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Yeast ecology of Kombucha fermentation

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Abstract

Kombucha is a traditional fermentation of sweetened tea, involving a symbiosis of yeast species and acetic acid bacteria. Despite reports of different yeast species being associated with the fermentation, little is known of the quantitative ecology of yeasts in Kombucha. Using oxytetracycline-supplemented malt extract agar, yeasts were isolated from four commercially available Kombucha products and identified using conventional biochemical and physiological tests. During the fermentation of each of the four products, yeasts were enumerated from both the cellulosic pellicle and liquor of the Kombucha. The number and diversity of species varied between products, but included *Brettanomyces bruxellensis*, *Candida stellata*, *Schizosaccharomyces pombe*, *Torulaspora delbrueckii* and *Zygosaccharomyces bailii*. While these yeast species are known to occur in Kombucha, the enumeration of each species present throughout fermentation of each of the four Kombucha cultures demonstrated for the first time the dynamic nature of the yeast ecology. Kombucha fermentation is, in general, initiated by osmotolerant species, succeeded and ultimately dominated by acid-tolerant species.
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Keywords: Yeast; Ecology; Kombucha

1. Introduction

Kombucha is a traditional fermented beverage with a history of several thousand years in the East and yet is quite popular today in the West. Current strong and increasing interest in and consumption of the product derives from its purported therapeutic benefits, which range from weight loss to curing cancer and AIDS (Frank, 1995; Dufresne and Farnworth, 2000; Greenwalt et al., 2000). While many of these claims are far from proven, the beverage does exert antimicrobial activity against a range of bacteria (Greenwalt et al., 1998, 2000; Sreeramulu et al., 2001).

Kombucha is typically prepared by fermenting black tea, sweetened with sugar, with what is popularly known as a ‘tea fungus,’ at room temperature for 10–12 days (Anken and Kappel, 1992). This so-called ‘tea fungus’ is actually a symbiosis of yeasts and acetic acid bacteria, the cellulosic pellicle formed by the latter commonly described as the ‘fungus’ (Kappel and Anken, 1993; Timmons, 1994; Steinkraus, 1996). The yeasts ferment the sugar in the tea medium to ethanol, which is then oxidised by the acetic acid bacteria to produce acetic acid. The

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resultant low pH and presence of antimicrobial metabolites reduces the competition of other bacteria, yeasts and filamentous fungi. While fermentation conducted in the home usually involves volumes of 1–2 l, commercial fermentation is conducted in 100-l volumes.

Analysis of the fermented liquid has revealed the presence of acetic, lactic and gluconic acids as major chemical compounds (Frank, 1995; Hobbs, 1995). Gluconic acid is considered by several workers to be the main therapeutic agent in Kombucha, as it functions in the liver as a detoxification agent (Frank, 1995; Loncar et al., 2000). Tietze (1995) described the presence of usinic acid, an antibacterial agent, in Kombucha cultures. More recently, acetic acid has been suggested as the major antimicrobial agent (Greenwalt et al., 1998), though other compounds, such as bacteriocins and tea-derived phenolic compounds, may be involved (Sreeramulu et al., 2001). A diverse range of flavour compounds, including a range of alcohols, aldehydes, ketones, esters and amino acids have been identified. However, the absence, or presence at diverse concentrations, of various metabolites determined in a range of studies (Jankovic and Stojanovic, 1994; Reiss, 1994; Siever et al., 1995; Blanc, 1996) suggest inconsistencies in metabolism during Kombucha fermentation. With respect to putative therapeutic substances, such inconsistencies call into question the use of the beverage as an alternative medicine. As a refreshment only, the variation of microbiological and chemical composition may be desirable, catering for individual taste. However, as a therapeutic substance or functional food, Kombucha should be defined and standardised with regard to its microbiological composition and consequently its chemical composition. The metabolic and ecological interactions occurring during Kombucha fermentation are not well understood. In particular, the role of yeasts in determining the sensory and health benefits of the beverage requires detailed investigation.

