Sequence-based analysis of the bacterial and fungal compositions of multiple kombucha (tea fungus) samples

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ABSTRACT

Kombucha is a sweetened tea beverage that, as a consequence of fermentation, contains ethanol, carbon dioxide, a high concentration of acid (gluconic, acetic and lactic) as well as a number of other metabolites and is thought to contain a number of health-promoting components. The sucrose–tea solution is fermented by a symbiosis of bacteria and yeast embedded within a cellulosic pellicle, which forms a floating mat in the tea, and generates a new layer with each successful fermentation. The specific identity of the microbial populations present has been the focus of attention but, to date, the majority of studies have relied on culture-based analyses. To gain a more comprehensive insight into the kombucha microbiota we have carried out the first culture-independent, high-throughput sequencing analysis of the bacterial and fungal populations of 5 distinct pellicles as well as the resultant fermented kombucha at two time points. Following the analysis it was established that the major bacterial genus present was *Gluconacetobacter*, present at >85% in most samples, with only trace populations of *Acetobacter* detected (<2%). A prominent *Lactobacillus* population was also identified (up to 30%), with a number of sub-dominant genera, not previously associated with kombucha, also being revealed. The yeast populations were found to be dominated by *Zygosaccharomyces* at >95% in the fermented beverage, with a greater fungal diversity present in the cellulosic pellicle, including numerous species not identified in kombucha previously. Ultimately, this study represents the most accurate description of the microbiology of kombucha to date.

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1. Introduction

Kombucha is a sweetened, black tea beverage, which is fermented to contain ethanol and carbon dioxide. Traditionally fermented for 8–10 days, kombucha has a sour taste, resembling that of sparkling apple cider, which develops over prolonged fermentation into a mild vinegar flavour (Blanc, 1996; Reiss, 1994). The tea is fermented by the presence of a cellulosic pellicle or mat that rests above the broth forming a fresh layer with each successful fermentation. The fermentation itself is performed by a symbiosis of bacteria and yeast embedded within the cellulosic matrix. Kombucha, known by several names, including tea fungus and Haipao (Liu et al., 1996), has been brewed in China for over 2000 years, where it was fermented by many households. Although probiotic effects have yet to be directly associated with kombucha-derived microorganisms (Kozyrovska et al., 2012), initial studies have shown promising health benefits in relation to the tea itself. Such health benefits include anti-carcinogenic (Jayabalan et al., 2011) and anti-diabetic (Aloulou et al., 2012; Hiremath et al., 2002) effects, treatment for gastric ulcers (Banerjee et al., 2010) and high cholesterol (Yang et al., 2009), and it also has been shown to impact immune response (Ram et al., 2000) and liver detoxification (Loncar et al., 2000).

The majority of microbiology-orientated studies of kombucha to date have been culture-based. These are limited in that certain species can be difficult to isolate and the exclusive reliance on phenotypic traits can lead to misidentification (Raspor and Goranovic, 2008). Additionally, culture-based studies tend to be low-throughput and thus only a certain proportion of isolates will ever be investigated. Traditionally, only a few genera of bacteria have been isolated from kombucha, most frequently *Acetobacter* (Chen and Liu, 2000; Dutta and Gachhui, 2006; El-Salam, 2012; Hesseltine, 1965; Liu et al., 1996; Sievers et al., 1996; Zhang et al., 2011), but species of *Gluconacetobacter* and *Lactobacillus* have also been identified (Trovatti et al., 2011; Wu et al., 2004; Yamada et al.,

1997; Yang et al., 2010; Zhang et al., 2011). The most important of these species are those which produce bacterial cellulose, such as *Komagataeibacter xylinus*, which was recently reclassified from *Gluconacetobacter xylinus* (Yamada et al., 2012) (also previously known as *Acetobacter xylinum* (Yamada et al., 1997)) and is considered the best studied and most efficient bacterial cellulose producer in kombucha (Mikkelsen et al., 2009; Strap et al., 2011). The yeast population is generally believed to be more varied in composition (Mayser et al., 1995; Teoh et al., 2004). Yeasts identified include species of the genera *Zygosaccharomyces*, *Candida*, *Kloeckera/Hanseniaspora*, *Torulaspora*, *Pichia*, *Brettanomyces/Dekkera*, *Saccharomyces* and *Saccharomycoides* (Chen and Liu, 2000; Hesseltine, 1965; Jankovic and Stojanovic, 1994; Liu et al., 1996; Markov et al., 2001; Mayser et al., 1995; Teoh et al., 2004). The role of yeasts in the fermentation of kombucha is to convert sucrose to organic acids, carbon dioxide and ethanol, with the latter then used by acetic acid bacteria to form acetaldehyde and acetic acid. Acetic acid bacteria also use yeast-derived glucose to synthesise bacterial cellulose and gluconic acid (Dufresne and Farnworth, 2000; Greenwalt et al., 1998). Bacterial and yeast numbers are generally thought to reach 10^4–10^6 cfu ml^{-1} in the kombucha after approximately 10 days of fermentation, with yeast slightly outnumbering bacteria (Chen and Liu, 2000; Goh et al., 2012; Teoh et al., 2004). Microbial counts have been reported to be greater in the tea broth than the cellulosic pellicle (Goh et al., 2012). It has also been found that the viability of the microbes present decreases gradually over the course of the fermentation due to oxygen starvation and extremely acidic (pH 2.5) conditions (Chen and Liu, 2000). While first generation (Sanger) sequencing of 16S rRNA genes has been used to identify kombucha bacterial isolates (Dutta and Gachhui, 2006; El-Salam, 2012; Trovatti et al., 2011), the significant developments in the field of microbial ecology that have been possible through the use of high-throughput, culture-independent techniques (Ercolini, 2013) have, to date, yet to be applied to kombucha populations.

