U. of K. J. Agric. Sci. 13 (1), 2005

Studies on the Microbiology of Kombucha (Tea Fungus)

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Abstract: Kombucha (tea fungus) is an acidic fermented beverage from tea extract and sugar. During the course of fermentation, yeasts declined in number in the beginning and then increased towards the end of fermentation. Bacteria began growing from the start and dominated the fermentation process, but their numbers dropped sharply at the end of fermentation. The fermentation products were mostly organic acids, with acetic acid giving the flavour of the drink. The yeasts isolated from Kombucha were mainly *Debaryomyces,* and the bacterium of importance was *Acetobacter xylinum*. Kombucha`s antimicrobial activity was conferred by the organic acids, and this is commonly known for other acidic fermented foods and drinks.

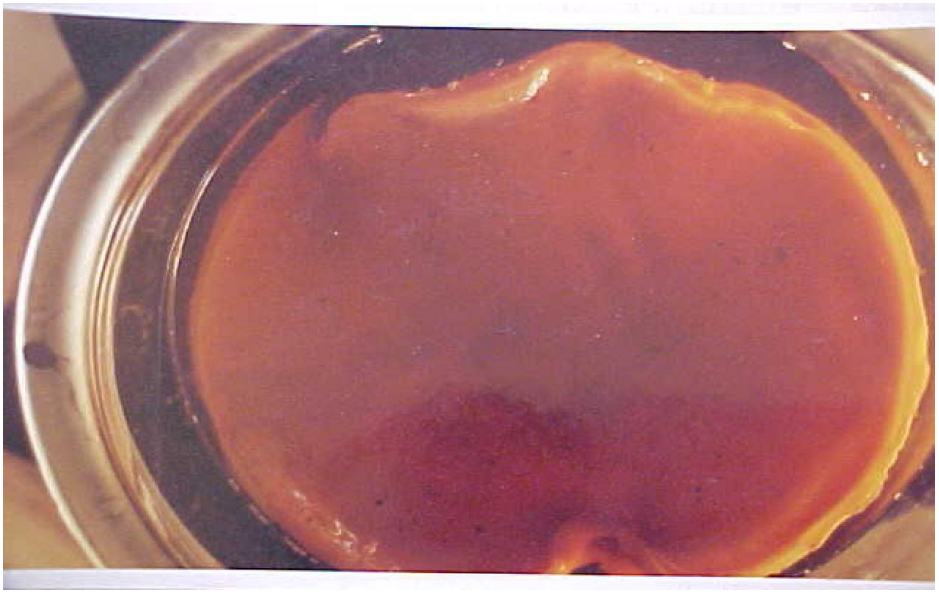
INTRODUCTION

Of all the traditional fermented foods and beverages none is more intriguing than Kombucha (tea fungus). It has gained worldwide fame as an alleged panacea for practically all chronic diseases of man. The product is better known in Russia, Japan, Bulgaria, Germany, Manchuria and Indonesia (Steinkraus 1983). It bears many names, including, honogo, wunderpilz, teekwass, magu, miracle fungus, fungus of a long life, Indian tea fungus, Indonesian tea fungus, fungus japonicus, etc. But the name Kombucha gained a wider recognition. It has been derived from the name of a Korean doctor, Kombo, who recommended it as medicine for an ailing Japanese emperor, Inkyo. Kombucha is said to have been used two thousand years ago by the Tsin dynasty of the ancient Chinese empire (Frank 1994).

Kombucha is a beverage which consists of a sugared aqueous extract of black tea on which a thick zoogloeal mat of a symbiotic growth of bacteria and yeasts develops. The mat could become as thick as 5 cm (Plates 1, 2 and 3). When consuming Kombucha, one drinks the liquor (the liquid part) and throws away the thick film. But, in the case of a similar Indonesian product called Nata, it is the thick mat that is eaten.

In the Sudan and Middle Eastern countries, the appearance of Kombucha is characterized by a cyclic nature. It appears suddenly and is consumed widely in a spate of activity for a year or two, and then it disappears just as sudden. For example, according to popular memory, it appeared in the Sudan in the late 1950s and early 1960s, when it was named *um-el-mihan*, and then disappeared for decades only to reappear in the period 1999-2001 when it was given the names *nebta* and *hajja-sukkara*. Consumers of Kombucha in the Sudan were often diabetics, hypertensives, barren women, malaria patients, depressed individuals and those suffering from gastritis. In the Sudan as well as in other Middle Eastern countries, the consumers of Kombucha attach to its alleged medicinal powers a nimbus of sanctity.

