LWT - Food Science and Technology 47 (2012) 71–77

Contents lists available at SciVerse ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Antimicrobial effect of Kombucha analogues

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ARTICLE INFO

Article history:
Received 7 May 2009
Received in revised form
24 December 2011
Accepted 31 December 2011

Keywords:
Kombucha
Fermented tea
Fermented medicinal herbal infusions
Antibacterial
Antifungal activity

ABSTRACT

Kombucha is a traditional refreshing beverage obtained by the fermentation of sugared tea with a powerful symbiosis of acetic bacteria and yeasts. This drink has been intensively consumed during a long time worldwide for its prophylactic and therapeutic properties. In the present study, we have screened traditional fermented black tea for antibacterial and antifungal activity against Gram (+) and Gram (−) human pathogenic bacteria ($n = 7$) and candida yeasts ($n = 7$) using agar diffusion method. Additionally, antimicrobial activity of five new fermented beverages produced from the following medicinal herbal extracts: *Thymus vulgaris* L., *Lippia citriodora*, *Rosmarinus officinalis*, *Foeniculum vulgare* and *Mentha piperita* noted as Kombucha-analogues were investigated, using agar diffusion method. Strong antimicrobial potentials were found with the new fermented beverages, particularly those prepared by the fermentation of *L. citriodora* and *F. vulgare* infusions, exhibiting the most important inhibition zone observed against the *Candida* strains tested (*Candida glabrata, Candida tropicalis, Candida sake, Candida dubliniensis and Candida albicans*). In view of their antimicrobial activity demonstrated against a range of pathogenic bacteria and against a number of clinical Candida species, the fermented *L. citriodora* and *F. vulgare* may be very healthful.

1. Introduction

Kombucha is a traditional fermented beverage with a history of several thousand years in the East and remains quite popular in the West. It is typically prepared by fermenting sweetened black tea with a popularly culture known as a "tea fungus", at room temperature for about 14 days (Anken & Kappel, 1992). The microbial ecology of the tea fungus has been investigated. Tea fungus is actually a symbiosis of yeasts and acetic bacteria (Kappel & Anken, 1993; Steinkraus, 1996). The main acetic acid bacteria found in the tea fungus were: *Acetobacter xylinum* (Balentine, 1997), *Acetobacter xylinoides*, *Bacterium gluconicum* (Reiss, 1994), *Acetobacter aceti*, *Acetobacter pasteurianus* (Liu, Hsu, Lee, & Liao, 1996). Yeasts identified in such fermented tea are *Schizosaccharomyces pombe*, *Saccharomycodes ludwigii*, *Kloeckera apiculata*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Torulaspora delbrueckii*, *Brettanomyces bruxellensis*, *Brettanomyces lambicus*, *Brettanomyces custersii*, *Candida stellata*. Furthermore, others *Candida* and *Pichia* species have been isolated from tea fungus (Balentine, 1997; Jankovic & Stojanovic, 1994; Liu et al., 1996; Mayser, Fromme, Leitzmann, & Gründer, 1995; Teoh, Heardb, & Cox, 2004).

Potential effects have increased the interest in Kombucha uses which ranged from weight loss to cancer and AIDS cure (Dufresne & Farnworth, 2000; Frank, 1995; Greenwalt, Ledford, & Steinkraus, 1998; Greenwalt, Steinkraus, & Ledford, 2000). However, only a few experimental investigations have demonstrated these mentioned properties. Indeed, recent studies have reported that Kombucha prevents the paracetamol induced hepatotoxicity (Pauline et al., 2001) and the chromate (VI) induced oxidative stress (Sai Ram et al., 2000). Moreover, regular Kombucha tea ingestion contributes significantly to weight gain inhibition and life elongation (Hartmann, Burleson, Holmes, & Geist, 2000). Particularly, Kombucha was proved to exert an antimicrobial activity against *Helicobacter pylori*, *Salmonella typhimurium, Staphylococcus aureus, Agrobacterium tumefaciens* (Greenwalt et al., 1998; Steinkraus, Shapiro, Hotchkiss, & Mortlock, 1996), *Bacillus cereus* (Greenwalt et al., 1998) *Shigella sonnei*, *Salmonella enteritidis* and *Escherichia coli* (Greenwalt et al., 1998; Sreeramulu, Zhu, & Knol, 2001; Steinkraus, 1996). Black and green teas are the usual and the best substrates known for the preparation of Kombucha drinks (Jayabalan, Marimuthu, & Swaminathan, 2006; Reiss, 1994). Recently, Velićanski, Dragoljub, Siniša, Vesna and Sladana (2007) reported the *Lemon balm* as an alternative cultivation media of