In this study, high-throughput amplicon sequencing was performed on DNA extracts from cellulosic pellicles sourced from 5 distinct geographic locations and from the corresponding kombuchas at two time points during fermentation. This analysis provides the most in-depth analysis of the kombucha microflora to date.

2. Materials and methods

2.1. Culture maintenance

Five kombucha cellulose pellicles with approximately 200 ml starter culture were acquired from suppliers from different geographic locations. Two kombucha samples were sourced from Canada and designated Ca1 and Ca2; other kombucha samples were sourced from the United Kingdom (UK), United States (US) and Ireland (Ire). All kombuchas were cultivated under uniform conditions. Two litres of tap water was sterilised at 121 °C for 15 min in a 3 L glass container. The water was brought to the boil and 0.49% w/v black tea (Barry's Original Blend) was added, and allowed to steep for 15 min.. After removal of the tea leaves, 10% sucrose was added and stirred to dissolve. Once the sucrose–tea solution had cooled to room temperature, 10% fermented tea (of the previous fermentation brew from kombucha with the same origin, corresponding to the aforementioned starter culture) was added to acidify the solution. The cellulose pellicle was placed in the culture, light side up. The container was covered with a 100% cotton towel and fixed with an elastic band. Cultures were fermented at room temperature (23 °C) and re-inoculated into fresh tea every 10 days. Samples were taken at days 3 and 10 of fermentation for DNA extraction.

2.2. Metagenomic DNA extraction

To extract DNA from the fermented kombucha at day 3 and 10 of fermentation, 1.8 ml of fermented tea was centrifuged to generate a pellet which was suspended in 450 μl of lysis buffer P1 from the Powerfood Microbial DNA Isolation kit (MoBio Laboratories Inc, USA). The resuspended pellet was subjected to enzymatic digestion with enzymes mutanolysin (100 U/ml) and lysozyme (50 μg/ml) at 37 °C for 1 h, followed by proteinase K (250 μg/ml) digestion at 55 °C for 1 h. Extraction was optimised with a 10 min 70 °C incubation (Quigley et al., 2012) prior to mechanical lysis using the Qiagen TissueLyser II (Retsch®). The Powerfood Microbial DNA Isolation kit was then used as per the manufacturer's instructions. Pure DNA was eluted in HPLC-grade sterile water. For extraction of DNA from the pellicle, 0.25 g of cellulosic pellicle was removed from the surface mat of a fresh fermentation, washed twice in sterile H$_2$O, and chopped into small fragments using a sterile blade. 0.3 g of sterile glass beads and 750 μl of cellulase (Sigma–Aldrich) were added to a microcentrifuge tube containing the pellicle, which was mechanically lysed for 10 min in a Qiagen TissueLyser II (Retsch®). The solution was incubated for 1 h at 40 °C, after which it was centrifuged to generate a pellet. The supernatant was discarded and the pellet was resuspended in 450 μl of pre-warmed buffer P1. The extraction was then subjected to enzyme digestion and the modified Powerfood extraction was performed as described above.

2.3. DNA amplification and high-throughput sequencing

Metagenomic DNA extracts were used as a template for PCR amplification. PCR amplification of the V4–V5 variable region (408 bp) of the 16S rRNA gene was performed using the universal primers F1 (5′-AYTGGGYDTAAAGNG) and R5 reverse (5′-CCGTCAATTYYTTTRAGTTT) to facilitate an investigation of the bacterial component of the microbial populations (Claesson et al., 2010). Unique multiplex identifier adaptors were attached between the 454 adaptor sequences and the forward primers to facilitate the pooling and subsequent differentiation of samples. Tagged universal primers were also used to amplify fungal DNA from the variable internal transcribed spacer (ITS)-1 rDNA region (Buee et al., 2009). In this instance the forward primer ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA) and ITS2 reverse (5′-GCTGCGTTCTTCATCGATGC) generated two different sets of PCR products of *circa* 410 bp and 250 bp. To prevent preferential sequencing of the smaller amplicons, the bands were extracted separately using the Roche High Pure PCR Cleanup Micro Kit, and two pools were created and sequenced separately. The PCR conditions used for 16S amplification were 94 °C denaturation for 2 min, 35 cycles of 94 °C for 1 min (denaturation), 52 °C for 1 min (annealing) and 72 °C for 1 min (extension) followed by a final 72 °C for 2 min. The PCR conditions used for ITS amplification were 94 °C denaturation for 4 min, 35 cycles of 94 °C for 30 s (denaturation), 50 °C for 1 min (annealing), and 72 °C for 1 min and 30 s (extension). A final annealing step of 72 °C for 10 min was performed. All DNA was subject to a 10 min hotstart at 94 °C prior to PCR amplification. Amplicons generated from three PCR reactions/template DNA were pooled and cleaned using the Agencourt AMPure® purification system (Beckman Coulter Genomics, Takeley, UK). Purified products were quantified using the Nanodrop 3300 Fluorospectrometer (Thermo Scientific) and the Quant-iT™ Picogreen® dsDNA Assay kit (Invitrogen). Equal concentrations of 16S or ITS amplicons were pooled, AMPure cleaned and assessed by an Agilent 2100 Bioanalyser (Agilent Technologies) to determine purity and to ensure the absence of primer dimers. Sequencing of the 16S rRNA V4–V5 and ITS-1 rDNA ribosomal amplicons was performed using a 454 Genome Sequencer FLX Titanium System

