International Journal of Food Microbiology 220 (2016) 63–72

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Kombucha tea fermentation: Microbial and biochemical dynamics

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ARTICLE INFO

Article history:
Received 28 June 2015
Received in revised form 24 December 2015
Accepted 31 December 2015
Available online 7 January 2016

Keywords:
Kombucha tea
Microbial community structure and dynamics
High throughput sequencing
T-RFLP
Change in fermentation
Radical scavenging

ABSTRACT

Kombucha tea, a non-alcoholic beverage, is acquiring significant interest due to its claimed beneficial properties. The microbial community of Kombucha tea consists of bacteria and yeast which thrive in two mutually non-exclusive compartments: the soup or the beverage and the biofilm floating on it. The microbial community and the biochemical properties of the beverage have so far mostly been described in separate studies. This, however, may prevent understanding the causal links between the microbial communities and the beneficial properties of Kombucha tea. Moreover, an extensive study into the microbial and biochemical dynamics has also been missing. In this study, we thus explored the structure and dynamics of the microbial community along with the biochemical properties of Kombucha tea at different time points up to 21 days of fermentation. We hypothesized that several biochemical properties will change during the course of fermentation along with the shifts in the yeast and bacterial communities. The yeast community of the biofilm did not show much variation over time and was dominated by *Candida* sp. (73.5–83%). The soup however, showed a significant shift in dominance from *Candida* sp. to *Lachancea* sp. on the 7th day of fermentation. This is the first report showing *Candida* as the most dominating yeast genus during Kombucha fermentation. *Komagateibacter* was identified as the single largest bacterial genus present in both the biofilm and the soup (~50%). The bacterial diversity was higher in the soup than in the biofilm with a peak on the seventh day of fermentation. The biochemical properties changed with the progression of the fermentation, i.e., beneficial properties of the beverage such as the radical scavenging ability increased significantly with a maximum increase at day 7. We further observed a significantly higher D-saccharic acid-1,4-lactone content and caffeine degradation property compared to previously described Kombucha tea fermentations. Our data thus indicate that the microbial community structure and dynamics play an important role in the biochemistry of the fermentation of the beverage. We envisage that combined molecular and biochemical analyses like in our study will provide valuable insights for better understanding the role of the microbial community for the beneficial properties of the beverage.

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1. Introduction

Kombucha tea is a traditional non-alcoholic fermented beverage originating in the Orient and its inception is shrouded in mystery (Teoh et al., 2004). The beverage has gained substantial popularity especially in the West because of a large number of claims regarding its therapeutic potential against a host of maladies. Some of its beneficial effects have already been demonstrated such as: anti-microbial, anti-oxidant, anti-carcinogenic (Jayabalan et al., 2011), anti-diabetic (Aloulou et al., 2012; Bhattacharya et al., 2013), treatment for gastric ulcers (Banerjee et al., 2010) and high cholesterol (Yang et al., 2009), etc. It has also shown to have impact on immune response (Ram et al., 2000) and liver detoxification (Loncar et al., 2000).

Kombucha tea fermentation is the product of microbial activity by a consortium of both yeast and bacteria (Jarrell et al., 2000). The microorganisms, produce in the course of the fermentation, a thick cellulosic biofilm on the liquid–air interface. In contrast, most of the biofilms that have been studied to date are formed either on liquid–solid or air–solid interface (Nikolaev and Plakunov, 2007).

The beverage is prepared by adding a small portion of the biofilm into sweetened (10% *w*/*v*) black tea. The broth also contains 10–15% of previously fermented Kombucha tea (called hereafter as old soup for convenience). The fermentation is static and the usual fermentation time is 7–12 days at room temperature (Dutta and Gachhui, 2006, 2007). Thus the Kombucha tea microbial community can be divided into two parts; the first one being the cellulosic biofilm and the second one thriving in the underlying liquid or soup. Studies have reported that the entire microbial spectrum of this beverage is dominated by acetic

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http://dx.doi.org/10.1016/j.ijfoodmicro.2015.12.015
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acid bacteria (AAB) and yeast (Jarrell et al., 2000; Jayabalan et al., 2014; Marsh et al., 2014). Previous explorations however indicated that the microbial community may vary between different Kombucha fermentations across the globe depending upon the source of the inoculum used (Malbaša et al., 2011). The majority of these analyses have been culture-based so far (Dutta and Gachhui, 2006, 2007; Hesseltine, 1965; Liu et al., 1996; Teoh et al., 2004). Only recently, Marsh et al. (2014) has performed sequence-based analysis of the bacterial and yeast populations of Kombucha tea.

The enhanced beneficial activities of Kombucha tea compared to that of the unfermented tea (Banerjee et al., 2010; Bhattacharya et al., 2013; Yang et al., 2009) indicated that some changes have been brought by the microbial community during the fermentation process. Most of the scientific literature available so far either deals with the general microbial community of the system or the beneficial effects of the drink. Extensive exploration of the microbial community dynamics of both the biofilm and the soup along with the dynamics of the fermentation is very scarce. Such an investigation may eventually allow the correlation of the microbial community to the fermentation parameters and thus improve the management and the control of the system.

In this paper we describe concomitantly for the first time the yeast and bacterial community dynamics in both the biofilm and the soup of a Kombucha tea maintained in our laboratory by culture independent methods and also the changes in the fermentation biochemistry and antioxidant property of Kombucha tea during 21 days of fermentation. We hypothesize that several biochemical properties will change during fermentation together with shifts in the microbial communities. High throughput sequencing (HTS) of the yeast internal transcribed spacer 2 (ITS2) and the D1–D2 region of the large sub-unit (LSU) ribosomal RNA (rRNA) gene, as well as of the V3 region of the bacterial 16S rRNA gene was performed for a detailed description of the microbial communities in both compartments. Terminal restriction fragment length polymorphism (T-RFLP) analysis was chosen to monitor bacterial community dynamics during the fermentation process.

2. Materials and methods

2.1. Preparation of Kombucha tea

The Kombucha tea system was maintained in our laboratory as described previously by Bhattacharya et al. (2013). For determining the structure and dynamics of the microbial community, a total of sixteen, 200 mL Kombucha tea batches were maintained. These 16 batches were divided into 4 sets, each set having 4 replicate Kombucha tea systems. Four replicates were prepared so that each replicate was completely harvested after its stipulated period of fermentation. The harvesting was done after 3, 7, 14 and 21 days of fermentation. One set was for the yeast, two sets were for the bacterial T-RFLP and the last set for bacterial next-generation sequencing. The batches were maintained at 28 ± 2 °C.

2.2. Isolation of total DNA

The isolation of DNA for bacteria from both compartments was done using the QIAamp DNA stool mini kit (Qiagen, USA) following the manufacturer's protocol. However for the yeast DNA, a combined protocol using Zymolyase (USBiologicals, USA) and QIAamp DNA stool mini kit was employed, as either of the protocols alone failed to produce quality yeast DNA for this analysis.

