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ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF LEMON BALM KOMBUCHA

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Kombucha is a beverage traditionally produced by metabolic activity of yeasts and acetic acid bacteria. The antimicrobial activity of lemon balm Kombucha as well as of particular control samples was determined by agar-well diffusion method. Antioxidant activity on stable 1,1-diphenyl-2-picrylhydrazyl radicals of lemon balm Kombucha and lemon balm tea was determined by electron spin resonance spectroscopy. Acetic acid, Kombucha samples and heat-denaturated Kombucha showed significant antimicrobial activity against bacteria. However, there was no activity against yeasts and moulds. Kombucha showed higher antioxidant activity than tea sample for all applied sample volumes.

KEYWORDS: antimicrobial activity, antioxidant activity, lemon balm Kombucha

INTRODUCTION

Kombucha is a fermented beverage with a history of several thousand years in the East and yet is quite popular today in the West. The beverage has been claimed to be a prophylactic agent and to be beneficial to human health – as a diuretic in edemas, in arterosclerosis, in case of gout, sluggish bowels, for stones, etc. (1-3). Experience has also shown the Kombucha beverage regulate the intestinal flora, strengthen the cells, harmonize the metabolism, act as a natural antibiotic and help maintain the pH, e.g. the acid-alkaline balance of the body (3). However, many of these claims remains have to be proved.

Kombucha is traditionally prepared by fermenting sweetened (sucrose) black tea (*Camelia sinensis* L.). This medium is usually inoculated with cellulose pellicle formed during the previous cultivation, popularly known as a ″tea fungus″, and incubated statically under aerobic conditions for 7-10 days (2-4).

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This so-called ″tea fungus″ is actually a symbiosis of acetic acid bacteria (*Acetobacter xylinum, Acetobacter aceti*, *Gluconobacter oxydans*) (5) and yeasts (*Saccharomyces* sp., *Zygosaccharomyces* sp., *Torulopsis* sp., *Pichia* sp., *Brettanomyces* sp.) (2,6). The yeasts ferment the sugar in the cultivation medium to ethanol, which is further oxidised by the acetic acid bacteria to acetic acid. The result is a reduced pH of the medium. The final product is a sour, slightly carbonated, acidic beverage, comprised of sugars, organic acids, tea components, vitamins, and minerals, resembling cider. Many flavour compounds, including alcohols, aldehydes, ketones, esters and amino acids have been identified (1,7,8).

The tea in the cultivation medium provides tea fungus with the necessary nitrogen compounds, of which especially important are purine derivatives (caffeine and theophylline), amply present in black tea (7). Because of that, sweetened black tea has been the traditional and almost only recommended medium for preparing Kombucha.

Studies of some alternative cultivation media have shown that green tea has more stimulating effect on the Kombucha fermentation than black tea, yielding the fermentation product in a shorter time frame (9). The stimulative effect of green tea on Kombucha culture was explained by a higher caffeine content compared to black tea (10). Sweetened tea of *Echinacea purpurea* L. can also be used for Kombucha fermentation and obtained beverage has outstanding antioxidant properties (11). It is known that *Echinacea* spp. herbal medicines and dietary supplements are traditionally used as immunostimulants in treatment of inflammatory and viral diseases. Also, Kombucha can be successfuly obtained from peppermint tea (12).

Our previous study have shown that the use of lemon balm tea (*Melissa officinalis* L.) as an alternative medium, yields Kombucha beverage in a shorter time than in case of black tea (13). Besides, lemon balm is a well-known herb used to give fragnance to the different food and beverage products. It has also been used as a medicinal plant for treatment of headaches, gastrointestinal disorders, nervousness and rheumatism. The esential oil is a well-known antibacterial and antifungal agent, and it is also responsible for the mild depressive and spasmolytic properties of the plant (14). Because of these effects, lemon balm tea was used as a medium for Kombucha fermentation in the present work.