Only a few key bacteria are involved in Kombucha fermentation, such as *Acetobacter* spp., especially cellulose-producing strains of *Acetobacter xylinum*, although strains of *Gluconobacter* and *Lactobacillus* may be encountered. On the other hand, a broad spectrum of yeasts has been reported including species of *Brettanomyces/Dekkera*, *Candida*, *Kloeckera*, *Pichia*, *Saccharomyces*, *Saccharomycoides*, *Shizosaccharomyces*, *Torulospora* and *Zygosaccharomyces* (Jankovic and Stojanovic, 1994; Frank, 1995; Mayser et al., 1995; Liu et al., 1996). Previous investigation of several different Kombucha cultures (Mossel, 1996) revealed a diversity of yeast species, although the identification of the species was incomplete. While the diversity of yeast species associated with Kombucha products has been well documented, and numerical populations of yeasts and bacteria have been reported (Chen and Liu, 2000), there has been no quantitative study of the development of different yeasts during the fermentation.

The aims of this study were therefore to identify the individual yeast species associated with several Kombucha cultures and the resulting beverages commercially available in Australia and, importantly, to determine changes occurring in yeast populations of the culture and the beverage throughout fermentation.

2. Materials and methods

2.1. Microbiological culture media

Bacteriological peptone (L37) and Malt Extract Agar (MEA, CM59) were obtained from Oxoid Australia (Melbourne, Australia). Oxytetracycline HCl (O5875) was obtained from Sigma (Sydney, Australia). MEA was prepared according to manufacturer's instructions. To prepare plates for enumeration of yeasts from Kombucha fermentations, a 0.1% aqueous solution of oxytetracycline was filter-sterilised (0.2 μm) and added to cooled, molten MEA to a final concentration of 0.01%.

2.2. Kombucha cultures

Kombucha cultures were obtained from commercial outlets and labelled Kombucha Brookvale or KB (The Kombucha Gold, Brookvale, New South Wales), Kombucha Gast or KG (GAST, Bermagui South, New South Wales), Kombucha Randwick or KR (Kombucha–the Miracle, Randwick, New South Wales) and Kombucha Queensland or KQ (Kombu Australia, Springwood, Queensland). The cultures were obtained as small pieces of pellicle in a minimal volume of

Kombucha liquor. All cultures were stored at 5 °C prior to fermentation.

2.3. Preparation and cultivation of Kombucha

The tea medium was prepared traditionally by boiling 1 l of distilled water, 100 g white sugar (CSR, Pyrmont) and 5.4 g black tea leaves (Lipton, Epping) for 5 min and allowing the mixture to steep for 15 min. The sweetened tea was strained to remove the leaves, during transfer to a sterile glass jar (2 l). Once cooled to the ambient laboratory temperature (20–22 °C), the tea was inoculated with the Kombucha culture (pellicle). At the completion of the first fermentation, the pellicle, covering the entire surface area of the liquor, was used to inoculate the next (second) fermentation. During subsequent fermentations, a 'daughter' pellicle similar to that of the 'parent' was used as the inoculum. For each of the second and subsequent fermentations, 100 ml of beverage was added from the previous fermentation, in addition to the pellicle. For each fermentation, the jar was covered with paper towel, secured with rubber bands.

In addition, a sterile sweetened tea medium was prepared by autoclaving separately 500-ml volumes of 20% sugar solution and steeped tea (5.4 g tea/500 ml) and then mixing the two solutions, once cooled, in a sterile 2-l jar.

Fermentation was conducted in a temperature-controlled laboratory (20–22 °C) for up to 14 days. The pH of the beverage was monitored throughout fermentation using a digital pH meter (TPS, Brisbane) calibrated at pH 4 and 7 at 22 °C.

2.4. Yeast isolation and enumeration

Samples of both the fermentation liquor and the pellicle were extracted separately for yeast isolation and enumeration. After gentle mixing, liquor samples (10 ml) were taken at day 0 and on every second day thereafter, throughout fermentation. Pellicle samples (10 g) were aseptically cut from the newest layer of cellulose formed, from day 4 of fermentation (prior to day 4, little new pellicle material had developed). The remaining pellicle was returned to the tea medium and fermented for a further 2 days, after which the next sample was extracted. The procedure was repeated for examination on days 8, 10, 12 and 14 of fermentation. All samples were diluted (1:10) with 0.1% peptone and homogenised using a Stomacher (Colworth, Stomacher 400). Homogenates were serially diluted in bacteriological peptone (0.1%) where necessary, and samples were spread onto duplicate plates of Malt Extract Agar (MEA) supplemented with oxytetracycline. After incubation at 25 °C for up to 4 days, plates were examined using a plate microscope, and the number of each individual colony type was counted. Based on the count and dilution factor, the population of each yeast type was determined at each sampling time. In preparation for identification, colonies representative of each type were isolated and purified by streak inoculation onto MEA. A pure culture of each isolate was subcultured to and maintained on MEA slants held at 5 °C. Each yeast growth curve experiment was performed twice.