DNA isolation from the biofilm required a pre-treatment, which included transfer of the entire biofilm (10 cm diameter) into a beaker and incubation with 750 μL cellulase (Sigma-Aldrich, Germany) and 25 mL of sterile distilled water at room temperature for 30 min. Subsequently the biofilm was manually homogenized. The sample was then centrifuged at 4588 ×g for 6 min and the supernatant was discarded. Five mL sorbitol buffer (1 M Sorbitol, 50 mM Tris pH 7.5) supplemented with 30 mM DTT was added to the biofilm and incubated for 10 min at 30 °C. The suspension was centrifuged at 4588 ×g for 6 min. Resuspension in 5 mL sorbitol buffer/2 mM DTT was followed by incubation with 400 U of Zymolyase at 30 °C for 45 min. The mixture was then used to isolate the DNA following the manufacturer's protocol. For isolation of total DNA from the soup, 200 mL of the beverage was centrifuged and the pellet was used subsequently using the manufacturer's protocol.

2.3. Next generation sequencing and data analysis

The total yeast DNA from all 4 time points served as templates to amplify the internal transcribed spacer ITS2 and the D1–D2 region of the LSU rRNA genes respectively, using Ion Torrent specific barcoded fusion oligonucleotide primers and PCR protocols as described previously by Tonge et al. (2014). Similarly the bacterial total DNA from the 7 day biofilm and soup were used to amplify the V3 region of the 16S rRNA gene using the primers 341F and 518R as described previously by Mühling et al. (2008). The amplified bacterial products were then attached with dual indices and Illumina sequencing adapters using the Nextera XT Index Kit (Illumina, USA) following manufacturer's protocol. All amplicons were quality checked for fragment size and DNA concentration in a Bioanalyser 2100 (Agilent Technologies). The yeast amplicons were sequenced by the Personal Genome Machine using a 400 bp sequencing kit with a 316v2 chip used for each amplicon in accordance with the manufacturer's standard protocol. The bacterial amplicons were sequenced in an Illumina MiSeq platform (Illumina) using manufacturer's protocol.

Following sequencing the yeast raw data was first filtered within the PGM software to remove low quality and polyclonal sequences. Sequences matching the PGM 3′ adaptor were also automatically trimmed. The reverse primer sequences were trimmed using CutAdapt software. Full length reads were processed using the USEARCH algorithm followed by de-replication of identical reads and individual reads de-noised and chimeric sequences were eliminated using the QIIME pipeline following default parameters (Caporaso et al., 2010). Similarly the bacterial reads were first assembled into candidate 16S rRNA genes from the paired end reads using tools from the UPARSE package and a maximum of two mismatches were allowed in the overlapping region. During this analysis the low quality and polyclonal sequences were filtered out. The reads were then subjected to de-replication using the tool from UPARSE Package in Prefixed De-Replication Mode. Minimum length cut-off of 64 base pairs was applied (Edgar, 2013). De-replicated reads were then de-noised followed by chimera checked using the RDP Gold Database (Cole et al., 2013). The OTU clustering was done at 97% similarities and the clustered OTUs were phylogenetically identified by using the Silva 108 release database (Quast et al., 2013) for the yeast and the bacterial identification was done using the SILVA Incremental Aligner (SINA) tool (Pruesse et al., 2012) against the SILVA and Greengenes databases. In both cases a minimum identity cutoff of 97% was applied for the phylogenetic assignments. The relative abundance was calculated on the basis of the number of sequences assigned to each taxa against the total number of sequences. The raw files were submitted to the SRA database under the BioSample IDs: SAMN02999971, SAMN02595524 and SAMN02602923.

2.4. Terminal restriction fragment length polymorphism (T-RFLP) analysis

T-RFLP analysis with 16S rRNA genes was performed as described previously (Giebler et al., 2013; Giebler et al., 2014). Briefly 16S rRNA genes were amplified using a 6-carboxyfluorescein (6-FAM)-labeled forward primer 27f and 1525r (Lane, 1991; Weisburg et al., 1991). Purified PCR products from each sample were digested overnight at 37 °C with 2 U MspI (NEB, USA). T-RFLP analysis was run on an ABI PRISM 3100 genetic analyzer system using the GeneScanTM 500

ROXTM size standard (both Applied Biosystems). Data were analyzed with GeneMapper V3.7 (Applied Biosystems). Noise removal, peak binning and normalization of signal intensity were conducted according to Abdo et al. (2006) in statistical software R version 2.10.0 (R Development Core Team, 2014; http://www.r-project.org/index.html).

2.5. Biochemical analysis of Kombucha tea

Soup samples taken after 7, 14 and 21 days of fermentation were centrifuged at 9176 ×g for 15 min using an Eppendorf 5810 R centrifuge. The supernatants were then lyophilized to dryness using Eyela UNI TRAP UT-1000. The dry powders were resuspended in HPLC grade water (SRL, India) to prepare multiple concentrations as per the requirement of the analysis performed. For each time point this was done in triplicate.

2.5.1. Total polyphenol and flavonoid content

The total phenolic and flavonoid compounds in Kombucha tea (50 mg/mL) at different time points of fermentation were measured by the Folin–Ciocalteu method (Singleton et al., 1999) and by a colorimetric method using aluminum chloride (Chang et al. 2002) respectively. Gallic acid and quercetin were used as standards. The total phenolic compounds were expressed as gallic acid equivalents (GAE) and the flavonoid content as quercetin equivalents (QE).

2.5.2. pH and titratable acidity

The pH of both the fermented beverage and the unfermented control was determined by a pH meter. Titratable acidity was similarly determined by titrating 100 mL of samples against 0.1 N NaOH (Bhusari et al., 2013). The titration was done till the pH was increased to 7.

2.5.3. Chemical composition

The samples were tested for acetic acid, gluconic acid, ethanol content, total reducing sugar, black tea polyphenols (theaflavin, thearubigin and caffeine) and D-saccharic acid-1,4-lactone (DSL) content.