EXPERIMENTAL

Cultural conditions of the tea fungus

Substrate for Kombucha fermentation was prepared by adding 70 g/l of commercial sucrose to tap water and after boiling 5 g/l of dry crushed leaves of lemon balm (*Melissa officinalis* L.) were added. The tea leaves were steeped for 15 minutes and removed by filtration. After cooling to about 30^0C, the inoculum (Kombucha beverage from previous process) was added in an amount of 10% (v/v). Then the 0.33 l of prepared medium was poured into small flasks (Ø=8 cm, capacity 0.72 l) and incubated under aerobic conditions at 28^0C.

Chemical analyses

The pH value of fermented liquid samples was determined by electronic pH-meter (HI 9321).

Total acidity of fermented beverage samples was determined by potentiometric titration with NaOH, c = 0.1 mol/l, after the removal of CO_2 (15).

Samples. Samples for the derermination antimicrobial activity were:

- Kombucha beverage (afrer three days of cultivation, total acidity=4.56 g/l),
- acetic acid solution at the same concentration as in fermented tea (4.56 g/l),
- unfermented tea sample (5 g/l), neutralized Kombucha sample (prepared by neutralizing Kombucha beverage with 0.1 M NaOH),
- heat-denaturated Kombucha (treated at 100°C for 10 min.).

Samples were filtered through a sterile microfilter (0.22 μm) to remove cells.

Test microorganisms. Gram negative bacteria: *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (ATCC 35659), *Escherichia coli* (ATCC 25922), *Erwinia carotovora* (NCPPB 595), Gram positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 10876)*, Sarcina lutea* (ATCC 9341), yeasts: *Saccharomyces cerevisiae* (112, Hefebank Weihenstephan), *Candida pseudotropicalis* (clinical isolate), *Rhodotorula* sp. (natural isolate) and moulds: *Penicillium aurantiogriseum* (natural isolate), *Aspergillus niger* (natural isolate) i *Aspergillus flavus* (natural isolate) were used as test microorganisms.

Antimicrobial activity. Antimicrobial activity was determined by agar-well diffusion method. The strains were grown on Mueller-Hinton (bacteria) or Sabouraud Dextrose (yeasts and moulds) slants 24 h at 37 or 25 ^{0}C and checked for purity. After incubation the cells were washed from the agar surface and suspended in sterile phisyological solution. The number of cells in 1 ml of the suspension for inoculation measured by Mc Farland nefelometer was 1×10^7 cfu ml^{-1}. A volume of 1 ml of this suspensions was homogenized with 19 ml of melted (45 ^{0}C) Mueller-Hinton or Sabouraud Dextrose Agar and poured into Petri dishes. Wells of 9 mm diameter were made with a sterile metal tube by means of a vacuum pump. Sterile samples (100 μl) were then transferred into the wells of agar plates inoculated with test microorganisms. Plates were incubated at 37°C (bacteria) or 25°C (yeasts and moulds) for 24 hours and diameter of halo zones were measured. The evaluation of antimicrobial activities of samples was carried out in three repetitions.

Antioxidant activity. Radicals scavening activity of lemon balm tea and Kombucha samples against stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was determined by electron spin resonance (ESR) spectroscopy.

Blank probe was obtained by mixing 400 μl of 0.4 mM methanolic solution of DPPH and 400 μl of water. A volume of x μl of investigated sample was added to (400-x) μl of water and 400 μl of 0.4 mM methanolic solution of DPPH. After that the mixture was stirred for 2 min and transferred to a quartz flat cell ER-160FT.

The ESR spectra were recorded on an ESR spectrometer Bruker 300E (Rheinstetten, Germany) under the following conditions: field modulation 100kHz, modulation amplitude 0.256G, receiver gain $2x10^4$, time constant 40.96 ms, conversion time 327.68 ms, center field 3440.00 G, sweep width 100.00 G, x-band frequency 9.45 GHz, power 7.96 mW, temperature 23ºC.

