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Different temperatures select distinctive acetic acid bacteria species and promotes organic acids production during Kombucha tea fermentation

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ABSTRACT

Kombucha is a traditional beverage produced by tea fermentation, carried out by a symbiotic consortium of bacteria and yeasts. Acetic Acid Bacteria (AAB) usually dominate the bacterial community of Kombucha, driving the fermentative process. The consumption of this beverage was often associated to beneficial effects for the health, due to its antioxidant and detoxifying properties. We characterized bacterial populations of Kombucha tea fermented at 20 or 30 °C by using culture-dependent and −independent methods and monitored the concentration of gluconic and glucuronic acids, as well as of total polyphenols. We found significant differences in the microbiota at the two temperatures. Moreover, different species of *Gluconacetobacter* were selected, leading to a differential abundance of gluconic and glucuronic acids.

1. Introduction

Kombucha is a traditional beverage usually consumed in Asia. It is prepared by fermentation of sweetened tea using a consortium of yeasts and bacteria as inoculum, also known as tea fungus or SCOBY ("Symbiotic Colony of Bacteria and Yeast"; Sreeramulu et al., 2000). It is usually fermented for 8–10 days and it contains acetic acid, small quantities of ethanol and CO_2. Acetic acid bacteria (AAB) and osmophilic yeasts are the dominant microorganisms during Kombucha fermentation. They develop a cellulosic pellicle (biofilm) floating on the fermented liquid, where they remain embedded and that can be transferred to propagate the inoculum. Few AAB species were identified by culture-dependent studies: species of *Acetobacter* usually dominate (Chen and Liu, 2000; Dutta and Gachhui, 2006; El-Salam, 2012), but also *Gluconacetobacter* and *Lactobacillus* were sometimes isolated (Trovatti et al., 2011). However, AAB are known to be particularly difficult to isolate through culture-dependent approaches (Sengun and Karabiyikli, 2011). Among AAB, *Acetobacter xylinum,* re-classified as *Gluconacetobacter xylinus* (Yamada et al., 2012), is considered the best cellulose producer in Kombucha (Strap et al., 2011). Also many yeast species were identified in Kombucha, mainly *Zygosaccharomyces* spp., *Saccharomyces* spp., *Dekkera* spp. and *Pichia* spp. (Marsh et al., 2014; Reva et al., 2015; Jayabalan et al., 2014).

Chemical profile of Kombucha is dominated by organic acids (mostly acetic, gluconic and glucuronic) and tea polyphenols (Jayabalan et al., 2014), that can be responsible for the multiple health benefits associated with the regular consumption of this beverage. Such effects include anti-carcinogenic (Jayabalan et al., 2011), anti-diabetic (Aloulou et al., 2012) and detoxifying potential (Lončar et al., 2000), improvement of the immune response (Ram et al., 2000) and treatment of gastric ulcers (Banerjee et al., 2011) and high blood cholesterol (Yang et al., 2009).

We aimed to characterize the microbiota involved in Kombucha fermentation and to explore the effects of two fermentation temperatures (20 vs 30 °C) on the microbiota, in order to define which conditions can be applied to boost gluconic and glucuronic acid production.

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2. Materials and methods

2.1. Kombucha culture maintenance

The Kombucha biofilm used as inoculum was purchased from Lexas Distribution sas (Étréchy, France) and shipped refrigerated in its fermented liquid. About 20 g of pellicle were inoculated in 1 L of ceylon black or bancha green tea. One litre of water added with 100 g of sucrose was sterilized at 121 °C for 15 min. Ten g/L of tea leaves were infused for 10 min in boiling water. Initial acidification of the brew was done with 30 ml/L of the fermented liquid shipped with the biofilm. Fermentation was carried out in duplicate for each type of tea (black or green), both at 20 or 30 °C. The biofilm coming from previous fermentation was used as inoculum in the subsequent fermentation.