The major organic acids, acetic acid and gluconic acid were determined in Kombucha tea by high performance liquid chromatography (HPLC) equipped with a PDA detector (SPD-M20A) using a C-18 column (100 Å column 30 × 20 mm Phenomenex, USA). The mobile phase was 20 mM potassium dihydrogen phosphate, pH 2.4 with a flow rate of 1.0 mL/min and running time of 40 min. The column temperature was maintained at 28 °C and the detection was carried out at 220 nm by comparing the retention time of the standard compounds. The concentrations of organic acids were quantified from standard curves. Ethanol contents (100 μL) were determined by Ethanol Assay kit (Megazyme, Ireland) following manufacturers protocol. Reducing sugars were determined by the DNS (dinitrosalicylic acid) method as described previously (Sengupta et al., 2000). Quantity of the main black tea polyphenols theaflavin and thearubigins were estimated in fermented beverage by the method of Takeo and Oosawa (1976) as modified by Ramaswamy (1978) and Thanaraj and Seshadri (1990). The caffeine content was determined by HPLC with methanol:water (70:30 *v*/*v*) as mobile phase (Skoog et al., 2007). The flow rate of the mobile phase was maintained at 1.0 mL/min at 28 °C. Detection of caffeine was monitored at 272 nm and the concentration was quantified from standard curves. D-Saccharic acid-1,4-lactone (DSL) was determined by HPLC analysis using a mobile phase of 40 mM borax buffer ($Na_2B_4O_7$–KH_2PO_4), pH 6.5 and 15% methanol (*v*/*v*) at a flow rate of 1.0 mL/min and the elution profile was monitored at 190 nm with column temperature of 25 °C (Wang et al., 2010). Standard DSL was run under the same conditions and its elution pattern was compared with that of Kombucha tea.

2.6. Determination of reducing power and radical scavenging activities

Reducing power of the beverage (100 mg/mL) at different time points of the fermentation was determined following the method of Ferreira et al. (2007).

Radical scavenging and anti-oxidant activities at three fermentation time points were measured with 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Blois, 1958), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Roy et al., 2013), hydroxyl (Nash, 1953) and nitric oxide radicals (Kumari et al., 2011). For this purpose multiple concentrations of the lyophilized Kombucha tea were used as samples and their radical scavenging activity was determined. Percent scavenging was measured and the effective concentration of sample required for scavenging radicals by 50% (IC_{50} value) was calculated graphically for the fermented beverage, unfermented control and the standard (vitamin C).

2.7. Determination of anti-glycation effect

The Anti-advanced Glycation End products (AGE) formation capability was measured according to the method of Harris et al. (2011). Quercetin was used as the positive control.

2.8. Statistical validation

The species richness for both the bacteria and the yeast was determined by calculating the Shannon index (Shannon and Weaver, 1948), Simpson index and/or Simpson index of diversity (Simpson, 1949). The alpha diversity (α-diversity) expressing the OTU level diversity for both the yeast and bacteria was calculated as described by Whittaker (1972). All biochemical assays were performed on three batches of Kombucha tea and all the values were expressed as mean ± S.D. (n = 3). Significant differences between means of two groups were determined using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using the Origin Pro v8 software (Origin Lab). A difference was considered significant at the $P < 0.05$ level.

3. Results

3.1. Yeast community dynamics

A total of eight DNA samples served as template to amplify and sequence the ITS2 and D1–D2 LSU genes. The average read lengths and the total number of reads for both ITS2 and LSU genes obtained from the eight samples are shown in Supplementary Table T1. The α diversity obtained from the ITS2 gene sequencing ranged from 1.4 to 3.4 OTUs (Table 1). On the other-hand the α diversity of the LSU genes was higher and ranged from 3.7 in Soup-Day-14 sample to 6.1 in the Soup-Day-7 sample. The other two diversity parameters showed a similar pattern. This clearly indicated that the community had an overall low diversity and that the yeasts were most diverse in the soup after 7 days of fermentation (Table 1).

The taxonomic composition of the yeast communities in both the biofilm and the soup at different time points is shown in Tables 2–4. At the phylum level, the Ascomycota dominated both sequence sets claiming >98% of the share. At the family level, according to the ITS2 dataset, the Saccharomycetaceae was found to constitute between 94.8 and 98.8% of the community while in the LSU dataset the same family was seen to represent between 91 and 95.1% of the community (Table 2). The biofilm ITS2 data show that sequences affiliating to the genus *Candida* increased until day 14 from 80 to 94.1%. After 21 days of fermentation, the proportion of sequences corresponding to *Candida* sp. slightly dropped to 88.3% of the community. In the soup, the genus *Candida* occupied the highest share of the community at all the time points except on the seventh day where *Lachancea* sp. represented 78.9% of the community. After 21 days of fermentation sequences

Table 1
Diversity indices for the yeast ITS2 and LSU sequences and the bacterial 16S rRNA gene T-RFLP datasets.

Sample	Marker sequence								
	ITS2			D1–D2 LSU			16S rRNA T-RFLP		
	α diversity	Shannon	Simpson	α diversity	Shannon	Simpson	α diversity	Shannon	Simpson index of diversity
Biofilm—day-3	3.1	1	0.7	5.4	1.6	0.6	0.6	1	0.6
Soup—day-3	3	1	0.6	4.6	1.4	0.6	0.7	1.1	0.6
Biofilm—day-7	1.6	0.5	0.9	3.9	1.3	0.7	2.1	1.4	0.7
Soup—day-7	2.3	1	0.6	6.1	1.9	0.4	3.2	1.7	0.7
Biofilm-day-14	1.4	0.4	0.9	3.8	1.3	0.7	0.6	1.1	0.6
Soup-day-14	2.3	0.4	0.9	3.7	1	0.7	0.6	1	0.6
Biofilm-day-21	2.8	0.7	0.8	4.4	1.4	0.6	0.6	1	0.6
Soup-day-21	3.4	1	0.6	4.5	1.4	0.5	1.7	1.3	0.6

affiliating to *Candida* sp. and *Lachancea* sp. made up 77.4% and 19% respectively. The other yeast genera identified from the ITS dataset included *Kluyveromyces*, *Waitea*, *Debaryomyces*, *Pichia*, etc. (Table 3). A similar picture was also obtained with the LSU data with more or less constant dominance of *Candida* sp. in both the biofilm and the soup. After 7 days of fermentation the soup community was dominated by *Lachancea* sp. (58.2%). The other yeast genera identified from the LSU gene sequencing in both compartments across the fermentation time points were *Kluyveromyces*, *Eremothecium*, *Starmera*, *Hanseniaspora*, *Debaryomyces*, *Kazachstania*, *Meyerozyma*, *Merimbla*, *Sporopachydermia*, *Pichia*, *Zygowilliopsis*, *Saccharomyces*, *Sugiyamaella* and *Saccharomycopsis* (Table 3). *Candida stellimalicola* was the most dominant species across all four time points studied in the biofilm (Table 4). In the soup this yeast species dominated three time points except for the seventh day of fermentation; *Lachancea fermentati* dominated the soup in this particular time point.

3.2. Bacterial community

Both sets of T-RFLP produced very similar data with little or no variance. The diversity indices obtained from the T-RFLP analysis also indicated a low diverse community (Table 1). The bacterial community had the maximum diversity on the seventh day of fermentation in both the biofilm and the soup. However the diversity indices in the soup were higher than those in the biofilm indicating an overall increased diversity in the soup compared to the biofilm.

