Determination and Characterization of the Antimicrobial Activity of the Fermented Tea *Kombucha*

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Early reports on Kombucha, *a traditional fermented tea beverage, suggested that it has antimicrobial activity against a spectrum of organisms, and that concentrates of unfermented tea components also have anti microbial properties. Therefore, the focus of this study was to determine and characterize* Kombucha's *antimicrobial activity using an absorbent disc method. Antimicrobial activity was observed in the fermented samples containing 33 g/L total acid (7 g/L acetic acid) against the tested Gram-positive and Gram-negative organisms (*Agrobacterium tumefaciens, Bacillus cereus, Salmonella choleraesuis *serotype* typhimurium, Staphylococcus aureus, *and* Escherichia coli. Candida albicans *was not inhibited by* Kombucha. *The contribution of tea itself to the antimicrobial activity of* Kombucha *proved to be significant in the tested organisms, even at the highest levels tested, 70 g/L dry tea. As a result, the antimicrobial activity of* Kombucha *was attributed to its acetic acid content.*

Keywords: Kombucha; tea fungus; antibacterial activity; acetic acid; tea

Introduction

Kombucha is a traditional fermented tea that has gained popularity in the U.S.A. as it is increasingly associated with health-promoting effects. *Kombucha* is a slightly sweet, acidic tea beverage currently consumed worldwide, but historically in China, Russia, and Germany (1). Numerous popular media features in the U.S.A. have highlighted the beverage and its uses, including the *New York Times* and *Miami Herald*, suggesting that *Kombucha* consumption can reduce blood pressure, relieve arthritis, increase the immune response, and cure cancer (2, 3).

Kombucha lends itself to expansive consumption as a healthful beverage as it is easily and safely produced at home (4). Production of the tea is achieved by infusing tea leaves in freshly boiled water and sweetening it with about 100 g/L sucrose or honey. Once the tea has cooled to room temperature the gelatinous surface growth/mat from a previous batch is added to the sweetened tea. After about a 7–10-d incubation at room temperature, the mat is transferred to a new fermentation and the *Kombucha* beverage is ready. The final product is a slightly carbonated, acidic beverage comprised of sugars, organic acids, tea components, vitamins, and minerals, resembling cider.

The *Kombucha* colony/mat represents a symbiotic relationship between bacteria and yeasts (5). *Acetobacter xylinum* has been shown to be the primary bacterium in the colony (6). Hesseltine reported the presence of *Acetobacter* sp. (NRRL B-2357) and two yeasts, *Pichia* and *Zygosaccharomyces* (NRRL Y-4810 and 4882), in *Kombucha* (7). Mayser *et al.* demonstrated that the yeast composition of the colony is highly variable, but that *Brettanomyces, Zygosaccharomyces* and *Saccharomyces* occurred most frequently in the German household samples studied (6). Roussin determined that the typical North American *Kombucha* microorganisms were *Acetobacter xylinum*, *Zygosaccharomyces* and *Saccharomyces cerevisiae* (8). In addition, Mayser *et al.* 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 (6). The acidity of the product, at around 33 g/L total acid, is relatively high which limits the ability of many other organisms, possible contaminants, to grow (9). However, if the fermentation is allowed to continue too long, the acidity can increase to very high levels which may pose a potential risk if consumed. This was speculated to be the cause of one woman's death in

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1995 when she died from acidosis and intestinal perforations after consuming large amounts of very acidic *Kombucha* (10).

Throughout the fermentation the yeasts break down sugar into glucose and fructose (8). Glucose is used by the yeasts to yield ethanol and carbon dioxide. The primary *Kombucha* bacterium, *Acetobacter*, initially oxides ethanol to acetaldehyde and then to acetic acid (11). The secondary biochemical activity of *Acetobacter* is the oxidation of glucose to gluconic acid (11). Glucose is also used by acetic-acid bacteria to synthesize microbial cellulose (11). Fructose remains part of the ferment broth and is utilized by the microorganisms to a lesser degree. Acetic-acid concentrations may rise to levels as high as 30 g/L if the tea is allowed to ferment for up to 30 days (12). The usual concentration of acetic acid consumed in *Kombucha* is 10 g/L (4). Gluconic acid is also present in substantial quantities, about 20 g/L (13).