The DPPH radical scavening activity (AA_{DPPH}) was calculated according to the formula:

$$AA_{DPPH}(\%)=100x(h_0-h_x)/h_0$$

where h_0 – the height of the second peak in the ESR spectrum of DPPH free radicals of the blank and h_x – the height of the second peak in the ESR spectrum of DPPH free radicals of the test solution.

RESULTS AND DISCUSSION

The antimicrobial activity of lemon balm Kombucha and control samples is shown in Table 1. Cultivation process lasted 3 days, and Kombucha had the following parameters: pH=2.89±0.05 and titratable acidity=4.56±0.03 g/l. The third day was chosen as the end of the process because on that day Kombucha acheived optimal consuming acidity (3.5-4.5g/l) (16).

It is obvious from Table 1 that Kombucha, acetic acid solution, and heat-denaturated Kombucha, have expressive bactericidal activity. Antimicrobial activity was determined toward *Sarcina lutea*, too, but there was no any activity. Acetic acid has the highest activity against all bacteria, but the differences are not high (diameter of the halo zone is about 2-3 mm larger than for the other samples). The largest halo zones showed *Erwinia carotovora*, and the smallest *Bacillus cereus.* Neutralized Kombucha showed bacteriostatic activity only against *Escherichia coli* (halo zone was 30±0.00 mm). Heat denaturated Kombucha was tested to check whether the active antimicrobial components are thermostable, to confirm whether the active components are large proteins. Samples were not inhibitory toward tested yeasts (*Saccharomyces cerevisiae*, *Candida pseudotropicalis*, *Rhodotorula* sp.) and moulds (*Aspergillus niger, Aspergillus flavus, Penicillium aurantiogriseum*), probably because the yeasts and moulds as acidophilic organisms are more resistant to organic acids. Because of that, potential danger from contamination with moulds exists in case of growing Kombucha at home. Although there are numerous reports that the polyphenols/tannins extracted from tea inhibit a broad spectrum of Gram-positive and Gram-negative bacteria (17), in this study, the unfermented tea did not show any antimicrobial activity against test microorganisms. It is probably because the concentration of tea broth was 0.5% and the polyphenol/tannin level at such a low concentration of tea was unlikely to have an inhibitory effect against test microorganisms. Namely, Greenwalt et al. (9) showed that inhibitory effects of Kombucha increased with the increasing tea concentration.

In previous studies, the antimicrobial activity of traditional Kombucha (from black tea) and control samples was determined (18).

Table 1. Antimicrobial activity of lemon balm Kombucha (diameter (mm) of the halo $zone_{mean}$ including well (9 mm) ± SD)

MICROORGANISM	Kombucha		Acetic acid C=4.56 g/l		Heat denaturated Kombucha	
	A	B	A	B	A	B
Salmonella enteritidis	13.85±0.54	28.12±1.2	17.23±0.36	Ø	15.25±0.32	28.67±0.89
Escherichia coli	13.67±1.54	30±0.0	16.67±0.58	Ø	14.4±0.89	30±0.0
Proteus mirabilis	15±1.0	Ø	17.75±0.96	Ø	17.0± 0.82	Ø
Pseudomonas aeruginosa	14.4±0.89	Ø	17.0±0.71	Ø	16±0.00	Ø
Staphylococcus Aureus	16.0±1.22	Ø	16.8± 2.17	Ø	15.8±1.64	Ø
Bacillus cereus	14.33±1.54	Ø	15.0±1.73	Ø	14.25±1.7	Ø
Erwinia carotovora	17.83±1.18	Ø	22.8±0.84	Ø	21.6±0.89	Ø

A – microbicidal activity; B – microbiostatic activity; Ø – no activity (growth inside the wells);
+/- -boundary antimicrobial activity (without growth inside and on brim of wells, zone about 9 mm)

Cultivation process lasted 8 days, and Kombucha had pH=2.87±0.01 and titratable acidity=3.55±0.03 g/l. Results are shown in Table 2.