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doi:10.1016/j.lwt.2011.12.033

Kombucha. To the author's knowledge, no previous studies reported the antibacterial and the antifungal activities of other fermented Kombucha herbs besides tea.

In this work, we studied and compared the antimicrobial activity of Kombucha tea produced with black tea to five fermented common Tunisian plants, used as broth in traditional medicine: *Thymus vulgaris* L., *Lippia citriodora*, *Rosmarinus officinalis*, *Foeniculum vulgare* and *Mentha piperita*. Their effect against pathogenic bacteria and yeasts incriminated in candidosis cases were investigated for the first time in order to evidence an effective treatment, particularly for multi-resistant species.

2. Materials and methods

2.1. Starter culture

The tea fungus used in this study was a Japanese traditional culture (originated from Kambucha Kamp, Beverly Hills, CA, USA), kindly provided from Mr. lan Ogino, expert of Japan International Cooperation Agency. The starter used was a symbiotic culture between yeast and acetic bacteria mainly *A. xylinum*.

2.2. Preparation of fermented beverages

Kombucha was prepared using black tea (Les jardins du thé, Office Tunisien du Commerce, Tunisia). Infusions were prepared after mixing 10 g of dry tea leaves with 20 g sucrose in 1 L of boiled water and steeped for 15 min. The broth was cooled to room temperature and then leaves were separated. The resulting clear filtrate was poured into 250 mL flask. Then, the preparation was inoculated with 10 g/L of actively growing Kombucha culture. The inoculated flask was covered and incubated at room temperature. After 21 days of incubation, the fermented liquid was centrifuged at 300 g (JOUAN, Centrifuge BR 4i, ThermoFisher Scientific, Maryland, USA) for 15 min to remove cell debris and the supernatant was used for analysis. Unfermented broth was incubated simultaneously, as previously mentioned, and centrifuged supernatant was used for control analysis.

The same process was applied for the preparation of the new Kombucha analogue beverages, using separately *T. vulgaris* L. (thyme), *L. citriodora* (lemon verbena), *R. officinalis* (rosemary), *F. vulgare* (fennel) and *Me. piperita* (peppermint). These herbs were purchased from a local market (Tunisia).

For control purpose, unfermented and acidified infusions were used. Acetic acid solution at the same concentration as that of the fermented tea after 21 days was prepared and filter-sterilized for antimicrobial test as described above. In the same way, neutralised samples of fermented extract were obtained by adjusting the pH with NaOH (1 mol/L). Heat-denatured fermented samples were prepared by autoclaving the fermented beverage supernatant at 120 °C for 20 min to investigate the active components nature.

2.3. Target strains and cultivation conditions

2.3.1. Bacterial strains and cultivation

The human pathogens reference strains used for antimicrobial activity test included Gram-positive cocci: *Staphylococcus epidermidis* CIP 106510, *S. aureus* ATCC 25923, *Mi. luteus* NCIMB 8166 and Gram-negative bacteria: *E. coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *S. typhimurium* LT2 and *Listeria monocytogenes* ATCC 19115. The bacterial strains were grown on Müller Hinton agar (MH) plates (bioMérieux, France) at 37 °C for 18 h.

2.3.2. Candida yeast strains and cultivation

The human pathogenic yeasts used were isolated from patients suffering from candidosis. These strains were isolated on Sabouraud chloramphenicol agar plates (bioMérieux, France) and identified by Api ID 32 C test strips (bioMérieux, France) according to the Manufacturer's recommendations. Seven strains were used in this study: *Candida albicans*, *Candida krusei*, *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata*, *Candida dubliniensis* and *Candida sake*. The *Candida* strains were grown on Sabouraud chloramphenicol agar plates at 37 °C for 48 h prior to use.

