable variability in regards to the substrate composition, environmental parameters, and geographical location (Jayabalan, Malbasa, Loncar, Vitas, & Sathishkumar, 2014; De Filippis, Troise, Vitaglione, & Ercolini, 2018). The kombucha consortium comprises yeasts (i.e., *Saccharomyces*, *Schizosaccharomyces*, *Torulaspora*) and acetic bacteria (i.e., *Komagataeibacter*, *Acetobacter*, *Gluconobacter*) (De Filippis et al., 2018). The microbial interaction produces ethanol, organic acids, vitamins, and cellulosic biofilm. Therefore, these conditions inhibit contaminant microbiota due to strong environmental pressure (Jayabalan, Malbaša, Lončar, Vitas, & Sathishkumar, 2014; Neffe-Skocińska, Sionek, Scibisz, & Kołożyn-Krajewska, 2017).

Moreover, chemical parameters interfere with microbial succession communities during fermentation. Thus, an integrative analysis of microbiota and chemical compounds is essential to understand the dynamic microbial kombucha.

The culture-independent methods play a pivotal role in characterizing the kombucha microbiota and in identifying the microbial communities. Nevertheless, these methods can quantify only culturable living microorganisms during the fermentation process.

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Thus, the present study aimed to assess the microbiota of black and green tea used for kombucha with an integrative analysis of fermented tea physicochemical parameters. We used culture-dependent and amplicon metagenomics approaches associated with chemical features for monitoring the fermentation process.

2. Material and methods

2.1. Kombucha inoculum, fermentation process, and sampling

The tea leaves (*C. sinensis*) were purchased from the local market at Belo Horizonte, Brazil. The starter cultures (fermented broth and cellulosic pellicle) were provided by an artisanal producer. The infusions and fermentation conditions were carried according to instructions by Barbosa et al. (2020).

The experimental design comprised two blocks, one for black tea and other for green tea. For each block, three biological replicates were carried out. The samples were collected on 0 (infusion just after the addition of the kombucha inoculum), 3, 7, 10, and 15 days of fermentation. The samples were composed of cellulose pellicle and fermented broth. All the samples included a pool of biological replicates. Thereafter, the samples were divided into three parts: to isolate culturable microorganisms, amplicon metagenomics, and chemical characterization. The samples for chemical analyses and metagenomics were stocked at 4 °C and −20 °C, respectively, until further use.

2.2. Culture-dependent approach

2.2.1. Isolation of culturable acetic acid bacteria (AAB), lactic acid bacteria (LAB), and yeasts

The AAB were isolated in reinforced acetic acid and ethanol (RAE) and mannitol, yeast extract, and Peptone (MYP), according to instructions by Oliveira et al. (2010) and Spinosa (2002, p. 191). LAB was detected on Man, Rogosa, and Sharpe MRS (MRS) medium according to Coton et al. (2017). For yeasts, glucose, yeast extract, malt extract, and peptone (GYMP) and Sabouraud agar supplemented with chloramphenicol (0.05%), were used (Spinosa, 2002, p. 191). In total, 20 colonies (bacteria and fungi) were randomly selected from all fermented samples. The strains were purified in MYP and GYMP for AAB yeast, respectively (Oliveira et al., 2010; Spinosa, 2002, p. 191). Purified microorganisms were stocked at −80 °C.

2.2.2. DNA sequencing of rRNA for bacteria and yeast

The bacterial DNA extraction, purification, and amplification were carried out according to Ruiz, Poblet, Mas, and Guillamon (2000), with certain modifications. The extraction and purification were carried out with GRS Genomic DNA and PCR & Gel Band Purification (GRiSP Research Solutions, Porto - Portugal). The primers used for bacteria were 16S–F (5′-GCTGGCGGCATGCTTAACACAT-3′) and 16S-R (5′-GGAGGTGATCCAGCCGCAGGT-3′). The DNA was amplified for 5 min at 94 °C to denature the target DNA, and cycled 35 times at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min. The amplification product was not digested with restriction endonucleases. The conventional Sanger 16S rDNA was used for sequencing the bacteria (Ruiz et al., 2000; Vaz-Moreira et al., 2009).

The yeasts identification was carried out according to the methodology described by Rademaker and De Bruijn (2003) and Kurtzman, Fell, and Boekhout (2011). Before molecular identification, these microorganisms were group by GTG_5 (Gomes et al., 2015; Lieckfeldt, Meyer, & Börner, 1993). The regions D1/D2 of 26S rRNA were amplified via PCR using NL1 and NL4 primers for yeast molecular identification.

Sequences were aligned using BLAST. Similar sequences were assessed using the National Center of Biotechnology Information databases (http://www.ncbi.nlm.nih.gov). The raw sequences were deposited in NCBI SAR under BioProjects PRJNA665807 and PRJNA686871.

2.3. Culture-independent approach—metagenomic analysis

2.3.1. Metagenomic DNA extraction

The DNA was extracted as described by Marsh, O'Sullivan, Hill, Ross, and Cotter (2014), with certain modifications: (i) use of DNeasy® PowerFood® Microbial Kit (Qiagen, Hilden - Germany); (ii) 1 mL of cellulase; (iii) mechanical lyse by two cycles of 40 s in a FastPrep (MP Biomedicals); (iv) the solution was incubated for 2 h at 40 °C; (v) enzymatic digestion with enzymes mutanolysin (100 $U{\cdot}mL^{-1}$) and lysozyme (50 mg mL^{-1}) at 37 °C for 1 h. Extraction was optimized for 10 min at 70 °C incubation.

