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RESEARCH ARTICLE

Unraveling microbial ecology of industrial-scale Kombucha fermentations by metabarcoding and culture-based methods

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ABSTRACT

Kombucha, historically an Asian tea-based fermented drink, has recently become trendy in Western countries. Producers claim it bears health-enhancing properties that may come from the tea or metabolites produced by its microbiome. Despite its long history of production, microbial richness and dynamics have not been fully unraveled, especially at an industrial scale. Moreover, the impact of tea type (green or black) on microbial ecology was not studied. Here, we compared microbial communities from industrial-scale black and green tea fermentations, still traditionally carried out by a microbial biofilm, using culture-dependent and metabarcoding approaches. Dominant bacterial species belonged to *Acetobacteraceae* and to a lesser extent *Lactobacteriaceae,* while the main identified yeasts corresponded to *Dekkera, Hanseniaspora* and *Zygosaccharomyces* during all fermentations. Species richness decreased over the 8-day fermentation. Among acetic acid bacteria, *Gluconacetobacter europaeus*, *Gluconobacter oxydans, G. saccharivorans* and *Acetobacter peroxydans* emerged as dominant species. The main lactic acid bacteria, *Oenococcus oeni,* was strongly associated with green tea fermentations. Tea type did not influence yeast community, with *Dekkera bruxellensis*, *D. anomala*, *Zygosaccharomyces bailii* and *Hanseniaspora valbyensis* as most dominant. This study unraveled a distinctive core microbial community which is essential for fermentation control and could lead to Kombucha quality standardization.

Keywords: Fermented tea; Kombucha; microbial ecology; metabarcoding, bacteria, yeast

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INTRODUCTION

Kombucha is a traditional fermented beverage with a long history of use dating back thousands of years, in particular in Asia (Teoh, Heard and Cox 2004). The etymology of the word 'Kombucha' may come from two Japanese words *kombu*, for seaweed, and *cha*, for tea; however, this remains unclear (Jarrell, Cal and Bennett 2000). Nowadays, this tea-based naturally sparkling beverage has become very popular in the Western world (North America and Europe) and is often claimed to exhibit healthful properties (i.e. detoxification, anti-oxidation or anti-carcinogenic) that may come from the tea itself or metabolites produced by microorganisms during fermentation. Despite marketing campaign alleges, studies are still lacking in the literature to fully assess such health claims.

Traditionally obtained by fermenting sweetened black or green tea (for, on average, 8 to 12 days under aerobic and static conditions) in the presence of indigenous microorganisms entrapped in a natural floating cellulosic biofilm also called 'pellicle', the final product is particularly rich in organic acids and CO_2 (Reiss 1994; Blanc 1996; Teoh, Heard and Cox 2004; Dutta and Gachhui 2006, 2007; Marsh *et al*. 2014b). Although considered as a non-alcoholic beverage, ethanol traces may also be observed according to variations in fermentation processes and/or duration (Sievers *et al*. 1995; Blanc 1996; Chen and Liu 2000; Jayabalan, Marimuthu and Swaminathan 2007). The biofilm used to carry out Kombucha fermentations is directly produced by indigenous microorganisms making the process quite similar to traditional vinegar production. Each fermentation leads to a new biofilm layer that can be used for future fermentations as a starter (back slopping). The final product, which has a slightly acidic or sour taste, is considered to be refreshing and typically effervescent. After prolonged periods, the final product also leads to a mild vinegar taste (Reiss 1994; Blanc 1996). This is due to organic acid (mainly acetic, lactic and to a lesser extent gluconic and glucoronic acids) as well as other metabolites released by microorganisms, along with CO_2 from yeast alcoholic fermentation (Sievers *et al*. 1995; Teoh, Heard and Cox 2004; Cvetkovic *et al*. 2008; Chakravorty *et al*. 2016). The fermented product will have an acidic pH generally close to 3, although some authors have described pH below 2 after prolonged fermentations (which may cause organoleptic defects).

The fermentation process occurs spontaneously and rapidly thanks to the microbiologically rich biofilm containing indigenous yeast and bacterial species. It is well recognized that these bacterial (especially acetic acid bacteria, AAB) and yeast communities have a symbiotic relationship during fermentation. Indeed, first AAB, such as *Komagataeibacter xylinus* (recently reclassified from *Gluconacetobacter xylinus* and *Acetobacter xylinum*), have been described to be involved in producing the cellulose rich biofilm in which both bacterial and yeast species are encountered (Sievers *et al*. 1995; Mikkelsen *et al*. 2009; Jayabalan *et al*. 2010; Strap *et al*. 2011). This biofilm, in direct contact with the atmosphere (Sievers *et al*. 1995), can also be viewed as a microbial niche, maintaining aerobic conditions during fermentation in open tanks, an essential condition for metabolic activities of strictly aerobic microorganisms (i.e. AAB). Then, at the beginning of fermentation, yeast, especially fermentative species, hydrolyze the added sucrose into simple sugars (glucose and fructose) and produce ethanol, CO_2 and organic acids. Then, bacteria, especially AAB, show intense activity and transform the simple sugars glucose and fructose into, among others, gluconic and acetic acids, respectively, but also ethanol into acetic acid (Reiss 1994; Sievers *et al*. 1995). Lactic acid bacteria (LAB) can also be metabolically active during this phase and produce organic acids such as lactic acid. As stated previously, the resulting fermented beverage is low in pH and contains multiple organic acids (mainly acetic and lactic, and to a lesser extent gluconic acid), trace amounts of ethanol and glycerol as well as phenolic compounds (Sievers *et al*. 1995; Teoh, Heard and Cox 2004; Cvetkovic *et al*. 2008; Chakravorty, Sarkar and Gachhui 2015; Chakravorty *et al*. 2016).

Culture-based and molecular methods have been used to identify microbial species present during the different stages of Kombucha fermentations (Jankovic and Stojanovic 1994; Mayser *et al*. 1995; Liu *et al*. 1996; Chen and Liu 2000; Markov *et al*. 2001; Teoh, Heard and Cox 2004; Marsh *et al*. 2014b; Chakravorty *et al*. 2016). However, all these studies describe lab-scale productions in low volume fermentations (typically ranging from 200 mL to 2 L) that, due to differences in related parameters such as oxygenation throughout fermentation tanks, sugar content distribution (osmotic stress) and biofilm surface area, do not necessarily and accurately represent real industrial-scale conditions. Moreover, the recent use of culture-independent methods to study microbial communities did not allow identifying bacterial communities to the species level (Marsh *et al*. 2014b; Reva *et al*. 2015; Chakravorty *et al*. 2016) which can be considered as key information needed for optimized and controlled fermentations. Yeast community dynamics appear quite complex with a succession of dominant species varying over time and including species affiliated to the *Zygosaccharomyces*, *Brettanomyces/Dekkara*, *Saccharomyces*, *Candida*, *Kloeckera/Hanseniaspora*, *Torulaspora*, *Torulopsis*, *Pichia*, *Saccharomycoides*, *Wallemia*, *Schizosaccharomyces* and *Lachancea* genera (Hesseltine 1965; Jankovic and Stojanovic 1994; Mayser *et al*. 1995; Liu *et al*. 1996; Chen and Liu 2000; Markov *et al*. 2001; Teoh, Heard and Cox 2004; Marsh *et al*. 2014b; Reva *et al*. 2015; Chakravorty *et al*. 2016;). Studies have also shown that osmotolerant fermentative species (i.e. *Zygosaccharomyces* spp., *Torulaspora delbrueckii*, *Schizosaccharomyces pombe*, etc.) often initiate the fermentation while acidotolerant species (i.e. *Brettanomyces* spp., *Candida stellata*, etc.) appear subsequently (Teoh, Heard and Cox 2004). Among bacterial communities, AAB are very common and present at high counts. The main species include *A. xylinum*, *A. aceti*, *A. pasteurianus*, *K. xylinus* and *Gluconobacter oxydans* (Jankovic and Stojanovic 1994; Liu *et al*. 1996; Greenwalt, Steinkraus and Ledford 2000; Kurtzman, Robnett and Basehoar-Powers 2001; Jayabalan *et al*. 2010; Reva *et al*. 2015). Some LAB have also been described in Kombucha but are not systematically present (Teoh, Heard and Cox 2004).

Variations in both chemical and microbial composition can be observed and are due to differences in tea varieties (black, green and geographical origin), microbial culture conditions (i.e. biofilm selection), process technologies and fermentation duration (Mayser *et al*. 1995; Blanc 1996; Jarrell, Cal and Bennett 2000). As for other fermented beverages such as cider, the natural and traditional fermentation conditions used must be properly monitored to ensure final product quality and stability. Taking into account the substantial interest of Western countries for Kombucha, better knowledge on key microbial drivers encountered during industrial-scale fermentations, coupled with an array of inherent parameters, would be a first step for better fermentation control. In this context, this study investigated, for the first time (i) the impact of tea type (green or black) on Kombucha microbial ecology and (ii) microbial diversity and dynamics in industrial-scale Kombucha fermentations. This was

done through culture-dependent and -independent methods (high-throughput sequencing) and by physical and chemical fermentation parameter follow-up. Together, the obtained data could allow for better fermentation control and product quality standardization.