The most recent chemical analysis to date is the investigation conducted by Roussin and his colleagues. Roussin determined by high-performance liquid chromatography (HPLC) and mass spectrophotometry identification that fructose, acetic acid and gluconic acid were the primary constituents of the fermented sweetened black tea (8). These and all constituents tested were shown to vary in batches and different colonies tested (8). Roussin also noted that the vitamin content of the fermented tea was not in sufficient concentrations to assist human health (8). This investigation also revealed that no glucuronic acid was present in the samples tested (8).

Numerous studies refer to *Kombucha*'s antimicrobial activity and suggest that it might influence the gastrointestinal microbial flora of humans. Steinkraus *et al.* showed that the antimicrobial activity of *Kombucha* against *Helicobacter pylori* (primary cause of gastritis and peptic ulcer disease), *Escherichia coli*, *Staphylococcus aureus*, and *Agrobacterium tumefaciens*, made with a low tea usage level (4.4 g/L), was attributable to the acetic-acid content (4). According to Levine and Fellers, acetic acid can inhibit and destroy microorganisms when used in sufficiently high concentrations (14). However, at as little as 1 g/L acetic acid pathogenic and spore-forming bacteria are inhibited (15).

Steinkraus *et al.* stated that they could not directly compare their results with other studies on the inhibitory activity of the tea, fermented and unfermented, because they used a substantially lower level of tea (4). DeSilva and Saravanapavan discussed the tea cider prepared with 10 g/L tea w/v (16). Gadd suggested preparing tea cider with 11–15 g/L (17). Hesseltine investigated the antimicrobial activity of *Kombucha* prepared with 37 g/L (7). Using this undrinkable level of tea, Hesseltine reported that *A. tumefaciens*, a common plant pathogen, was inhibited in the fermented tea and neutralized samples (7). Hesseltine did not report the effect of the unfermented substrate; therefore, it is unknown whether the tea components contributed to the neutralized ferment's antimicrobial activity.

Toda *et al.* (18, 19) demonstrated that unfermented tea at high concentrations (using 20% dry tea) inhibits *Staphylococcus aureus*, *Staph. epidermidis*, *Salmonella typhi*, *Salm. typhimurium*, *Salm. enteritidis*, *Shigella flexneri*, *Shig. dysenteriae* and *Vibrio* spp. At concentrations of 200 g/L w/v tea, Diker and colleagues (20, 21) showed that black and green tea extracts (50 times the usual level of tea used for consumption) had bacterial activity against *Campylobacter jejuni*, *Camp. coli* and *H. pylori*.

Yokihiko and Watanabe (22) found that *Clostridium botulinum* spores were killed when inoculated into tea drinks. This investigation demonstrated that the inhibitory effects observed could have been due to the catechin content of the tea. Later, it was determined that most of the bactericidal activity of tea itself may be attributed to the polyphenols, specifically catechins (23–25). The polyphenolic group that is most reactant during the enzymatic fermentation of fresh green leaves to black tea leaves are the catechins (26). Green tea has a much higher catechin content than black tea. As a result, green tea may have more antimicrobial activity than black tea (27). Kodama *et al.* demonstrated that crude catechins may be useful in the prevention of some bacterial plant diseases (28). The catechin fraction of black tea, at about 0.4 mg/ml, was shown to be antimicrobial against *Streptococcus mutans*, related to dental carries found in human teeth (25). Clearly, high levels of tea have antimicrobial effects; however, it has yet to be shown whether drinkable levels have similar properties.

The focus of this study was to test the antimicrobial activity of *Kombucha* brews made with increasing tea concentrations (black and green), thereby characterizing the contributions of unfermented tea and fermentation components. An objective was, thus, to determine whether pathogenic growth can be prevented by the consumption of the fermented tea, which may be important to aid immunity and prevent illness and could lead to better overall health.

Materials and Methods

Sample preparation

Kombucha was prepared by adding 100 g/L w/v sucrose and tea leaves of desired dry weight to boiling water. Samples of normal drinkable tea with 4.4 g/L weight of dry tea per volume of boiled water, and of tea with increased levels of 8.7 g/L, 17 g/L, 35 g/L, and 70 g/L, were prepared in duplicate. Both black (Lovers Leap Orange Pekoe Tea, Pure Premium Ceylon Tea) and green (Japanese Sencha Tea, Pure Premium Green Tea) tea leaves from the Metropolitan Tea Company Ltd, Buffalo, NY, U.S.A. were tested. The leaves were steeped for 30 min and removed. After the tea reached room temperature (about 25 °C) the colony/mat was added from the previous batch. The original colony/mat was kindly provided by Professor Keith Steinkraus, Department of Food Science, Cornell University. The fermentation continued until the desired taste and

acidity were reached. The fermentation was terminated at the organoleptically pleasing total acidity of about 33 g/L. This end-point was determined previously (data not published) by analyzing the sensory attributes, pH, and acidity of a variety of *Kombucha* ferments. The fermentation time averaged 9 d at 25 °C.