2.3.2. Sample preparation, PCR, amplicon sequencing, and metagenomic analysis of 16S rRNA gene and ITS2

Samples were prepared for Illumina Sequencing for 16S rRNA gene and ITS1 region amplification of the microbial communities in Illumina MiSeq® sequencer (Illumina, 2013; Marsh et al., 2014).

2.4. Chemical characterization

All the samples were centrifuged at 2240×*g* for 5 min, and the supernatant was used for further analyses. The concentrations of ethanol (E), total reducing sugars (TRS), total titratable acidity (TTA), pH, and polyphenols were carried out as described by Barbosa et al. (2020).

Volatile compounds were analyzed via gas chromatograph mass spectrometer (GC-MS) (Agilent 7890B; 5977A -MSD). Based on the peaks obtained and their respective retention times, those with the highest signal were selected, and at least 50% of these were identified (Rigobello, Scandelai, Corso, & Tavares, 2015).

2.5. Bioinformatics and statistical analysis of the microbiome, quantitative and qualitative chemical variables, and their integration

Bioinformatic analyses were performed in a customized workflow using VSEARCH v.2.7.1 (Rognes, Flouri, Nichols, Qiince, & Mahe, 2016) and the databases Silva (for Bacteria) (Quast et al., 2012) and UNITE (for Fungi) (Nilsson et al., 2018), as well as customized scripts in Python that were applied for amplicon metagenomic analysis.

In brief, VSEARCH was used to merge pairs, filter, dereplication, preclustering, quimera removal, and clustering. Erging pairs was done using **fastq_mergepairs** function; (ii) filtering was done using **fastq_filter** function with the flag fastq_maxee and fastq_minlen with parameters 0.5 and 300, respectively; (iii) two scripts (join_files and abundance) were built to join all the samples in one file and to create abundance table, (iv) BLAST was used to identify all the centroids in cluster analysis. We used 90% coverage and 97% identity with the flags -qcov_hsp_perc 90.0 and -perc_identity 97.0, respectively.

In order to integratively analyze all the data of amplicon metagenomics with physicochemical attributes, we used the multivariate statistics ordination method of Principal components analysis (PCA), which finds hypothetical components, accounting for majority of the variance in the data (Legendre & Legendre, 1998). A normalized variance–covariance matrix was constructed with the multivariate data for input of Singular Value Decomposition (SVD) algorithm. Furthermore, we performed a pairwise correlation analysis, using the parametric correlation coefficient (Pearson's r), with all the quantitative variables of both amplicon metagenomics with physicochemical traits (Schilling, 1984). Ordination (PCA) and correlation analyses were carried out in Past 4.03 (Hammer, Harper, & Ryan, 2001).

2.6. Statistical methods for chemical variables

Each variable was deregressed according to the block effects on time (0, 3, 7, 10, and 15) by applying multiple linear regression with dummy variables (GK and BK). Multiple linear regression models of 1° and 2° for time were analyzed (Fox et al., 2019). The statistical analyses were

performed in R software (R Core Team, 2020).

3. Results

3.1. Microbiological analysis

The metagenomics and culture-dependent approaches revealed that *Komagataeibacter* and *Zygosaccharomyces* were the dominant genera of bacteria and yeast, respectively. These genera are predominant during all the kombucha fermentation in both GK and BK (Suppl.Table 1). Moreover, the bacteria *Sphigomonas melonis* was identified in the analyzed samples (Suppl.Table 1). In this study, lactic acid bacteria were not isolated.

In total, 133 strains ($n = 102$ bacteria, $n = 31$ fungi) were isolated during 15 days of fermentation. The bacterial and fungal strains were isolated from BK ($n = 63$ and $n = 23$, respectively) and GK ($n = 39$ and $n = 8$, respectively) (Figs. 1 and 2). The AAB corresponded to 92.16% ($n = 94$), whereas genus *Sphingomonas* was 7.84% ($n = 8$). Moreover, *Komagataeibacter hansenii* (41.2%), *Komagataeibacter europaeus* (23.5%), and *Komagataeibacter xylinus* (14.7%) were most frequently observed (Suppl.Table 1). These species are frequently isolated from kombucha (Gaggìa et al., 2018; Marsh et al., 2014). For yeasts, *Zygosaccharomyces bailli* exhibited high frequency ($n = 24$), followed by *Rhodotorula mucilaginosa* ($n = 4$), and *Saccharomyces cerevisiae* ($n = 3$) (Suppl.Table 1). The yeast isolates were grouped by GTG_5, resulting in 27 profiles (data not shown).

Bacteria and fungi revealed 48.19% and 0.13% similarity for BK, respectively, and 51.45% and 0.23% similarity for GK, respectively (Suppl.Table 1). Despite the similarity for both kombuchas, the number of reads and the relative abundance of bacteria were higher than fungi (Fig. 2A and B). The total number of reads was 217,618 reads for bacteria and 775 reads for fungi (Fig. 1). The number of reads was higher for BK ($n = 112,374$ bacteria and $n = 492$ fungi) than for GK ($n = 105,244$ for bacteria and $n = 283$ for fungi) (Fig. 1).

In total, 11 operational taxonomic units of fungi were detected and two of them belonged to the *Malassezia* genus. This genus occurred only in the beginning and at the end of fermentation (Suppl.Table 1). *Zygosaccharomyces* exhibited the highest total number of fungal reads ($n = 352$), followed by Malassezia ($n = 262$) and *Rhodotorula* ($n = 58$) (Fig. 1; Suppl.Table 1). Seven OTUs of bacteria were found, and six of these belonged to the *Komagataeibacter* genus and one to *Stenotrophomonas*. The genus *Stenotrophomona* was only detected at 7 days of fermentation in BK (Suppl.Table 1).