Table 2. Antimicrobial activity of traditional Kombucha (diameter of the halo $zone_{mean}$ (mm) including well (9 mm) ± SD)

Microorganism	Kombucha		Acetic acid c=3.55 g/l		Kombucha pH=7		Heat denaturated Kombucha
	A	B	A	B	A	B	A
Salmonella enteritidis	12.33±0.58	29±1.73	13±0.58	Ø	Ø	24.67±0.58	12.67±0.92
Escherichia coli	13.67±0.58	Ø	13±0.5	Ø	Ø	Ø	13.25±1.12
Proteus mirabilis	Ø	15.67±0.58	Ø	17.33±0.58	+/-	20±0.0	Ø
Pseudomonas aeruginosa	12±0.0	Ø	12±0.0	Ø	Ø	Ø	11.33±0.0
Staphylococcus aureus	12.33±0.58	Ø	Ø	14±0.0	Ø	Ø	13.2±0.63
Bacillus cereus	9.33±1.53	10.33±0.71	10.33±1.5	10.67±0.58	9.33±1.35	Ø	9.2±1.23
Erwinia carotovora	14.33±1.23	19.3±0.0	15.45±1.25	Ø	9.55±1.05	22.33±0.0	14.5 ±0.5
Penicillium aurantiogriseum	+/-	Ø	+/-	Ø	Ø	Ø	Ø

A– microbicidal activity; B – microbiostatic activity; Ø – no activity (growth inside the wells); +/- - boundary antimicrobial activity (without growth inside and on brim of wells, zone about 9 mm)

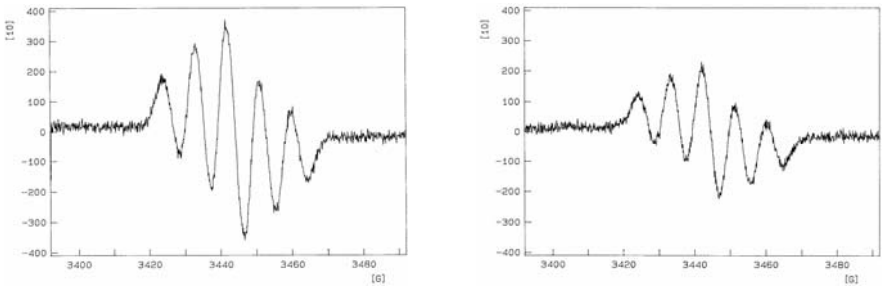
As can be seen from Table 2, Kombucha, acetic acid solution, and heat denaturated Kombucha have the most expressive antimicrobial activity. Their activity is similar against all bacteria except for *Staphylococcus aureus*, where acetic acid solution does not have bactericidal activity, only bacteriostatic. Neutralized Kombucha (pH=7) has bactericidal activity toward *Bacillus cereus* and *Erwinia carotovora*, while against *Salmonella enteritidis* and *Proteus mirabilis* has only bacteriostatic activity. None of the samples have antimicrobial activity against *Sarcina lutea.* Samples are not inhibitory toward yeasts (*Saccharomyces cerevisiae*, *Candida pseudotropicalis*, *Rhodotorula* sp.) and moulds (*Aspergillus niger* and *Aspergillus flavus*), except for *Penicillium aurantiogriseum*, where boundary microbicid activity of Kombucha and acetic acid was obtained. Unfermented tea does not show any antimicrobial activity against test microorganisms.

It is obvious that Kombucha from lemon balm tea has higher activity toward all bacteria than Kombucha from black tea. Also, Kombucha from lemon balm tea shows bactericidal activity against *Proteus mirabilis* and acetic acid solution against *Staphylococcus aureus.*

In both cases, acetic acid may be assumed to be the major antimicrobial agent. In some cases traditional Kombucha has higher activity than acetic acid, which implies the presence of an antimicrobial component other than acetic acid and large proteins. In both cases there is no effects against yeasts and moulds.

