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ANTIMICROBIAL ACTIVITY OF FERMENTED GREEN TEA LIQUID

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ABSTRACT

Green tea kombucha made of sugar and single yeast was investigated for its antimicrobial activity and composition changes during fermentation. Individual and total tea polyphenols, caffeine, sucrose, reducing sugar, total sugar, pH, acetic acid and total acids, ethanol concentration, superoxide dismutase and hexokinase activities, yeast yield and antimicrobial activity were measured systematically at the intervals of 12 to 24 hours of fermentation. Results shown that a 36-hour sample had total growth inhibition on *Escherichia coli* and *Staphylococcus aureus,* but almost no effects on *Bifidobacterium* and *Lactobacillus delbrueckii* subspecies *bulgaricus*. In order to see the effects of the main components on the microbial growth, a mixture of tea polyphenols, a mixture of acids and ethanol were tested respectively and showed various degrees of antimicrobial activities which were much less than the activity of the 36-hour kombucha, suggesting that there was a synergy effect among the components.

Keywords: Antimicrobial activity; Composition change; Fermentation; Green tea kombucha; Tea polyphenols.

INTRODUCTION

Kombucha is a fermented tea beverage which origin is traceable in China more than two thousand years ago. It is produced from fermentation of sugared tea by a symbiosis of yeasts and bacteria (Dufresne and Farnworth 2000, Teoh *et al.* 2004). It has been reported many health benefits mainly based on personal observation and testimonials (Greenwalt, *et al.* 1998, Dufresne and Farnworth 2000) and getting quite popular today in the West. Kombucha has been studied intensively since 1852, few of the health properties have been demonstrated by scientific and experimental studies (Dufresne and Farnworth 2000).

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Black tea is usually used for kombucha preparation, green tea and herbs are also used. It has been shown that green tea has a more stimulating effect on the kombucha fermentation than black tea, yielding the fermentation in a short time frame (Greenwalt, *et al.* 1998). It is not known how the composition of the tea itself is affected during the fermentation or how it is transformed (Dufresne and Farnworth 2000).

Kombucha is a home-brewing product which means the preparation conditions are not sterile. Majority of the tests for kombucha indicated that a low rate of contamination from spoilage and pathogenic microorganisms, suggesting that kombucha has antimicrobial properties to pathogenic and other "bad" microorganisms (Mayser *et al.* 1995). Some studies reported that antimicrobial activity of kombucha against a range of bacteria, made with a low tea usage

level (4.4 g/L), was attributable to the acetic-acid content (Steinkraus *et al.* 1996, Greenwalt *et al.* 1998). While Sreeramulu *et al.* (2001) revealed that the antimicrobial components of kombucha are compounds other than organic acids, ethanol, proteins or tannins in tea or their derivates after systematic investigation. Therefore, it is necessary to conduct further study on antimicrobial components of kombucha.

The objective of this study was to investigate compositional changes of sugared green tea kombucha and its antimicrobial activities during formation with a systematic approach.

2. MATERIALS AND METHODS

2.1. PREPARATION OF SUGARED GREEN TEA INFUSION

Green tea (40 g, produced in Zhejiang Province, PR China) was infused in 3000 mL boiling distilled water for 10 min with stirring, and then vacuum filtered with a paper filter. The tea residue was washed twice with 500 mL boiling water. Sucrose (600 g) was added into the combined tea infusion. The sugared tea infusion (STI) was adjusted to 5000 mL, which made 120 g/L of the final sugar concentration and 8 g dry green tea per liter. The STI was transferred into 36 × 250 mL conic flasks, with each flask containing 120 mL STI. They were sterilized at 121 °C for 15 min.

2.2.YEAST AND FERMENTATION

A yeast strain, Saccharomycodes ludwigii sp. (provided by Shanghai Institute of Microbiology Research, PR China) was used for the STI fermentation. The yeast was added into 100 mL sterilized STI and developed over 24 hours as a pre-culture. The pre-culture was used for inoculating STI at the level of 6-8 × 10^8 cells/mL. STI fermentation was carried out at 25 °C for up to 156 hours. At an interval of 12 hours, three flasks of the fermenting STI were random sampled for analysis. The samples were centrifuged at 15000g for 20 min. The supernatant was filtered through a 0.45 μm filter and stored at 4 °C until analysis. The residual was collected and disrupted with methylbenzene (1:0.8) for 2 hours at 37 °C. After being centrifuged at 15000g for 20 min, the aqueous phase was filtered through a 0.45 μm filter and stored at 4 °C until analysis.