3.2. Chemical analysis and microbiota integration

The quadratic linear regression coefficient ranged from 0.41 to 0.99 for kombucha fermentation kinetics parameters (Suppl. Fig. 2).

The concentration of *total reducing sugars* was similar in both teas, ranging from 32.3 to 74.5 g L^{-1} (Fig. 3; Suppl. Fig. 2; Suppl. Table 2). The pH decreased from 3.8 to 3.1 in GK and BK fermentations (Fig. 3; Suppl. Table 2). Moreover, the total titratable acid increased in both fermented teas from 2.5 to 24 g L^{-1} (BK) and from 4.5 to 27.3 g L^{-1} (GK (Fig. 3; Suppl. Table 2). The total concentration of *ethanol* at the end of fermentation was lower than 0.7 g L^{-1} for both kombucha.

The *gallic acid* concentration in GK was 7.6 mg L^{-1}; whereas, that in BK was 8-fold higher (68.6 mg L^{-1}). Furthermore, *caffeine* concentration was almost 3-fold higher in BK (205 mg L^{-1}) than that in GK (78·2 mg L^{-1}). The *quercetin* concentrations were 0.226 and 1.16 mg L^{-1}, for GK and BK, respectively; whereas, those of *rutin* were 16 0.5 and 27.8 mg L^{-1}, for GK and BK, respectively. All phenolic compounds analyzed have always been higher in BK (Suppl. Table 3). The exception was *catechin* that did not exhibit any difference for both kombucha (6.54 and 6.73 mg L^{-1}, for GK and BK, respectively) (Barbosa et al., 2020).

In total, 18 compounds were identified via GC-MS in BK ($n = 16$), GK ($n = 13$), and in the inoculum ($n = 12$) (Suppl. Table 5). Five compounds were detected exclusively in BK (acetal, glycolaldehyde, hydroquinone, paromomycin, furyl hydroxymethyl ketone and D-Melezitose) (Suppl. Table 6).

Principal component analysis was applied to integrate chemical and microbial analysis. Fig. 4 depicts a clear separation of chemical parameters of GK from those of BK, but not of the microbiota. Furthermore, regarding microbial metagenomic results, the correlation analysis illustrated in Fig. 5 depicted that the presence of *Komagataeibacter* was positively correlated ($p < 0.05$) with ethanol and catechins. For *Zygosaccharomyces*, no correlation was significant when paired with all variables studied ($p > 0.05$). *Malassezia*, *Rhodotorula*, and other fungi were negatively corelated to *Komagataeibacter* (Fig. 5).

4. Discussion

4.1. Microbiological analysis

The chemical compounds, such as caffeine, gallic acid, and chlorogenic acids which occurred in higher concentrations in BK, exert selective pressure on the microbiota found in this type of kombucha. Figs. 1 and 2 illustrate the number of isolates (bacteria and yeasts) in GK ($n = 86$) and BK ($n = 47$). These results emphasize that the aforementioned compounds or their synergistic effects could influence the microbial viability. Moreover, they decrease the microbial recovery via culture-dependent methodology considering that the cells are in a viable but noncultivable (VBNC) state (De Roos & De Vuyst, 2018).

The presence of *Z. bailli*, *R. mucilaginosa,* and *S. cerevisiae* has already been described in kombucha fermentation (Teoh, Heard, & Cox, 2004; Chakravorty et al., 2016; Coton et al., 2017), and these organisms are recognized as ethanol producers (Martorell, Stratfrod, Steels, Fernández-Espinar, & Querol, 2007; Kurtzman et al., 2011). *Z. bailii* exhibits high resistance for low pH and high sugar content (Erickson & McKenna, 1999) and preferentially metabolizes fructose to produce ethanol (Kurtzman et al., 2011).

The acetic acid bacteria *K. hansenii*, *K. europaeus,* and *K. xylinus* identified in this study are frequently isolated from kombucha and are characterized as strong cellulose producers (Gaggìa et al., 2018; Li et al., 2019; Marsh et al., 2014). Although *K. xylinus* is frequently correlated as the dominant microorganism in kombucha (Marsh et al., 2014) fermentation, our results indicated that the occurrence of this microorganism is less than 15% of the total isolates. The low presence of *K. xylinus* could be explained by the temperature used in our work (28 °C). De Filippis et al. (2018) stated that the fermentation temperature could alter the kombucha's microbial composition. In that study, the authors reported the dominance of *K. xylinus* in fermentation at 20 °C and abundant multiplication of *K. saccharivorans* at 30 °C.

The *S. melonis* isolated from GK at 0 and 10 days of fermentation are described as gellan and welan gum (exopolysaccharides) producers and have industrial applications (Kaur, Bera, Panesar, Kumar, & Kennedy, 2014). Thus, in the present study, these isolates also produced polysaccharides.

Moreover, in other previous studies (Arıkan, Mitchell, Finn, & Gürel, 2020; Marsh et al., 2014; Villarreal-Soto et al., 2020), the presence of bacterial reads was markedly higher than the fungal reads during entire fermentation process in both kombuchas. The total number of bacterial reads was higher in GK and then in BK during metagenomics findings. Nevertheless, in the culture-dependent method findings, the total number of bacterial and fungi reads was higher in BK than in GK. The total microbial diversity (Shannon index = 0.5 and 0.6 for GK and BK, respectively) was similar between both kombuchas; however, at 15 days of fermentation, this value was lower (0.12) for BK. This can be explained due to the high dominance of few groups. The predominant yeast *Z. balli* metabolizes acid acetic even under glucose-fermentative conditions, which presumably leads to its predominance in the fungal microbial kombucha (Rodrigues et al., 2012).