MATERIALS AND METHODS

Sample preparation and microbial enumerations

Both sweetened black and green tea industrial-scale Kombucha fermentations were performed by a Kombucha producing SME (Brittany, France) using the same biofilm divided in 2. All samples (biofilm and juice) were analyzed at days 0, 2, 4 and 8 during fermentation. Juice samples were directly obtained from 1000 L tanks by harvesting juice at two different depths (top and bottom of the fermentation vessel) in order to obtain representative samples. This sampling procedure is critical to ensure a substantial array of inherent parameters (i.e. dissolved oxygen content, sugar content, etc.) was considered. Biofilm samples were harvested by cutting out at least 100 g samples from the outermost section to the center point from the floating multilayer biofilm to ensure full microbial community representation. For each sampling date and sample type, three repetitions were performed. Each biofilm sample was first homogenized with an Ultra-Turrax (IKA, Germany) then placed in a sterile stomacher bag and mixed for 2 min in 80 mL tryptone salt buffer using a Stomacher (AES, France). Liquid samples were directly diluted with 40 mL tryptone salt buffer in a stomacher bag then mixed for 2 min. In order to numerate total aerobic bacterial counts, homogenates were serially diluted and plated on plate count agar (AES, France) and then incubated for 72 h at 30°C. For AAB, serial dilutions were plated onto mannitol medium (D-mannitol 25g/L, yeast extract 5 g/L, universal peptone 3 g/L and agar 15 g/L) supplemented with 0.1 g/L pimaricine to inhibit yeast growth and incubated for 24–48 h at 30°C. LAB were enumerated on De Man Rogosa Sharpe (MRS) (AES, France) acidified with 10% (v/v) citric acid to pH 4.8 and supplemented with 0.1 g/L pimaricine to inhibit yeast growth; plates were incubated at 30°C under anaerobic conditions for 72–96 h. Yeasts were enumerated on yeast extract glucose Chloramphenicol (YGC) agar (AES, France) and plates were incubated at 25°C for 72 h. Finally, filamentous fungi were isolated on malt extract agar (M2Lev) (malt extract 20 g/l, yeast extract 3 g/L) supplemented with penicillin (5 mg/L) and streptomycin (5 mg/L), and incubated at 25°C for 1 week. For each Petri dish and at each sampling date, colonies were selected according to their colony morphology, and then microscopic evaluation by Gram-staining (bacteria) or methylene blue staining (yeast), and/or catalase and oxidase determinations were performed in order to select representative isolates for each microbial group. In general, on plates displaying between 50 and 300 colonies, a number of colonies corresponding to the square root of each morphological type were analyzed (in total, 92 LAB isolates, 204 AAB isolates and 127 yeast isolates). In parallel, the prepared Kombucha juice or biofilm homogenates were also used for molecular analyses (see section 'Yeast FTIR spectroscopy clustering and identification').

Regarding plate count analyses, growth potentials were also calculated for each microbial group using the $A_j = \log(D8) - \log(D0)$ formula, where A_j is the growth potential value and D is the day of analysis, to determine how microbial groups evolved during fermentation.

Liquid culture conditions

All representative AAB strains were cultivated in mannitol broth (D-mannitol 25g/L, yeast extract 5 g/L, universal peptone 3 g/L) and incubated at 30°C for 24 h under agitation. LAB were cultivated in MRS broth (AES, France) and incubated at 30°C for 24–48 h under static conditions. Yeast isolates were cultivated in tryptic soy broth (AES) supplemented with 2.5 g/L yeast extract and incubated at 25°C for 48 h. Bacterial and yeast strains were conserved in cryotubes with 30% or 40% v/v glycerol, respectively, at –80°C.

Bacterial M13-PCR clustering and identification

Preparation of template DNA

DNA was extracted from bacterial cultures grown to stationary phase using 1 mL of culture with the NucleoSpin Tissue Kit (Macherey Nagel, France) according to the manufacturer's instructions. Purified DNA samples were stored at –20°C.

PCR amplifications of bacterial isolates

For bacterial clustering, genetic profiles of each strain were generated using the method described by Guinebretière (Guinebretiere and Nguyen-The 2003). Briefly, 1 μL purified DNA (50–100 ng) was used for each reaction in the presence of 2.0 μM M13 primer (5′-GAGGGTGGCGGCTCT-3′), 400 μM dNTP, 2 mM $MgCl_2$ in the presence of 1.25U *Taq* polymerase (GoTaq Promega, France). Amplification conditions were as follows: 95°C, 5 min; 45 cycles of 95°C, 1 min, 36°C, 1 min; 72°C, 4 min. The repeatability and reproducibility of all experiments was also evaluated by using a laboratory control strain in all M13-PCR experiments.

For bacterial identification, 16S rRNA gene amplification and sequencing were carried out using the universal primers BSF8 and BSR1541 (Wilmotte, Van der Auwera and De Watcher 1993) in the presence of ~50 ng DNA, 0.2 μM each primer, 1.5 mM $MgCl_2$, 200 μM dNTPs and 1U *Taq* polymerase (GoTaq, Promega, France). Amplification conditions were 94°C for 5 min, 30 cycles of 94°C for 45 s, 59.5°C for 45 s, 72°C for 2 min with a final extension at 72°C for 5 min. All amplifications were performed using a peqSTAR 2X Gradient Thermocycler (PEQLAB Biotechnologie GMBH, Erlangen, Germany).

PCR sample aliquots (10 μL for M13 PCR products or 16S rDNA fragments) were analyzed using 1.2% (w/v) (M13-PCR fragments) or 0.8% (w/v) (16S rRNA gene fragments) agarose gels (Promega, France) in 1X TBE buffer at 110 V for 1 h and then visualized with GelRed staining (Biotium, France).

M13-PCR genetic profile clustering and sequence analysis

M13-PCR genetic profile banding patterns were analyzed for acetic and lactic acid bacterial isolates using BioNumerics fingerprinting software version 5.1 (Applied Maths, Belgium). For 16S rRNA gene fragment sequencing, amplicons were sent to Eurofins MWG Operon for sequencing (Ebersberg, Germany). Alignments were performed using ClustalX program or Bionumerics software as required and sequence similarity was determined using the BLAST program (Altschul *et al.* 1990) in GenBank database.

Yeast FTIR spectroscopy clustering and identification

Sample preparation and FTIR analyses

Sample preparation, measurement and Fourier transform infrared (FTIR) spectral analyses were performed according to

Kümmerle, Scherer and Seiler (1998) on a FTIR high-throughput system comprising a spectrometer (Tensor 27, Bruker Optics, Champs sur Marne, France) coupled to a high-throughput module (HTS-XT, Bruker Optics). FTIR analyses were performed on three technical triplicates. Dendrograms were created with the OPUS software program (Bruker, France). Yeast isolates were first grouped based on their FTIR spectrum and then presumptive identifications were done using the Technical University of Munich reference database comprising about 2500 FTIR spectra of type and reference strains of yeasts.

Molecular identification of yeasts

Representative yeast isolates were also identified at the species level using molecular tools. The D1/D2 region of the 26S rRNA gene was amplified and sequenced using the primers NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) (Kurtzman and Robnett 1997) directly on yeast colonies resuspended in ultrapure molecular biology grade water (Sigma, France) in the presence of 0.2 μM each primer, 1.5 mM $MgCl_2$, 200 μM dNTPs and 0.625U *Taq* polymerase (GoTaq, Promega, France). Amplification conditions were 95°C for 25 min, 35 cycles of 95°C for 60 s, 52°C for 90 s, 72°C for 2 min with a final extension at 72°C for 5 min. All amplifications were performed using a peqSTAR 2X Gradient Thermocycler (PEQLAB Biotechnologie GMBH, Erlangen, Germany). PCR sample aliquots (9 μL) were analyzed using 0.8% (w/v) agarose gels (Promega, France) in 1X TBE buffer at 110 V for 50 min and then visualized with GelRed staining (Biotium, France). Sequencing and alignments were carried out as described above.

16S and 26S amplicon profiling

DNA extraction

Total DNA extractions were performed on either 20 g Kombucha biofilm or 10 mL Kombucha tea samples. Biofilm samples were treated with an Ultraturrax for 1 min at 14 000 rpm and then placed in a stomacher bag for 15 min before recuperating a 10 mL liquid sample. Liquid samples were then centrifuged at 8800 rpm at 4°C. The supernatant was discarded and cell pellets were stored at –20°C until use. Each sample was performed in triplicate.

Cell pellets were thawed overnight at 4°C. The pellet was resuspended in 400 μL lysis solution (Tris-HCl 20 mM, pH 8.0, EDTA 2 mM, Triton X-100 1.2%, lysozyme 20 mg/mL, lyticase 200 U) and incubated at 37°C for up to 3 h. Lysed samples were divided in 2 and proteinase K (20 mg/mL) and RNAse A (10 μL of 1 mg/mL stock solution) treatments were performed in 200 μL of DNeasy Blood and Tissue kit AL buffer (Qiagen, France). Samples were incubated at 56°C for 1 h with agitation. DNA extraction and purification was then performed using the DNeasy Blood and Tissue kit (Qiagen, France) according to the manufacturer's instructions. DNA quality and quantity was verified by gel electrophoresis and a Nanodrop (Labtech, France), respectively, prior to storage at –20°C.

Targeted 16S and 26S rRNA gene metagenomic analyses

PCR amplification of the V1–V3 region of the 16S rDNA and library preparation were performed with the following primers (with Illumina overhand adapters): forward (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′) and reverse (5′-GTVTVGTGGGCTCGGAGATGTGTATAAGAGACAG-3′). Similarly, 26S D1/D2 region was amplified with the following primers (forward 5′-GCATATCAATAAGCGGAGGAAAAG-3′ and reverse 5′-GGTCCGTGTTTCAAGACGG-3′). Each PCR product was purified with the Agencourt AMPure XP beads kit (Beckman Coulter, Pasadena, USA) and submitted to a second PCR round for indexing, using the Nextera XT index primers 1 and 2. After purification, PCR products were quantified using the Quant-IT PicoGreen (ThermoFisher Scientific, Waltham, USA) and diluted to 10 ng/μL. A final quantification, by qPCR, of each sample in the library was performed using the KAPA SYBR FAST qPCR Kit (KapaBiosystems, Wilmington, USA) before normalization, pooling and sequencing on a MiSeq sequencer using v3 reagents (ILLUMINA, USA).

Bioinformatic analyses of metabarcoding data

Sequence read processing for 16S and 26S was used as previously described using MOTHUR software package v1.35 (Schloss *et al.* 2009), Pyronoise algorithm and UCHIME algorithm (Edgar *et al.* 2011) for alignment and clustering, denoising and chimera detection, respectively (Rodriguez *et al.* 2015; Degrune *et al.* 2016). Amplicon reference alignment and taxonomical assignation were based upon the SILVA database (v1.15) of full-length SSU (16S) and LSU (26S) rDNA sequences (Quast *et al.* 2012). All biosample raw reads were deposited at the National Center for Biotechnology Information (NCBI) and are available under a Bioproject ID.