2.3. ANALYSIS OF TEA POLYPHENOLS

The concentrations of tea polyphenols were analyzed on a Diamonsil C18 column (4.6 × 250 mm, 5 μm particle size, Japan) using a Shimadzu LC-2010 HPLC (Shimadzu, Japan) equipped with a UV detector. Wavelength was set at 280 nm. Mobile phase A and B were all made of acetic acid/acetonitrile/water but with different ratios (A: 0.5:3:96.5 and B: 0.5:30:69.5, by vol.). The flow rate used was 1 mL/min and 10 μL sample was injected into the column. The column was equilibrated initially with 100% A, and then eluted with a gradient up to 100% B in 45 min. The column temperature was 35 °C. Peaks were identified by comparison with the retention time of authentic standards of (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG), (-)-catechin(C), (-)-gallocatechin gallate (GCG), (-)-catechin gallate (CG), and (-)-gallocatechin (GC) (Mitsui Norin Co. Ltd, Japan). Concentration of the individual polyphenol was calculated using the corresponding standard curve.

2.4. DETERMINATION OF ORGANIC ACIDS

The same HPLC and column as described above were used to determine the concentrations of organic acids in the supernatant of the fermenting STI. The mobile phase was 2.5% $NH_4H_2PO_4$ (pH 2.5). Flow rate was 0.9 mL/min and 10 μL sample was injected. Wavelength was set at 280 nm. Peaks were identified by comparison with the retention time of authentic standards of organic acids (Sigma). Concentration of the individual organic acid was calculated using the corresponding standard curve.

2.5. ANTIMICROBIAL ACTIVITY

STI samples were centrifuged at 40000g (Beckman J2-HS, USA) for 15 min to remove cell debris. Sterile supernatant was obtained by filtering the supernatant through a sterile microfilter (0.22 μm, Millipore). The antimicrobial activity of the STI was tested at their natural pH.

A mixture of tea polyphenol solution was prepared according to tea polyphenol composition and individual concentrations of the STI fermented for 36 hours. The mixture was made of 136 mg/L GC, 111 mg/L EGC, 15 mg/L C, 39 mg/L EC, 249 mg/L EGCG, 52 mg/L GCG, 86 mg/L ECG, 17 mg/L CG with total polyphenols of 705 mg/L.

Organic acid solution was made of 10.5 g/L acetic acid, 0.36 g/L succinic acid, 0.69 g/L isocitric acid, and 0.39 g/L L-malic acid, which was the individual concentrations of the STI fermented for 72 hours.

Escherichia coli, *staphylococcus aureus*, *Bifidobacterium* and *Lactobacillus delbrueckii* subspecies *bulgaricus* were purchased from Shanghai Institute of Microbiology Research, PR China. They were grown and maintained on MRS medium containing agar (15 g/kg). MRS medium with agar (20 mL) was poured into each Petri dish (90 mm diameter). The tea polyphenol solution, organic acid solution, 1.97 % ethanol (the highest concentration of the fermented STI at 120 hr) or the sterile STI sampling at different fermentation time (0.9 mL) was mixed with the target strain culture (0.1 mL) and settled for 30 min before spreading on the agar plates uniformly. Unfermented STI was used as a control. The plates of *E. coli* and *S. aureus* were then incubated at 37 °C for 24 hours. The plates of *Bifidobacterium* and *L. bulgaricus* were incubated anaerobically at 37 °C for 24 hours. Antimicrobial activity was determined by counting the average numbers of colonies of the target strain grown on five agar plates.

2.6. THE ACTIVITIES OF SUPEROXIDE DISMUTASE AND HEXOKINASE

The assay for superoxide dismutase (SOD, EC 1.15. 1.1) activity was based on the method of Beauchamp and Fridovich (1971). The activity of hexokinase in ferment solution were spectrophotometrically measured at 37 °C according to the method of Uyeda and Racker (1965).

2.7. MEASUREMENT OF ETHANOL CONTENT

The fermented STI (0.5 mL) was mixed with 1 mL *n*-butanol and voltaxed for 1 min. Then 0.5 mL trichloroacetic acid was added in and voltaxed for another 1 min. After centrifuged at 5000g for 10 min, the supernatant was used for GC assay. The ethanol was analyzed using a Shimadzu GC 14-B gas chromatograph equipped with flame ionization detector and a PEG-Wax capillary column (0.25 mm ID × 50 m). The GC conditions were as follows: Nitrogen was a carrier gas (200 kPa). Injector and detector temperatures were 220 and 250 °C, respectively. Injection volume was 1 μL. Ethanol was identified by comparison with the retention time of authentic standard and the concentration was calculated using a corresponding standard curve.