2.5. Yeast identification

Identification of yeast isolates was attempted using two approaches, the Biolog Microstation system and conventional identification tests. For the Biolog system (Oxoid Australia), each isolate was cultured and tested in accordance with manufacturer's instructions. Results were interpreted using Microlog 3 software. Conventional tests were performed as described elsewhere (Kreger-van Rij, 1984; Barnett et al., 2000). A modified microtitre tray method (Heard and Fleet, 1990) was used to conduct assimilation tests. Assimilation tests were monitored and optical density measured at 610 nm using a CERES UV 900 Hdi plate reader (Bio-Tek Instruments, Winooski, VT), an OD reading of greater than 0.4 scored as a positive reaction. Each microtitre tray was prepared so that

Table 1
Predominant yeast species isolated from four Kombucha cultures

Yeast species	Found in Kombucha culture
Zygosaccharomyces bailii	KB[a], KQ, KR
Schizosaccharomyces pombe	KG, KQ
Torulospora delbreuckii	KG, KR
Rhodotorula mucilaginosa	KG, KR
Brettanomyces bruxellensis	KG
Candida stellata	KB

[a] KB, Kombucha Brookvale; KG, Kombucha GAST; KQ, Kombucha Queensland; KR, Kombucha Randwick.

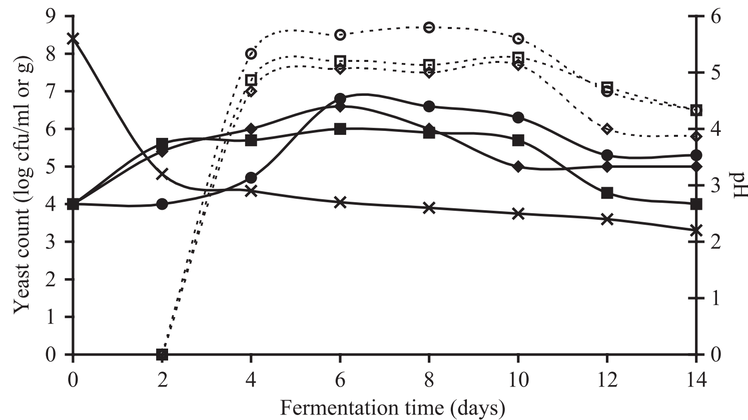

Fig. 1. Growth of yeasts and pH (×) during fermentation of Kombucha GAST: *B. bruxellensis* in the liquor (●) and in the pellicle (○); *S. pombe* in the liquor (■) and in the pellicle (□); *T. delbrueckii* in the liquor (♦) and in pellicle (◇).

each well contained the same carbon or nitrogen source, and each well was inoculated with a different yeast isolate.

3. Results

3.1. Yeast identification

Of the 163 yeast isolates obtained, 9 isolates representative of each colony type were tested using the Biolog identification system, of which only *Schizosaccharomyces pombe* was correctly identified. All 163 yeast isolates from Kombucha liquors and pellicles were characterised by physiological and morphological tests (Kreger-van Rij, 1984; Barnett et al., 2000), resulting in identification of six predominant species (Table 1). *Zygosaccharomyces bailii* was isolated from three of the four cultures and *S. pombe*, *Torulaspora delbrueckii* and *Rhodotorula mucilaginosa* from two fermentations. The remaining species *Candida stellata* and *Brettanomyces bruxellensis* were each isolated from only one of the fermentations. For each Kombucha culture, species

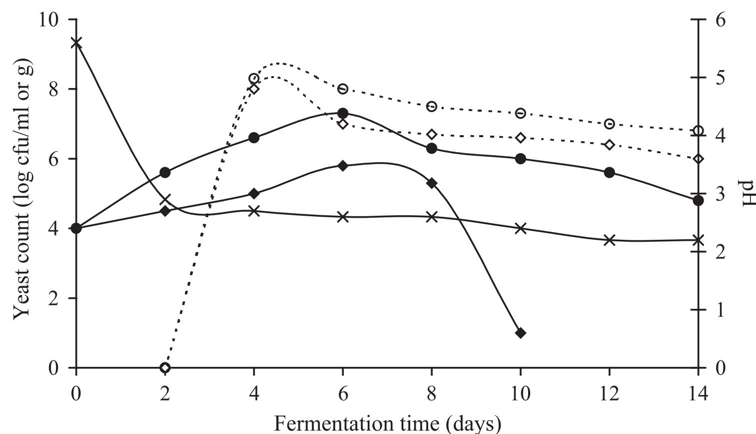

Fig. 2. Growth of yeasts and pH (×) during fermentation of Kombucha Brookvale: *Z. bailii* in the liquor (●) and in pellicle (○); *C. stellata* in the liquor (♦) and in the pellicle (◇).