(Roche Diagnostics Ltd) at Teagasc Food Research Centre, Moorepark, according to 454 protocols.

2.4. Amplicon sequencing data analysis

Raw sequences were quality trimmed and filtered using the Qiime Suite of programs (Caporaso et al., 2010); any reads not meeting the quality criteria of a minimum quality score of 25 and sequence length shorter than 150 bps for 16S amplicon reads and 200 bps for ITS amplicon reads were discarded. The maximum homopolymer limit was increased to 10 for ITS amplicons as ITS sequences are known to harbour long homopolymer runs. Chimeras were removed by ChimeraSlayer (http://microbiomeutil.sourceforge.net/#A_CS). Trimmed fasta sequences were assessed by BLAST analysis against the SILVA database (version 100) for 16S reads and a previously published ITS-specific database for ITS samples (Pruesse et al., 2007; Santamaria et al., 2012). MEGAN was used to parse BLAST outputs (Huson et al., 2007) and a bit-score of 86 was employed for 16S ribosomal sequence data, with a bit-score of 35 for ITS sequence data. The QIIME suite of programs was used to calculate alpha diversity including Choa1 richness, Shannon diversity, Simpson index, Phylogenetic Diversity and Observed species (Caporaso et al., 2010). Sequences were clustered into operational taxonomical units, chimera checked (0.11% of 16S and 0.02% of ITS sequences removed) and aligned using the default pipeline within Qiime for 16S sequences and a modification to implement MUSCLE (Edgar, 2004) for the alignment of ITS sequences. The FastTree package (Price et al., 2009) was used to generate phylogenetic trees for both sequence types. For 16S sequences, default clustering and alignment methods were implemented within Qiime, whilst the default pipeline was modified for the ITS sequences. Sequencing depth was estimated using rarefaction analysis. QIIME was also used to generate weighted UniFrac, unweighted UniFrac and Bray—Curtis distances matrices. Principal Co-ordinate Analysis plots based on these distance matrices were generated with Qiime and visualised using King software (Chen et al., 2009). Reads were deposited in the SRA database under the accession number ERP002661.

3. Results and discussion

3.1. High-throughput sequencing reveals the α and β diversity of the microbial population in kombucha

In advance of carrying out this study, a number of steps were taken to ensure that the results generated would be as representative as possible. Five kombuchas were acquired from individual and commercial suppliers from Canada ([Ca1] and [Ca2]), Ireland [Ire], the United Kingdom [UK] and the United States [US], in order to mitigate against geographical bias. Although a sample number of five kombuchas will not provide a definitive microbial analysis, this is the first time high-throughput, culture-independent analysis has been applied to the microbial population of kombucha and, as such, represents the most in-depth study to date. As it has been proposed that the microbiological composition of the fermented product can vary with time (Sreeramulu et al., 2000; Teoh et al., 2004), DNA was extracted from the fermented kombucha tea (fermentate) at days 3 and 10 of the fermentation and from the pellicle. Given that the pH of the fermented product dropped as the process continued, reaching pH 3—3.5 by day 10, the microorganisms detected at this time point reflect a more acid tolerant population. It was also decided that the sucrose-tea solution should not be sterilised by autoclaving in advance of inoculation as this has in the past been shown to generate toxic chemicals inhibitive of kombucha growth (Teoh et al., 2004). Instead, culturing conditions adhered to were

those closest to that which occur in the home, with black tea and sucrose considered the best substrates for metabolic and microbial activity (Kallel et al., 2012).

Using universal tagged primers, amplicons corresponding to the V4—V5 region of the bacterial 16S rRNA gene were generated. This region was chosen as it provides longer reads which enable more confident assignments than shorter products generated by amplification of other variable regions. Following high-throughput sequencing of the amplicons, a total of 15,209, 13,409 and 13,400 reads were successfully assigned to the pellicle, day 3 and day 10 extracts, respectively, equating to a respective average of 3042, 2682 and 2680 reads per individual sample (Table S1). To identify fungal populations present, ITS rDNA amplicons were generated from the variable ITS-1 region using primers adapted from Buee et al. (2009). These primers produced amplicons of circa 410 bps in all samples. Additional amplicons of circa 250 bps from UK (the pellicle, day 3 and day 10) were also sequenced. In order to prevent preferential sequencing of the smaller-sized reads, the respective amplicons were extracted individually via gel extraction and separated into two pools according to approximate size, and sequenced independently, with the quality-approved reads subsequently combined to form an overall total. Due to the approach taken, comparisons between ITS amplicons of specific sizes, rather than of all amplicons, was carried out. It was found that reads from the 250 bp pool were assigned specifically to the genus Dekkera whereas amplicons from other fungi were approx. 400 bp in length. In total, 11,795 reads were obtained for the pellicle, corresponding to an average of 2949 reads per individual sample. Total reads for day 3 and day 10 were 18,592 and 37,868, respectively (Table S1).