Samples of fermented liquid were collected at the beginning and after 1, 7, 9, 14, 21 days of fermentation. The biofilm was analysed at the beginning and at the end of each fermentation. pH of the fermented liquid was monitored at each sampling point using a bench pH-meter (XS Instruments, Carpi, Italy).

2.2. Culture-dependent analysis

Serial dilutions were prepared starting from 10 ml of liquid or 10 g of biofilm homogenized in Ringer's solution (Oxoid, Milan, Italy) by using a stomacher (Stomacher400 circulator; Seward Medical, London, United Kingdom). Dilutions from 10^{-2} to 10^{-8} were plated on two different media for AAB enumeration: Frateur (Frateur, 1950) and Kappeng & Pathom-Aree (Kappeng and Pathom-Aree, 2009) media added with 5 ml of a 1% cycloheximide solution. Plates were incubated at 30 °C for 48 h and typical colonies were counted. Analyses were performed in triplicate.

2.3. DNA extraction, library preparation and sequencing

Total DNA extraction from samples collected at the different sampling points was carried out by using the Biostic Bacteremia DNA extraction kit (MoBio Laboratories Inc., Carlsbad, CA), starting from the pellet obtained from 2 ml of liquid (12,000 g for 2 min). Samples of biofilm were cut with sterile scalpel, washed with sterile deionized water and then diluted 1:10 in Phosphate Buffered Saline (pH 7.4) and homogenized in a stomacher (Stomacher400 circulator; Seward Medical, London, United Kingdom), for 1 min at 230 rpm at room temperature. The pellet obtained from centrifugation (12,000 g for 2 min) of 2 ml of this suspension was used for DNA extraction as described above. Bacterial diversity was studied through pyrosequencing of the V1-V3 regions of the 16S rRNA gene (about 520 bp). Primers and PCR conditions were previously reported (De Filippis et al., 2014). PCR products were purified with the Agencourt AMPure kit (Beckman Coulter, Milano, Italy) and quantified using a Plate Reader AF2200 (Eppendorf, Milano, Italy). Equimolar pools were obtained prior to further processing and sequenced on a GS Junior platform (454 Life Sciences, Roche Diagnostics, IT), according to the manufacturer's instructions.

2.4. Determination of gluconic and glucuronic acid

Fermented liquid samples were centrifuged (2500 g, 4 °C, 10 min) and the aqueous layer was filtrated by nylon filters (0.45 μm, Millipore, Billerica, MA); 20 μl were diluted 50 times in acetonitrile and 10 μl were injected by using a Series 200 autosampler equipped with two micropumps Series 200 (PerkinElmer, Waltham, MA). Separation of gluconic and glucuronic acid was achieved on a TSKgel Amide-80 column (250 mm × 2.0 mm, 5 μm, Tosoh Bioscience, Tokyo, Japan) using the following mobile phases: A, acetonitrile and B, water 13 mM ammonium acetate (pH 5.8). The compounds were eluted at 200 μl/min using the following gradient of solvent B (t in [min]/[%B]): (0/10), (3/10), (7/90), (11/90). Negative electrospray ionization was used for the detection and the source parameters were optimized by infusing the two standards (20 ppm) dissolved in a mixture acetonitrile/water/acetic acid (90:9.9:0.1, v/v/v) directly in the ion source: spray voltage, −4.5 kV; capillary temperature, 250 °C; dwell time, 100 ms; declustering potential, −25 V; cad gas and curtain gas were set to 6 and 40, while ion source gas 1 and 2 were 35 and 5 respectively (arbitrary units). The chromatographic profile was recorded in multiple reaction monitoring (MRM) mode in order to improve selectivity by using an API 2000 triple quadrupole mass spectrometer (ABSciex, Carlsbad, CA). The two compounds were analyzed by using the mass transitions and collision energy (CE) given in parentheses, with the quantitative transition in bold: gluconic acid (*m/z* $[M-H]^-$ 195 → **129**, CE: −16 V; 195 → 75, CE: −24 V) and glucuronic acid (*m/z* $[M-H]^-$ 193 → 73, CE: −21 V; 193 → **59**, CE: −29 V). Gluconic and glucuronic acid were quantified using a linear calibration curve with the external standard technique. The limit of detection (LOD) and the limit of quantitation (LOQ) were monitored according to the signal to noise ratio (Armbruster et al., 1994). The coefficients of determination r^2 were tested plotting the area counts towards known concentration of the pure compounds dissolved in acetonitrile/water (90/10, v/v). The relative standard deviation (RSD%) of intraday and interday assay was monitored three times each day and three times in different days (reproducibility and repeatability test). The recovery test was performed by spiking three samples with the lowest concentration of gluconic and glucuronic acid (T0) with three concentrations of standard mix (0.1 mg/L, 1 mg/L and 10 mg/L).