Ordination analysis and 3D plots were performed with Vegan, Vegan3d and rgl packages in R [Dixon, Philip]. Non-metric dimensional scaling, based on the Bray-Curtis dissimilarity matrix (a measure of community structure which considers shared OTUs and their relative abundances) (Bray and Curtis 1957), was applied to visualize the biodiversity between the groups. An AMOVA test was performed to assess the diversity clustering of treatment groups with the Bray-Curtis matrix using MOTHUR (Martin 2002).

All the biosample raw reads have been deposited at the NCBI and are available under a Bioproject ID PRJNA371699.

Statistical analyses

Statistical difference of population abundance between treatment groups were assessed with ANOVA, corrected for multitesting (Benjamini-Hochberg False Discvery Rate) using STAMP software (Parks and Beiko 2010). Statistical paired differences between treatment groups of specific bacterial populations were assessed by the two-way ANOVA and Tukey-Kramer post-hoc test using PRISM 6 (Graphpad Software); differences were considered significant for a *P*-value of < 0.05.

Biochemical parameter analyses

Three milliliters of each Kombucha tea sample were centrifuged for 10 min at 7500 g at 4°C, and then the collected supernatant was kept for 1 h at 4°C before filter sterilization (0.45 μm pore size). Samples were stored at −80°C for organic acid and ethanol analyses. Sample pH values were determined using a standard pH meter (Eutech Instruments, The Netherlands) and tea density was measured with a 1.000–1.100: 0.001 g/mL range densitometer at 20°C (Fisher Scientific, France).

Organic acid quantification

Gluconic and glucuronic acid concentrations were determined for all tea samples using enzymatic kits (D-Gluconic acid/D-Glucono-d-lactone Assay Kit, D-Glucuronic acid/D-Galacturonic Assay Kit, Libios, France).

Lactic and acetic acids were quantified using an Agilent 1100 series high-performance liquid chromatograph (Agilent

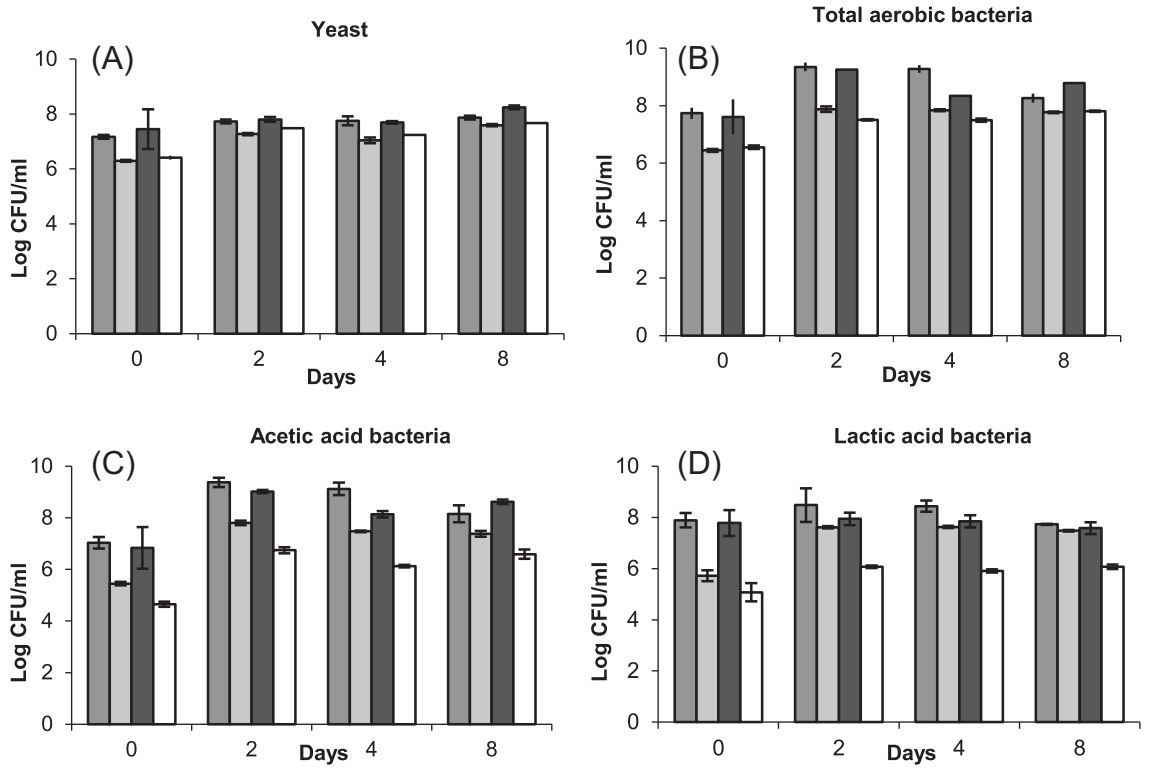

Figure 1. Enumeration of bacteria and yeast associated with green and black tea Kombucha juice and biofilm samples during 8 days of fermentation. The following microbial groups were numerated at days 0, 2 4 and 8: yeast (**A**), total aerobic bacterial counts (**B**), acetic acid bacteria (**C**), lactic acid bacteria (**D**). Green tea biofilm: dark gray box; green tea juice: light gray box; black tea biofilm: black box; black tea juice: white box.

Technologies, USA) equipped with a Rezex ROA-Organic Acid analysis column (300 × 7.8 mm, Phenomenex, Australia), a refractive index detector and a UV detector set at 210 nm. The mobile phase was 0.01M H_2SO_4 and the flow rate was set to 0.6 mL/min. The volume injected was 20 μL. The temperature was set to 60°C and 40°C in the column oven and refractive index detector, respectively. Lactic and acetic acid concentrations were determined in triplicate using external standard curves of lactic acid and acetic acid (Sigma, USA).

Ethanol quantification

Ethanol was quantified with a GC 3900 Varian gas chromatograph (Varian Analytical Instruments, USA) equipped with a CP Sil 8CB LB/MS #CP8752 column (30 m x 0.32 mm, FT 0.25 μm, Chrompack Capillary Column, Varian, USA), an FID detector and an EFC detector. The mobile phase was nitrogen and the flow rate was set to 1.5 mL/min. The volume injected was 1 μL. The temperature was set to 150°C, 30°C and 250°C in the injector, column oven and FID detector, respectively. EFC detector was programmed to obtain an air flow of 300 mL/min, a di-hydrogen flow of 30 mL/min and a nitrogen 'make-up' flow of 28 mL/min. Supernatants were first directly injected into the gas chromatograph for ethanol concentration estimation. Then, 500 μL of sample were mixed with 500 μL of isopropanol (at the estimated ethanol concentration in each sample) in a vial. In parallel, 0.2%, 0.5% and 1% ethanol standards were prepared and mixed with isopropanol at the same concentration. The same protocol was then used for samples: 500 μL of ethanol were mixed (v/v) with isopropanol at the same concentrations. For each sample, three analyses were performed.

RESULTS

Microbial dynamics of green and black tea Kombucha fermentations

Microbial population kinetics were monitored for two industrial-scale black and green tea fermentations on both the fermented tea and biofilm samples. Sampling was performed at four different dates (D0, D2, D4 and D8) until the fermentation was complete. Results showed 1 to 2 log CFU/g higher microbial counts in biofilm samples at day 0 when compared to tea (juice) samples (Fig. 1). Then, the microbial communities slowly mixed and by day 8, green tea samples showed similar microbial counts for all microbial groups while major differences in LAB and AAB counts were observed between black tea biofilm samples and fermented black tea samples (1.5–2 log CFU/mL differences). No molds were ever detected on the selected agar media during both green and black tea fermentations. Overall, higher microbial counts were observed in all biofilm samples over time. Calculated growth potentials for LAB and yeasts were –0.15/0.70 (green tea biofilms) and –0.20/0.79 (black tea biofilms) log CFU/g, respectively, while AAB and total plate count growth potentials increased by 1.93/1.33 (green tea biofilms) and 1.78/1.18 (black tea biofilms) log CFU/g, respectively (Fig. S1, Supporting Information). AAB and LAB were much more abundant in biofilms than in teas in the first days of fermentation, and then equilibrated to similar levels by the end of fermentation, especially in green tea fermentations.

Population dynamics were much more important over time for bacterial groups (highest changes at D2 and D4) in comparison to yeasts. Indeed, yeast counts were highly stable in all sample types regardless of tea type during fermentation despite an approximate 1 log increase over time (Fig. 1).

Total mesophilic aerobic bacteria counts were higher in biofilm versus tea samples at day 0 (>1 log CFU/g) for both tea types and remained higher over 8 days although 8 log CFU/mL were numerated in all sample types by the end of fermentation.

Overall, results indicated that microbial communities were much more abundant in biofilm samples in both green and black tea fermentations. Initial counts in biofilms were determined to be at least 7 log CFU/g while initial microbial counts in sweetened teas were lower, in particular, for lactic (~5 log CFU/mL) and acetic acid (~4 to 5 log CFU/mL) bacteria regardless of the considered tea type. Growth kinetics were observed to be highly similar for all microbial groups in both black and green tea Kombucha fermentations but with higher values in teas compared to biofilms, supporting the relative stability of the biofilms. Noteworthy, AAB displayed highest growth potentials reaching close to 2 log CFU/mL in teas and between 1.12 and 1.78 log CFU/g in biofilms suggesting intense activity during fermentations.

Culture-based cell counting provides first insights into the community composition and the dynamics of microorganisms during the first stages of the Kombucha fermentation. Molecular identifications of microbial isolates were then performed to evaluate the species and strain-level diversities.

M13-PCR typing and bacterial species identifications

Dereplication of 296 representative lactic and acetic acid bacterial isolates, gathering respectively 92 and 204 isolates, was performed by generating genetic profiles using the coliphage M13 sequence-based PCR (M13-PCR) method (Henderson, Duggleby and Turnbull 1994; Guinebretiere and Nguyen-The 2003). The profiles were integrated and analyzed in a Bionumerics database to group isolates, analyze their genetic diversity and generate proximity dendrograms (Fig. S2, Supporting Information, presents LAB profiles during green (A) and black (B) tea fermentations as an example). M13-PCR profiles for the quality control strain indicated that repeats were highly similar (data not shown).