To determine species richness and diversity, Chao1 values, Shannon and Simpson indices, Phylogenetic Diversity and Observed Species were calculated (Tables S2—S3). The values for each were reflective of the naturally low bacterial and fungal diversity in fermented beverages (Marsh et al., 2013) relative to other environmental samples, where bacterial communities can consist of >1000 and >3000 OTUs in the human gastrointestinal tract and soil, respectively (Nacke et al., 2011; Stearns et al., 2011), with fungal populations containing >850 OTUs in the soil (Buee et al., 2009). Rarefaction curves, calculated at 97% similarity, are approaching parallel to the x-axis for all samples, indicating sufficient reads were obtained to adequately assess the populations (Fig. S1). Averages for Observed Species and Phylogenetic Diversity suggest that bacterial diversity was greatest at day 3 of the fermentation. Across all measures of alpha diversity, except Chao1, diversity was shown to be greater within the fungal component of the kombucha population than the bacteria.

Beta diversity was measured using three different distance matrices i.e. weighted UniFrac, unweighted UniFrac and Bray—Curtis (weighted UniFrac and Bray—Curtis data not shown). Visualisation of the populations, reflecting the different samples and time points, was performed by generating Principal Co-ordinate Analysis (PCoA) plots (Fig. 1). From this analysis it was shown that the bacterial populations of the pellicles were distinct from one another, as evidenced by a lack of clustering. However, there was some clustering amongst day 3 samples, and by day 10, the bacteria had converged on a more comparable population, denoted by clustering in blue. Thus the pellicle populations were shown to possess the greatest diversity, with only the most competitively fit populations dominating within the fermented medium.

3.2. 16S rRNA sequence analysis reveals a dominant Gluconacetobacter, with a prominent LAB, presence in kombucha

Five bacterial phyla were identified across the various samples, including Actinobacteria, Bacteroidetes, Deinococcus-Thermus,
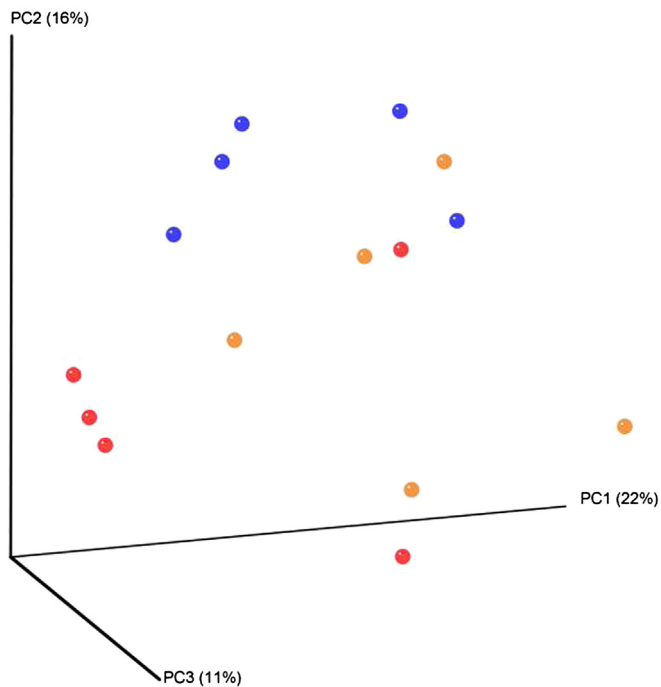
Fig. 1. Principle Co-ordinate Analysis (PCoA) plots, based on unweighted UniFrac distance matrices, show clustering of the bacterial populations from several kombuchas, where Red = day 3 extracts, Blue = day 10 extracts and Orange = pellicle extracts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Firmicutes and Proteobacteria. Of these, Proteobacteria were the most abundant, accounting for >90% of reads in each sample, with the exception of Irish-sourced kombucha, where this phylum constituted 87.9% of the kombucha population at day 3 of the fermentation and 60% of the pellicle (Table S4). Members of this phylum consisted of 2 genera, *Acetobacter* and *Gluconacetobacter*. In every kombucha, and in every sample type, reads assigned to *Gluconacetobacter* greatly exceeded those of *Acetobacter* (Fig. 2; Table 1). Indeed, *Gluconacetobacter* accounted for >85% of assigned reads from all samples, with the exception of Ire, where it is present

