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Characterization of Antimicrobial Activity in Kombucha Fermentation

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Summary

Fermented tea drink, Kombucha, can inhibit the growth of *Shigella sonnei*, *Escherichia coli*, *Salmonella enteritidis* and *Salmonella typhimurium*. Several metabolites were analyzed every two days during a 14-day Kombucha fermentation. Levels of acetic acid and gluconic acid were found to increase with fermentation time. No lactic acid or ethanol was detected. Systematic investigation of the antimicrobial activity in Kombucha revealed the presence of antimicrobial compounds other than organic acids or proteins (enzymes) produced during fermentation or the tannins originally present in the tea broth.

Introduction

Kombucha is a traditional beverage known to be associated with a number of health benefits [1, 2]. It tastes slightly sour-sweet due to the presence of a number of organic acids produced by acetic acid bacteria and yeasts and sugar residuals. These acids are presumed to be causative agents for a number of speculative curative and antimicrobial effects of Kombucha [2, 3]. Little is known about the detailed composition of Kombucha and the antimicrobial compounds present in this traditional beverage. The effect of carbon source concentration on metabolite production has been investigated by BLANC [4]. It has been reported that the ethanol concentration increases for six days of fermentation and declines during subsequent fermentation. Acetic acid and gluconic acid levels were found to enhance with increasing fermentation time [2, 4]. Very low levels of glucuronic acid were noticed by BLANC [4]. This acid may likely have a number of health potentials by virtue of its binding characteristics with toxins in the liver.

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Unfermented tea has also several health benefits such as anti-carcinogenic, anti-tumour, anti-microbial, anti-oxidant and anti-inflammatory effects [5]. Several groups [6–10] have studied the antimicrobial properties of tannins in tea extracts against a number of pathogenic organisms. However, the influence of these tannins in Kombucha may have no effect because the concentration of tea extract used is very low. On the other hand, the tannins present in the tea extract used to make Kombucha may likely be metabolized into pyrogallol groups. Hence, the antimicrobial properties of Kombucha are supposed to be largely governed by organic acids as well as other unknown antimicrobial compounds. Recent reports revealed that acetic acid might be one of the causative agents for the antimicrobial activity of Kombucha [2]. However, our preliminary investigations [11] demonstrated that Kombucha possesses antimicrobial activity even at neutral pH values as well as after heat denaturation against *Escherichia coli*, *Shigella sonnei*, *Salmonella typhimurium* and *Salmonella enteritidis*. In this paper, we report about the metabolite composition of Kombucha and the preliminary characterization of its supposed antimicrobial activities.

Materials and Methods

Kombucha Preparation

Tea fungus was obtained from a local pharmacy in Utrecht, Netherlands, and had been produced at PHARMA IMPORT, B-3581 Beverlo-Berigen, Belgium. Demineralized water was boiled for 15 min, and 10% [w/v] sucrose and 2.5% [w/v] glucose were added. Subsequently, 0.5% [w/v] black tea (C'estbon, Lapsang souchon) was added, allowed to steep for 15 min and filtered through a sieve. The tea was cooled to 25 °C and 400 ml was transferred into a 750-ml glass bottle. The tea broth was inoculated with 5 g of freshly grown tea fungus and covered with tissue paper towels. Fermentation was carried out in an incubator at 25 °C. Samples were analyzed every two days for physiological parameters.

Estimation of Metabolites

Glucose, sucrose, gluconic acid, acetic acid, lactic acid and ethanol were analyzed on an autoanalyzer (Cobas Mira Plus, ROCHE, Basel CH) by using BOEHRINGER MANNHEIM test kits.

Bacterial Strains and Cultivation Conditions

Shigella sonnei (ATCC 29930), *Escherichia coli* (ATCC 8739), *Salmonella enteritidis* (TNO own collection B308) and *Salmonella typhimurium* (ATCC 13311) were grown and maintained on TSBA (40 g/l tryptone soya broth, 15 g/l agar; Oxoid, UK).