2.8. MEASUREMENT OF SUGAR CONTENT

Total sugar and reducing sugar contents were determined by the phenol sulfuric acid method (Dubois *et al.* 1956) and the dinitrosalicylic acid method, respectively (Miller 1959, Roesser *et al.* 1996).

2.9. DATA ANALYSIS

Data were analysed and all figures were generated using MS Excel 2000.

3. RESULTS AND DISCUSSION

Green tea infusion, sugar and yeast were the three components in the sugared green tea kombucha and subjected to a series of compositional changes during fermentation. The composition of tea polyphenols in sugared tea

infusion (STI) decreased rapidly in 24 hours of fermentation (Table 1), with GC, EGC, C, EC, EGCG, GCG, ECG, and CG decreased 33.6%, 47.6%, 40.7%, 35.4%, 36.5%, 30.1%, 32.8%, and 29.6%, respectively. Total polyphenols decreased 37%. This suggests that all the tea polyphenols are sensitive to acidic pH. By the end of the formation, the individual polyphenol decreased 51.2%, 58.1%, 55.6%, 55.4%, 58.1%, 59%, 95.5%, and 59.3%, respectively. Total polyphenols decreased 60.9%. ECG was the only tea polyphenol decreased 95.5% and all the others decreased less than 60%. Caffeine decreased 33.7% in 24 hours of fermentation and 64.3% at the end of fermentation.

Time (hr)	GC[a]	EGC[b]	C[c]	EC[d]	EGCG[e]	GCG[f]	ECG[g]	CG[h]	Total Catechings	Caffeine
0	216.9	201.1	26.9	64.7	426.6	82.9	133.9	27.05	1253.5	392.2
24	144.1	110.4	16.3	42.3	270.8	57.8	90.4	18.5	793.0	260.2
36	136.3	111.3	15.4	39.4	248.5	52.3	85.8	17.0	745.3	244.2
48	126.9	86.8	14.9	34.9	247.7	54.6	83.5	17.7	701.9	251.0
72	118.2	86.6	14.0	32.4	233.6	49.2	78.7	14.9	659.9	246.9
96	113.3	88.1	13.2	24.5	200.4	36.0	64.8	11.8	576.4	204.8
120	105.7	85.1	11.8	30.6	178.0	35.2	59.5	11.4	547.9	196.4
156	105.7	87.6	12.4	28.9	179.1	33.5	6.0	11.4	493.4	193.9

[a] GC: (-)-gallocatechin, [b] EGC: (-)-epigallocatechin, [c] C: (-)-catechin, [d] EC: (-)-epicatechin, [e] EGCG: (-)-epigallocatechin gallate, [f] GCG: (-)-gallocatechin gallate, [g] ECG: (-)- epicatechin gallate and [h] CG: (-)-catechin gallate.

Table 1. Changes of individual tea polypheols, toal polyphenols and caffeine during fermentation of the sugared green tea (μg/ml).

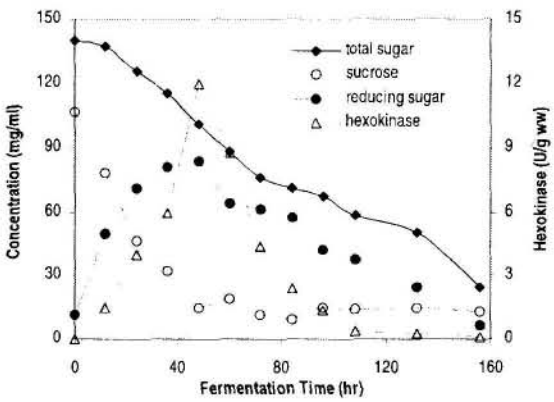

Figure 1. Change of sucrose, reducing sugar, total sugar and hexokinase during fermentation of the suygared green tea.

Yeast cells convert sucrose into fructose and glucose. Glucose is primarily used up by the yeast to produce ethanol and carbon dioxide. The sucrose in STI decreased rapidly from 107 mg/mL to 15 mg/mL during the first two days of fermentation and then decreased slightly during the rest time of fermentation. Reducing sugar which includes fructose and glucose increased rapidly in 48 hours from 11.5 mg/mL to 83.9 mg/mL and then decreased to 6.7 mg/mL at the end of fermentation. Total sugar content decreased linearly during fermentation. Hexokinase (ATP: D-hexose 6-phospho-transferase) is an enzyme responsible for the catalysis of the phosphorylation of glucose, the first step in glycolysis. It also has the ability to phosphorylate different hexoses. The activity of hexokinase in the yeast cells increased rapidly to maximum in the first two days of fermentation and then decreased rapidly to a very low level at 108 hours.