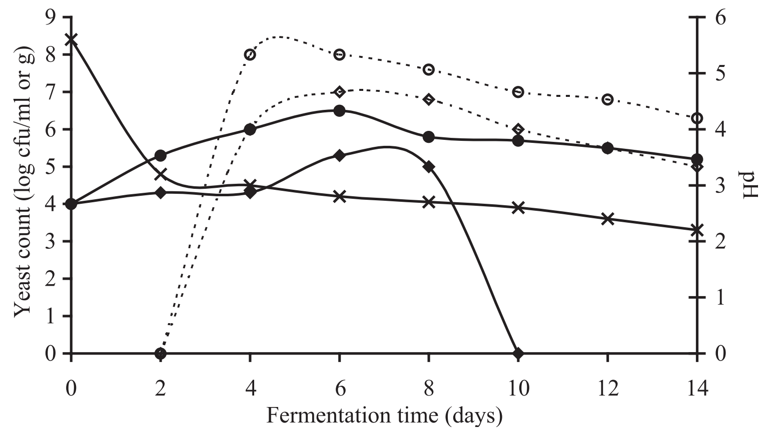

Fig. 3. Growth of yeasts and the pH (×) during fermentation of Kombucha Randwick: *Z. bailii* in the liquor (●) and in the pellicle (○); *T. delbrueckii* in the liquor (♦) and in the pellicle (◇).

isolated from liquors were the same as those found in the pellicle.

3.2. Yeast growth curves

Growth curves were determined only from Kombucha cultures cultivated in the traditionally prepared medium. The medium composed of sterile tea and sugar solutions was quickly abandoned, as it did not support the growth of any Kombucha culture.

During Kombucha fermentation, the predominant yeast species in the liquor of each of the four products reached a peak population between days 6 and 8 of the fermentation (Figs. 1–4). The population of each species of yeast ranged from 10^4 to 10^6 cells/ml in the liquor. The growth of yeasts in the pellicle from each of the four products remained constant at 10^6–10^8 cells/g throughout the fermentation, varying from product to product. The pH of the beverage decreased rapidly within the first 24 h, decreasing to pH 2.2 by the completion of fermentation.

Figs. 1–4 show growth curves for individual yeast species throughout the fermentation of the four Kombucha cultures. *R. mucilaginosa* was present only at the beginning of fermentation. In KG, *S. pombe* and *T. delbrueckii* initially outgrew the slower growing *B.*

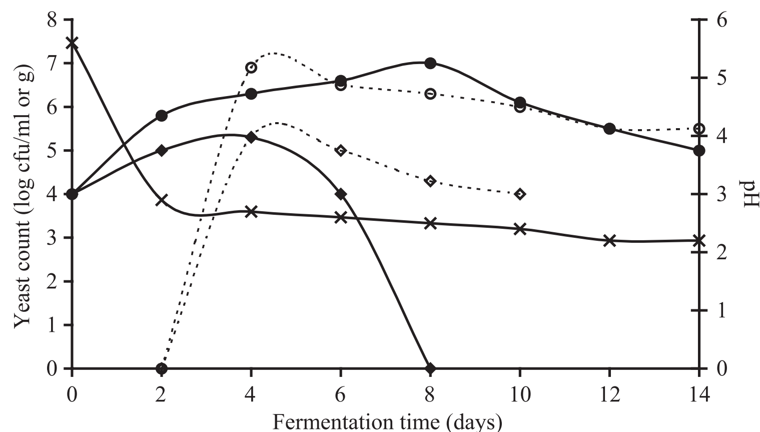

Fig. 4. Growth of yeasts and the pH (×) during fermentation of Kombucha Queensland: *Z. bailii* in the liquor (●) and in the pellicle (○); *S. pombe* in the liquor (♦) and in the pellicle (◇).