The objective of this study was to throw some light on the nature of the product by studying the microorganisms involved in the fermentation and their salient metabolic products, touching only lightly on the antimicrobial activity.



Plat 1. Surface view of Kombucha

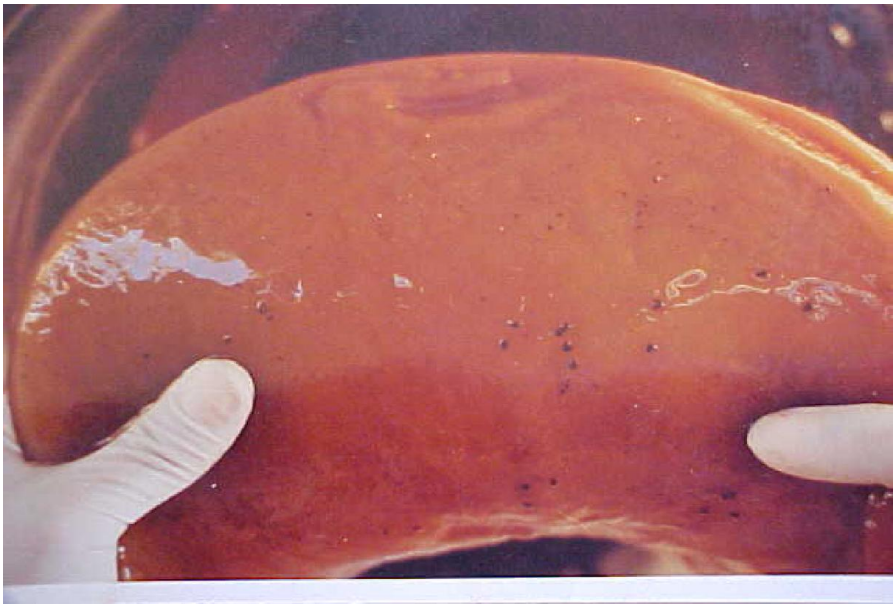


Plate 2. Kombucha mat held by hand

Plate 3. Kombucha mat cut by a pair of scissors

MATERIALS AND METHODS

Materials: The tea brand used in this study was the popular black tea *Algazaltain* (Cofftea, Khartoum) originally imported from Kenya. In preparing tea extract for Kombucha, 100 g of sugar were boiled in one litre of distilled water and then one gramme of tea leaves was added. After three minutes, the leaves were sieved off using an alcohol-sterilized plastic mesh sieve. This extract was cooled down to room temperature before it was inoculated with the starter, without further sterilization. The sugar used for growing Kombucha was white, refined sugar, available in the local market and originally produced by Kenana Sugar Company (Sudan).

The starter employed was a Kombucha sample obtained from the town of Halfa–el–Jedida, in eastern Sudan. This sample was preferred over

11 others that were collected from various parts of the country but which were grossly contaminated with moulds.

The acetic acid and lactic acid used in the antimicrobial tests (7.0 and 33.0 g/l, respectively) were pure Analar products.

Preparation of Kombucha: Kombucha`s zoogloeal mat usually grows in layers, floating on the surface of the tea extract. The youngest layers are found on the top of the mat, the older layers below, and may eventually drop to the bottom of the container.

To prime Kombucha for growth, a starter from the liquor of a previous batch (10% v/v) was added to the new tea extract using a sterile pipette. Attempts to use the part of the mat as starter were not as successful. It was found that teasing the layers apart or cutting the mat with sterile scissors was not convenient, and the process of obtaining such a starter subjected it to contamination. Moreover, it was observed that Kombucha from a liquor starter grew faster than that from a mat starter, but both types of starter gave the same final product.

In most cases, Kombucha was grown in a 500-ml sterile beaker and covered with a clean cheesecloth kept in place with a rubber band. The preparation was incubated at winter room temperature (25°C-28°C) for 7 days. This situation mimicked the commonly followed home process of Kombucha preparation which is not strictly sterile. Sampling was done by using sterile pipettes for the liquid part and sterile scissors for the mat. All samples were collected in sterile flasks or Petri dishes

Analytical methods: Moisture, ash, crude fibre, crude protein and carbohydrates content of Kombucha mat were determined according to AOAC (1985). Titratable acidity (as lactic acid) and total volatile acids (TVA) (as acetic acid) were determined according to AOAC (1975). Ethanol was estimated using the Veritable Ebulliometre (DUJARDIN-Salleron, Paris) technique. pH was measured with an AMEL Instruments model 338 pH meter. Samples for the titratable acidity, TVA, ethanol and pH determinations were taken from the liquor.