Acidified MRS media allowed successful isolation and identification of LAB (n = 92) from both tea fermentations. LAB showed lower M13-PCR genetic profile diversity with only eight distinct clusters (>80% similarity) containing from as few as 1 or 2 isolates to up to 39 isolates (Fig. S2, Supporting Information). At least one representative strain (19 in total) for each cluster was further identified at the species level by partial 16S rRNA gene sequencing (~1500 bp). Among the eight clusters, four were assigned to *Oenococcus oeni*, three to *Lactobacillus nagelii* and one to *L. satsumensis* (Table 1). Interestingly, all three species were identified during black tea fermentations (*O. oeni* 57.1%, *L. nagelii* 32.7% and *L. satsumensis* 10.2%) while only *O. oeni* (62.8%) and *L. nagelii* (37.2%) could be identified from green teas (Fig. 2). *Oenococcus oeni* was clearly the dominant species in both fermentations. Noteworthy, lower intraspecies diversity was observed for strains belonging to *O. oeni* when compared to the second most dominant species *L. nagelii* (Fig. S2, Supporting Information). The presence of multiple strains per bacterial species highlighted the intraspecific diversity.

AAB M13-PCR genetic profile diversity (n = 204) was much higher with 34 and 41 clusters observed from green and black tea isolates, respectively, all showing at least 80% similarity. These clusters contained 1 to 16 isolates and each cluster represented one species as determined by species level identifications on 35 representative isolates able to grow by partial 16S rRNA gene sequencing (~1500 bp) (Table 1). Overall, high species diversity was observed for AAB in both tea types with at least 16 different species identified (Fig. 2) belonging to the *Acetobacter, Gluconoacetobacter, Gluconobacter* and *Tanticharoenia* genera (52.4%, 24.1%, 22.0% and 1.5%, respectively). Dominant species corresponded to *Gluconobacter oxydans* (25.5% and 13.8% of all isolates in black and green teas, respectively), *Acetobacter okinawensis* (14.7% and 20.6% of all isolates in black and green teas, respectively), *A. tropicalis* (9.8% and 21.6% of all isolates in black and green teas, respectively), *A. syzygii* (11.8% and 15.7% of all isolates in black and green teas, respectively) and *Gluconacetobacter europaeus* (11.8% and 4.9% of all isolates in black and green teas, respectively) while less dominant species were identified as *G. hansenii*, *G. intermedius* and *A. peroxydans* (Fig. 2). In some cases, tea-specific species diversity was also observed with *G. xylinus* and *A. lovaniensis* only identified in green tea fermentations while *G. saccharivorans*, *G. oboediens*, *G. liquefaciens* and *Gluconobacter cerinus* were only identified from black tea fermentations highlighting that tea type influences microbial diversity and dynamics (Fig. 2). When comparing both fermentations, higher AAB species diversity was identified during black tea fermentation (14 species identified) versus green tea fermentation (12 species identified). Interestingly, the most dominant species also differed according to tea type as *G. oxydans* was found in black tea while *A. tropicalis* was identified in green tea followed by similar dominant species (*A. okinawensis* and *A. syzygii*) in both tea types. Finally, as described for LAB, multiple genetic profiles were often observed within a given cluster, but also a large proportion of distinct genetic profiles represented by a single isolate suggesting high intra-specific diversity. Indeed, 11 clusters detected in both tea types were associated to a single species, *G. oxydans*.

FTIR spectroscopy yeast species dereplication and identification

FTIR spectroscopy was used for yeast isolate dereplication and presumptive species identifications. Generated FTIR spectra were analyzed using the OPUS software, and repeatability and reproducibility verified for all experiments. Presumptive species identifications were performed by comparing spectra to a yeast species library and allowed identifying 15 yeast species among the 127 isolates from both tea fermentations (data not shown). From these data, 44 representative yeast isolates (based on presumptive FTIR species identifications and spectra dendrograms) were selected for further molecular identifications based on sequencing the D1/D2 domain of the 26S large ribosomal subunit (LSU) rRNA gene. Overall, high genus and species diversity was identified with eight genera represented by 11 species in both tea fermentations (Fig. 2C). Dominant genera were *Dekkera* (50%) and *Hanseniaspora* (26%) and to a lesser extent *Zygotorulaspora* (10%) and *Zygosaccharomyces* (6%) in both tea types. The main species identified were *Dekkera anomala* (33.9% and 18.5% in green and black tea, respectively), *D. bruxellensis* (17.8% and 30.8% in green and black tea, respectively), *Hanseniaspora valbyensis* (~26% in both green and black tea) followed by *Zygotorulaspora florentina* (8% and 12% in green and black tea, respectively) and *Zygosaccharomyces bailii* (4.9% and 6.2% in green and black tea, respectively). Interestingly, the same species were observed at similar abundances with the exception of the most dominant species, *D. anomala* and *D. bruxellensis*, which were more abundant in green and black tea, respectively. Minor species were also encountered and rather associated to a given tea type such as *Pichia membranifaciens*, *P. anomala* (*Wickerhamomyces anomalus*) or

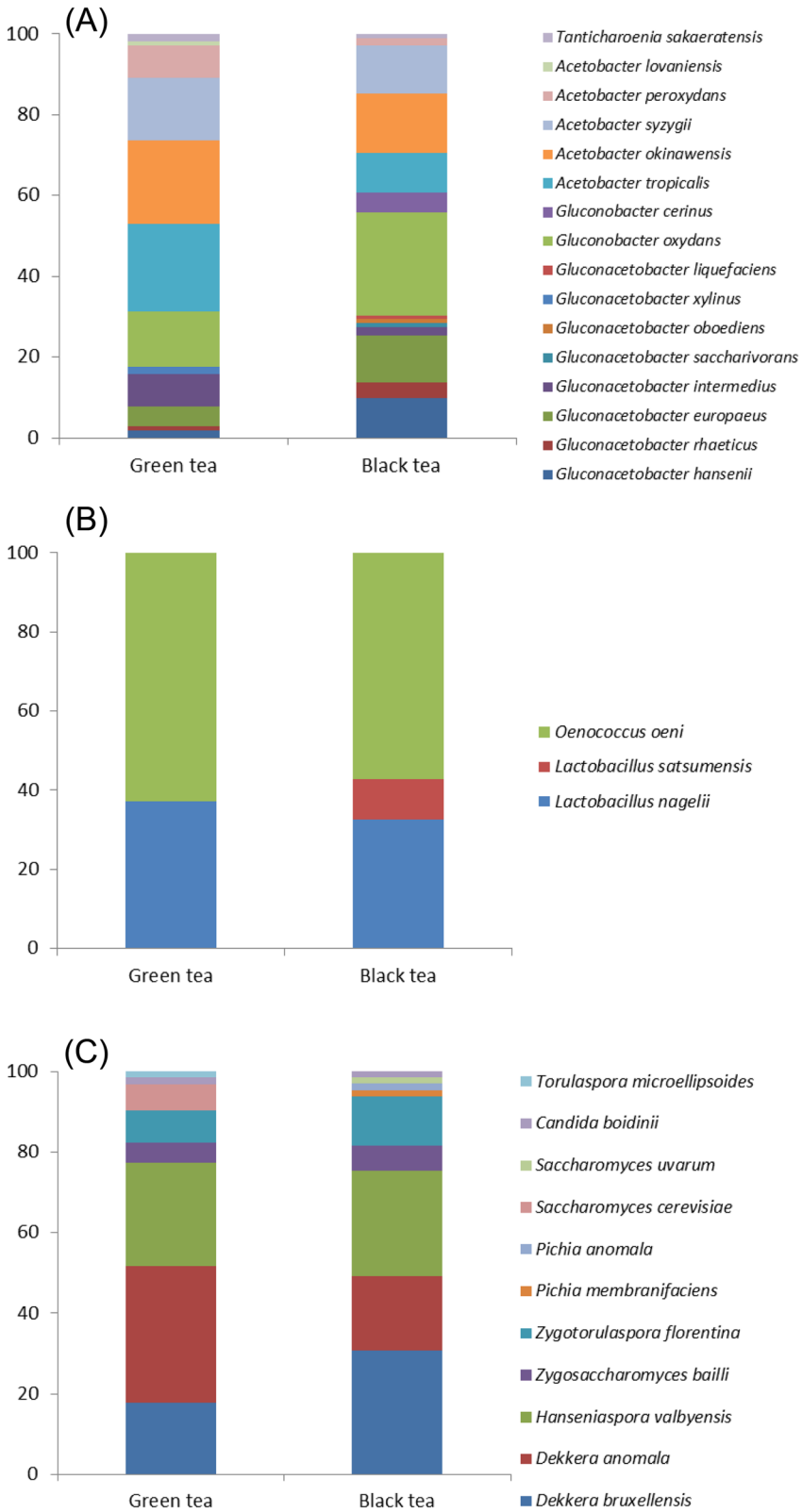

Figure 2. Bacterial and yeast species diversity associated with green and black tea Kombucha fermentations determined using culture-dependent analyses. Analyses based on 204 acetic acid bacteria isolates (**A**), 92 lactic acid bacteria isolates (**B**) and 127 yeast isolates (**C**) and results are presented as percentage of total numbers.

Table 1. Species diversity observed by the identification of 19 representative LAB isolates (16S rRNA gene sequence), 35 representative AAB isolates (16S rRNA gene sequence) and 44 representative yeast isolates (D1/D2 large ribosomal subunit region sequence).