bruxellensis to reach a population of 10^5–10^6 cfu/ml (Fig. 1). *B. bruxellensis* and *T. delbrueckii* grew to 10^7 cfu/ml, to complete the fermentation. *Z. bailii* was the predominant species throughout the three remaining fermentations, reaching populations of 10^7–10^8 cfu/g in the pellicle (Figs. 2–4). *C. stellata* was present throughout the fermentation of KB (Fig. 2), whereas in KR (Fig. 3), *T. delbrueckii* was a minor contributor, reaching a population of 10^5 cfu/ml before dying off. Similarly, *S. pombe* was a minor contributor in KQ, reaching 10^5 cfu/ml (Fig. 2). *T. delbrueckii* and *S. pombe* died off in the beverage after 8–10 days (Figs. 3 and 4). In both KR and KQ, *Z. bailii* dominated the beverage fermentation until completion. Yeast populations in the pellicle were similar for these cultures, and in both fermentations, highest populations were reached early in fermentation, declining gradually as the pellicle thickened. The KR and KQ fermentations were similar to that of KB, both initiated by the growth of *Z. bailii*. In KR, *T. delbrueckii* was a minor contributor to the fermentation, the population reaching as high as 10^5 cfu/ml before dying off (Fig. 3). In KQ, *S. pombe* was the minor contributor, also reaching 10^5 cfu/ml before dying off (Fig. 4). In both cases, *Z. bailii* eventually took over the fermentations, to their completion. The viable populations of the yeasts in the pellicle for both Kombucha cultures were also similar. The highest counts were reached at the early stages of the fermentation and declined gradually as the pellicle thickened towards the end of the fermentation.

4. Discussion

This study has determined not only the yeast species associated with four different Kombucha cultures and the subsequent fermentation associated with each product, but has, for the first time, reported the dynamics of each yeast species throughout Kombucha fermentation.

Whilst there have been no yeast species associated universally with Kombucha fermentation, osmotolerant, fermentative and acid-producing species are among the most common isolates. The five fermentative species isolated in this study have all been reported in previous single-point studies of Kombucha cultures or fermentation processes (Jankovic and Stojanovic, 1994; Frank, 1995; Mayser et al., 1995; Liu et al., 1996; Mossel, 1996), and all exhibit the characteristics required to participate in Kombucha fermentation. The aerobic species *R. mucilaginosa* has not been reported previously in Kombucha fermentations; however, *Rhodotorula* spp. have been described (Jankovic and Stojanovic, 1994; Stamets, 1995; Mossel, 1996). *R. mucilaginosa* is a common contaminating yeast in foods and beverages and occurs naturally in the atmosphere (Gentles and La Touche, 1973).

A range of acid-producing yeast species, such as *Brettanomyces* (*Dekkera*), are well adapted to an environment such as Kombucha fermentation. *Brettanomyces* spp. have been reported to participate in the spontaneous fermentation of Belgian beer and are commonly isolated as contaminants of other beers during fermentation (Kreger-van Rij, 1984; Mayser et al., 1995). They contribute to the flavour of the beer by producing acetic acid and acetic acid esters under anaerobic conditions (Kreger-van Rij, 1984; Gancedo and Serrano, 1989). *C. stellata*, also a producer of acetic acid, is commonly found during the early to mid-stages of wine fermentation (Heard and Fleet, 1993). The remaining fermentative species, *Z. bailii*, *S. pombe* and *T. delbrueckii*, are also suited to the Kombucha environment because of their ability to tolerate high-sugar foods and, in the case of *Z. bailii*, acetic acid concentrations of up to 20 g/l (Thomas and Davenport, 1985).

An extensive range of yeast species and immense variation in the yeast composition of Kombucha is reported in the literature. The variations could be due to geographic, climatic and cultural conditions as well as on local species of wild yeasts and bacteria (Danielova, 1959 cited in Frank, 1995; Mayser et al., 1995) or possibly from cross-contamination between cultures. In the current study, all factors other than origin of culture were constant, yet variation of species was observed between cultures. This observation supports the theory of Phaff et al. (1978), who attributed the variation in Kombucha ecology to the origin of the culture. With this perception, the bacterial spectrum and chemical components would also be expected to differ due to these variations. Hence, it is not possible to state an exact microbial composition for Kombucha. Mayser et al. (1995) investigated 41 specimens of Kombucha collected throughout Germany and concluded from the diversity of samples that Kombucha

did not contain a characteristic yeast species or genus but a range of yeasts. However, that study did not investigate the diversity of yeast species associated with each product during fermentation. Clearly, Kombucha ecology is more complex than a single yeast and acetic acid bacteria symbiosis described in earlier literature (Hesseltine, 1965; Steinkraus, 1996).