Greenwalt et al. (9) determined antimicrobial activities of Kombucha from black tea. Bactericidal effects toward *Escherichia coli*, *Staphylococcus aureus* and *Bacillus* sp. were much higher (3-5 times) than our results for lemon balm Kombucha. It is probably because acetic acid concentration in their Kombucha was 7 g/l, while in case of lemon balm Kombucha total acidity was 4.56 g/l, and it is known that acetic acid is the main antimicrobial agent. If acetic acid concentration in black tea Kombucha was 8.5 g/l, only bacteriostatic activities were measured (17). Comparing with lemon balm Kombucha, the activity is higher toward *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, but lower toward *Bacillus* sp.

The antioxidant activity of lemon balm tea and lemon balm Kombucha were investigated by ESR method in the DPPH model system. The ESR specta of stable DPPH radicals (blank) and DPPH radicals obtained in the presence of 100 μl lemon balm Kombucha are shown in Figure 1.



(a) (b)

Fig. 1. ESR spectra of stable DPPH free radicals: a) blank and b) in the presence of 100 µl of lemon balm Kombucha.

The influence of different volumes of lemon balm tea and lemon balm Kombucha on stable DPPH radicals is presented in Figure 2.

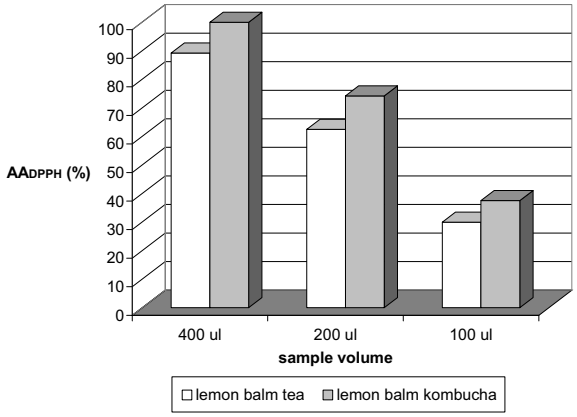


Fig. 2. Antioxidant activity of lemon balm tea and lemon balm Kombucha on stable DPPH radicals

It is visible that for tea samples and Kombucha samples with the increasing sample volume, antioxidant activity is increasing, too. Independent of sample volume, Kombucha has higher antioxidant activity than lemon balm tea. It is probably because of some metabolic products that are formed during the fermentation process (like vitamins C and B). Antioxidant activities on DPPH radicals of Kombucha made from echinacea tea and black tea, as well as of control samples, have been determined previously (19). For the same applied sample volumes (100 µl), Kombuchas from echinacea tea and black tea had higher activity (AA_{DPPH} is about 80%) than lemon balm Kombucha. As for tea samples, lemon balm tea had higher activity than black tea, but less then echinacea tea.

CONCLUSION

Kombucha, acetic acid solution, and heat-denaturated Kombucha samples showed significant antimicrobial activity against all bacteria except for *Sarcina lutea*, but there was no activity against moulds and yeasts. Neutralized Kombucha and unfermented tea did not show antimicrobial activity against test microorganisms. Lemon balm tea samples and Kombucha samples showed antioxidant activity against DPPH radicals in all applied sample volumes. Kombucha samples had higher antioxidant activity than tea samples.

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АНТИМИКРОБНА И АНТИОКСИДАТИВНА АКТИВНОСТ КОМБУХЕ ОД МЕЛИСЕ

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Комбуха је напитак који се традиционално добија метаболичком активношћу квасаца и бактерија сирћетног врења. У раду је испитана антимикробна активност Комбухе од мелисе и одговарајућих контролних узорака модификованом диск-дифузионом методом и антиоксидативна активност (на стабилне DPPH радикале) комбухе и чаја од мелисе електрон спин резонантном (ESR) спектроскопијом. Сирћетна киселина, комбуха и топлотно денатурисана комбуха показују значајну антимикробну активност према бактеријама, док није било деловања на квасце и плесни. Комбуха је показала већу антиоксидативну активност на DPPH радикале него узорци самог чаја, при свим примењеним количинама узорака.

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