Analytical performances of both acids were tested for reproducibility, repeatability, limit of detection (LOD), limit of quantification (LOQ), linearity, precision and carry-over effect. Before and after each batch, a solution of acetonitrile:water (50:50, v/v) was injected in order to verify the absence of any contaminants. The concentration 10 ppb resulted in no signal, the LOD was 50 ppb and the LOQ was 100 ppb for both analytes. Linearity was achieved in the range 0.1–10 mg/L and after each calibration curve set, two blank samples (fermented samples at T0) were injected to verify the absence of carry-over effects. The RSD (%) among the six curves was always lower than 10% (reproducibility and repeatability test). Typical retention times of glucuronic acid and gluconic acid were 9.50 min and 9.75 min, respectively. The analytical performances were in the same order of magnitude of those previously obtained by LC-DAD (Jayabalan et al., 2007) or by anion exchange chromatography coupled to a copper modified electrode (Casella and Gatta, 2001).

2.5. Total polyphenol analysis

The method described by Singleton et al. (1999) with brief modifications was used. Samples were diluted 15 times in water and 0.125 ml were further dissolved in a 20% (v/v in water) of Folin-Ciocalteu reagent. The solutions were stirred for 30 s and allowed to stand 6 min, then 1 mL of a 7.5% aqueous sodium carbonate solution, along with 1.250 mL of water was added to the flasks. Stirring and incubation of the flasks at room temperature for 90 min was performed. Absorbance was read at 765 nm by using a T92 + UV double beam spectrophotometer (PG Instruments, Leicester, UK). Specific calibration curve of (−)-epicatechin was built in the range 10–100 mg/L and the activity of the polyphenol compounds was expressed as mg (−)-epicatechin equivalent/mL, whereas water was used as blank sample.

2.6. Data analysis

Raw reads were firstly filtered according to the 454-processing pipeline. Sequences were then analyzed and denoised by using QIIME 1.9.1 software (Caporaso et al., 2010). Quality filtering, Operational Taxonomic Units (OTU) picking and taxonomic assignment was carried out as previously described (De Filippis et al., 2016). In order to avoid biases due to the different sequencing depth, OTU tables were rarefied to the lowest number of sequences per sample and imported in R (http://www.r-project.org) for statistical analyses and visualization.

Spearman's pairwise correlations were computed between OTU or oligotypes abundance and gluconic and glucuronic acids concentration (*corr.test* function in *psych* package). Correction of p-values for multiple testing was performed (Benjamini and Hochberg, 1995). Alpha-diversity analysis was carried out in QIIME on rarefied OTU tables. Kruskal-Wallis and pairwise Wilcoxon tests were used to determine significant differences in alpha diversity parameters or in OTU abundance. Permutational Multivariate Analysis of Variance (non-parametric MANOVA) based on Jaccard and Bray Curtis distance matrices was carried out by using 999 permutations to detect significant differences in the overall bacterial community composition as affected by the type of tea (black or green) or temperature (20 or 30 °C) by using the *adonis* function in *vegan* package.