In our study, *Rhodotorula* and *Malassezia* as well as other amplicon

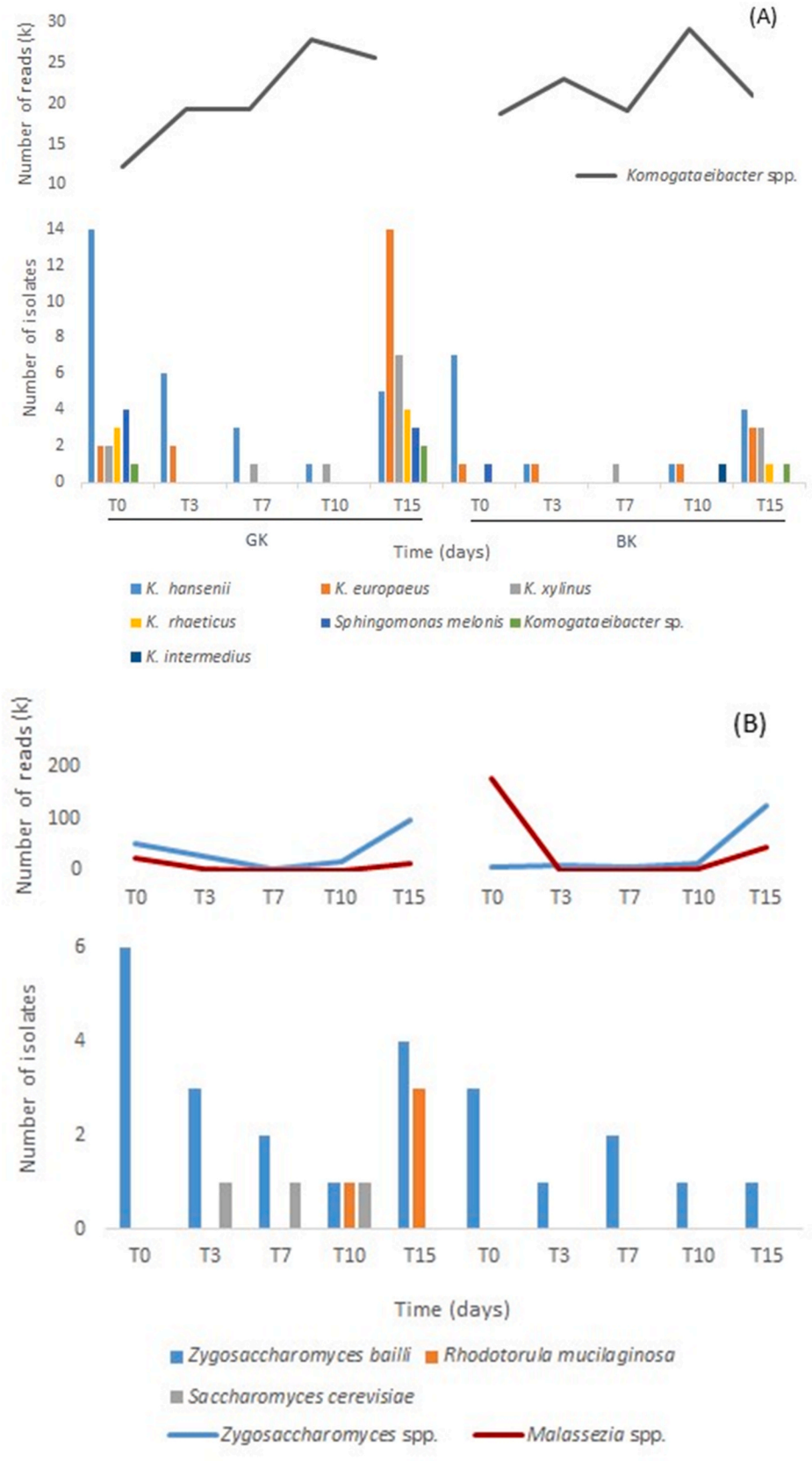

Fig. 1. Number of isolates and reads in green kombucha (GK) and black kombucha (BK) of bacteria (A) and Fungi (B), along 15 days of fermentation, at 28 °C. (COLOR SHOULD BE USED). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