Antimicrobial Activity

Antimicrobial activity was demonstrated by agar diffusion assay. TSBA (20 ml) was poured into each PETRI dish (90 mm in diameter) and allowed for solidification of agar. Overnight freshly grown target strain suspension (100 μl) was uniformly spread onto the plates, and wells of 9-mm diameter were made with a sterile metal tube with help of a vacuum pump. Samples of Kombucha were centrifuged at 40000 × g (DU PONT centrifuge, Sorvall RC-5B) for 15 min to remove all cell debris. Sterile samples were obtained by microfiltration (Millex-GV filter, 0.22 μm pore size; MILLIPORE).

A 100 μl pre-treated sample was transferred into the wells of seeded agar plates. After pre-diffusion of tea sample into the agar for 2 h at 4 °C, the plates were incubated at 37 °C. The diameter of the inhibition zone was measured after 12 h of incubation.

Electrophoresis

Freeze-dried Kombucha samples were dissolved in SDS-PAGE sample buffer (pH 6.8). Four native PAGE samples were dissolved in sample buffer without SDS and 2-mercaptoethanol. Samples were loaded onto the gel slots and separated at 15 mA for 1 h. Gels were stained with Coomassie Brilliant Blue R-250 for 2 h and destained with 3% sodium chloride solution according to SREERAMULU and SINGH [12].

Protease Treatment

Kombucha samples were independently treated with 100 μg of trypsin, chymotrypsin and proteinase K at 37 °C according to a standard procedure [13].

Preparation of Tannin Free Tea Samples

Kombucha (100 ml) was added to 50 ml 0.25% [w/v] gelatin prepared in saturated sodium chloride solution. To this mixture 100 ml of acid sodium chloride solution was added (25 ml of concentrated sulphuric acid added to 375 ml of saturated sodium chloride solution). A 20-g portion of kaolin powder was added and the mixture was shaken for several minutes. The mixture was allowed to settle down and filtered through WHATMAN No. 1 filter paper. The filtrate was free of tannins. Filtrate was adjusted to pH 7.0 before antimicrobial activity testing.

Extraction of Antimicrobial Compounds

Extraction of antimicrobial compounds was performed with chloroform, diethyl ether, isoamyl alcohol, propanol and butanol. Equal volumes of Kombucha and organic solvents were mixed thoroughly and centrifuged at 500 × g for 10 min for phase separation. The aqueous and organic phases were dried under vacuum and dissolved in an original volume of 0.85% NaCl for testing antimicrobial activity. When propanol was used for extraction, 5% sodium chloride was added for achieving phase separation.

Results and Discussion

Metabolite Composition of Kombucha

Kombucha samples were analyzed every two days during fermentation for glucose, sucrose, pH, lactic acid, ethanol, protein, acetic acid and gluconic acid. The results are shown in Fig. 1. No lactic acid and ethanol were detected. The sucrose level decreased with fermentation time whereas glucose levels increased for 6 days and stayed fairly constant thereafter. Sucrose was completely metabolized in 14 days. The reason for glucose increase is that sucrose is converted into glucose by microbial fermentation. The protein concentration increased very little until Day 4 and stayed fairly constant thereafter.