Reads assigned to *Gluconoacetobacter* genus were extracted and entropy analysis and oligotyping were carried out as described by the developers (Eren et al., 2013). After the initial round of oligotyping, high entropy positions were chosen (-C option): 4, 541, 553. To minimize the impact of sequencing errors, we required an oligotype to be represented by at least 1000 reads (-M option). Moreover, rare oligotypes present in less than 5 samples were discarded (-s option). These parameters led to 182,914 (99.91%) sequences left in the dataset and to the identification of two different oligotypes. BLASTn was used to query the representative sequences against the NCBI nr database, and the top hit was considered for taxonomic assignment.

The 16S gene sequences produced in this study are available at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI), under accession number SRP112753.

3. Results

3.1. Monitoring of AAB by culture-dependent analysis

Initial loads of AAB detected on both the media used were below 10^5 CFU/ml. AAB increased of about 1.5 log in the fermented liquid after one week and their levels remained stable until the end of the process (Fig. 1). Consistently, pH rapidly decreased from about 3.5 to 2.5 after 7 days. No significant effect on AAB loads was found for neither the type of tea used (black or green) nor the fermentation temperature ($P > .05$). On the contrary, AAB levels in the biofilm at the end of the fermentation period (21 days) were significantly ($P < .05$) higher at 30 °C compared to 20 °C (about 5.5 and 8.5 log CFU/g, at 20 and 30 °C, respectively; Fig. S1A).

3.2. Higher fermentation temperature affects microbial diversity and selects different species of Gluconacetobacter promoting organic acids production

No differences in the overall bacterial community composition were found by MANOVA according to the type of tea used ($P > .05$). On the contrary, samples fermented at 30 °C showed higher diversity ($P < .05$) and clearly clustered apart from those fermented at 20 °C (Fig. 2). *Gluconacetobacter* dominated both the fermentations, while *Acetobacter* was found at lower levels, already after 24 h of fermentation (around 80 and 10%, respectively) regardless of the temperature used. After 7 days, *Gluconacetobacter* accounted more than 90% of the bacterial population and its abundance remained unchanged till the end of the fermentation, while Acetobacter abundance decreased over the time, reaching levels of around 3–5% after 21 days. However, higher temperature promoted the growth of several Firmicutes (*Lactobacillus, Lactococcus, Streptococcus*) and Actinobacteria (*Propionibacterium, Corynebacterium*; Fig. 2), although their abundance was always lower than 2% and decreased as the fermentation proceeded. The biofilm bacterial community was dominated by the same genera at both the temperatures tested, with higher diversity in the biofilm coming from the fermentation at 30 °C (Fig. S1B). Interestingly, *Gluconobacter* was present in the original biofilm with an abundance of about 2%, but it was not detected in the biofilm samples coming from the laboratory fermentations (Fig. S1B). We oligotyped *Gluconacetobacter* sequences in order to explore sub-genus diversity. Two different oligotypes were found, identified as *G. saccharivorans* and *G. xylinus* and the prevalence of either of them was strongly affected by the fermentation temperature ($P < .05$; Fig. 3). Indeed, *G. xylinus* prevailed at 20 °C, while *G. saccharivorans* was boosted by higher temperature. Although *G. saccharivorans* was the only oligotype detected in the original biofilm used as first inoculum, *G. xylinus* quickly took over already after 24 h at 20 °C (Fig. 3). Gluconic acid was always more abundant than glucuronic acid. Interestingly, both acids showed higher concentrations in samples fermented at 30 °C and were positively correlated to *G. saccharivorans* levels ($P < .05$).

While no effect of the tea used was observed neither on the microbiome nor on the organic acid concentration, the variation of total polyphenol content showed a matrix-dependent evolution, when the fermentation was carried at 20 °C. Although higher in green compared to black tea at the beginning of the fermentation ($P < .05$), the content of polyphenols did not change in green tea during 21 days of fermentation at 20 °C, while it increased over time in black tea ($P < .05$, Fig. S2). The matrix-effect was not observed at 30 °C, where total polyphenols did not change up to 15 days, while decreasing from 15 to 21 days (data not shown).