Strain code	Closest relative	Identity (%)[a]	Genbank accession number	Other strain codes associated to this identification
LAB				
D8 JB1 LAB5	*Oenococcus oeni*	99	NR_075030.1	D0 PG1 LAB2; D0 PB1 LAB3; D0 JB1 LAB3; D2 PG3 LAB1; D2 JG3 LAB6; D2 PB2 LAB4; D4 PG3 LAB2; D4 PB1 LAB1; D8 JG2 LAB1; D8 PB3 LAB1;
D2 PB2 LAB6	*Lactobacillus nagelii*	99	NR_112754.1	D0 JG3 LAB5; D0 PB1 LAB4; D4 PG3 LAB6; D4 JG2 LAB5; D8 PG2 LAB5
D2 JB3 LAB5	*Lactobacillus satsumensis*	99	NR_028658.1	D4 JB3 LAB7
AAB				
D8 JG2 AAB 8.2	*Acetobacter lovaniensis*	99	NR_114845.1	
D0 PB1 AAB 3.1	*Acetobacter okinawensis*	100	AB906403.1	D2 PB2 AAB 2.1; D8 PG2 AAB 2.2; D8 PB3 AAB 2.1
D0 JG3 AAB 2.2	*Acetobacter peroxydans*	99	AB906404.1	
D2 JG3 AAB 2.2	*Acetobacter syzygii*	99	KJ469777.1	
D2 JG3 AAB 6.2	*Acetobacter tropicalis*	99	AJ419842.1	D0 JG3 AAB 6.1; D0 JG3 AAB 7.1; D2 PG3 AAB 7.2; D2 PB2 AAB 1.1; D4 PG3 AAB 1.1
D8 PG2 AAB 9.2	*Gluconacetobacter eurapaeus*	99	AB680040.1	D0 PB1 AAB 5.1; D2 JB3 AAB 4.1; D8 PB3 AAB 5.1
D4 PB1 AAB 4.2	*Gluconacetobacter hansenii*	99	AB166735.1	D2 PB2 AAB 3.2; D2 PB2 AAB 4.2
D8 PG2 AAB 5.1	*Gluconacetobacter intermedius*	99	AB166739.1	D8 PG2 AAB 5.2; D8 PB3 AAB 5.2
D0 JB1 AAB 4.2	*Gluconacetobacter liquefaciens*	99	NR_113406.1	
D0 JG3 AAB 5.1	*Gluconacetobacter xylinus*	99	AB680512.1	D0 PG1 AAB 5.2
D4 JB3 AAB 3.1	*Gluconobacter cerinus*	100	NR_041048.1	D2 JB3 AAB 5.1
D0 PB1 AAB 6.1	*Gluconobacter oxydans*	99	CP004373.1	D2 PB2 AAB 5.2; D2 JB3 AAB 6.2; D4 JB3 AAB 5.2; D8 PG2 AAB 6.1; D8 JG2 AAB 8.1
D4 JG2 AAB 6.1	*Tanticharoemia sakaeratensis*	99	NR_041601.1	
Yeast				
D0 JG1 Y4.1	*Candida boidinii*	99	KC442246.1	D8 PB3 Y5.1
D4 PB1 Y2.1	*Dekkera anomala*	100	AY969093.1	D0 JG3 Y1.2 ; D2 PG3 Y2.2 ; D2 JG3 Y2.2 ; D2 PB2 Y2.1; D8 PG2 Y1.1 ; D8 PG2 Y1.2 ; D8 JB1 Y1.1
D8 JB1 Y1.2	*Dekkera bruxellensis*	100	GU291284.1	D0 JG3 Y1.1 ; D0 JB1 Y1.1 ; D2 PG3 Y1.1 ; D2 PB2 Y1.2 ; D2 JB3 Y1.1 ; D4 JG2 Y1.2 ; D4 PB1 Y1.2
D2 JG3 Y4.1	*Hanseniaspora valbyensis*	100	KR075686.1	D0 PG1 Y4.1 ; D0 JB1 Y3.1 ; D4 PG3 Y4.1 ; D4 PB1 Y4.1 ; D4 JB3 Y3.1 ; D8 JG2 Y3.1 ; D8 PB3 Y4.1 ; D8 JB1 Y3.1
D0 JB1 Y2.1	*Wickerhamomyces anomalus*	99	KP171608.1	
D8 PB3 Y7.1	*Pichia membranifaciens*	100	KF459941.1	
D4 PG3 Y3.1	*Saccharomyces cerevisiae*	100	KR014241.1	
D8 PB3 Y3.1	*Saccharomyces uvarum*	100	EU145759.1	
D0 JG3 Y3.1	*Torulaspora microellipsoides*	99	JN641754.1	
D0 PG1 Y2.1	*Zygosaccharomyces bailii*	100	KP171612.1	D0 PG1 Y2.2 ; D0 PB1 Y2.1 ; D0 PB1 Y3.1 ; D0 PB1 Y3.2
D2 PG3 Y3.1	*Zygotorulaspora florentina*	100	FJ527242.1	D0 PG1 Y5.1 ; D0 PB1 Y5.1 ; D0 PB1 Y5.2 ; D4 JG2 Y3.1 ; D4 JB3 Y2.2 ; D8 PB3 Y3.2

[a]Identical nucleotides percentage in the sequence obtained from the 16S rRNA or D1/D2 region of the 26S rRNA gene and the sequence found in Genbank.
D: day, J: juice; P: biofilm (pellicle), B : black tea ; G : green tea; Number corresponds to replicate number; LAB: lactic acid bacteria; AAB: acetic acid bacteria; Y: yeast; Number corresponds to isolate number.

Saccharomyces uvarum in black tea or *S. cerevisiae* or *Torulaspora microellipsoides* in green tea. Overall, nine species were identified during black tea fermentation while eight were found in green tea fermentation. Yeast species identifications for all sequenced strains are provided in Table 1 as well as strain codes associated to the same species based on presumptive FTIR clusters and identifications.

As culture-based approaches may be biased due to species that remain refractory to culture due to the experimental design (i.e. slow growth, non-cultivable states, subdominant populations, etc.), a complementary molecular approach using high-throughput sequencing was performed.

Microbial community diversity and dynamics during Kombucha fermentations determined by metabarcoding analyses

High-throughput sequencing analyses using Illumina technology were carried out by targeting both 16S and 26S ribosomal DNA fragments to study in-depth bacterial and fungal diversity and dynamics during industrial-scale black and green tea fermentations. For bacteria, the V1–V3 regions of the 16S rRNA gene were amplified while for fungi (in particular yeasts) the D1/D2 region of the 26S rRNA gene was specifically targeted for genus and/or species-level identifications.

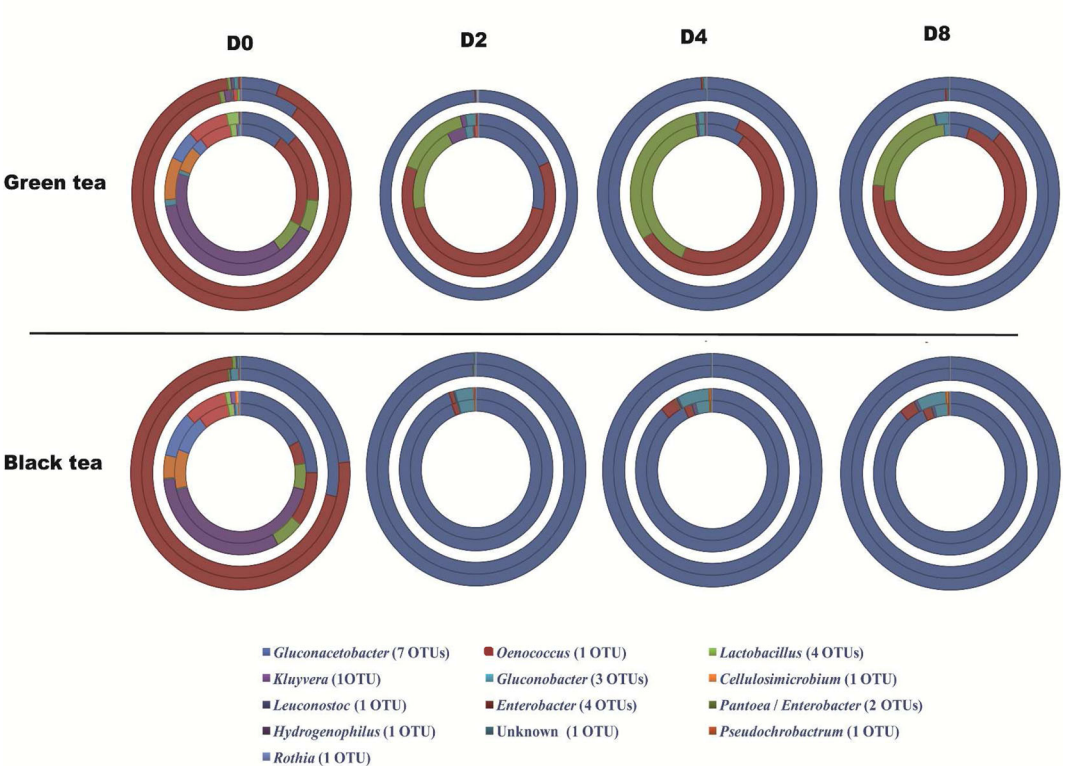

Figure 3. Relative abundance of bacterial species in green and black tea Kombucha fermentations based on 16S rDNA metabarcoding. The 28 most abundant OTUs are represented on this figure (>1% relative abundance) out of 354 OTUs in total. Sampling was performed at days (D) 0, 2, 4 and 8 on both tea (two inner circles) and biofilm (two outer circles) samples. The two circles represent replicate samples for each sample type.

Metabarcoding analyses were performed on both fermented tea juice (sampled from the top and bottom of tanks and then combined) and biofilm samples at the same four sampling dates (D0, D2, D4, D8). The number of observed operational taxonomic units (OTUs) was 354 for the 16S target and 20 for the 26S target. Quality analysis of read data is presented in Table S1 (Supporting Information).

