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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.

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.,

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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_2O, 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