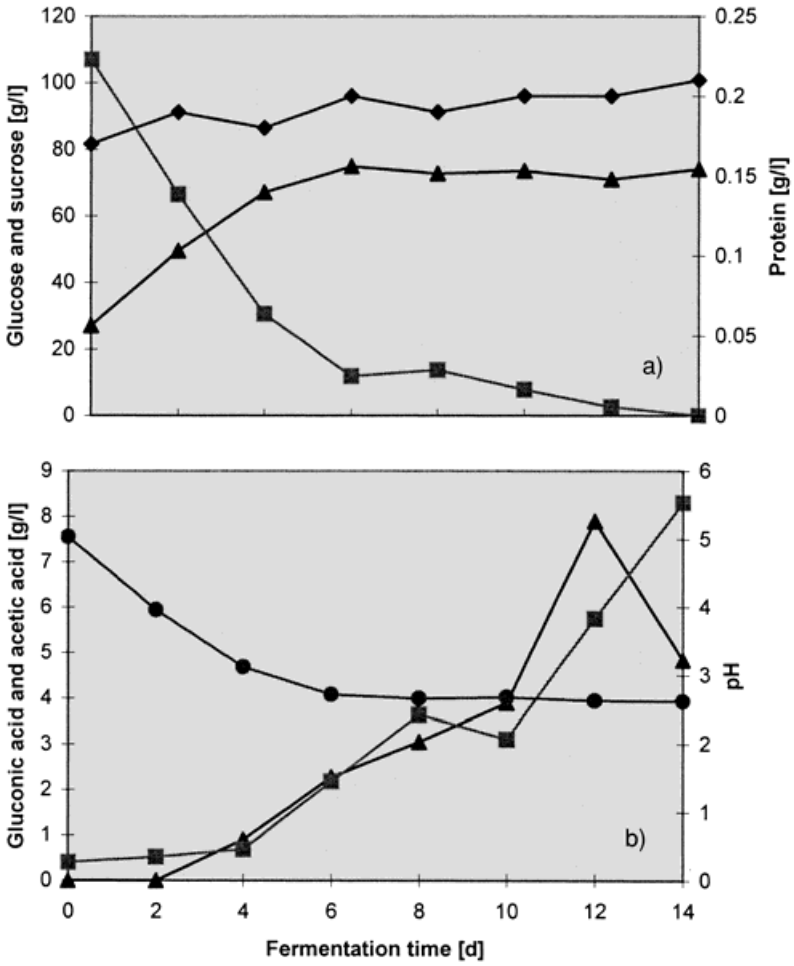

Fig.1. Kinetics of Kombucha fermentation
a) ▲ glucose; ■ sucrose; ◆ protein
b) ▲ gluconic acid; ■ acetic acid; ● pH

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Testing the Possibility of a Protein as the Antimicrobial Agent

Kombucha was found to exert antimicrobial activity at neutral pH and after thermal denaturation against *E. coli*, *S. sonnei*, *S. enteriditis* and *S. typhimurium* [11]. This suggests the presence of an antimicrobial component other than organic acids and proteins. This was further investigated in the following experiment. Kombucha sam-

ples were analyzed by electrophoresis before and after the addition of 2-mercaptoethanol and SDS. Antimicrobial activity of proteins present in Kombucha was investigated by laying the gel containing proteins separated by electrophoresis in agar in which target organisms were plated. No inhibition was noticed. This suggests that proteins present in Kombucha are unlikely to be the components responsible for antimicrobial activity. These results were confirmed by testing the antimicrobial activity of Kombucha samples after treatment with chymotrypsin, trypsin and proteinase K (Tab. 1). Antimicrobial activity was not affected by these treatments. This excludes the possibility that the antimicrobial component in Kombucha is a protein.

Tab. 1. Effects of protease and heat treatment on the antimicrobial activity of Kombucha*

Sample tested	Target organism			
	E. coli	*S. sonnei*	*S. typhimurium*	*S. enteritidis*
Kombucha treated with chymotrypsin	+++	+++	++++	++++
Kombucha treated with trypsin	+++	+++	++++	++++
Kombucha treated with proteinase K	+++	+++	++++	++++
Chymotrypsin (blank)	–	–	–	–
Trypsin (blank)	–	–	–	–
Proteinase K (blank)	–	–	–	–
Unfermented tea	–	–	–	–
Heat-denatured Kombucha	+++	+++	++++	++++
Kombucha (pH 2.6)	+++	+++	++++	++++
Kombucha (pH 7.0)	+++	+++	++++	++++

* Kombucha samples after 14 days of fermentation.
Diameter of the inhibitory zone: +++ 20–25 mm; ++++ 25–30 mm.

Synergistic Effects

The supposed synergistic effects of organic acids produced in Kombucha were investigated with the respective concentration present in Kombucha (Tab. 2). Interestingly, no antimicrobial activity was noticed with acetic acid and gluconic acid at their natural pH (2.95 and 6.3, respectively) and after adjusting their pH to 7. When the two acids were mixed, there was a synergistic effect at their natural pH (3.71) against *E. coli*, *S. enteritidis* and *S. typhimurium*. No antimicrobial activity of the mixture was observed at pH 7.0 against *E. coli*, *S. sonnei* or *S. typhimurium*. This implies that the synergistic effect of these two acids is only active at low pH, not under neutral conditions. On the other hand, the antimicrobial activity of Kombucha samples was not

affected at pH 7. This phenomenon excludes the synergistic effect of the two acids for the antimicrobial activity of Kombucha.