For bacteria, metabarcoding analyses showed high diversity although mainly dominated by AAB and LAB belonging to *Gluconacetobacter*, *Gluconobacter*, *Oenococcus* and *Lactobacillus* genera (Fig. 3). Other genera were also identified, especially at the start of fermentation and likely originating from the raw materials. For example, *Kluyvera* was relatively abundant at day 0 (30%–40% according to sample) then drastically decreased to trace levels from day 2 onwards while other genera (i.e. *Leuconostoc*, *Enterobacter*, etc.) were only periodically identified at low levels in samples during fermentation (Fig. 3). Among the 354 OTUs obtained for the 16S target, up to 28 species could potentially be determined per fermentation at >1% relative abundance based on the observed 16S gene target polymorphisms (OTUs) and BLAST analyses. Diversity was found to be highest at the start of both fermentations, in particular in tea juice samples. These samples were dominated by acetic acid and lactic acid bacterial species closely related to *G. europaeus* (among the seven OTUs affiliated to *Gluconacetobacter*), *G. oxydans* (among the three OTUs linked to *Gluconobacter*), *O. oeni* (genus represented by one OTU), *L. nagelii* (among the four OTUs linked to *Lactobacillus*), *Leuconostoc* sp. (genus represented by one OTU) as well as some *Enterobacteriaceae* (i.e. *Kluyvera ascorbate*, *Enterobacter* sp., *Pantoea* sp.) and the *Cellulosimicrobium* genus (represented by only one OTU closely related to the cellulolytic species *Cellulosimicrobium cellulans*) belonging to the suborder *Micrococcineae*. Interestingly, dominant genera abundances were quite similar in both green and black (Fig. 3) tea juice samples at D0 while from D2 to D8, relative genus and species abundances were highly different in these sample types. Indeed, when comparing tea types, major differences in lactic acid and acetic acid bacterial species were observed and results clearly showed a strong matrix effect (Fig. 4). Green tea samples were highly dominated by two lactic acid bacterial species closely related to *O. oeni* and *L. nagelii*, and to a lesser extent *G. europaeus*, while black tea samples were dominated by acetic acid bacterial species, especially *G. europaeus* during fermentation, but also at the end of both fermentations. Green and black tea biofilm samples also presented higher species diversity at the start of fermentation with similar dominant species encountered. *Oenococcus oeni* was represented by 89% and 72% relative abundances in green and black tea biofilms at D0, respectively, while *G. europaeus* was represented by 7% and 22.5%, respectively. From day 2 to 8, both green and black tea biofilms were dominated by *G. europaeus* (98%–99% species abundance). At the family level, a matrix effect was clearly observed and *Acetobacteraceae* were highly abundant in biofilm samples when compared to tea samples while this difference was less apparent for *Lactobacillaceae* and *Enterobacteriaceae*. Finally, ordination analyses were carried out to determine the effect of sampling time and matrix type (biofilm or juice) during fermentation and clearly highlighted strong differences in bacterial diversity between juice and biofilm samples, especially at the start of fermentation (Fig. 5).

Fungal diversity was relatively low in comparison to bacterial diversity with only 20 OTUs for fungi while 354 OTUs were detected for bacteria regardless of tea type (Table S1, Supporting Information). For fungal diversity, three dominant taxa were observed along with some minor taxa. These taxa were

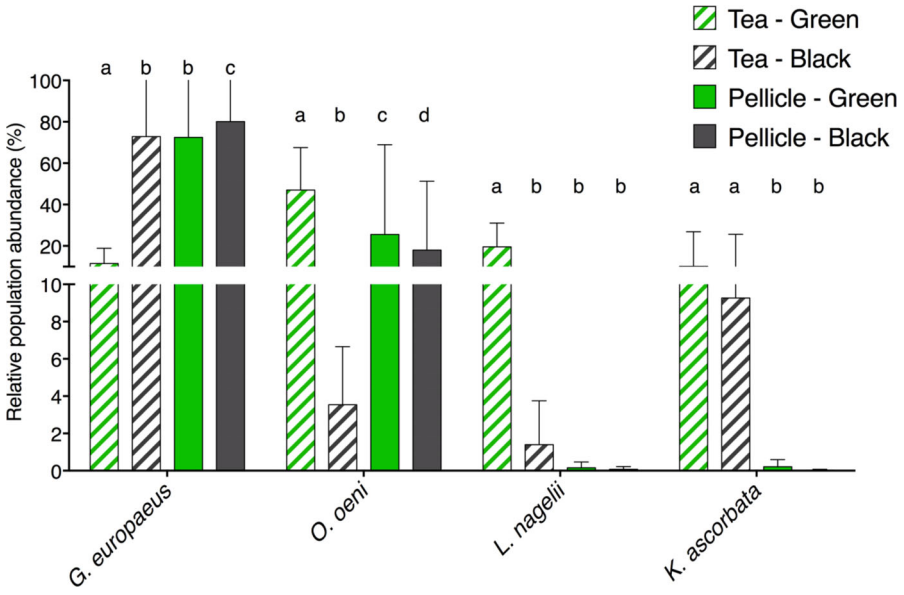

Figure 4. Bar plot showing selected bacterial species whose relative abundance is statistically different between tea type and matrix groups, based on 16S rDNA profiling. Data with different superscript letters are significantly different at $P < 0.05$ according to the one-way analysis of variance statistical analysis followed by the Tukey post hoc test.

systematically identified in both biofilms and juices and in both tea types during the 8-day fermentation. The three dominant taxa corresponded to yeast species closely related to *D. bruxellensis*, *D. anomala* and *H. valbyensis*. They were followed by *S. cerevisiae*, *S. bayanus* and *Torulapsora delbrueckii* (Fig. 6A and B). Other yeast species were also periodically found in certain samples such as *W. anomalus* (only black tea fermentation, D0 to D8, in both tea and biofilm samples), *Candida boidinii* (D8 black tea biofilm), *P. membranifaciens* (D8 black tea biofilm), *Zygoascus hellenicus* (D8 black tea biofilm), *Kregervanrija* sp. (D8 black tea biofilm) and *Clavispora* sp. (D4 black tea biofilm). Metabarcoding analyses also identified traces of filamentous fungi belonging to the *Aspergillus* genus in two biofilm samples while no filamentous fungi were found using culture-dependent analyses. Interestingly, when biofilm and tea sample types were compared at the start of fermentation, dominant species were clearly different regardless of tea type (Fig. 6A and B). Ordination analyses according to matrix type, time and tea type clearly showed that yeast diversity was clearly different between juice and biofilm samples and for each day of fermentation; however, tea type did not strongly affect yeast species diversity (Fig. 7). *Dekkera bruxellensis* was identified as highly dominant in biofilm samples, while *H. valbyensis* was the major species identified in tea samples. Dominant species dynamics then changed over the 8-day fermentation in both tea types and the microbial community slowly mixed, and for some species was re-equilibrated, during this period (Fig. 6). Regardless, *D. bruxellensis*, *D. anomala* and *H. valbyensis* were all identified among the major taxa at the end of fermentation in both biofilm and tea samples (Fig. 6). Noteworthy, *D. bruxellensis* dynamics showed a decrease in the biofilm over time coupled to a direct increase in tea samples while the complete opposite was observed for *H. valbyensis*. When closely comparing microbial communities identified in the two tea types, no statistical differences were observed for dominant species contrary to minor taxa. In particular, from day 0 to 4, *S. uvarum*, *W. anomalus* and *C. boidini* were identified within the microbial community in green and black tea biofilm samples while their presence was no longer detected at day 8 in green tea samples. Minor taxa were more frequently identified in black tea biofilm samples when compared to green tea biofilm samples. Finally, some minor taxa such as *Saccharomyces* spp. slowly increased during fermentation and were detected in both black and green teas with higher relative abundances in black tea fermentation.

Biochemical properties of green and black tea Kombucha fermentations

The pH of unfermented black and green tea preparations were 5.20 and 5.26, respectively; then slowly decreased over the 8-day fermentation period to reach 3.55 and 3.30, respectively, thus highlighting the increase in organic acid production during Kombucha production (Table 2). In a similar manner, density values also decreased from close to 1019 at D0 to 1013 in both tea types after 8 days showing that fermentable sugars were consumed over time (Table 2).

Ethanol content was also determined at each sampling date and remained very low from D0 (0.08%) to D4 (0.41% and 0.47%) in green and black tea fermentations, respectively, and then continued to slowly increase at D8 to just over 1% in both fermentations (Table 2). Tea types had no impact on the ethanol content over the 8-day period.

Concerning the production of four major organic acids (acetic, lactic, gluconic and glucuronic acids), clear differences in both acetic acid and lactic acid production were observed between tea types from D2 to D8 (Table 2). Maximal acetic acid levels reached 15.72 ± 0.36 mM in the green tea fermentation while only 8.17 ± 0.26 mM was quantified at the end of the black tea fermentation despite higher AAB abundance in black tea. Similar observations were also found for lactic acid concentration at D8 in green and black tea fermentations with 15.37 ± 0.13 mM and 10.46 ± 0.12 mM, respectively. Gluconic and glucuronic acids were quantified at low to trace levels at the end of both green and black tea fermentations (Table 2). No major differences were observed between samples for these two organic acids.