4. Discussion

The effect of fermentation temperature on the bacterial community during Kombucha tea fermentation was explored by using both culture-dependent and independent approaches. We decided to monitor AAB using two different growth media, since these populations are known to be extremely difficult to cultivate in laboratory media (Mamlouk and Gullo, 2013). Nevertheless, no differences in cultivable AAB were detected according to the medium used, or to the fermentation temperature. The increase in AAB loads was consistently associated with a decrease of pH up to about 2.5. AAB loads reached about 5.5 and 9 log CFU/g in the Kombucha biofilm, and 6.5–7.5 log CFU/ml in the liquid at the end of the fermentation at 20 and 30 °C, respectively. These values are in line with a recent study on industrial-scale Kombucha, although the temperature of the process was not reported by the authors (Coton et al., 2017). HTS revealed *Gluconacetobacter* as dominating genus in both the fermentations, regardless of the temperature, as previously reported in other studies (Marsh et al., 2014; Reva et al., 2015). Nevertheless, the higher fermentation temperature allowed the development of sub-dominant bacterial populations, mainly characterized by environmental contaminants (e.g. *Acinetobacter*, *Propionibacterium*) and lactic acid bacteria (LAB, e.g. *Lactobacillus*, *Streptococcus*). In this study, the maximum LAB abundance was 0.1%. This level was lower than that previously