at a low of 58% in the pellicle, making it the dominant bacterial genus in every sample. The genus *Acetobacter* was consistently found in Irish-sourced kombucha only, where proportions were highest in the pellicle (1.9%) but represented <1% of the bacterial population in the fermentates. Although 16S reads of this length cannot be accurately assigned beyond genus level due to the high level of sequence homology, the reclassification of *A. xylinum* to *G. xylinus* (Yamada et al., 1997), a species considered to be the dominating bacterium and principal contributor of bacterial cellulose in kombucha (Hesseltine, 1965; Mikkelsen et al., 2009; Sievers et al., 1996; Strap et al., 2011), might partially account for the high frequency of the genus *Gluconacetobacter*, and the relative lack of *Acetobacter*. Moreover, the genus *Gluconacetobacter* has recently been subdivided into three genera: *Nguyenibacter*, *Komagataeibacter* and *Gluconacetobacter* (Vu et al., 2013; Yamada et al., 2012). Most noteworthy is the fact that *G. xylinus* is now the type strain of the genus *Komagataeibacter*, and is known as *K. xylinus*. It is possible that reads assigned to *Gluconacetobacter* may actually belong to the genus *Komagataeibacter* as, at the time of writing, microbial databases have yet to be updated to accommodate the reclassification. In addition, while there are risks associated with assigning short 16S amplicons at the species level, it was noted here that the most prominent *Gluconacetobacter* hits were to an uncultured species and *Gluconacetobacter* sp. Rh1-MS-CO.

More recently, there have also been several other species of *Komagataeibacter* and *Gluconacetobacter* identified in kombucha including *Komagataeibacter kombuchae* (Dutta and Gachhui, 2007), *Gluconacetobacter* sp A4 (Yang et al., 2010) and *Gluconacetobacter sacchari* (Trovatti et al., 2011). The high proportions of *Gluconacetobacter* also indicate that other species of *Acetobacter*, such as *A. aceti* (El-Salam, 2012; Liu et al., 1996), its close relative *A. nitrogenificans* (Dutta and Gachhui, 2006), *A. pasteurianus* (Chen and Liu, 2000; Liu et al., 1996) and *A. liquefaciens* (Zhang et al., 2011) that have been found in the past to be a dominant component in kombucha are not as common in the kombuchas prepared in this study or were inaccurately classified in the past (Sreeramulu et al., 2000). Similar acetic acid species have been found in related wine and vinegar cultures (De Vero et al., 2006; Gonzalez et al., 2004).

The detection of considerable proportions of lactic acid bacteria (LAB), such as *Lactobacillus* and *Lactococcus*, in kombucha pellicles is another key observation. Reads representative of the corresponding phylum, the Firmicutes, were consistently detected in the
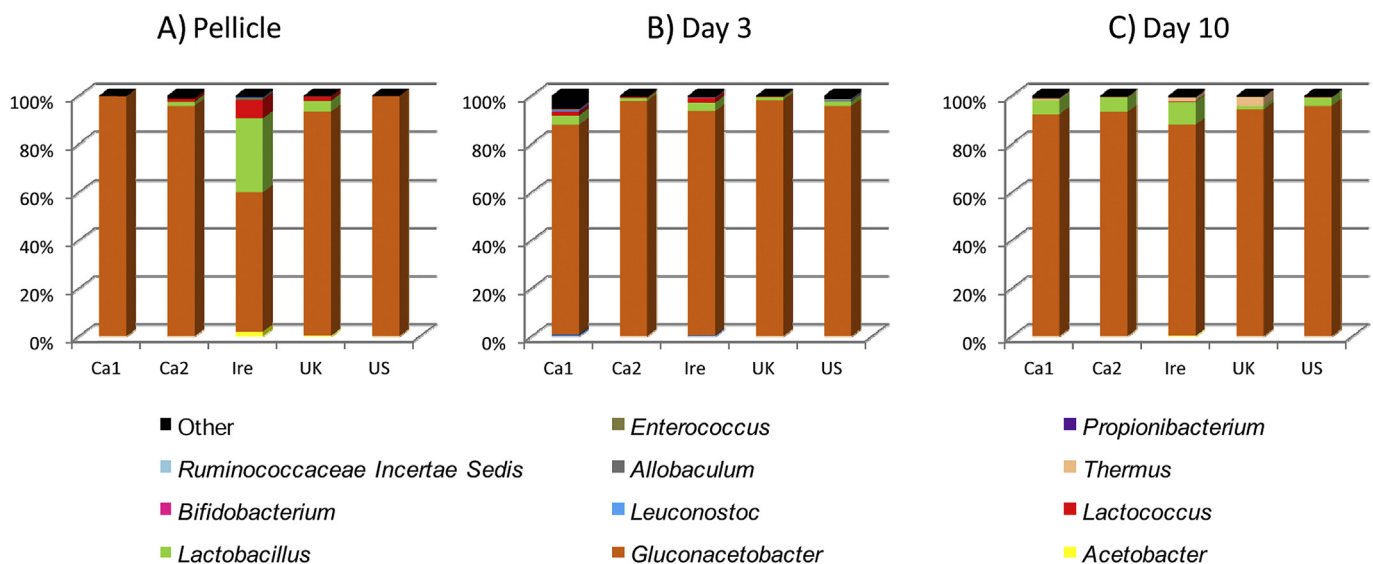

Fig. 2. 16S phylogenetic composition of the bacterial component of the kombucha pellicle (A) and the tea at day 3 (B) and day 10 (C) of fermentation, at genus level.

Table 1
Relative abundances of the 16S bacterial genera at day 3, day 10 and in the pellicle.