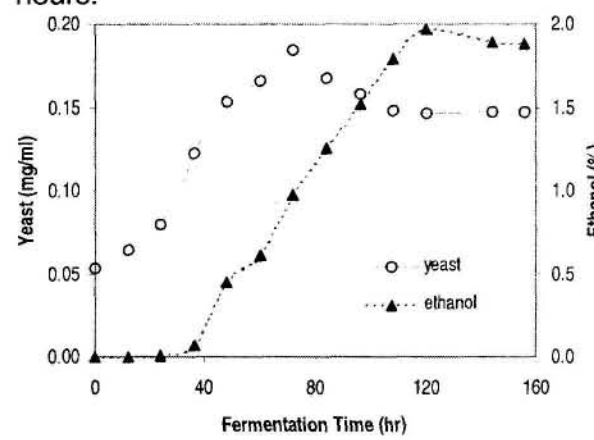

Figure 2. Changes of yeast as wet weight and ethanol concentration during fermentation of the sugared green tea.

Yeast yield as wet weight increased from 5.4 mg/L to 18.4 mg/L in 72 hours and then decreased slightly during the rest of fermentation. The final yield was 14.7 mg/L. Ethanol increased rapidly after one day of fermentation, reached to maximum at day five (1.9%) and decreased a little at the rest of the fermentation.

The pH value of STI dropped rapidly from 5.16 to 2.48 within 48 hours, then changed slightly during the rest of the fermentation (Figure 3). The pH change was correlated to the increase of acids in STI. Acetic acid was the main acid and its concentration increased rapidly to 12 g/L in STI in 84 hours and increased a little at the rest of the fermentation. Total acids showed a similar pattern of change during fermentation.

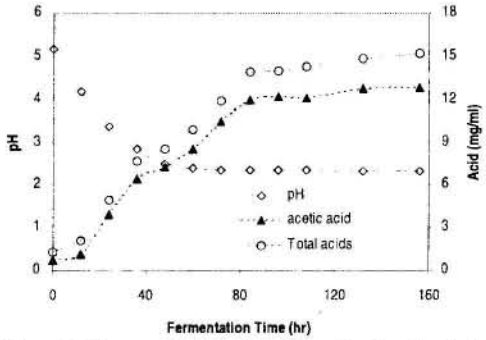

Figure 3. Change of pH, acetic acid and total acids during fermentation of the sugared green tea.

Aerobic organisms possess antioxidant defense systems that deal with reactive oxygen species (ROS) produced by aerobic respiration and substrate oxidation. Superoxide dismutase (SOD) is an antioxidant enzyme to prevent cell and tissue damage initiated by ROS. Yeast produced a large amount of SOD within 12 hours, of which a large proportion of SOD was secreted outside the yeast. SOD activity in the fermenting STI increased from 14.5 to 25 unit/mL in 72 hours and then decreased to 20 unit/mL in 108 hours and kept at this level until the end of the fermentation. The change of SOD activity in the fermenting STI had the same pattern as the growth curve of the yeast (Figure 4), which reached maximum at 72 hours. While SOD activity in yeast cells increased continuously during fermentation.

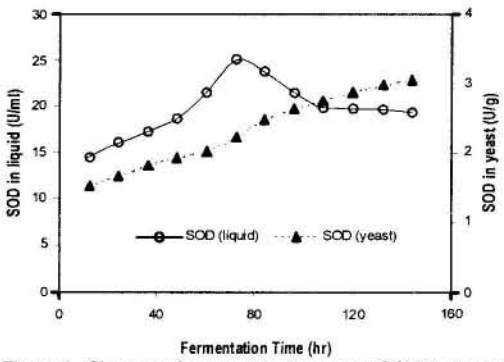

Figure 4. Changes of superoxide dismutase (SOD) in fermented green tea and in yeast cells as wet weight during fermentation of the sugared green tea.

The antimicrobial activities of tea polyphenols are well documented, while the tea concentration used in those studies usually exceeded normal human consumption levels (see review of Dufresne and Farnworth 2000). In order to see the effects of green tea kombucha with different degrees of fermentation on microbial growth, STI samples were tested systematically. Unfermented STI (0 hr) was used as a control. The antimicrobial activities of the fermenting STI samples increased with fermentation time. The 36-hour STI showed total inhibition on the growth of *S. aureus,* almost total inhibition on *E. coli,* but only slightly on *Bifidobacterium*, and had no effect on *L. bulgaricus*.