Since the bacterial community was most diverse in the seventh day of fermentation, the HTS analysis of the V3 region of 16S rRNA gene was performed for that day only in both the biofilm and the soup. Similar to the T-RFLP analysis, the diversity indices from the HTS indicated an overall low diversity of the bacteria, especially in the biofilm. However, the soup community was more diverse (Supplementary Table T2). Unlike the yeast community, where the phylum and family level distribution of the sequences were dominated by mainly one type of yeast in both the biofilm and the soup, the picture was somewhat different for the bacterial community, especially in the soup. In the biofilm, sequences affiliating to the class Proteobacteria and within this class the family Acetobacteraceae were dominating with more than 88% of the sequences. However, in the soup, at the phylum level, in addition to the dominating Proteobacteria (65.7%), sequences affiliating to the Firmicutes (11.2%), the Cyanobacteria (7.1%) and the Actinobacteria (4.1%) were also obtained. Similarly, at the family level, although Acetobacteraceae was the single largest family comprising 63.5% of the reads, other families such as, Oscillatoriaceae (5.5%), Bifidobacteriaceae (2.3%), Ruminococcaceae (2.1%) were also observed (Table 5). Overall the soup bacterial community was more diverse than the biofilm community. *Komagataeibacter* sp. and *Gluconobacter* sp. were the most prominent genera identified in the system. Reads corresponding to *Komagataeibacter* sp. made up about 50% abundance in both the biofilm and the soup, which made this genus to be the most dominant bacteria present in this system. The other genera identified in the soup were *Bifidobacterium*, *Collinsella*, *Enterobacter*, *Weissella*, *Lactobacillus*, etc. However, in both cases a substantial portion of the population at the genus level remained unclassified with ~33% in biofilm and 34% in soup (Table 5).

3.3. Total polyphenol and flavonoid content

The concentrations of total polyphenols and flavonoids increased progressively with fermentation time in Kombucha tea. The relative amounts of total polyphenols and flavonoids in the fermented Kombucha tea, compared to the unfermented tea, increased by 54% and 24% respectively, across the span of 21 days of fermentations (Fig. 1).

3.4. Reducing power, anti-oxidant property and anti-glycation effect

Fermentation resulted in a gradual increase in the reducing power of the beverage by about 3.7%, 35% and 44.4% than the unfermented one after 7, 14 and 21 days of fermentation respectively. The DPPH, ABTS, hydroxyl and NO radicals scavenging abilities of Kombucha tea were assayed and the IC_{50} values were calculated. As anticipated, the fermented beverage showed less scavenging activity compared to the standard ascorbic acid. But it is interesting to note that the radical scavenging activities were augmented in Kombucha tea in comparison to that of the unfermented control. The DPPH and ABTS radicals scavenging activity increased by 39.7% and 38.36%, respectively, after 21 days. The increase in hydroxyl radical scavenging activity of the fermented product was also evident from the decrease in the IC_{50} values by about 45.8% (Table 6). Similarly in case of the NO scavenging activity, the IC_{50} value of Kombucha tea after 21 days of fermentation was found to be reduced by 43.8%.

In the present study, we evaluated the ability of tea and Kombucha tea to inhibit AGE formation using the BSA-glucose assay. The results showed that both unfermented black tea and Kombucha tea fermented for 21 days exhibited anti-glycation properties with IC_{50} 7.37 ± 0.48 mg/mL and 5.49 ± 0.73 mg/mL, respectively. This indicated that the anti-glycation properties of black tea were augmented after fermentation by about 25.5% after 21 days of fermentation. The increase was also significant (18.4% and 22.6%) after 7 and 14 days of fermentation, respectively.

3.5. Biochemical analysis

3.5.1. pH, titratable acidity and acid content

The initial pH before fermentation was about 5.03 which decreased abruptly to 2.28 after 7 days of fermentation (Table 7). After that the decrease in pH was not so prominent and the pH was found to be 1.88 at day 21 of the fermentation. In contrast, the titratable acidity was found to be increasing linearly and progressively with the fermentation time (Table 7). The titratable acidity was recorded to be 0.045 ± 0.002 (M) after 21 days of fermentation.

Table 2
Relative distribution of yeast families in the biofilm and the soup (% abundance).

Family	Marker gene sequenced															
	ITS2								D1–D2 LSU							
	Biofilm—day-3	Biofilm—day-7	Biofilm—day-14	Biofilm—day-21	Soup—day-3	Soup—day-7	Soup—day-14	Soup—day-21	Biofilm—day-3	Biofilm—day-7	Biofilm—day-14	Biofilm—day-21	Soup—day-3	Soup—day-7	Soup—day-14	Soup—day-21
Saccharomycetaceae	94.8	96.4	97.2	95.2	97.6	98.2	98.8	98.2	93.6	92.6	91.1	94.1	94.4	95	93.3	95.1
Davidiellaceae	3.0	0.8	0.8	0.6	0.4	0.1	0.7	0.1	–	–	–	–	–	–	–	–
Agyriaceae	0.5	1	0.7	0.9	1.4	0.3	0.5	1	–	–	–	–	–	–	–	–
Debaryomycetaceae	0.1	–	–	0.6	0.2	0.6	–	0.1	2.5	2.7	1.3	2.2	2.3	1.5	3.1	1.1
Pichiaceae	–	–	–	–	–	0.4	–	0.3	0.7	0.3	0.7	1	0.9	0.6	0.7	0.7
Saccharomycopsidaceae	–	–	–	–	–	–	–	–	0.7	0.6	0.4	0.6	0.6	0.7	0.6	0.7
Saccharomycodaceae	–	–	–	–	–	–	–	–	0.6	0.8	2.5	0.7	1	1.6	1.1	1
Schizosaccharomycetaceae	–	–	–	–	–	–	–	–	0.1	–	–	–	–	–	–	–
Trichomonascaceae	–	–	–	–	–	–	–	–	–	–	0.9	–	–	–	–	–
Dipodascaceae	–	–	–	–	–	–	–	–	–	0.8	1.1	0.3	-	0.2	0.8	0.6
Others	1.6	1.8	1.3	2.7	0.4	0.4	–	0.3	1.8	2.2	2	1.1	0.8	0.4	0.4	0.8

Note: '–' signifies below 0.1% or absent.

Table 3
Relative distribution of yeast genera in the biofilm and the soup (% abundance).