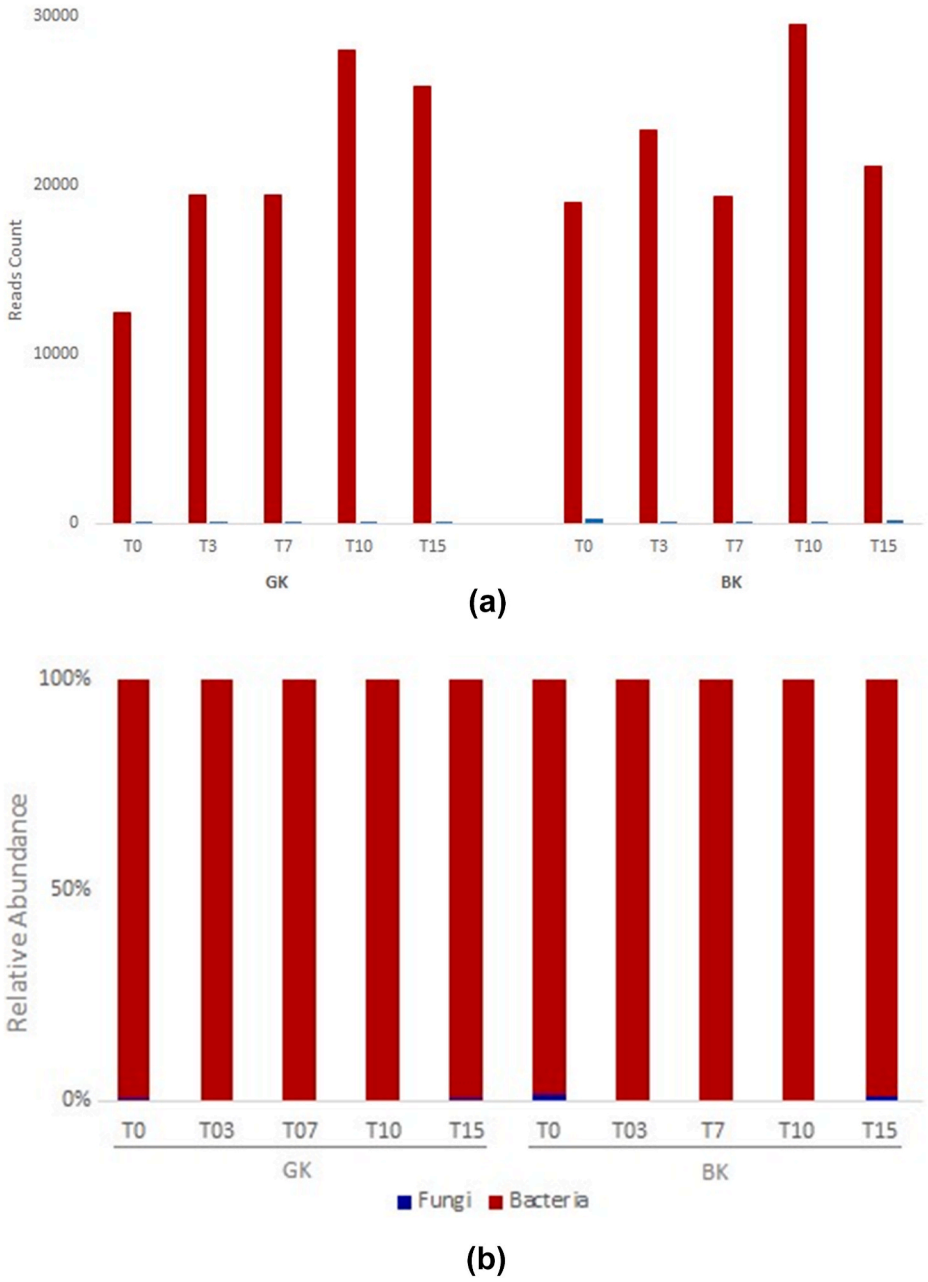

Fig. 2. Total reads count (A) and relative abundance (B) of bacteria (red) and fungi (blue) in cellulosic matrix in green kombucha (GK) and black kombucha (BK) along 15 days of fermentation achieved by metagenomics (amplicon). (COLOR SHOULD BE USED). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

sequences were detected specifically at the beginning and at the end of fermentation (Suppl. Table 1). The larger population of *Rhodotorula* and *Malassezia* and other ethanol nonproducer yeasts in BK in competition with *Zygosaccharomyces* presumably contributed to the lower ethanol production (Fig. 3).

The presence of *Malassezia* was previously limited to the dermis of humans and animals and was intricately linked to pathogenicity. In particular, molecular analysis of the environmental sequences revealed that the members of *Malassezia* are among the most widespread fungi in the world. *Malassezia* has been found in fermented food, marine environments, and other substrates (Góes-Neto, Marcelino, Verbruggen, Da Silva, & Badotti, 2020; Amend, 2014).

Certain studies report the presence of LAB in kombucha fermentation (Coton et al., 2017; Marsh et al., 2014), and in our study, LAB were not detected in any kombucha tea.

In general, our results are in accordance with those observed in the studies by Marsh et al. (2014) and Arıkan et al. (2020). These studies used a similar DNA isolation method. They found 90% of bacteria belonging to the genus *Komagataeibacter* (formerly *Gluconacetobacter*) and 95% of the total fungi belonging to the genus *Zygosaccharomyces*. These genera are adapted to environmental selective pressures in kombucha tea fermentations, such as low pH, high acetic acid and ethanol concentrations, and the possible positive interactions between them (Teoh et al., 2004; Kurtzman et al., 2011; Marsh et al., 2014; Reva et al., 2015; Nakano & Ebisuya, 2016; Matsushita, Toyama, Tonouchi, & Okamoto-Kainuma, 2016; Arıkan et al., 2020).