Variation in the species reported may also in part be related to proper identification. In this study, identification was attempted using the Biolog system, proving generally unsuccessful. The system proved variable in a previous study when used to identify a range of food- and beverage-related yeasts (Praphailong et al., 1997). In that study, *S. pombe* was correctly identified, while *Zygosaccharomyces* spp. were identified correctly only 50% of the time, consistent with findings here. In contrast, *B. bruxellensis* was poorly identified in the present study, while previously identified correctly.

This study has established that the microbial ecology of the liquor is similar to that of the pellicle. However, growth patterns were not identical. During fermentation of the liquor, the viable population of yeasts in Kombucha beverage followed a standard growth curve pattern, in which yeast grew exponentially for up to 8–10 days, dying off as nutrients became limiting and the pH decreased. However, the yeast population in the Kombucha pellicle remained constant, at 10^6–10^8 cfu/g, throughout the fermentation. This may be due to an encapsulation effect arising from the arrangement of yeasts and bacteria within the pellicle. Studies of the pellicle structure (Fontana et al., 1991; Anken and Kappel, 1992) showed it to be laminar, primarily formed by the bacteria. However, yeasts may be arranged in islets or bands and are embedded in the laminae.

Variation in the yeast species reported in previous studies may also be due to the point in time at which samples have been taken, whether from the inoculant pellicle, or the liquor at the end of fermentation. Such studies may not have determined fully the diversity of yeast species associated with a single Kombucha culture. The present study has shown the variation in yeast species and their respective populations throughout the fermentation, highlighting the fact that sampling at any single point may provide a different profile of the yeast species present. The changes in yeast populations during fermentation can be attributed to the growth characteristics of individual species and are similar to patterns observed in other fermentations. For example, the current Kombucha fermentations were initiated by osmotolerant yeasts (Figs. 1–4). Isolates of *S. pombe*, *T. delbrueckii* and *Z. bailii* exhibit tolerance up to 60% glucose concentration (Kreger-van Rij, 1984; Barnett et al., 2000) and are commonly associated with alcoholic fermentation for wine and champagne production (Heard and Fleet, 1993). As the fermentation progressed, species with low acid tolerance decreased in population. Isolates of *C. stellata* and *T. delbrueckii* decreased in population after day 8. Growth of these species also declines after 6–8 days during wine fermentation (Heard and Fleet, 1993). In the current Kombucha study, species such as *S. pombe*, with moderate tolerance to acidic conditions, died off after day 10. The exception was in KQ, where *S. pombe* died off after only 4 days fermentation. The species may have been outgrown by *Z. bailii* which, as mentioned previously, is well suited to these conditions.

In an attempt to study fermentation under more controlled microbiological conditions, a medium consisting of sterile tea and sugar solutions was prepared, though it did not support growth of any the four Kombucha cultures. Other studies of Kombucha have been conducted successfully using autoclaved solutions, either as a sterilised composite of all ingredients (Hesseltine, 1965) or as a mixture of sterile solutions (Blanc, 1996), so the reasons for failure here are unclear. It is suggested that phenolic compounds in the tea formed complexes with other components to form toxic substances during autoclaving. The antimicrobial role of phenolics such as tannins has been suggested previously (Sreeramulu et al., 2001). Autoclaving at a lower temperature and pressure for a longer period would be a useful approach to minimising or eliminating the toxic effect.

This study demonstrated variability of the yeast ecology in Australian Kombucha cultures, consistent with previous studies and, as a novel contribution, quantified the populations of individual yeast species during Kombucha fermentation. It may be that the diversity of yeasts reportedly associated with Kombucha may be related to the material sampled and the stage of production. It is clear that the desirable characteristics of yeast species for Kombucha fermentation are osmotolerance, tolerance of acid conditions

and production of alcohol and other flavour compounds. Although several workers have isolated and grown *A. xylinum* strains to analyse metabolic products (Reiss, 1994; Siever et al., 1995), investigation of yeast metabolites is lacking. Mixed culture interactions are complex, and hence, it is difficult to determine the exact contribution of the yeasts to the chemical composition of the fermented tea. The factors affecting these interactions, and the metabolism of both yeasts and bacteria, require further investigation. A better understanding of the ecology of fermentation will enable the development of combinations of yeast and bacterial strains to provide a product of predictable taste and consistent quality.

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