Strain	Fermentation hour of SGT						Organic acids	Poly-phenols	Ethanol
	0	12	24	36	48	108			
L. bulgaricus	165	160	163	181	165	99	162	168	159
Bifidobac.	254	249	245	243	247	183	146	226	221
E. coli	318	247	15	3	0	0	164	154	79
S. aureus	692	516	68	0	0	0	376	494	191

Table 2. Antimicrobial effects of the main components and the sugared green tea (SGT) at different fermentation time on *S. aureus. E. coli, Bifidobacterium and L. bulgaricus.* The units listed in the table were the average numbers of colonies of the target strain grown on five agar plates.

After 108-hour fermentation, the STI showed 40% inhibition on *Bifidobacterium* and 28% on *L. bulgaricus*. Mayser *et al.* (1995) noted a low rate of contamination from harmful microorganisms (spoilage and pathogenic) and concluded that kombucha can be prepared safely at home without pathogenic health risk. The antimicrobial results from this study support that conclusion that the green tea kombucha inhibits the growth of pathogenic bacteria.

The green tea used in this study was 8 g dry tea per liter which is about a normal consumption level of green tea. Since the 36-hour fermented STI had total growth inhibition to pathogenic bacteria, a mixture of tea polyphenols prepared according to the same concentrations of the individual tea polyphenols as the 36-hour fermented STI was used to test its antimicrobial activity. The tea polyphenol mixture had no inhibition on the growth of *L. bulgaricus,* light inhibition on *Bifidobacterium* (11%), but stronger inhibition on *S. aureus* (28.6%) and *E. coli* (51.6%). Unfermented STI (0 hr) had sucrose (120 g/L) and higher concentration of tea polyphenols (1.25 g/L) than the tea polyphenol mixture (0.71 g/L). This could only be explained that sucrose in the STI might have some protective effects on microbial against the antimicrobial activity of tea polyphenols.

Ethanol, using the highest concentration of 1.97% in the fermented STI, had strong inhibition on *S. aureus* (72.4%) and *E. coli* (75.2%), but light inhibition on *Bifidobacterium* (13%), no inhibition on *L. bulgaricus*. AS the ethanol concentration of the 36-hour STI was 0.07%, it would have much less antimicrobial effect.

Some studies reported that antimicrobial activity of kombucha against a range of bacteria, made with a low tea usage level (4.4 g/L), was attributable to the acetic-acid content (Greenwalt *et al.* 1998, Steinkraus *et al.* 1996). Acetic acid at as little as 1 g/L concentration can inhibit pathogenic and spore-forming bacteria (Adams 1985). Greenwalt *et al.* (1998) demonstrated that fermented kombucha at about 7 g/L acetic acid (33 g/L total acid) had antimicrobial activity against most of the test organisms in all green and black tea kombucha. The antimicrobial activity observed was due to the organic acids, primarily acetic acid, and was eliminated when samples were neutralized. In this study, a mixture of organic acids, the same concentrations of the individual acids as the 72-hour STI, had a similar inhibitory effect on *Bifidobacterium* (42.5%), *S. aureus* (45.7%) and *E. coli* (48.4%) except *L. bulgaricus* which was not affected. This tested mixture of the acids had higher acid concentration (11.9 g/L) than that at 36-hour STI (7.7 g/L). While the 36-hour STI had total inhibition on *S. aureus* and *E. coli,* indicating that acetic acid plays an important role on the antimicrobial activity of the kombucha, but it is not the sole contributor to the antimicrobial activity of the kombucha.

These results in this study shown that the main components along had much less antimicrobial activities on *S. aureus* and *E. coli* than the 36-hour STI, suggesting that there was a synergy effect among the components in the green tea kombucha.

L. bulgaricus and *Bifidobacterium* are "friendly" bacteria found in human gastrointestinal tract. The fermented STI had no or light inhibition on their growth, suggesting that it will benefit to the balance of "friendly" bacteria by inhibiting the growth of pathogenic bacteria in our digestive system after consumption of the kombucha.

In summary, a systematic approach to investigate composition changes and antimicrobial activity of the green tea kombucha shown that there was a series of composition changes during fermentation, the 36-hour fermented STI had total growth inhibition on the pathogenic bacteria, and the antimicrobial activity was a synergy effect of the kombucha components.

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