	Ca1	Ca2	Ire	UK	US
Day 3					
Acetobacter	0.86	0	0.43	0	0
Gluconacetobacter	86.91	97.79	93.09	98.14	95.73
Lactobacillus	3.93	1.13	3.57	1.19	1.77
Lactococcus	1.56	0.51	1.77	0.37	0
Leuconostoc	0.52	0	0	0	0
Bifidobacterium	0.3	0	0	0	0
Thermus	0.22	0	0.2	0	0
Allobaculum	0	0	0	0	0.88
Ruminococcaceae Incertae Sedis	0	0	0	0	0.19
Propionibacterium	0	0	0.2	0	0
Other	5.75	0.58	0.75	0.3	1.42
Day 10					
Acetobacter	0	0	0.19	0	0
Gluconacetobacter	92.17	93.16	87.62	94.26	95.73
Lactobacillus	5.96	6.17	9.59	1.44	3.47
Lactococcus	0	0.23	0.19	0	0.18
Leuconostoc	0	0	0	0	0
Bifidobacterium	0	0	0	0	0
Thermus	0.66	0	1.62	3.73	0
Allobaculum	0	0	0	0	0
Ruminococcaceae Incertae Sedis	0	0	0	0	0
Other	1.22	0.45	0.79	0.58	0.61
Pellicle					
Acetobacter	0	0	1.93	0.28	0
Gluconacetobacter	99.77	95.73	58.02	92.97	99.82
Lactobacillus	0	1.72	30.57	4.64	0
Lactococcus	0	1.29	7.76	1.62	0
Leuconostoc	0	0	0	0	0
Bifidobacterium	0	0	0	0	0
Thermus	0	0	0	0	0
Allobaculum	0	0	0	0	0
Ruminococcaceae Incertae Sedis	0	0	0	0	0
Enterococcus	0	0	0.69	0.21	0
Propionibacterium	0	0	0.38	0	0
Other	0.23	1.26	0.66	0.28	0.18

fermentates, increasing in each instance from day 3 to 10, and were also detected in the pellicles of Ca2, Ire and UK at abundances of 3.3%, 39.4% and 6.6%, respectively. Of the fermented teas, the Irish kombucha contained the greatest proportions of Firmicutes at 5.6% and 10.5% in the day 3 and day 10 extracts, respectively. The Firmicutes-associated reads were predominantly assigned to the genus *Lactobacillus*, which was detected in all fermentates, but only in the pellicles of Ca2, Ire and UK. Proportions of lactobacilli were lowest in the day 3 extracts, with proportions being higher at day 10 in all instances. Lactobacilli have been isolated in only two previous kombucha studies, both of which focused on Chinese kombuchas (Wu et al., 2004; Zhang et al., 2011), and thus the frequency and abundance at which lactic acid bacteria were detected in this study is particularly novel. The Irish-sourced pellicle stood out in this regard with >35% of reads assigned at genus level corresponding to lactic acid bacteria. The data presented here suggests that lactobacilli are more prevalent in kombucha than was previously understood, particularly at the latter stages of fermentation. Studies have indicated that one role for these microorganisms might be to assist in the growth of acetic acid species in that *Lactobacillus* has been shown to greatly increase cellulose production by *Gluconacetobacter* in co-culture (Seto et al., 2006) and, even more relevantly, LAB have been shown to support the growth of *Gluconacetobacter* in kombucha (Yang et al., 2010). The abundance of *Lactococcus* varied but was generally lower, consistently representing <2% of reads across all fermentates and pellicles with the exception of the Irish pellicle (7.8%). Proportions were greatest in the three pellicles in which it was detected, and numbers reduced from <2% in four of the five day 3 fermentates to <1% in three day 10 fermentates, suggesting poor lactococcal survival as the

fermentation proceeded. Following a random inspection of approximately 100 hits across the *Lactobacillus*-assigned reads, it was revealed that the most common corresponded to a community that were 99% identical to *Lactobacillus kefiranofaciens* subsp. *kefirgranum*. A number of other genera from the Firmicutes were detected at low proportions, which have not been identified in kombucha previously, including *Leuconostoc* (Ca1, day 3 – 0.5%), *Allobaculum* (US, day 3 – 0.9%), *Ruminococcaceae Incertae Sedis* (US, day 3 – 0.3%) and *Enterococcus* (Ire and UK, pellicle – 0.7% and 0.2% respectively) (Table 1). The number of different genera detected was greatest among the day 3 samples.