4.2. Chemical characterization and microbiota integration

For most of the analyzed parameters, changes were observed during

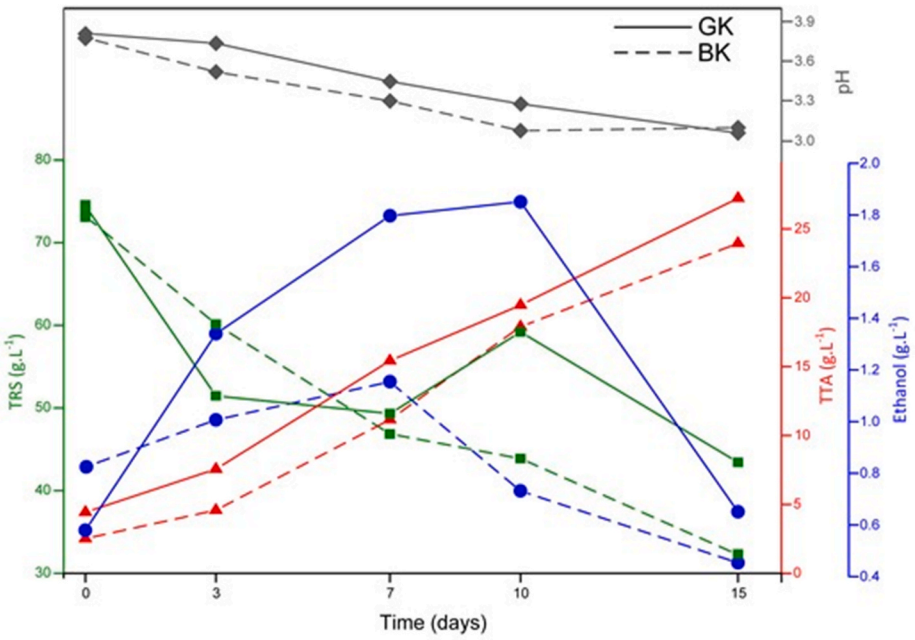

Fig. 3. pH and ethanol, organic acid, and total reducing sugar concentrations for 15 days to monitor kombucha fermentation process produced with green kombucha (GK) (filled lines) and black kombucha (BK) (dashed lines) teas, both at 28 °C. (COLOR SHOULD BE USED). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

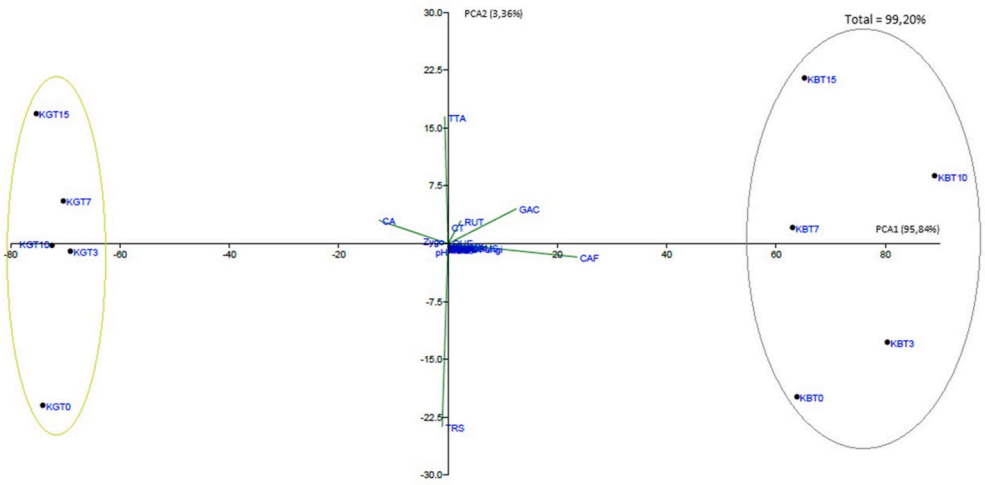

Fig. 4. Principal component analysis (PCA) of bacterial and fungal reads at genera level and physicochemical variables of green kombucha (GK) and black kombucha (BK) fermentations at different time periods (T0, T3, T7, T10, T15). (COLOR SHOULD BE USED). Legend: TRS: total reducing sugars; ETOH: ethanol detected quantitatively; pH: hydrogen potential; TTA: total titratable acidity; GAC: gallic acid; CT: catechin detected quantitatively; CA: chlorogenic acid; CAF: caffein; RUT: rutin; QUE: quercetin; Komag: *Komagataeibacter* spp.; Zygo: *Zygosaccharomyces* spp.; Malas: *Malassezia* spp.; Rhodo: *Rhodotorula* spp.; Other Fungi: other fungi; Acet: Acetal; EtOH: ethanol detected by GC-MS; PpO: propanol; HQ: hydroxyacetone; GlyAl: glycolaldehyde; AA: acetic acid; FurfAL: furfural; FA: formic acid; furfYL: furfuryl alcohol; DHA: dihydroxyacetone; 5A2F: 5-acetoxymethyl-2-furaldehyde; HMF: 5-hydroxymethylfurfural; HQ: hydroquinone; CAF-GCMS: Caffeine detected by GC-MS; Paro: Paromomycin; FurHMK: furyl hydroxymethyl ketone; 4HPyran: 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-; Mel: D-Melezitose. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the entire fermentation and indicated changes in chemical dynamics during fermentation in both kombucha (Fig. 3 and Suppl. Tables 2–5).

The concentration of *total reducing sugars* decreased along with fermentation (Fig. 1). This behavior indicates the sugar consumption by the kombucha microbial community, which is important in microbial metabolism and cellulose matrix production (Tonouchi, 2016). *Komagataeibacter* can use sucrose, glucose, and fructose (Komagata, Ino, & Yamada, 2014) to obtain energy and grow, thereby competing with yeasts for sugar since the commencement of fermentation. Moreover, this genus can produce acid from glucose and ethanol (Komagata, Iino, & Yamada, 2014) and cellulose matrix through fructose (Wang et al., 2018). The pH decreases are a consequence of organic acid production in both kombuchas (GK and BK). The *total titratable acidity* was also similar for both kombuchas (Suppl. Table 2). The Additionally, the TTA concentration values increased throughout the fermentation time. GK exhibited higher values for TTA than BK (Fig. 3; Suppl. Table 2). According to the literature on kombucha fermentation, weak acids such as acetic, glucuronic, and gluconic acids are produced by AAB in high concentrations (Jayabalan et al., 2014). Acetic acid may induce stimulatory effects on the growth and fermentative metabolism of *Z. bailii* (Prudencio, Sansonetty, & Corte-Real, 1998). Furthermore, *Z. bailii* seems to be more resistant than *S. cerevisiae* toward the inhibitory effects of ethanol in the presence of acetic acid that allowed them to referment sugars in wines (Fernandes, Corte-Real, Loureiro, Loureiro-Dias, & Leao, 1997).