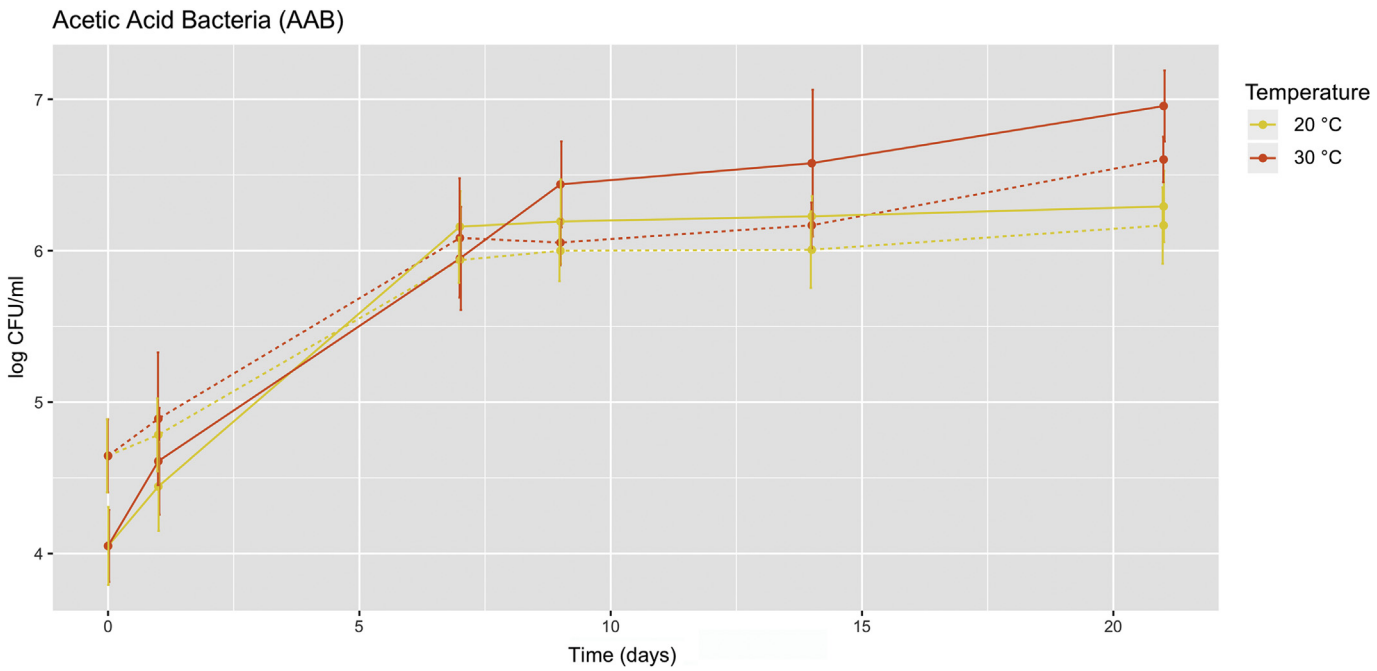

Fig. 1. Microbial loads (log CFU/ml) of Acetic Acid Bacteria (AAB) in Kombucha tea fermented at 20 °C (yellow) or 30 °C (orange) measured on Kappeng (continuous line) or Frateur (dashed line) medium. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

reported in Kombucha tea and this was likely due to the origin of the biofilm used (Marsh et al., 2014). Indeed, the same authors reported different microbiota in pellicles from Europe and US and observed the highest levels of LAB in the Irish one (Marsh et al., 2014). Chakravorty et al. (2016) reported *Komagataeibacter* (formerly *Gluconacetobacter*) as dominating genus in Kombucha biofilm and soup, although the species level taxonomy was not reached in that study. *Gluconacetobacter* taxonomy was recently updated and this genus has been subdivided into three genera: *Nguyenibacter*, *Komagataeibacter* and *Gluconacetobacter* (Yamada et al., 2012). Thus, it is possible that reads assigned to *Gluconacetobacter* may actually belong to *Komagataeibacter*, since microbial databases have not been updated yet to accommodate the reclassification.

Moreover, we used the oligotyping technique in order to explore a possible diversity within the dominating genus *Gluconacetobacter*. While fermentation temperature did not seem to affect the composition of the dominant bacterial community at genus level, a strong effect was found at the sub-genus level. We found two different *Gluconacetobacter* oligotypes, whose abundance was strongly correlated with the fermentation temperature and that were identified as two different species (*G. xylinus* and *G. saccharivorans*). Higher temperature promotes the growth of *G. saccharivorans*, while *G. xylinus* dominated the fermentation at 20 °C. Interestingly, Coton et al. (2017) reported *G. europaeus* as dominating species in industrial Kombucha (although the fermentation temperature was not reported), while *Komagataeibacter xylinus* (formerly *Gluconacetobacter*) was found as dominant in Ukrainian Kombucha fermented at 28 °C (Reva et al., 2015). This may support the hypothesis of a species-level selection driven by the different fermentation conditions, as well as a diversity associated with the geographical origin of the biofilm. *Gluconacetobacter* species previously showed different phenotypic traits, such as the ability of producing acids from several sources, growing at different concentration of acetic acid and synthesizing cellulose, although many traits seem to be strain-specific as well (Lisdiyanti et al., 2006; Dutta and Gachhui, 2007). This difference at sub-genus level may partially explain the higher abundance of gluconic and glucuronic acids in Kombucha tea fermented at 30 °C. Indeed, gluconic acid production was previously found to be a strain-specific trait (Sainz et al., 2016). This study firstly shows that the modulation of the temperature during Kombucha tea fermentation may be used to select the AAB species that will drive the process, influencing organic acids concentration, which may contribute to the health-promoting effects of the habitual consumption of Kombucha tea (Jayabalan et al., 2007, 2014). Glucuronic acid levels reached in the samples analysed here are similar to those previously reported in fermented black and green tea (Jayabalan et al., 2007), while gluconic acid concentration was lower than that reported by Chakravorty et al. (2016).