Antimicrobial activity

When the fermented samples reached the desired endpoint, the antimicrobial activities were tested in duplicate with the following organisms: *Staphylococcus aureus* NRRL B-1317, *Staphylococcus aureus* NRRL B-1318, *Escherichia coli* serotype H10 (non-pathogenic) NRRL B-2207, *Escherichia coli* serotype H48 (pathogenic) NRRL B-3704, *Salmonella choleraesuis* serotype *typhimurium* NRRL B-4420, *Bacillus cereus* NRRL B-14720, *Bacillus cereus* NRRL B-14725, *Candida albicans* NRRL Y-12983, *Agrobacterium tumefaciens*. The NRRL test organisms were kindly supplied by the Northern Regional Research Laboratory of the United States Department of Agriculture–Agriculture Research Service (USDA-ARS) in Peoria, IL. *Agrobacterium* was kindly provided by Dr Stephen Winans, Section of Microbiology, Cornell University. All bacterial species were cultivated on agar plates prepared with 25 g/L mannitol, 15 g/L Bacto agar, 5 g/L Difco yeast extract, and 3 g/L Bacto peptone as recommended by the American Type Culture Collection. *C. albicans* was cultivated on YM agar plates prepared with 20 g/L Bacto agar, 10 g/L glucose, 5 g/L Bacto peptone, 3 g/L malt extract, and 3 g/L Difco yeast extract as recommended by the Northern Regional Research Laboratory of the USDA-ARS. The bacterial species were chosen to represent the most common pathogenic and undesirable organisms associated with food. *A. tumefaciens* is a common plant pathogen that is not associated with food but was chosen as a test organism in this investigation because it was used in previous studies. Both Gram-negative and Gram-positive organisms were tested to allow observation of differences among the two types of prokaryotes. *C. albicans* was included because of its predominance as a common human pathogen.

Sterile cotton applicator swabs were used to inoculate the surface of the agar with the test organisms rather than a constant volume of culture because the swab method yielded a uniform mat of growth more consistently. A 2.5-cm cellulosic absorbent pad (Millipore AP 1002500) was used as the antimicrobial disc to estimate the zone of inhibition (clearing). The 2.5-cm disc was saturated with *Kombucha* or other test solution and placed on the freshly inoculated agar with sterile forceps. All plates were incubated at 37 °C for 72 h. The antimicrobial activity of each test solution was estimated by measuring the zone of inhibition around the disc. The diameter of the disc was subtracted from the measured clear zone.

Test solutions included duplicates of unfermented controls, samples fermented to about 33 g/L total acidity, and fermented samples neutralized to pH 7 with 1 g/L NaOH of both black and green tea preparations at the various concentrations (4.4 g/L, 8.7 g/L, 17 g/L, 35 g/L and 70 g/L dry tea weight per volume of boiled water). Tested control solutions included duplicates of commercial vinegar at 50 g/L acetic acid and prepared 10 g/L acetic-acid samples.

Kombucha *composition*

The pH and titratable acidity of each sample were checked daily to follow and characterize the fermentation progression. The pH was determined using Baxter S/P pH indicator strips or a Beckman ϕ 10 pH Meter and the titratable acidity was determined by titrating 10-ml samples with 1 g/L NaOH using 500 µl of 10 g/L phenolphthalein as the visual endpoint indicator. The total acidity was then calculated (as gluconic acid) by multiplying the volume (ml) of 1 g/L NaOH needed to titrate the sample by 1.96. Gluconic acid was used as the reference acid because it was demonstrated by Roussin that gluconic is typically the primary acid component (8).

Total acidity was broken down into volatile and nonvolatile acid components by boiling 10 mL samples for 10 min to drive-off the volatiles. The boiled samples were brought back to volume and titrated with 1 g/L NaOH. The volatile acidity was calculated as the total acidity minus the nonvolatile acidity.