Genus	Marker gene sequenced															
	ITS2								D1–D2 LSU							
	Biofilm—day-3	Biofilm—day-7	Biofilm—day-14	Biofilm—day-21	Soup—day-3	Soup—day-7	Soup—day-14	Soup—day-21	Biofilm—day-3	Biofilm—day-7	Biofilm—day-14	Biofilm—day-21	Soup—day-3	Soup—day-7	Soup—day-14	Soup—day-21
Candida	80.4	93	94.1	88.3	75.7	16.2	93.5	77.4	73.5	83	82.3	79.6	73.7	26.5	86.4	70.6
Lachancea	11.9	3	1.8	6.3	20.7	78.9	4.7	19	11.8	2.8	1.9	6.9	16.5	58.2	3.9	21.0
Kluyveromyces	2.6	0.4	1.1	0.6	1.1	3	0.4	1.7	2.3	2.7	2.6	3.0	2.2	4.9	2.0	1.1
Debaryomyces	–	–	–	0.6	–	0.6	–	–	0.6	2.0	0.9	1.5	1.5	0.7	2.2	0.6
Pichia	–	–	–	–	–	0.4	–	–	0.7	0.3	0.7	1.0	0.9	0.6	0.7	0.7
Waitea	–	–	–	1.6	–	–	–	0.3	–	–	–	–	–	–	–	–
Eremothecium	–	–	–	–	–	–	–	–	2.9	0.8	2.9	1.0	0.1	0.1	0.2	0.6
Meyerozyma	–	–	–	–	–	–	–	–	1.8	0.7	0.4	0.7	0.8	0.9	0.8	0.5
Zygowilliopsis	–	–	–	–	–	–	–	–	1.0	0.4	0.4	0.2	0.4	0.9	0.3	–
Saccharomyces	–	–	–	–	–	–	–	–	0.9	0.4	0.2	–	0.2	0.1	–	–
Saccharomycopsis	–	–	–	–	–	–	–	–	0.7	0.6	0.4	0.6	0.6	0.7	0.6	0.7
Hanseniaspora	–	–	–	–	–	–	–	–	0.6	0.8	2.5	0.7	0.9	1.6	1.1	1.0
Kazachstania	–	–	–	–	–	–	–	–	0.5	–	0.6	0.4	–	2.0	-	0.2
Starmera	–	–	–	–	–	–	–	–	0.3	2.1	–	2.6	1.0	2.1	0.4	1.2
Merimbla	–	–	–	–	–	–	–	–	–	1.3	1.2	–	–	–	–	–
Sporopachydermia	–	–	–	–	–	–	–	–	–	0.8	1.1	0.3	–	0.2	0.8	0.6
Sugiyamaella	–	–	–	–	–	–	–	–	–	–	0.9	–	–	–	–	–
Others	5.1	3.6	3	2.6	2.5	0.9	1.4	1.6	2.4	1.3	1	1.5	1.2	0.5	0.6	1.2

Note: '—' signifies below 0.1% or absent.

Table 4
Relative distribution of yeast species in the biofilm and the soup as per D1–D2 LSU gene sequencing (% abundance of reads).

Species	Sample							
	Biofilm—day-3	Biofilm—day-7	Biofilm—day-14	Biofilm—day-21	Soup—day-3	Soup—day-7	Soup—day-14	Soup—day-21
Candida stellimalicola	59	72.2	71.3	65.3	61.5	17	68.5	56.5
Candida tropicalis	11.9	6.8	8.4	11.8	8.4	6	11.4	7.2
Candida parapsilosis	2	4	2.6	2.5	2.9	3.3	6.5	6.5
Lachancea thermotolerans	7.2	–	–	2.5	1.3	4.8	0.2	1
Lachancea fermentati	2.3	2.5	1.9	4.2	15.1	51.1	3.6	20
Lachancea kluyveri	–	0.3		0.1	–	1.5	–	–
Eremothecium cymbalariae	2.4	0.8	2.9	0.9	0.1	–	0.2	0.6
Eremothecium ashbyii	–	–	–	–	–	0.1	–	–
Kluyveromyces marxianus	2.3	2.6	2.5	2.5	2.1	4.8	2	0.9
Debaryomyces hansenii	0.6	2	0.9	1.5	1.5	0.6	2.2	0.6
Pichia mexicana	0.7	0.3	0.7	0.9	0.9	0.6	0.7	0.7
Meyerozyma caribbica	1.5	0.7	0.3	0.7	0.8	0.9	0.8	0.5
Meyerozyma guilliermondii	0.3	–	–	–	–	–	–	–
Zygowilliopsis californica	1	0.3	0.4	0.2	0.4	0.9	0.3	–
Saccharomyces cerevisiae	0.8	0.4	0.2	–	0.2	0.1	–	–
Saccharomycopsis fibuligera	0.7	0.6	0.4	0.6	0.6	0.7	0.6	0.7
Hanseniaspora uvarum	0.5	0.7	2.5	0.3	0.8	0.4	0.2	0.5
Hanseniaspora meyeri	–	–	–	0.1	–	–	0.8	–
Hanseniaspora vineae	0.1	0.1	–	0.3	0.2	1.2	–	0.5
Merimbla ingelheimense	–	1.2	1	–	–	–	–	–
Sporopachydermia lactativora	–	0.8	1	0.3	–	0.2	0.8	0.6
Kazachstania telluris	0.5	–	0.6	0.4	–	1.7	–	0.2
Kazachstania exigua			–	–	–	0.3	–	–
Starmera amethionina	0.3	1.4	–	2.6	1	1.8	0.4	–
Starmera caribaea		0.7	–	–	–	–	–	–
Unclassified	3.5	0.3	1.4	0.8	1.0	1.5	0.2	1.8
Others	2.4	1.3	1	1.5	1.2	0.5	0.6	1.2

Note: "Unclassified" indicates the percentage of reads that could not be assigned to any species. Note: '–' signifies below 0.1% or absent.

The low pH is attributed to the production of various organic acids during fermentation. The major organic acids found are acetic acid and gluconic acid. The concentration of both the acids increased steadily with time and reached 16.57 ± 0.9 g/L and 7.36 ± 0.87 g/L respectively after 21 days of fermentation (Table 7).

3.5.2. Ethanol and reducing sugar content

The ethanol concentration increased initially with time to reach a maximum value of about 0.28 ± 0.014 g/L at day 7 of fermentation, followed by a decrease to about 0.073 ± 0.003 g/L after 21 days of fermentation (Table 7). This decrease in ethanol concentration is due to its utilization by acetic acid bacteria to produce acetic acid. We observed a period of significant production of reducing sugar (8.2 ± 0.7 g/L) in the first 7 days of fermentation when sucrose was hydrolyzed to glucose and fructose by yeast invertase. This was followed by an intensive utilization of reducing sugar as indicated by the reduction of its concentration to 2.25 ± 0.3 g/L after 21 days of fermentation.

3.5.3. Black tea polyphenol and caffeine content

Theaflavin was found to increase by about 88.63% whereas thearubigin was decreased by about 47.02% after 21 days of fermentation. The caffeine content was decreased by about 40% after 21 days of fermentation (Table 7).

3.5.4. DSL production

The fermentation of sugared black tea might result in the production of certain metabolites which were not present in unfermented black tea. In our study we have detected DSL in Kombucha tea which was not found in unfermented black tea. The DSL concentration increased gradually with fermentation time and reached a concentration of 2.24 ± 0.1 g/L after 21 days of fermentation (Table 7).

Table 5
Relative distribution of bacterial taxa in the biofilm and soup (% abundance).