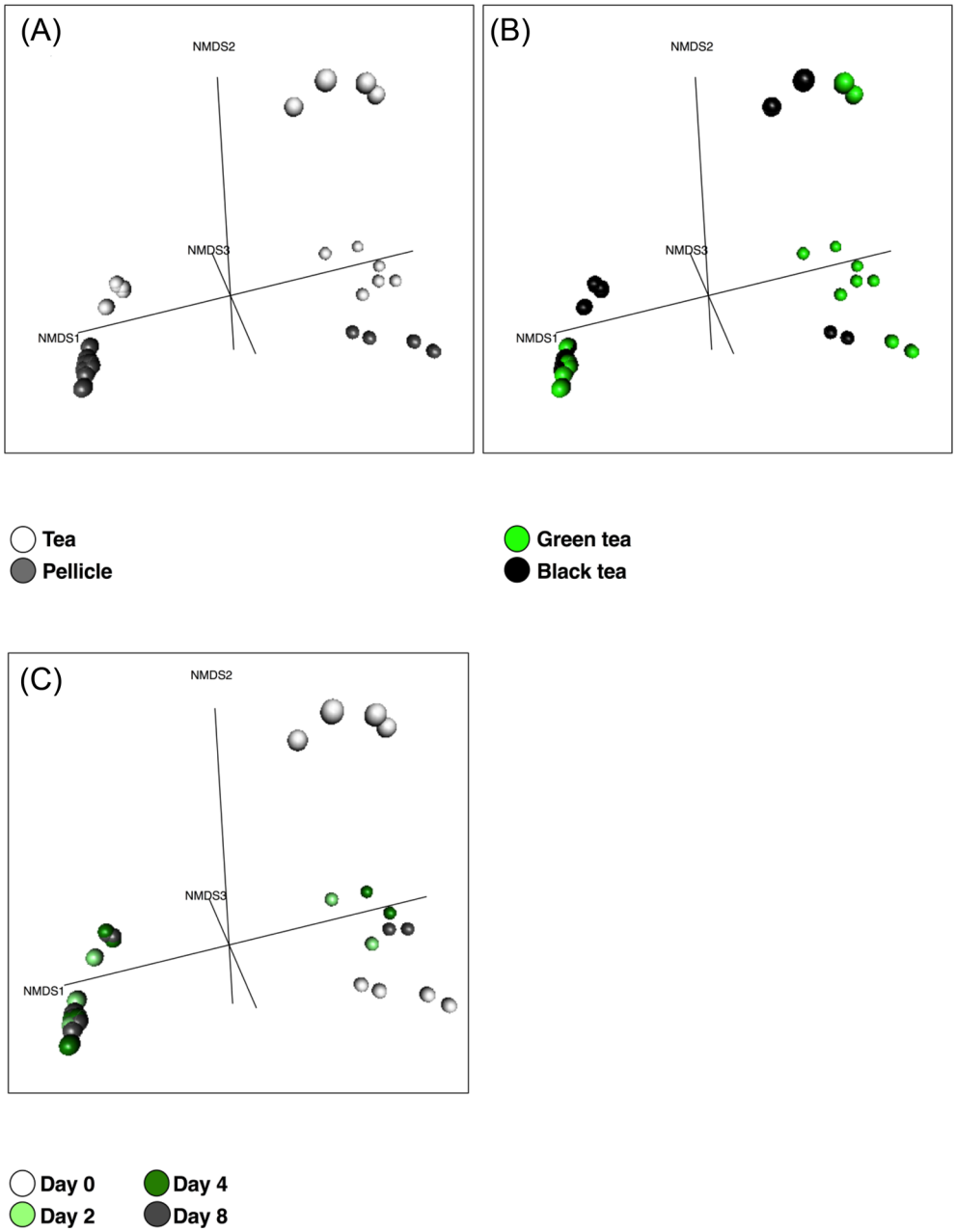

Figure 5. Spatial ordination of samples deduced from 16S rDNA metabarcoding. Non-metric dimensional scaling is based on a Bray-Curtis dissimilarity matrix built at the species level. Samples were colored according to tea matrix (**A**), tea type (**B**) and days of fermentation (**C**).

DISCUSSION

Although previous studies on Kombucha fermentations have reported microbial and/or biochemical properties (Reiss 1994; Mayser *et al.* 1995; Sievers *et al.* 1995; Blanc 1996; Chen and Liu 2000; Greenwalt, Steinkraus and Ledford 2000; Jarrell, Cal and Bennett 2000; Kurtzman, Robnett and Basehoar-Powers 2001; Teoh, Heard and Cox 2004; Jayabalan, Marimuthu and Swaminathan 2007; Cvetkovic *et al.* 2008; Malbasa *et al.* 2008, 2011; Marsh *et al.* 2014b; Reva *et al.* 2015; Chakravorty *et al.* 2016), to the best of our knowledge, this is the first study describing microbial community diversity and dynamics during industrial-scale green and black tea Kombucha fermentations using both culture-dependent and -independent techniques. The determined microbial communities were linked to key biochemical parameters that are monitored during fermentation including density changes, organic acid production, pH decrease and potential ethanol production by fermentative yeasts. This study also provided in-depth bacterial and yeast species-level identifications based on the polyphasic approach combining both culture-dependent and -independent data which has not been extensively described in recent Kombucha-related studies in lab-scale fermentations (Marsh *et al.* 2014b; Chakravorty *et al.* 2016). Furthermore, literature data suggest that all previous studies evaluating Kombucha fermentations have been carried out in laboratory-scale conditions using containers ranging from 200 mL to 2 L (Jarrell, Cal and Bennett 2000; Teoh, Heard and Cox 2004; Cvetkovic *et al.* 2008; Yang *et al.* 2010; Malbasa *et al.* 2011; Marsh *et al.* 2014b; Chakravorty *et al.* 2016), thus not

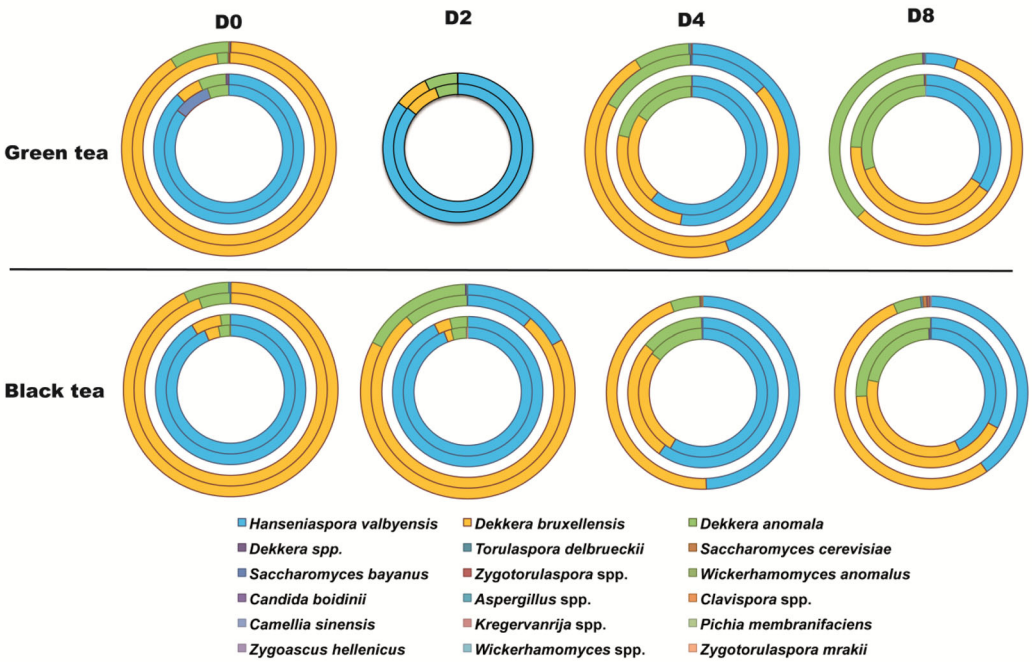

Figure 6. Relative abundance of yeast species in green and black tea Kombucha fermentations based on 26S rDNA metabarcoding. Sampling was performed at days (D) 0, 2, 4 and 8 on both tea (two inner circles) and biofilm (two outer circles) samples. The two circles represent replicate samples for each sample type.

taking into account real industrial-scale fermentation conditions that may be encountered and impact microbial community dynamics and, moreover, were usually linked to only one tea type. In this context, to thoroughly study microbial communities at the industrial scale, 1000 L fermentation vessels were followed over time at a Brittany SME industrial production site and sampling was performed from a mixture of the upper and lower tank levels and from the floating biofilms for Kombucha black and green tea samples. This allowed us to ensure that industrial-scale microbial stress factors and important scale-up conditions were accounted for such as oxygenation (upper level of fermentation vessel highly oxygenated vs. lower level), osmotic stress related to sugar content (lower levels of fermentation vessels richer in sugar content than upper level), static fermentation conditions and to distinguish between liquid and solid samples.

Coupling of metabarcoding and culture-dependent approaches in a dynamic follow-up study provides a clear overview of the microbial diversity, including microorganisms that are refractory to culture, and their potential roles during Kombucha fermentation. Moreover, by following two different tea fermentations (black and green), and by using the same biofilm base that was divided into two sections for each fermentation, tea matrix effects impacting microbial diversity and dynamics were clearly highlighted. Concerning the culture-independent method, the two targets (16S V1-V3 region, 26S D1/D2 region) are commonly used in metabarcoding analyses to study microbial communities in diverse niches. However, they may have some limitations in certain cases. In particular, the 16S gene target can present low discriminatory power for species-level identifications due to the lack of polymorphism. However, in this study, a very strong correlation between metabarcoding and culturing data was observed suggesting that, in some cases, accurate genus- and species-level identifications can be made, as also recently confirmed on Italian cheeses (Giello *et al.* 2017). Indeed, in our study, similar dominant yeast and bacterial species were identified by both approaches which led to determining a core microbial community. The advantage of culture-dependent methods is that the conserved isolates may further be used for technological strain selection. In particular, *Dekkera* spp. and *Hanseniaspora valbyensis* were clearly dominant during both green and black tea fermentations as shown via both approaches while for bacteria, although acetic acid bacterial species were systematically dominant in all fermentations, LAB and more precisely *Oenococcus oeni* were associated to green tea Kombucha fermentations. This result was clearly highlighted using metabarcoding and this strategy also showed higher levels of lactobacilli, especially *Lactobacillus nageli*, that were only identified at low levels using cultural techniques. Moreover, metabarcoding showed very different bacterial communities according to tea type as LAB were mostly present during green tea fermentations while AAB largely dominated black tea fermentations. In this context, it appears that tea type has a major impact on bacterial species dominance in both liquid and biofilm samples during fermentation. This observation raises questions about the molecules impacting the microbiota in both green and black tea. The main difference between these teas is the processing of *Camelia sinensis* leaves, as for black tea they are rolled and aerated to allow for endogenous enzymatic oxidation and a full fermentation while for green tea they are steamed, rolled and dried. From a compositional point of view, this mainly leads to differences in terms of the type and concentration of polyphenols (Graham 1992). The presence of dominant LAB and AAB (i.e. *Gluconacetobacter europaeus* in both liquid and biofilm samples and *O. oeni*, *L. nageli* and *G. europaeus* in liquid samples) during green tea fermentation was directly correlated with higher levels of two organic acids, lactic and acetic acid. This finding can also be related to the presence of acidotolerant yeast species