The total concentration of *ethanol* detected along all the fermentation

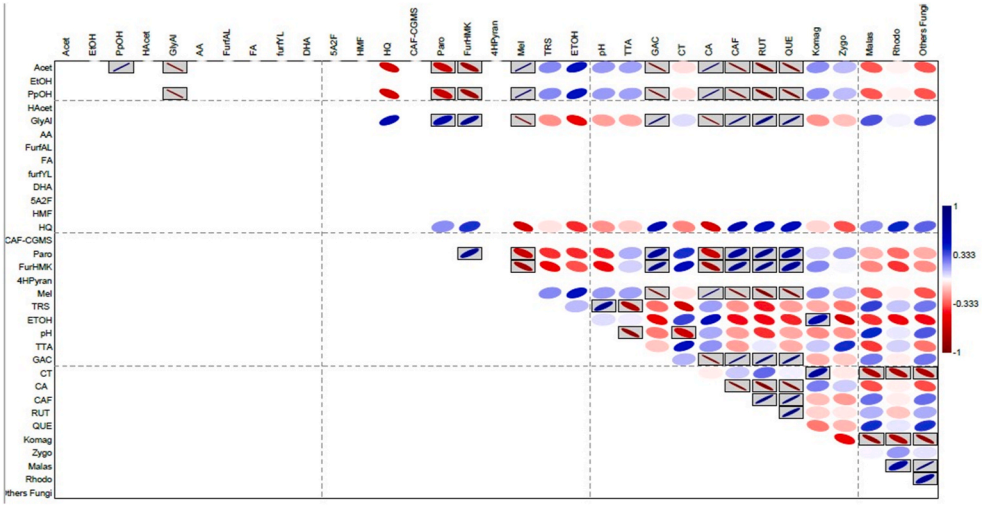

Fig. 5. Correlation analysis of bacterial and fungal reads at genus level and physico-chemical variables in each stage from green kombucha (GK) and black kombucha (BK) fermentation sample groups. (COLOR SHOULD BE USED). **Legend:** TRS: total reducing sugars; ETOH: ethanol detected quantitatively; pH: hydrogen potential; TTA: total titratable acidity; GAC: gallic acid; CT: catechin detected quantitatively; CA: chlorogenic acid; CAF: caffein; RUT: rutin; QUE: quercetin; Komag: *Komagataeibacter* spp.; Zygo: *Zygosaccharomyces* spp.; Malas: *Malassezia* spp.; Rhodo: *Rhodotorula* spp.; Others Fungi: others fungi; Acet: Acetal; EtOH: ethanol detected by GC-MS; PpO: propanol; HQ: hydroxyacetone; GlyAl: glycolaldehyde; AA: acetic acid; FurfAL: furfural; FA: formic acid; furfYL: furfuryl alcohol; DHA: dihydroxyacetone; 5A2F: 5-acetoxymethyl-2-furaldehyde; HMF: 5-hydroxymethylfurfural; HQ: hydroquinone; CAF-CGMS: caffeine detected by GC-MS; Paro: paromomycin; FurHMK: furyl hydroxymethyl ketone; 4HPyran: 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-; Mel: D-Melezitose. Blue ellipses are positive correlation, red ellipses are negative correlations, and ellipses (either blue or red) inside grey boxes are statistical significant correlations ($p < 0.05$). Note: As this test is not applicable to qualitative variables (e.g.,: FurA, FAL, etc.), no ellipses are depicted in the plot for those qualitative variables. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

processes was at least 2.3-fold higher in GK when compared to BK, particularly at times T7 and T10. In BK fermentation, no statistical difference ($p > 0.05$) was observed in ethanol production throughout the fermentation (Fig. 3; Suppl. Table 2; Suppl. Fig. 2). The microbial composition, particularly yeasts, may have contributed to this result and will be discussed further. The lowest *ethanol* concentrations were observed in T0 and T15, during the growth of various microorganisms (Figs. 1 and 3; Suppl. Table 1). Yeasts belonging to *Zygosaccharomyces* genus reveal the ability to ferment hexose sugars, such as glucose and fructose (Martorell, Stratford, Steels, Fernández-Espinar, & Querol, 2007; Wang et al., 2018). Therefore, at the beginning of the kombucha fermentation, the fermenting yeasts *Zygosaccharomyces* converts the available sugar into ethanol, and acetic acid bacteria *Komagataeibacter* metabolizes ethanol and produces acetic acid.

The antimicrobial activity of *polyphenols* of plants has been extensively investigated (Granato et al., 2016). In our study, most of the detected polyphenol concentration was higher in BK than GK during the entire fermentation. Nonetheless, carboxylic acid was the only polyphenol higher in GK than BK (Suppl. Fig. 2; Suppl. Fig. 2). These polyphenols can influence microbial composition when GK and BK are compared, which could explain certain differences among the read counts of bacteria by amplicon metagenomics (Fig. 1A; Suppl. Table 3). The presence of polyphenols in kombuchas, particularly gallic acid and caffeine in black tea, presumably interfered negatively in the microbial composition and growth during the fermentation process (Fig. 4; Suppl. Table 3).