Phylum	Sample		Family	Sample		Genus	Sample	
	Biofilm	Soup		Biofilm	Soup		Biofilm	Soup
Proteobacteria	88.5	65.7	Acetobacteraceae	88.5	63.5	*Komagataeibacter*	50.3	49.9
Firmicutes	–	11.2	Oscillatoriaceae	–	5.5	*Gluconobacter*	16.8	2.5
Cyanobacteria	–	7.1	Bifidobacteriaceae	–	2.3	*Lyngbya*	–	4.4
Actinobacteria	–	4.1	Ruminococcaceae	–	2.1	*Bifidobacterium*	–	2.3
Unclassified	11.4	11.4	Peptostreptococcaceae	–	1.7	*Collinsella*	–	0.7
Others	0.1	0.5	Coriobacteriaceae	–	1.3	*Enterobacter*	–	0.6
X	X	X	Lachnospiraceae	–	1.2	*Weissella*	–	0.5
X	X	X	Enterobacteriaceae	–	1.1	*Lactobacillus*	–	0.3
X	X	X	Leuconostocaceae	–	0.5	Unclassified	32.8	34.6
X	X	X	Erysipelotrichaceae	–	0.4	Others	0.1	4.2
X	X	X	Rhodobacteraceae	–	0.4	X	X	X
X	X	X	Lactobacillaceae	–	0.3	X	X	X
X	X	X	Unclassified	11.4	17.5	X	X	X
X	X	X	Others	0.1	2.2	X	X	X

Note: '–' signifies below 0.1% or absent. 'X' signifies not applicable.

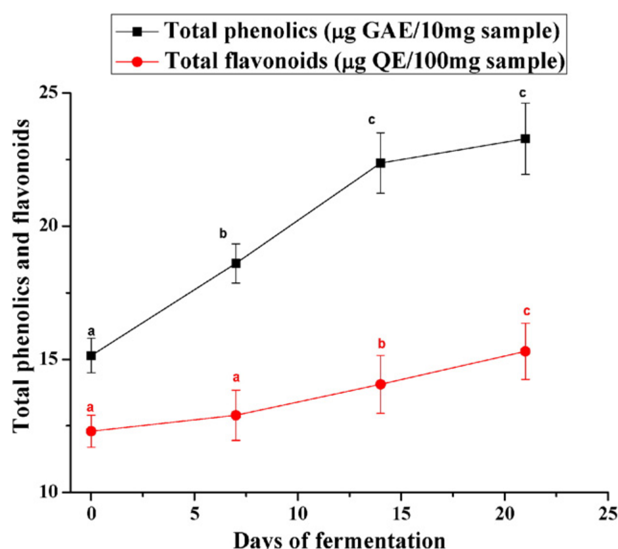

Fig. 1. Changes in total polyphenol and flavonoids in Kombucha tea during the course of its fermentation values are expressed as mean ± SD (n = 3). Values not sharing a common superscript letter differ significantly at P < 0.05.

4. Discussions

The Kombucha tea microbial community has rarely been studied in depth using modern next generation sequencing tools. The majority of the explorations reported till date have been done solely based on culturable methods (Chen and Liu, 2000; Dutta and Gachhui, 2006; El-Salam, 2012; Hesseltine, 1965; Jankovic and Stojanovic, 1994; Liu et al., 1996; Mayser et al., 1995; Sievers et al., 1995; Teoh et al., 2004). Using culture dependent methods we have been able to identify cultures pertaining to *Candida*, *Pichia*, *Acetobacter* and *Enterobacter* (Chakravorty et al. unpublished data). However, in comparison to the recent high throughput exploration of Kombucha tea by Marsh et al. (2014), these results indicated that a culture dependent analysis might not be sufficient to describe the overall microbial community structure and to also depict the rare microbes of the systems. This disparity in the data along with the absence of investigations describing the microbial community structure and dynamics at different time points, prompted us to examine the communities of the Kombucha tea thoroughly using-high throughput sequencing. Additionally, a simultaneous exploration of biochemical properties and chemical composition at different time points, to the best of our knowledge, has not been performed so far.

The analysis clearly showed that the results obtained from the D1–D2 LSU gene sequencing was capable of elucidating the yeast community at more depth compared to the ITS2 gene. Moreover, sequencing only one of the ITS or the LSU gene may have provided a bias towards either the fungi or yeast respectively. The LSU gene in tandem with the ITS gene fragment has been reported to be the best candidate for species level demarcation of yeast like organisms (Tonge et al., 2014). A similar approach has been undertaken in this exploration.

Table 6
Free radical scavenging activities of Kombucha tea during the course of its fermentation.

Sample	IC_{50} (mg/ml)			
	DPPH	ABTS	Hydroxyl	Nitric oxide
KT-0	1.53 ± 0.1^a	1.59 ± 0.1^a	1.83 ± 0.14^a	3.2 ± 0.11^a
KT-7	1.27 ± 0.08^b	1.32 ± 0.09^b	1.39 ± 0.16^b	2.3 ± 0.23^b
KT-14	0.95 ± 0.05^c	1.14 ± 0.09^c	1.1 ± 0.09^c	1.94 ± 0.08^c
KT-21	0.92 ± 0.05^c	0.98 ± 0.1^d	0.99 ± 0.1^d	1.8 ± 0.1^d
Ascorbic acid	0.07 ± 0.01^d	0.05 ± 0.01^e	0.15 ± 0.03^e	0.12 ± 0.03^e

Values are expressed as mean ± SD (n = 3). Values not sharing a common superscript letter along a column differ significantly at P < 0.05.

From this study, we can infer that the dominating yeast of this community is different from those described previously by Marsh et al. (2014). The dominance of the genus *Candida* has not been observed so far, as previous studies have shown a clear dominance of e.g. the genera *Zygosaccharomyces*, and *Saccharomyces* (Marsh et al., 2014; Teoh et al., 2004). While *Zygosaccharomyces* was not identified in our system, *Saccharomyces* was present in both compartments (i.e. biofilm and soup) in very low proportions. The biofilm harbored the *Saccharomyces* genus up to 14 days after which it fell below the threshold of 0.1% of the reads. On the other-hand the soup only maintained the genus above this threshold for up to 7 days. The other significant yeast genera identified in this system were *Lachancea* and *Kluyveromyces*. The presence of these genera has hardly been reported previously except by Marsh et al. (2014). In our particular Kombucha tea system, *Lachancea* sp. was the second most dominant yeast at almost all time points except on the seventh day soup sample where it occupied the maximum share of the community. This is the first time a Kombucha culture dominated by these yeast genera is being described. As the microbial community composition plays a vital role in the properties of Kombucha tea (Malbaša et al., 2011), such distinction in the yeast community is very interesting and needs to be studied further to understand its implication. We believe that comparative transcriptome analyses of different Kombucha systems may provide valuable information towards this direction in future.