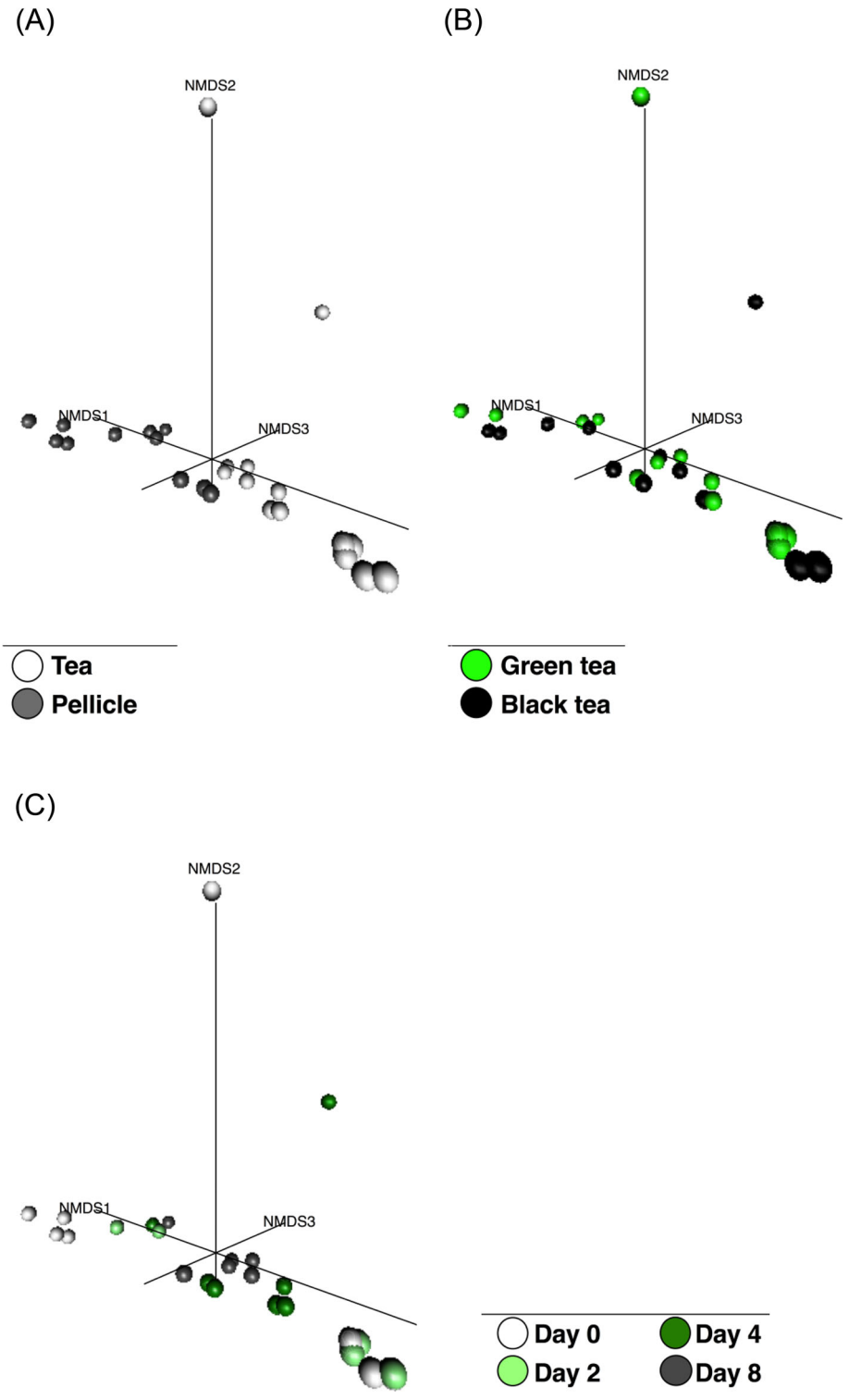

Figure 7. Spatial ordination of samples deduced by 26S rDNA metabarcoding. Non-metric dimensional scaling is based on a Bray-Curtis dissimilarity matrix built at the species level. Samples were colored according to tea matrix (**A**), tea type (**B**) and days of fermentation (**C**).

such as *Dekkera* spp. during the fermentation. Furthermore, the two lactic acid bacterial species were systematically correlated to the liquid samples (P-value < 0.005) and interestingly their proportions increased over time in these samples. On the other hand, black tea samples were largely dominated by AAB, especially *G. europaeus*, from D2 onwards; however, lower levels of acetic acid were quantified in these samples at D8 and the final pH was slightly higher in the final product. As sampling was performed at both upper (higher oxygen concentrations adapted to AAB) and lower (lower oxygen concentrations well adapted to LAB development) levels of the fermentation vessel, a clearer description of the bacterial community was obtained in this study.

Table 2. Evolution of different physico-chemical parameters during green and black tea Kombucha fermentations.

	Days			
	0	2	4	8
Acetic acid (mM)				
Green tea	ND	12.29 ± 0.25	11.02 ± 0.55	15.72 ± 0.36
Black tea	ND	8.18 ± 0.21	5.77 ± 0.35	8.17 ± 0.26
Lactic acid (mM)				
Green tea	0.22 ± 0.06	2.63 ± 0.01	6.14 ± 0.03	15.37 ± 0.13
Black tea	ND	2.51 ± 0.09	4.99 ± 0.09	10.46 ± 0.12
Gluconic acid (mM)				
Green tea	0.51 ± 0.19	0.20 ± 0.02	0.25 ± 0.02	0.15 ± 0.08
Black tea	0.10 ± 0.08	0.31 ± 0.08	0.25 ± 0.12	0.20 ± 0.05
Glucuronic acid (mM)				
Green tea	0.15 ± 0.07	0.15 ± 0.02	0.10 ± 0.07	0.05 ± 0.07
Black tea	0.15 ± 0.01	0.10 ± 0.05	0.05 ± 0.04	0.05 ± 0.03
Ethanol (%)				
Green tea	0.08 ± 0.00	0.22 ± 0.01	0.41 ± 0.00	1.04 ± 0.00
Black tea	0.08 ± 0.00	0.27 ± 0.01	0.47 ± 0.00	1.01 ± 0.00
pH				
Green tea	5.26	4.03	3.66	3.30
Black tea	5.20	4.32	3.95	3.55
Density				
Green tea	1019.8	1019.1	1018.8	1013.0
Black tea	1019.8	1019.5	1017.8	1012.2

Also, although high species diversity was identified at D0 in both sample and tea types, as stated above bacterial species diversity rapidly evolved during fermentation. Up to three key microbial species (bacteria and yeast), namely *Dekkera* spp., *H. valbyensis* and *G. europaeus* dominated from as early as D2 to D8 according to sample type.

Dominant yeast species diversity and dynamics was not majorly affected by tea type; however, subdominant taxa such as *Saccharomyces* spp., *Wickerhamomyces anomalus*, *Candida boidinii*, *Pichia membranifaciens, Zygoascu shellenicus, Kregervanrija* sp. and *Clavispora* sp. were clearly associated to a given tea type. Yet, due to their sporadic presence and low abundances, they are not likely to play a key role during fermentation. More interestingly, two dominant yeast species identified in this studied, namely *Dekkera anomala* and *D. bruxellensis*, are considered in other fermented beverages such as wine, cider and beer to be spoilage microorganisms as they can produce substantial quantities of volatile phenols inducing distinct animal and leather-like off-odours and flavours in the final product (Heresztyn 1986a,b; Oelofse, Lonvaud-Funel and Du Toit 2009; Buron *et al.* 2011, 2014; Smith and Divol 2016). On the other hand, in lambic beers, *D. bruxellensis* was suggested to potentially play a positive role in flavour development (DeKeersmaecher 1996) probably providing a very typical and characteristic taste to this type of beer. In the case of Kombucha, these species appear to play a key and important role during fermentation and their presence, along with other fermentative yeast species identified, can be correlated or associated with the low levels of ethanol encountered in the final product that would not exhibit microbial inhibitory effects. It can also be assumed that some ethanol produced by these yeast species is directly transformed into organic acids by a consortium of AAB. Supplementary experiments in our laboratory have indeed shown that these *Dekkara* spp. strains are capable of producing ethanol in both tea types (after inoculation into sterile teas) but concentrations remain low (data not shown). Previous studies have identified other yeast species as being the most dominant although *Dekkera* spp. were almost always identified. For example, in lab-scale black tea fermentations (200 mL vessels), high-throughput sequencing analyses identified *Zygosaccharomyces* as the most dominant genus (Marsh *et al.* 2014b) while other authors have described both *Zygosaccharomyces* and *Dekkera* spp. among the most abundant in similar lab-scale fermentations but using culture-dependent analyses (Mayser *et al.* 1995; Liu *et al.* 1996; Teoh, Heard and Cox 2004). More recently, *Dekkera* and *Pichia* genera were described as the most dominant in different studied microenvironments (Reva *et al.* 2015).

To date, this natural fermentation is carried out using the indigenous microbial community highly present in the surface biofilm under aerobic conditions in open fermentation vessels. Natural fermentations are still relatively common, especially for traditional fermented beverages such as cider (Coton *et al.* 2006; Coton, Coton and Guichard 2016), cereal-based beverages (Marsh *et al.* 2014a), kefir (Nalbantoglu *et al.* 2014) and of course Kombucha to name a few. In general, these natural fermentations are considered to be more difficult to control (i.e. batch to batch effects that can impact final product quality, seasonal effects) in comparison to directed fermentations. These changes are often due to modifications in indigenous microbial communities over time, to changes or modifications in raw material quality or to difference in fermentation technologies and conditions (i.e. fermentation vessels, lack of sufficient temperature control, agitation, hygiene and quality, etc.). This study highlighted that *Dekkera* spp., *H. valbyensis*, *G. europaeus* and possibly *O. oeni* could be of technological importance for Kombucha fermentations but their respective contribution to the final organoleptic properties of the product remains to be elucidated. Better knowledge of Kombucha microbiota should lead to better fermentation control and product quality in the future.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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