Microbiological methods: Total viable count of bacteria was carried out by the pour-plate method using Oxoid Plate Count Agar. Ten grammes of a blend of equal quantities of mat material and liquor were added to 90 ml of sterile peptone water to give the initial dilution from which further serial dilutions were made. The plates were incubated at 37°C for 48 hrs. Colony-forming units (cfu) were counted with the help of a colony counter (Scientifica and Cook, Ltd).

Isolation of bacteria for identification proved very difficult at first, because these organisms were embedded in the thick tough mat of Kombucha. It was only after blending equal weights of mat material and liquor in sterilized domestic blender (Moulinex, Paris) that the bacteria lended themselves for counting and isolation. Tentative identification of bacteria was done according to Bergey`s Manual of Systematic Bacteriology (Krieg and Holt 1984).

Yeasts were counted on Difco Malt Extract Agar, containing 0.1g of cholramphenicol per litre to inhibit bacterial growth. The spread-plate method was used, where 0.1 ml of a suitable peptone water dilution was spread evenly on the surface of the plate with the aid of a sterile bent glass rod. The plates were incubated at 28°C for 72 hrs. Colony-forming units were counted with a colony counter.

Isolation, purification and maintenance of yeast were done using Difco Wort Agar and Oxoid Potato Dextrose Agar in addition to Malt Agar. Tentative identification of yeast isolates was done according to Barnett *et al*. (1983) and Kreger van-Rij (1984).

Antimicrobial activity of Kombucha: Four materials were used to test for antimicrobial activity: Kombucha liquor, tea-acetic-lactic combination, acetic-lactic combination and tea extract alone. The combination of each component in these materials represented, more or less, its concentration in a three month-old Kombucha liquor.

The pathogenic bacteria used for the tests were *Salmonella typhi, Staphylococcus* sp., *Klebsiella* sp and *Pseudomonas* sp. They were all kindly provided by the Department of Microbiology of the Faculty of Veterinary Medicine, University of Khartoum. To run the test, each

organism was grown separately for 48 hrs in Oxoid Nutrient Broth at 37°C. Then, with a sterile cotton swab, part of the resulting growth was evenly spread on the surface of a petri plate of solidified Nutrient Broth with the aim of giving a lawn of bacterial growth following incubation.

In the meantime, Whatman filter paper discs (4 mm diameter), soaked in each of the test materials, were placed equidistant from each other, each on one quarter of a plate carrying one of the test organisms. The plates were then incubated for 24 hrs at 37°C, and the diameter of the clear zone around each disc was measured. The wider the clear zone the more powerful was the antimicrobial effect. Preliminary tests showed that distilled water as control had no antimicrobial effect.

RESULTS AND DISCUSSION

Proximate composition of Kombucha mat: As it is a product based on tea extract and some sugar, Kombucha is not considered a nourishing food. But because the thick mat of material on the surface of the product is intriguing to most people, it was decided to undertake proximate analysis of it.

Nine determinations gave the following average values after one month of fermentation: fibre 49.5%, carbohydrates 43.2%, crude protein 4.8%, ash 1.5% and fat 1%, on dry matter basis. The composition of a one year-old Kombucha was carbohydrates 57.59%, crude fibre 30.21%, protein 8.7%, ash 3% and fat 0.5%. Clearly, the fibre and the carbohydrates formed the bulk of the mat. The Kombucha that was kept for one year stayed alive during all that period even though it was furnished with no additional nutrients, other than distilled water. The changes in the proportions of the various components of the mat suggest that Kombucha survived by consuming the fibre which it had accumulated earlier. Fibre content dropped within one year from 49.50% to 30.21%. Carbohydrates, however, increased from 43.2% to 57.59%. Apparently, the organisms hydrolyzed the fibre to smaller carbohydrates faster than they consumed the latter. The breakdown of fibre needs extra-cellular enzymes, which are, of course, proteins. The increase of the protein fraction of the mat during the course of the year from 4.80% to 8.7% attests to this. Hoffman

(2000) is of the opinion that proteins of Kombucha must consist of the enzymes needed to break down complex nutrients.