Actinobacteria were less common amongst the kombuchas, with reads corresponding to the genera *Propionibacterium* (Ire, day 3 – 0.2%; pellicle – 0.4%) and *Bifidobacterium* (day 3 – 0.3%). To our knowledge this marks the first time that *Bifidobacterium* has been identified in natural kombucha, though it should be noted that there are instances of it having been added to commercially brewed kefir. The low abundance of both bifidobacteria and propionibacteria, and their absence from day 10 samples, suggests that these bacteria do not thrive in the fermented environment. It should be noted that the presence of these traditionally gut-associated organisms may reflect their introduction through poor sanitation or some other means at some point during the formation of specific pellicles and thus there is no guarantee that these populations are active in this environment. However, in the case of the Actinobacteria, it's worth mentioning that these microorganisms have been detected at similar abundances in fermented milk (Dobson et al., 2011), and bifidobacteria can constitute a significant proportion of the microbiota of fermented water beverages (Gulitz et al., 2013). Further investigations will be required to ultimately determine the source of individual populations and their contribution to the formation and health-promoting characteristics of kombucha. This point also relates to other populations detected at low frequencies. Reads corresponding to the phylum Deinococcus–Thermus were only consistently detected across Ca1 samples (0.2%, 0.9% and 0.2% at day 3, day 10 and pellicle, respectively), but were also present in the fermented Irish (day 3 – 0.2%; day 10 1.6%) and UK (day 10 – 3.8%) teas. Reads from this phylum were assigned to the genus *Thermus*. The presence of bacteria corresponding to this genus is interesting, since these species are traditionally regarded as being associated with thermophilic niches (Beffa et al., 1996; Vajna et al., 2012). The specific requirements for culturing such bacteria, in this case, high temperature, might explain why these and other such species have not been detected through culture-based approaches previously. Reads corresponding to the phylum Bacteroidetes were found in the US sample only at day 3 of the fermentation at 0.4% abundance. Reads that could not be assigned at the phylum level consistently represented <1% across all samples, and reads categorised as "other" in Fig. 2 and Table 1 refer to those that could not be assigned to the genus level. Finally, it was notable that a number of 16S reads corresponding to the order Sphingomonadales and the family *Lachnospiraceae*, respectively, could not be further assigned. Again, this marks the first occasion upon which these microorganisms have been identified in kombucha, though *Sphingobium* and *Zymomonas*, genera of Sphingomonadales, have been identified in beverages fermented by milk and water kefir grains respectively (Hsieh et al., 2012; Ninane et al., 2007).

3.3. The fungal population in kombucha is dominated by Zygosaccharomyces with a variable sub-dominant population

From a fungal perspective, the use of culture-independent approaches was particularly beneficial as the difficulties associated with slow growth on yeast-specific media makes culture-based

assessments difficult (21). The only fungal phylum detected in the fermented tea was Ascomycota, while Basidiomycota, the other phylum of the sub-kingdom Dikarya, was also detected in the cellulose pellicle extracts of Ca1, Ca2, and UK at sub-dominant levels.

Zygosaccharomyces was the dominant genus in all pellicle and broth extracts from Irish and Canadian-sourced kefirs (Fig. 3; Table 2), accounting for >95% of each population with the exception of the pellicle of Ca1, which contained a lower abundance (79%) as a consequence of containing a higher proportion of *Pichia* (8.3%) and unassigned (10.9%) reads. Genera proportions of the fermented teas matched that of the corresponding pellicle, thereby suggesting that the fungal composition of the cellulosic pellicle used to inoculate the tea is the key determinant.

The occurrence of amplicons of *circa* 250 bp in the pellicle, day 3 and day 10 extracts of UK-sourced kombucha necessitated sequencing of these smaller-sized reads separately from the regular 400 bp pool. Most reads from this pool were assigned to the genus *Dekkera* (Table S5). Mayser et al. (1995) have previously found that *Brettanomyces* (the anamorph of *Dekkera*) and *Zygosaccahromyces* were the most common yeast genera in a culture-based study of German kombucha samples. There have also been some other instances upon which both *Zygosaccharomyces* and *Dekkera* have been identified (Liu et al., 1996; Teoh et al., 2004).

Analysis of ITS sequence data also facilitated assignment at the species level. The *Zygosaccharomyces* population consisted of two species, *Zygosaccharomyces lentus* and *Zygosaccharomyces bisporus*, with *Z. lentus* present in consistently higher abundance, and in the case of Irish-sourced kombucha, was the only species detected. It has been proposed that *Z. lentus* isolates have probably been phenotypically misidentified as *Zygosaccharomyces bailii* in the past (Steels et al., 1999a), which may account for the reported presence of *Z. bailii* in some culture-based studies (Chen and Liu, 2000; Liu et al., 1996; Teoh et al., 2004). The dominant *Dekkera* species in UK samples was *Dekkera bruxellensis*, which out-numbered the *Dekkera anomala*, present in the pellicle. The identification of this particular species is consistent with some previous studies (Liu et al., 1996; Teoh et al., 2004). *D. bruxellensis*, often considered a spoilage yeast, is commonly found in fermented beverages such as fruit juices, red wine, beer and cider where it is responsible for the development of unpleasant odour and taste (Heresztyn, 1986a, b). Conversely, it is thought to have a beneficial contribution to the

flavour of lambic beer (DeKeersmaecker, 1996). Similarly, *Zygosaccharomyces* sp. are problematic in the brewing and juice industries with *Z. lentus* in particular being considered a food spoilage yeast due to its osmotolerance, ability to grow at low pH, and resistance to preservatives (Steels et al., 1999b). *Kazachstania* was the only other genus detected among the tea broths, being present in UK day 10, and is solely represented by the genus *Kazachstania unispora* which has not been linked with kombucha previously. Considering the variation in kombucha-associated yeast, it is likely that the role of yeast in kombucha fermentation can be performed by several, non-specific fermentative yeast, with *Zygosaccharomyces*-dominated populations being particularly common. Investigation of fermentations produced by defined starters is required to elucidate the exact contribution of each yeast to the final flavour and biochemical composition of kombucha.

Table 2
Relative abundances of the ITS fungal genera at day 3, day 10 and in the pellicle.