Tab. 2. Synergistic effect of gluconic acid and acetic acid and the influence of tannins on the antimicrobial activity of Kombucha

Sample tested	Target organism			
	E. coli	*S. sonnei*	*S. typhimurium*	*S. enteritidis*
Gluconic acid (pH 6.3)	–	–	–	–
Gluconic acid (pH adjusted to 7.0)	–	–	–	–
Acetic acid (pH 2.95)	–	–	–	++
Acetic acid (pH adjusted to 7.0)	–	–	–	–
Gluconic acid + acetic acid (pH 3.71)	++	–	++	++
Gluconic acid + acetic acid (pH adjusted 7.0)	–	–	–	–
Kombucha (pH 2.6)	+++	+++	++++	++++
Kombucha (pH adjusted to 7.0)	+++	+++	++++	++++
Kombucha after removal of tannins	+++	+++	++++	++++
Unfermented tea	–	–	–	–

Diameter of the inhibitory zone: ++ 15–20 mm; +++ 20–25 mm; ++++ 25–30 mm.
Acid concentration was the same as in Kombucha after 14 days of fermentation.

Tannins

The role of tannins present in tea in the antimicrobial activity of Kombucha was investigated by removing the tannins from Kombucha (Tab. 2). It had been observed that there is no antimicrobial activity in unfermented tea extract that contains tannins. This excludes the possibility that tannins in the concentration used for making Kombucha are an antimicrobial component. However, tannins present in tea extract could be metabolized during Kombucha fermentation into tannin derivatives that might be supposed to exert antimicrobial activities. Removal of tannins did not influence the antimicrobial activity of Kombucha. This suggests that tannins present in Kombucha do not play a role in antimicrobial activity, either in their original form or as a derivative.

Tab. 3. Effect of extraction with organic solvents on the antimicrobial activity of Kombucha

Extraction solvent	Testing phase	Target organism			
		E. coli	*S. sonnei*	*S. typhimurium*	*S. enteritidis*
n-Butanol	organic phase (pH 7.0)	–	–	–	–
	aqueous phase (pH 7.0)	++++	++++	+++++	+++++
	organic phase (pH 7.0 and heated)	–	–	–	–
	aqueous phase (pH 7.0 and heated)	++++	++++	+++++	+++++
Diethyl ether	organic phase (pH 7.0)	–	–	–	–
	aqueous phase (pH 7.0)	++++	++++	++++	+++++
	organic phase (pH 7.0 and heated)	–	–	–	–
	aqueous phase (pH 7.0 and heated)	++++	++++	++++	+++++
n-Propanol	organic phase (pH 7.0)	–	–	–	–
	aqueous phase (pH 7.0)	++++	++++	+++++	+++++
	organic phase (pH 7.0 and heated)	–	–	–	–
	aqueous phase (pH 7.0 and heated)	++++	++++	+++++	+++++
Chloroform	organic phase (pH 7.0)	–	–	–	–
	aqueous phase (pH 7.0)	++++	++++	+++++	+++++
	organic phase (pH 7.0 and heated)	–	–	–	–
	aqueous phase (pH 7.0 and heated)	++++	++++	+++++	+++++

Diameter of the inhibitory zone: ++++ 25–30 mm; +++++ 30–35 mm.
Acid concentration was the same as in Kombucha after 14 days of fermentation.

Organic Solvent Extraction

Antimicrobial activity of Kombucha was further characterized by extracting Kombucha with different organic solvents (Tab. 3) to investigate whether the active component is water-soluble or not. It is clear that, under the conditions of using the organic solvents listed in Tab. 3, the organic phase of the extracts did not exert any antimicrobial activity. Furthermore, the antimicrobial activity of the aqueous phase was not influenced by heat treatment.

In conclusion, the active antimicrobial components that are formed during Kombucha fermentation and inhibit the growth of *Shigella sonnei*, *Escherichia coli*, *Salmonella enteritidis* and *Salmonella typhimurium* are substances other than organic acids and their synergistic effect, ethanol, proteins or tannins originally present in tea or their derivatives. The active components are water-soluble. They are very likely microbial metabolites produced by bacteria and yeasts during fermentation with tea and sugar as substrates.

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