Many factors were found to influence the weight and thickness of the Kombucha mat. For the tea concentrations of 0.38%, 1.5% and 3.0%, the corresponding Kombucha dry weights were 2.0, 2.5 and 3.0 g, respectively, after 7 days. Sugar concentrations of 1.25%, 5.0% and 10% gave mat dry weights of 0.7, 1.1 and 1.4 g. Inoculum percentages of 3, 11.25 and 16 gave dry weights of 0.7. 0.9 and 2.3 g, respectively. The thicknesses of the mats produced in this experiment were 4.0, 4.8 and 6.0 cm, respectively.

The progress of the fermentation process: Fig. 1 gives an overview of the progress in the fermentation process in Kombucha. At the beginning of a new fermentation batch, the yeast count was high but continued to decrease till the fifth day when it picked up growth once more and continued to rise to the end of the experiment. The bacterial population, on the other hand, started at a lower level but continued to grow, dominating the fermentation till the sixth day when it dropped sharply. At the end of the fermentation, the yeast count was higher than that of the bacteria. This explains the high count of yeast at the beginning of the experiments, since the starter was taken from Kombucha at the end of its fermentation.

Paralleling the increase in bacterial counts there was a steady increase in titratable acids (as lactic). The increase in total volatile acids (as acetic) was slow at the beginning, but the rate of production of this group of acids began to increase at day 5 and the increase continued to the end of the experiment. The pH value of Kombucha remained around 4.0 throughout the experiment and all attempts to demonstrate the presence of ethanol failed.

The fermentation products: Kombucha is an acidic product. It was repeatedly noticed that once the 10% inoculum from a previous fermentation batch was added to the fresh tea extract (pH 6.2), the pH dropped immediately to around 4.0 or even less. There is no plausible explanation to fully account for this. Possibly, when the hydrogen ion concentration of a starter of pH 3.0 was diluted 10 times, the pH rose one unit to pH 4.0. However, it is possible that Kombucha has a powerful

buffering capacity around pH 4.0 that buffers any sudden changes in pH. In this respect, it was noticed that the value to which the pH drops is close to the pKa of the two most common fermented acids, viz, lactic (pKa 3.86) and acetic (pKa4.7). However, the low pH of Kombucha must contribute substantially to its safety aspects, as most foodborne pathogens do not survive such acidity (Adams and Moss 2000). In fact, Mayser *et al*. (1995) noted the low rate of contamination of Kombucha with harmful microorganisms and Stone (1998) found no evidence of pathogen contamination in this product.

Since Kombucha is often described as an acetic acid-flavoured fermented tea beverage (Mayser *et al*. 1995), an attempt was made to find out the effect of fermentation temperature on the pattern of the production of total volatile acids production. As depicted in Fig. 2, wavy curves were obtained at all degrees of temperature tested. Production of volatile fatty acids was high at first then dropped, only to rise once more. The undulations were more pronounced at the lower temperatures (25°C and 30°C) than at the higher temperatures (37°C and 40°C). The rise in acetic acid production is understandable, as a known activity of the microorganisms involved, but the drop cannot be easily explained. Perhaps these changes reflect some microbial succession. However, evaporation of acetic acid does take place. It was noticed that, when Kombucha was placed in an incubator, the evaporated acetic acid dissolved the lacquer coat of the inner walls of the incubator.

The titratable acidity (Fig. 3) did not show the same phenomenon as the volatile acids. Titratable acidity (as lactic) remained at a constant level for each degree of temperature, and very little variation was found between temperatures.

The microorganisms of Kombucha: According to the literature, the microorganisms of Kombucha consist of yeasts and acetic acid bacteria. The yeast breaks down sucrose into glucose and fructose. It, then, uses the sugars to produce ethanol and carbon dioxide under anaerobic conditions. The bacteria initially oxidize ethanol to acetaldehyde and then to acetic acid, which accumulates. A secondary activity of the bacteria is the oxidation of glucose to gluconic acid and conversion of the same sugar to

microbial cellulose which forms the matrix of the Kombucha mat (Asai 1968; Krieg and Holt 1984; Greenwalt *et al.* 1998).

In the initial experiments, it was very difficult to isolate the bacteria, while the yeasts were easy to isolate. Under the microscope, the bacteria were found embedded inside the fibre strips, while the yeasts were arranged on the outside of it. This explained the difficulty of isolating the bacteria. Blending the sample solved the problem.