The yeast HTS of this system along with the previous report by Marsh et al. (2014) on other Kombucha systems indicated that the overall bacterial diversity of the habitat may also be low. Instead of performing bacterial HTS for all time points, we first performed T-RFLP analysis to obtain a general overview of the bacterial community dynamics. The T-RFLP indicated a diversity peak in both compartments on the seventh day. Hence, only this time point was sequenced to obtain a definitive picture of the community. Thus the bacterial community study was done by a combination of a low resolution analysis (T-RFLP) followed by a high resolution (HTS) analysis. Such practice has previously been described by Camarinha-Silva et al. (2012) and Pilloni et al. (2012). However, as also reported by Pilloni et al. (2012), while both approaches show similar results regarding the community shifts in response to increasing fermentation time, amplicon sequencing is capable of revealing the diversity of the bacterial community at a greater depth. Hence the diversity indices from the HTS analysis were significantly higher than those obtained from T-RFLP especially in the soup sample.

The bacterial community of this Kombucha tea followed the expected trend of being dominated overwhelmingly by the Acetobacteraceae family especially in the biofilm (Jarrell et al., 2000; Jayabalan et al., 2014; Marsh et al., 2014). The soup showed a slightly higher diversity than the biofilm. However the initial diversity of the soup after 7 days of fermentation plummeted and then finally established itself similar to that of the biofilm. The major prokaryotic genus identified in this system was *Komagateibacter*. Previous reports have shown that the bacterial community in Kombucha tea was dominated by the genera *Acetobacter* and *Gluconacetobacter* (Jarrell et al., 2000; Konovalov and Semenova, 1955; Sievers et al., 1995). Traditional culture independent exploration with this Kombucha tea biofilm has indicated the presence of a number of species of *Komagataeibacter*, *Acetobacter* and *Gluconacetobacter* (Chakravorty et al., 2015). It is noteworthy that similar to our HTS-based analysis, in that report, the abundance of *Komagataeibacter* exceeded those of the other two genera. However, the genus *Komagataeibacter* has been reported to be a reclassification from *Gluconacetobacter* (Yamada et al., 2012). A very recent report has shown that the previously described V3 region of the 16S rRNA gene among the Acetobacteraceae family, instead of being variable, is actually highly conserved (Chakravorty et al., 2015). As a result the V3 region may not be able to provide enough variance to distinguish between the closely related genera of *Komagataeibacter* and *Acetobacter*. This may be the reason behind not identifying the latter genus in this Kombucha tea through HTS.

Table 7
Changes in the major chemical components of Kombucha tea during the course of its fermentation.

Parameters	Fermentation days			
	0	7	14	21
pH	5.03 ± 0.05[a]	2.28 ± 0.03[b]	1.98 ± 0.04[c]	1.88 ± 0.03[c]
Titratable acidity (M)	0.0018 ± 0.00065[a]	0.01637 ± 0.0012[b]	0.03492 ± 0.0025[c]	0.045 ± 0.002[d]
Acetic acid (g/L)	0.65 ± 0.06[a]	5.72 ± 0.21[b]	12.53 ± 0.83[c]	16.57 ± 0.9[d]
Gluconic acid (g/L)	0.33 ± 0.03[a]	2.44 ± 0.2[b]	6.38 ± 0.5[c]	7.36 ± 0.87[d]
Ethanol (g/L)	0.046 ± 0.002[a]	0.28 ± 0.014[b]	0.14 ± 0.007[c]	0.073 ± 0.003[d]
Reducing sugar (g/L)	6.8 ± 0.9[a]	8.2 ± 0.7[b]	3.5 ± 0.67[c]	2.25 ± 0.3[d]
Theaflavin (g/L)	1.76 ± 0.08[a]	1.84 ± 0.09[a]	2.84 ± 0.13[b]	3.32 ± 0.15[c]
Thearubigin (g/L)	21.5 ± 1.1[a]	19.08 ± 0.9[b]	17.6 ± 0.8[c]	11.39 ± 0.5[d]
Caffeine (g/L)	1.06 ± 0.095[a]	0.948 ± 0.09[b]	0.713 ± 0.05[c]	0.639 ± 0.03[d]
DSL (g/L)	0.48 ± 0.02[a]	0.85 ± 0.04[b]	1.32 ± 0.06[c]	2.24 ± 0.1[d]

Values are expressed as mean ± SD (n = 3). Values not sharing a common superscript letter along a row differ significantly at $P < 0.05$.

The genus *Lactobacillus* has been reported in the Kombucha system previously (Marsh et al., 2014). However the role of this genus in the fermentation is yet to be determined. The presence of other genera such as *Bifidobacterium*, *Collinsella*, *Enterobacter* and *Weissella* was probably transient in the system as indicated by the low diversity indices of the T-RFLP analysis at the rest of the fermentation time points. These bacteria were probably outcompeted subsequently with the progression of fermentation. A similar trend has also being reported previously (Marsh et al., 2014). Moreover, it has been reported that autoclaving sweetened black tea resulted in the formation of toxic chemicals that hampered the Kombucha fermentation (Teoh et al., 2004). Therefore in this study, the black tea was not autoclaved and the fermentation was done similar to the normal household practice for the beverage using normal municipality drinking water. This may have resulted in the incidence of these bacteria in the system.

Next generation sequencing (NGS) along with other culture independent methods are powerful tools to understand the in-depth community structure of any microbial niche (Caporaso et al., 2012). However, as the methods do not provide physical cultures of microbes, they often tend to overestimate the actual diversity of the niche (Dr. Luca Cocolin, personal communication). Neither can we describe the physiological properties of the identified microbes in the habitat only through a DNA based HTS analysis (Mayo et al., 2014). The culture dependent analysis on the other hand, although opening a window to understand the property of the cultured microbes, are inherently handicapped as often only a minor part of a community from complex habitats is culturable (Moyer et al., 1994). Moreover, the dominating microbes often tend to overshadow the rare ones which remain severely under-represented (Mayo et al., 2014). Another important aspect of culturing is that the cultures often show properties distinct from those exhibited in the actual consortium (Hugenholtz et al., 1998). Although both methods are important tools of understanding microbial community of a habitat, they do suffer from these inherent flaws.

In this exploration, the culture independent analysis has provided an in-depth picture of the Kombucha tea system across various fermentation time points. However, comparing the NGS data with that of the culture dependent analysis, we may infer that the most dominant microbes of the system (*Candida* and *Acetobacter*) have clearly outcompeted most the rarer organisms when we tried to culture them. The data that we obtained from this exploration will in future pave the way for combining NGS with other high throughput “omics” technologies viz. transcriptomics, metabolomics, proteomics, or glycomics and other system biology approaches (Mayo et al., 2014). Such a combination would help to determine and eventually control the physiologically active microbes at the different time points of the Kombucha fermentation.

The microbial community of Kombucha tea was studied at four time points during the fermentation. However when the chemical composition and the anti-oxidant properties were initially studied at these four time points, the 3rd day system failed to show any significant distinction from the unfermented control (data not shown). Hence the biochemical dynamics were studied from the seventh day onward. The microbial community was additionally sampled on the third day for further analysis because a distinguishable biofilm started to develop from that day onwards.