The production of ethanol and glucose, in addition to acidity, was quantified. The Sigma 333-A alcohol test kit was used for the determination of the ethanol content. Alcohol dehydrogenase catalyzed the conversion of ethanol, and the absorbance at 340 nm was recorded and used to calculate ethanol concentrations. Glucose was estimated with Stanbio's Glucose LiquiColor 1070 enzymatic test kit from Sigma. Glucose oxidase and peroxidase developed a colored product from glucose. The absorbance of the colored product at 500 nm was used to calculate the amount of glucose present in the sample.

Results and Discussion

The *Kombucha* colonies used in this investigation had a tendency to produce about 3.3% total acid, 0.7% acetic acid, 4.8% glucose, and 0.6% ethanol after a 9-d fermentation. There was no lactic acid produced by these colonies (verified with HPLC; (9). The average pH of the fermented samples tested was 2.5. The pH of the neutralized samples was 7.0. When the fermentation was allowed to continue beyond the desired endpoint, the acidity reached levels as high as 24 g/L acetic acid, with 14 g/L ethanol.

Tables 1 and 2 show the antimicrobial activities of the tested solutions in black and green tea preparations, respectively. The unfermented tea samples showed no antimicrobial properties against most of the test organisms even at 70 g/L dry tea, but *Staph. aureus* was minimally inhibited when the dry weight of tea reached 35 g/L and higher. Levels of tea above 4.4 g/L had an

Table 1 Antibiotic activity in black tea samples given as diameter of clearing around disc (cm)[a]

	Control tests		4.4 g/L Dry tea			8.7 g/L Dry tea			17 g/L Dry tea			35 g/L Dry tea			70 g/L Dry tea		
Test organisms	Vinegar (50 g/L)	Acetic acid (10 g/L)	Unfermented (0 g/L)	Fermented (7.2 g/L)	Neutralized (0 g/L)	Unfermented (0 g/L)	Fermented (6.9 g/L)	Neutralized (0 g/L)	Unfermented (0 g/L)	Fermented (6.8 g/L)	Neutralized (0 g/L)	Unfermented (0 g/L)	Fermented (6.6 g/L)	Neutralized (0 g/L)	Unfermented (0 g/L)	Fermented (6.6 g/L)	Neutralized (0 g/L)
Staphylococcus aureus 1317	2.3	1.3	0.0	1.1	0.0	0.0	1.0	0.0	0.0	1.0	0.0	0.4	1.1	0.0	0.6	1.2	0.2
Staphylococcus aureus 1318	2.5	1.3	0.0	1.4	0.0	0.0	1.6	0.0	0.2	1.2	0.0	0.2	1.3	0.0	0.5	1.1	0.2
Escherichia coli 2207	4.1	2.1	0.0	1.5	0.0	0.0	1.4	0.0	0.0	1.4	0.0	0.0	1.4	0.3	0.0	1.6	0.0
Escherichia coli 3704	3.0	1.4	0.3	1.5	0.3	0.4	1.4	0.3	0.4	1.3	0.2	0.1	1.3	0.3	0.3	1.1	0.0
Bacillus cereus 14720	3.0	1.5	0.0	0.3	0.0	0.0	0.1	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	1.0	0.2
Bacillus cereus 14725	4.3	1.0	0.0	1.2	0.0	0.0	1.1	0.0	0.0	1.2	1.0	0.1	0.6	0.0	0.1	1.7	0.2
Salmonella 4420	3.5	1.6	0.1	1.2	0.3	0.1	1.5	0.1	0.1	1.6	0.0	0.2	1.4	0.0	0.1	1.3	0.0
Agrobacterium tumefaciens	3.3	1.5	0.0	1.2	0.0	0.0	1.3	0.0	0.0	1.3	0.0	0.0	1.4	0.0	0.0	1.4	0.0
Candida albicans 12983	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a]Clearing of 0.2 cm or less is negligible. All measurements are averages and have an error of ±0.2 cm.
Figures in parentheses give the acetic acid concentration.