The bacteria isolated from Kombucha were of limited types. Five of seven isolates belonged to the genus *Acetobacter,* while the remaining two were *Bacillus* spp. As the *Acetobacter* was capable of forming a thick pellicle on liquid media, it was assumed that the organism was *A. xylinum.* According to Stanier *et al.* (1987), *A. xylinum* is one of a few procaryotes that synthesize cellulose. Steinkraus *et al*. (1996) and Mayser *et al.* (1995) stated that *A. xylinum* is the characteristic and the principal organism in Kombucha. As far as we know, *Bacillus* has not been reported to be a component of the micro-biota of Kombucha. Therefore, it is considered a contaminant from the dust commonly encountered in the Sudan.

Four of the six yeast isolates belonged to the genus *Debaryomyces*; the remaining two were *Candida* and *Pichia*. Although the presence of the latter two organisms in Kombucha has been reported in the literature (Mayser *et al.* 1995; Andre, 1998), to our knowledge, there is no report on the presence of *Debaryomyces.* The yeasts most commonly reported are *Zygosaccharomyces rouxii* and *Saccharomyces cerevisiae* (Blanc 1996; Greenwalt *et al*. 2000; Kurtzman *et al.* 2001*).*

The antimicrobial activity of Kombucha: The antimicrobial activity of Kombucha has been a controversial matter. Hesseltine (1965) reported antimicrobial activity of neutralized Kombucha against *Agrobacterium tumefaciens*. Greenwalt *et al*. (1998) demonstrated antimicrobial effect of Kombucha against many bacteria including some food-borne pathogens such as *Bacillus cereus, Salmonella typhimurium, Staphylococcus aureus and Escherichia coli.* Guttapadu and Sreeramulu (2000) also reported antimicrobial effect of Kombucha against foodborne pathogens such as *Camplylobacter jejuni, Salmonella enteritidis, Staphylococcus aureus,*

Listeria sp., *Yersinia enterocolitic,, Helicobacter pylori* and *Pseudomonas aeruginosa.*
On the other hand, Steinkraus *et al*. (1996) stated that no antimicrobial effect was observed when Kombucha was tested against *E. coli., S. aureus, H. pylori* and *A. tumefaciens.* Karamadin *et al.* (2001) showed that although Kombucha had antimicrobial activity in its normal acidic reaction against *Staphylococcus* sp. and *Streptococcus* sp, it had no such effect when its acidity was neutralized.

A limited research was carried out to check the extent of the antimicrobial activity of Kombucha and its components. Both the combinations lactic-acetic-tea extract and lactic-acetic exhibited more antimicrobial activity against the test organisms than did Kombucha (Table 1). It was concluded that the alleged antimicrobial activity of Kombucha is basically conferred by the organic acids produced during fermentation. This occurs in many acid-fermented foods (Jay 1992) and is not unique to Kombucha which thus seems not to have a unique antimicrobial capacity.

Table 1. Diameter (cm) of clear zone of antibacterial activity of Kombucha liquor and its components

Tested microorganisms	Tested solutions			
	Lactic acid+ acetic acid+ tea	Lactic acid + acetic acid	Kombucha	Tea
Klebsiella sp.	4.4	3.4	2.7	0.4
Salmonella sp.	6.8	6.1	2.2	3.2
Pseudomonas sp.	7.3	5.4	4.2	2.0
Staphylococcus sp.	4.8	4.4	2.2	1.8
Average	6.3	5.9	2.8	1.8

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مجلة جامعة الخرطوم للعلوم الزراعية: المجلد الثالث عشر- العدد الأول ، 2005م

دراسات على ميكروبيولوجيا الكمبوشا (فطر الشاى)

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موجز البحث: الكمبوشا (فطر الشاى) مشروب يعد بتخمير مستخلص الشاى والسكر تخميراً حمضياً . أثناء عملية التخمير ، تتناقص أعداد الخمائر فى بداية العملية ولكنها تأخذ فى التزايد بنهاية التخمير . أما أعداد البكتيريا فتأخذ فى الازدياد من البداية وتسود عملية التخمير ولكن أعدادها تنخفض بسرعة فى آخر العملية . منتجات التخمير هى الأحماض العضوية وتعزى إليها نكهة المشروب لوجود حمض الخليك بينها . وتنتمى معظم الخمائر التى عزلت لجنس *Debaryomyces* ، وكانت البكتيريا ذات الأهمية هى *Acetobacter xylinum* . أما مقدرة الكمبوشا على قتل الأحياء فتعزى للأحماض العضوية وهذا معروف فى التخمرات الحمضية الأخرى .