The polyphenols and flavonoids present in black tea have been reported to be responsible for its anti-oxidant capacities (Bancirova, 2010; Chan et al., 2011; Chen et al., 2008; Lee and Lee, 2002). In the present study, the concentrations of total polyphenols and flavonoids increased progressively with fermentation time, which is in good agreement with other studies (Chu and Chen, 2006; Jayabalan et al., 2008a, 2008b). This increase in total polyphenols and flavonoids content in Kombucha tea might be due to the degradation of complex tea polyphenols and flavonoids to smaller molecules by some enzymes liberated by the inhabitants of the Kombucha consortium. Microbes like *Candida tropicalis* are known to be capable of degrading various polyphenols (Ettayebi et al., 2003). As this Kombucha tea system is dominated by the genus *Candida*, it is quite possible that this yeast may have enzymatically degraded the complex polyphenols and flavonoids to smaller molecules resulting in their overall increase in content with the progression of the fermentation.

The reducing power of a beverage describes its ability to donate electrons to another compound and thus reflect its potential anti-oxidant activity (Ferreira et al., 2007). The reducing power of Kombucha tea increased significantly in the fourteenth day of fermentation and that increase continued until the twenty-first day as well. However the degree of increase from the fourteenth to the twenty-first day of fermentation was considerably lower than that between the seventh to fourteenth day. This increase in reducing power may be due to the increase in the polyphenol and flavonoid content of the fermented beverage.

The radical scavenging activity of Kombucha tea is an indication of its potential as an anti-oxidant rich beverage. The IC_{50} values clearly indicated that the fermentation results in increased ability of the beverage to scavenge free radicals. However in all four radicals the increase in scavenging ability of Kombucha tea was found to be highest on the seventh day of fermentation. The microbial community was also observed to be most diverse on the seventh day of fermentation. This might be an indication that the increase in diversity of the microbial community played an important role in the increased anti-oxidant property of Kombucha tea. Moreover, the shift in dominance from *C. stellimalicola* to *L. fermentati* in the soup on the seventh day might also be responsible for the increased anti-oxidant activity. Further experiments are required to confirm these hypotheses.

In this study, we measured the anti-glycation property of Kombucha tea for the first time and observed an increase in the property with progression of the fermentation. Thus the results indicate for the first time that the microbial community of Kombucha tea might play an important role in augmenting the anti-diabetic property of Kombucha tea. Moreover, the presence of AGEs with diabetic complications is well

established (Ahmed, 2005). The recent claimed anti-diabetic properties of Kombucha tea (Aloulou et al., 2012; Bhattacharya et al., 2013) may as well be attributed to the anti-glycation activity of the beverage.

The microbial growth is directly related to the pH and titratable acidity of the medium in any fermentation (Hur et al., 2014). We observed that although the pH of the medium plateaued after 7 days of fermentation, the titratable acidity kept increasing till the 21st day. This might be due to the formation of carbon-di-oxide during fermentation which may result in some buffering effects (Kallel et al., 2012). The low pH and high titratable acidity allows the growth of only those microbes that are capable of sustaining such niche and thus may provide some protection against invasive contaminants (Greenwalt et al., 2000).

The initial increase in reducing sugar content may be attributed to the hydrolysis of sucrose to glucose and fructose by yeast. With the progression of the fermentation, the yeast utilizes the sugar anaerobically to produce ethanol (Jayabalan et al., 2010). The acetic acid bacteria are believed to utilize the sugar and ethanol to produce gluconic acid and acetic acid respectively (Dufresne and Farnworth, 2000). Other acids that have been previously reported were glucuronic acid and lactic acid (Jayabalan et al., 2007). However in this system neither of these two acids has been detected. This difference in composition of this particular Kombucha tea variety may have been due to the overall distinction in the microbial community and/or the varying lengths of fermentations for this Kombucha brew.

The main polyphenols found in black tea are theaflavin and thearubigin (Chan et al., 2011). As black tea is one of the chief ingredients of Kombucha tea, the amounts of these two polyphenols were studied in the course of the fermentation. Our results are in agreement with the pattern described by Kallel et al. (2012). The data indicated that a part of thearubigin may have been converted to theaflavin during Kombucha fermentation and this conversion might have brought about the change in color of Kombucha tea from reddish brown to light brown with the progression of fermentation.

Earlier studies have reported that the caffeine content remains more or less constant across the fermentation in black tea Kombucha (Kallel et al., 2012). However, in this study we observed a decrease in caffeine content by 40% after 21 days of fermentation. It remains to be proven if this increased caffeine degrading ability may in fact be attributed to the increased diversity of the microbial community on the 7th day of fermentation. Thus this particular property of our Kombucha microbial community needs to be studied in-depth in future as it may act as a potential source of novel cost effective biological caffeine degraders.

Fermentation also resulted in the formation of some molecules like DSL in Kombucha tea which was not found in the unfermented control. The presence of DSL in Kombucha tea has been reported by other researchers also (Wang et al., 2010; Yang et al., 2009). The compound is believed to be key ingredient behind the hypocholesterolemic effects (Yang et al., 2009) and also the hepatoprotective property of Kombucha tea against acetaminophen induced hepatotoxicity (Wang et al., 2014). In our exploration we found that the content of DSL in this particular Kombucha tea was significantly higher than that reported previously (Wang et al., 2010). Such variation may thus be attributed to the difference in microbial community of this Kombucha brew. Thus as DSL is believed to be one of the key ingredients behind the beneficial properties of the beverage, such a finding is a significant step forward in Kombucha tea research.

We thus concluded that the beneficial properties of black tea were augmented by fermentation with the Kombucha consortium. The results of the present study showed that the microbial community was most diverse on the seventh day of fermentation and the bioactivity of Kombucha tea also gradually increased after 7 days of fermentation which continued up to 21 days. We inferred that the microbial community plays a major role in this fermentation and may be directly related to the enhanced activity of this fermented tea over unfermented one. We envisage that this study will pave the way to correlate in the future the microbial community to the various beneficial properties of Kombucha tea.

Conflict of interest

The authors declare no conflict of interest currently.

Acknowledgments

The authors are thankful to Indian Council of Medical Research, Govt. of India for the financial support (Index No: 80/713/2011-ECD-I). We also thank: Verna Jaschik (UFZ) for technical assistance during the T-RFLP analysis; Dr. Paltu Kumar Dhal (Jadavpur University), Dr. Joydeep Mitra (GCC Biotech Pvt. Ltd.) and Dr. Arnab Pramanik (University of Calcutta) for their assistance with next generation sequencing; Dr. Luca Cocolin for providing valuable insight on data integration; Shyamasree Basu Majumdar and Aritri Majumdar for their help in language editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2015.12.015.

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