Table 2 Antibiotic activity in green tea samples given as diameter of clearing around disc (cm)[a]

	Control tests		4.4 g/L Dry tea			8.7 g/L Dry tea			17 g/L Dry tea			35 g/L Dry tea			70 g/L Dry tea		
Test organisms	Vinegar (50 g/L)	Acetic acid (10 g/L)	Unfermented (0 g/L)	Fermented (6.6 g/L)	Neutralized (0 g/L)	Unfermented (0 g/L)	Fermented (6.9 g/L)	Neutralized (0 g/L)	Unfermented (0 g/L)	Fermented (6.8 g/L)	Neutralized (0 g/L)	Unfermented (0 g/L)	Fermented (6.4 g/L)	Neutralized (0 g/L)	Unfermented (0 g/L)	Fermented (6.9 g/L)	Neutralized (0 g/L)
Staphylococcus aureus 1317	2.3	1.3	0.0	1.1	0.0	0.0	1.2	0.0	0.0	1.1	0.0	0.2	1.0	0.0	0.5	1.2	0.3
Staphylococcus aureus 1318	2.5	1.3	0.0	1.0	0.0	0.0	1.1	0.0	0.2	1.1	0.0	0.3	1.1	0.1	0.4	1.1	0.2
Escherichia coli 2207	4.1	2.1	0.0	1.4	0.0	0.0	1.3	0.0	0.0	1.2	0.0	0.0	1.6	0.0	0.0	1.6	0.0
Escherichia coli 3704	3.0	1.4	0.0	1.2	0.3	0.0	1.3	0.3	0.0	1.3	0.1	0.0	1.1	0.3	0.0	1.1	0.2
Bacillus cereus 14720	3.0	1.5	0.0	0.2	0.0	0.0	0.6	0.0	0.0	0.8	0.0	0.0	1.0	0.0	0.0	1.3	0.0
Bacillus cereus 14725	4.3	1.0	0.0	0.6	0.0	0.0	0.8	0.0	0.0	0.9	0.0	0.0	1.3	0.0	0.0	1.2	0.0
Salmonella 4420	3.5	1.6	0.0	1.2	0.0	0.0	1.3	0.0	0.0	1.4	0.0	0.0	1.3	0.0	0.0	1.5	0.0
Agrobacterium tumefaciens	3.3	1.5	0.0	1.8	0.0	0.0	1.3	0.0	0.0	1.4	0.0	0.0	1.3	0.0	0.0	1.4	0.0
Candida albicans 12983	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a]Clearing of 0.2 cm or less is negligible. All measurements are averages and have an error of ±0.2 cm.
Figures in parentheses give the acetic acid concentration.

offensive bitter taste and were undrinkable. Therefore, drinkable levels of tea (4.4 g/L) possess no observable antimicrobial effects. In addition, the contribution of tea itself to the antimicrobial activity of *Kombucha* proved to be insignificant, despite the antimicrobial activity found in extracts and concentrates by researchers such as Toda *et al*., Ahn *et al*., and Kawamura and Takeo (18, 23, 25).

Fermented *Kombucha* at about 7 g/L acetic acid (33 g/L total acid) had antimicrobial activity against all test organisms in all green and black tea preparations, except *C. albicans*. *C. albicans* was not inhibited by any test solutions except tested commercial vinegar (50 g/L acetic acid). The antimicrobial activity observed was due to the organic acids, primarily acetic acid, and was eliminated when samples were neutralized.

The test organisms reacted similarly in all black and green tea *Kombucha* preparations, even in the highest level of tea used (70 g/L). There appeared to be a proportional similarity between the 10 g/L acetic-acid control and the fermented samples that contained about 7 g/L acetic acid. Therefore, the antimicrobial activity was primarily a result of the acetic-acid components of the ferment, as Steinkraus *et al*. found in their *Kombucha* prepared with 4.4 g/L dry tea (4).

Hesseltine reported the inhibition of *A. tumefaciens* in neutralized *Kombucha* at about 40 g/L dry weight/volume tea (7). However, in this investigation, *A. tumefaciens* had no vulnerability to the unfermented tea components, even at 70 g/L dry tea, but it was inhibited in all the fermented samples.

Conclusions

The antimicrobial activity observed in the fermented samples containing 33 g/L total acid (7 g/L acetic acid) was significant against the tested Gram-positive and Gram-negative pathogenic organisms. *C. albicans* was not inhibited by *Kombucha*. Tea, at drinkable levels, demonstrated no antimicrobial properties. The contribution of tea itself to the antimicrobial activity of *Kombucha* proved to be insignificant for the tested organisms, even at the highest levels tested. As a result, the antimicrobial activity of *Kombucha* was considered to result from the acetic-acid composition.

Kombucha may be a healthful beverage in view of its antimicrobial activity against a range of pathogenic bacteria. This may promote immunity and general well-being. It is recommended that *Kombucha* be consumed at 33 g/L total acid, 7 g/L acetic acid, to obtain these beneficial attributes.

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