Diversity at species-level may also explain the difference found in polyphenols concentration between 20 and 30 °C fermentations. Polyphenols concentration increased in black tea during the fermentation at 20 °C, consistently with other reports (Chakravorty et al., 2016; Kallel et al., 2012), and suggesting a role of the microbiota in releasing these compounds. The differences between the matrices may depend on the polyphenol content of black and green tea, the manufacturing conditions and particle size of the leaves (Astill et al., 2001; Chen and Sang, 2014). On the contrary, when fermentation was carried out at 30 °C, total polyphenols significantly decreased after 15 days. This may be due to a direct metabolizing activity of the microbiota on polyphenols leading to other not monitored bioactive compounds as well as to chemical transformation of polyphenols due to modification of the environmental conditions (Cardona et al., 2013; Wang and Chi-Tang, 2009). Accordingly, Jayabalan et al. (2007) monitored the levels of 4 epicatechin isomers and found a biotransformation during Kombucha fermentation, possibly driven by microbial enzymes.

We proposed an in-depth characterization of the bacterial populations during Kombucha tea fermentation and highlighted the effect of a modulation of the temperature during the process in selecting different *Gluconacetobacter* species, possibly associated

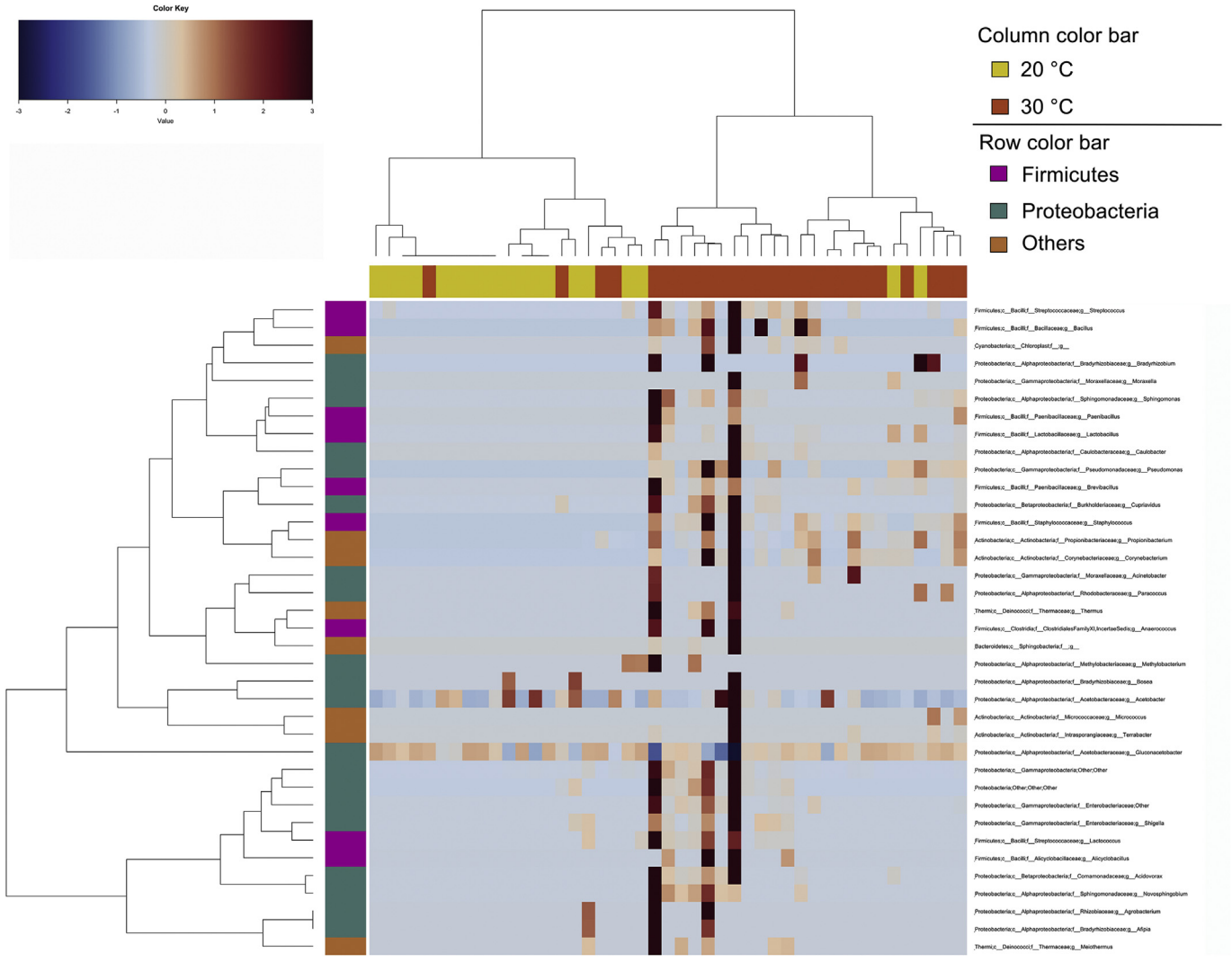

Fig. 2. Hierarchical Ward-linkage clustering of samples based on the Spearman's correlation coefficient of the proportion of genera. Column bar is color-coded according to the fermentation temperature (20 or 30 °C) and row bar is colored according to the bacterial phylum. The color scale represents the scaled abundance of each variable, denoted as Z-score, with red indicating high abundance and blue indicating low abundance.

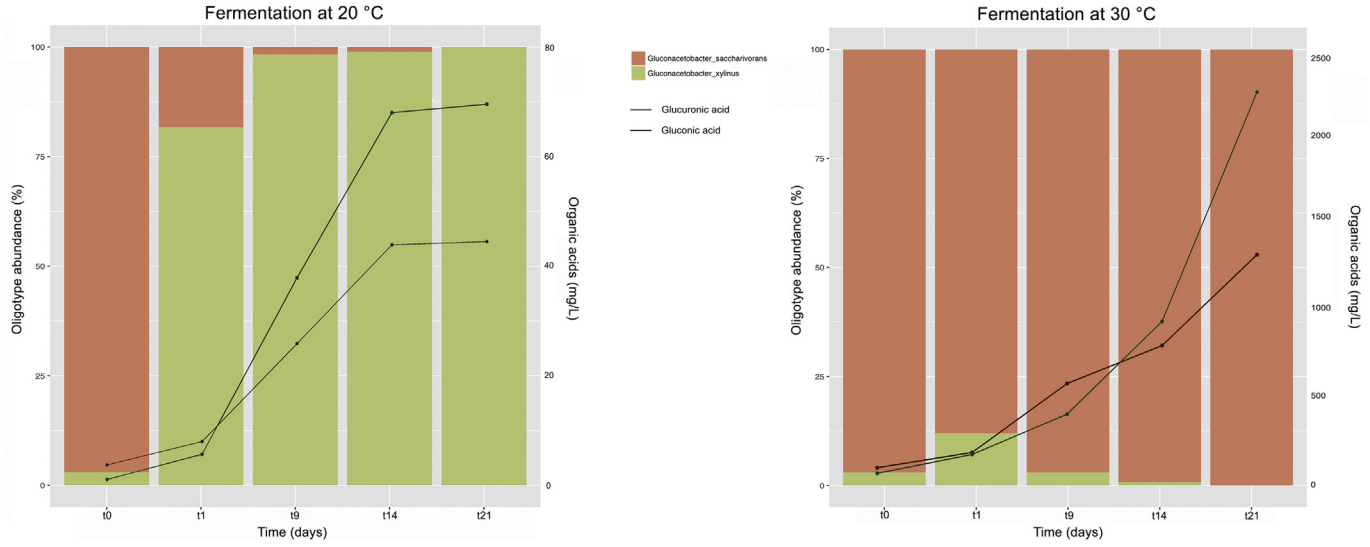

Fig. 3. Stacked bar chart showing the abundance of *Gluconacetobacter* oligotypes and line plot reporting the concentration of gluconic and glucuronic acids in Kombucha tea samples fermented at 20 °C (left panel) or 30 °C (right panel).

with the production of health-promoting metabolites. These results may be useful for the optimization of industrial-scale Kombucha fermentations, to improve process efficiency and product quality.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2018.01.008.

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