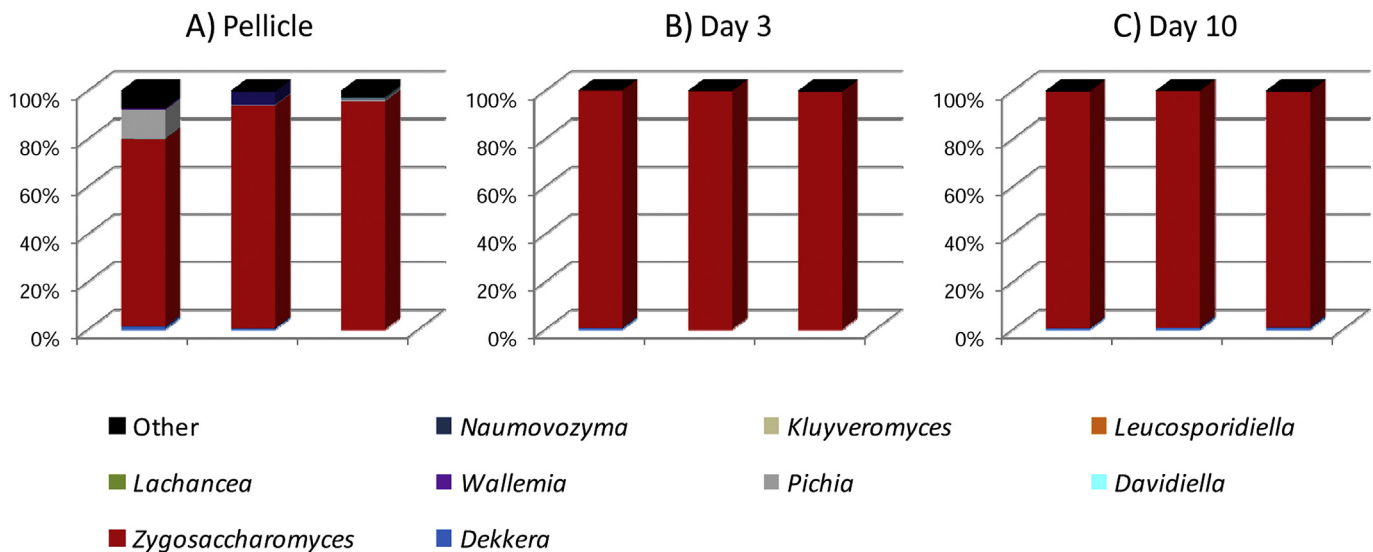|	Ca1	Ca2	Ire1
Day 3			
Dekkera	0.57	0	0
Zygosaccharomyces	99.43	99.8	99.57
Kazachstania	0	0	0
Other	0	0.2	0.43
Day 10			
Dekkera	0.43	0.74	0.83
Zygosaccharomyces	99.19	99.13	98.69
Kazachstania	0	0	0
Other	0.38	0.14	0.49
Pellicle			
Dekkera	1.07	0.41	0
Zygosaccharomyces	78.96	93.26	95.39
Davidiella	0.16	0	0
Pichia	11.54	0	0.62
Wallemia	0.58	0	0
Lachancea	0	0.3	0
Leucosporidiella	0	5.56	0
Kazachstania	0	0	0.33
Kluyveromyces	0	0	0.25
Naumovozyma	0	0	0.54
Meyerozyma	0	0	0
Saccharomyces	0	0	0
Hanseniaspora	0	0	0
Other	7.68	0.48	2.87

Fig. 3. ITS phylogenetic composition of the fungal component of the kombucha mat (A) and fermented tea at day 3 (B) and day 10 (C) of fermentation, at genus level, and the kombucha mat (D) and fermented tea at day 3 (E) and day 10 (F) of fermentation, at species level.

While *Zygosaccharomyces*, *Dekkera* and *Kazachstania* were the only genera detected across the various fermented tea samples, several other genera were detected in the pellicle samples and were represented by one species. Of these, *Davidiella tassiana*, *Lachancea fermentati*, *Kluyveromyces marxianus*, *Naumovozyma castelli* and the Basidiomycota representatives, *Wallemia sebi* and *Leucosporidiella fragaria*, have not been found in kombucha previously. Of the others, *Hanseniaspora* has previously been identified in one kombucha study (Mayser et al., 1995) while *Pichia kudriavzevii* and other *Pichia* and *Pichia*-like species such as *P. fermentans* and *P. membranaefaciens* and *Hyphopichia burtonii*, have been detected on a number of previous occasions (Chen and Liu, 2000; Jankovic and Stojanovic, 1994; Mayser et al., 1995). Although there were a number of hits that could not be assigned to a species within the fungal database, it is believed that, as more sequences are deposited, it will be possible to assign greater proportions of fungal ITS sequences from studies such as this one.

Ultimately, it would appear that the naturally low pH and ethanol content of the beverage generated under regular, household brewing conditions, combined with other forms of competition involving the indigenous microbial population, is sufficient to limit contamination from undesirable populations. This indigenous population appears robust, with those microbes contained within the matrix of the cellulosic pellicle dictating the microbiota of the eventual beverage. Further investigations will reveal the link between the different populations, their bioactive peptides and the purported health benefits of kombucha. It is believed that the information presented here will assist in future microbiology-focused kombucha studies and may ultimately assist in the development of defined kombucha starter cultures.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2013.09.003.

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