Chlorogenic acid is one of the most available phenolic acid compounds naturally found in plant food and beverage as green coffee extracts and tea (Cheynier, 2005). The *chlorogenic acid* concentration was at least 25 times higher in GK (average of 65 mg L^{-1}) than in BK (average of 2.5 mg L^{-1}) during all analyses (Suppl. Table 3). Furthermore, antioxidant, antibacterial, antiviral, antimicrobial, and free radical scavenger activities exercised interference on the microbial community of kombuchas (Fig. 4).

In general, the compounds identified by GC-MS during kombucha fermentation (GK and BK) are responsible for the sensory formation of the product and may also exhibit antimicrobial activity. Notably, paromomycin and furyl hydroxymethyl ketone are products of fermentation in BK since they were detected only from T3 of BK (Suppl. Table 6). Moreover, the integration by PCA revealed a clear separation of GK chemical parameters from BK, but not by the microbiota (Fig. 4). *Caffein, gallic acid,* and *chlorogenic acids* were the chemical variables that majorly divided GK and BK in two distinct groups (Suppl. Fig. 3A). Furthermore, the *total reducing sugars* and *total titratable acidity* remarkably contributed to sample separation based on the fermentation time point (Suppl. Fig. 3B). These clear, distinct, and dynamic profiles of GK and BK fermentation seem to be the result of the intrinsic characteristics of each GK and BK teas, as well as the contribution of the kombucha microbial metabolism (Fig. 4).

Regarding microbial metagenomic results, correlation analysis (Fig. 5) indicated that, in general, the presence of *Komagataeibacter* was positively correlated ($p < 0.05$) with ethanol and catechins. For *Zygosaccharomyces*, no correlation was significant when paired with all variables studied ($p > 0.05$). *Malassezia*, *Rhodotorula*, and other fungi were negatively related to *Komagataeibacter*, indicating that these bacteria presumably play a pivotal role in controlling the contaminating community kombucha (non-*Zygosaccharomyces* fungi) (Fig. 5).

Eventually, kombucha seems to be a stable microbial system in which every adapted microorganism is responsible for the low diversity (composition, richness, and abundance), particularly during the fermentation time. A little higher diversity was observed in the initial and end times (T0 and T15) of both kombucha fermentations. Initially, these organisms can reflect the plant and production process-associated microbiota and the practices during harvesting and handling of tea leaves. Microorganisms that can remain viable but not cultivable (VBNC) during fermentation have the chance to reappear at the end of fermentation when selective pressures, such as ethanol concentrations, decrease in the system. Several studies reported that the microbiota that makes up the kombucha inoculants could vary according to the source and inoculum origin, the fermentation temperature, and the inoculum manipulation (De Filippis et al., 2018; Jayabalan et al., 2014; Marsh et al., 2014). Our results are consistent with those of May et al. (2019) for kombucha bacterial diversity. These findings indicated that there is no apparent "canonical" species composition. Moreover, the environment (i.e., substrates, culture conditions) has an impact on the composition of kombucha microbiota.

Our results revealed that the GK and BK chemical composition does not interfere with the relative total abundance of bacteria and yeasts

during kombucha fermentation. Coton et al. (2017) reported differences in the relative abundance only for bacteria but not for fungi when comparing green and black tea kombucha. Furthermore, the microbial diversity, particularly the fungi found by Coton et al. (2017) differed from that used in the present study.

5. Conclusion

Komagataeibacter and *Zygosaccharomyces* genera were the most frequently observed bacterial and fungal genera in GK and BK kombucha during all the fermentation times, considering both dependent- and independent-culture methods.

Notably, most of the compounds detected by GC-MS mentioned herein, whether from the tea itself or the fermentative microbiota, have an antimicrobial activity already described in the literature. Their presence, associated to the increasing and high concentration of organic acids, low pH, and high concentrations of alcohols and phenolic compounds, make kombucha an extremely selective environment where only the well adapted microorganisms can persist.

This study importantly contributes to the knowledge about the profile of the integrated microbiota to the chemical results of the kombucha fermented in green and black teas. Based on our results and published literature on kombucha microbiota, we suggest, for future studies, the use of a starter culture for the production of kombucha comprising species of *Komagataeibacter*, producers of cellulose biofilm, combined with an acid resistant yeast, like *Z. bailii*, or another one that is able to carry alcoholic fermentation from sucrose, fructose, and/or glucose.

CRediT authorship contribution statement

Cosme Damião Barbosa: Conceptualization, Methodology, Formal analysis, Writting. **Ana Paula Trovatti Uetanabaro:** Methodology, Writing. **Wildon César Rodrigues Santos:** Methodology, Formal analysis. **Renata Gomes Caetano:** Methodology, Formal analysis. **Helena Albano:** Formal analysis, Supervision, Writting-Review. **Rodrigo Kato:** Methodology, Metagenomics approaches. **Gustavo Pereira Cosenza:** Formal analysis. **Antonio Azeredo:** Writting-Review. **Aristóteles Góes-Neto:** Metagenomics approaches, Writting-Review. **Carlos Augusto Rosa:** Writting-Review and supervision. **Paula Teixeira:** Funding acquisition, Writting-Review. **Verônica Ortiz Alvarenga:** Formal analysis, Writting-Review and supervision. **Inayara Cristina Alves Lacerda:** Conceptualization, Methodology, Formal analysis, Writting-Review and supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.111788.

Declarations of interest

None.

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