2.4. pH determination

The pH of the fermented beverage was measured with an electronic pH-meter (Inolab, Level1, Weilheim, Germany).

2.5. Antimicrobial activity

2.5.1. Antibacterial activity of Kombucha analogue extracts

Antimicrobial activity was screened by agar diffusion assay as described by Sreeramulu et al. (2001). Fresh suspensions of target strain cultured for 24 h were spread on the MH agar plates (20 mL) uniformly at a rate of 10^6 CFU/mL, and wells of 6 mm diameter were performed with a sterile metallic-tube. Sterile supernatant was obtained by filtering the centrifuged infusions through a sterile 0.22 μm microfilter (Sterile microfilter Sartorius; Minisart, Göttingen, Germany). Sterile fermented samples of beverages (100 μL) were then transferred into the wells performed within the pre-inoculated plate with the target strain. The plates were cooled at 4 °C for 2 h to make a pre-diffusion of the beverages into the agar. The plates were then incubated at 37 °C for 18 h.

2.5.2. Anti-Candida activity of Kombucha analogue extracts

The anti-Candida activity was performed by the well agar diffusion method just described to evaluate the antibacterial potential but using the Sabouraud chloramphenicol agar plates (bioMérieux, France).

The diameter of the inhibition zone was measured after 18 h of incubation. The antimicrobial activity was evaluated by measuring inhibition diameter surrounding the wells. Each experiment was carried out in triplicate and results were expressed as means value ± standard deviation.

3. Results

3.1. pH variation

The initial pH of the various extracts varied from acidic pH (black tea) to slightly alkaline pH in the case of *L. citriodora* (Table 1). However, after 21 days of fermentation with Kombucha consortium, all the final products were characterized by their acidic

Table 1
pH of the different studied infusions at initial time and after 21 days of fermentation.

Beverage	*Camellia sinesis* (Black tea)	*Foeniculum vulgare*	*Mentha piperita*	*Rosmarinus officinalis*	*Thymus vulgaris*	*Lippia citriodora*
Unfermented	5.14	6.27	6.34	6.30	6.12	7.94
Fermented	2.59	2.58	2.71	2.40	3.15	2.88

pH, varying from 2.4 to 3.1; the extreme pH values were respectively of *R. officinalis* and *T. vulgaris*. Since all the extracts included sugar, their fermentations led to the formation of many organic acids which could be responsible of the biological activities of the resulting beverages. Furthermore, the acidification degree noted was variable according to the fermented plant, *R. officinalis* was the most acidified beverage and the highest pH modification was noticed with *L. citriodora* extract; the difference between initial and final fermented beverage was of 5 pH units. Indeed, organic acids are commonly used for food preservation against some food-borne microorganisms (Guiraud, 1998, pp.70–76).

3.2. Antibacterial activity of fermented infusions

The antibacterial activity of the studied fermented beverages against the pathogenic microorganisms tested is presented in Table 2. The results show that all the tested bacteria were sensitive to the fermented beverages resulting from *Camellia sinesis*, *L. citriodora* and *Me. piperita*, while 6 out of 7 of the strains were sensitive to *R. officinalis* and 5 out of 7 to the fermented *F. vulgare*. However, *T. vulgaris* fermented infusion did not exhibit any antibacterial activity. Particularly, stronger antibacterial potentials were noticed with these new Kombucha analogue beverages compared to the traditional Kombucha tea. The agar wells diffusion method indicated that Kombucha originated from *L. citriodora* showed the highest antibacterial activity against the seven bacterial strains tested, with inhibition zones ranging from 12.5 mm (*S. typhimurium*) to 27.5 mm (*L. monocytogenes*). In this respect, the second most effective Kombucha beverage was the *Me. piperita* fermented broth, which showed inhibition zones ranged between 10 mm (*E. coli*) and 28.5 mm (*L. monocytogenes*). *L. monocytogenes* was also strongly susceptible to *F. vulgare* as well as *R. officinalis* fermented broth. Furthermore, the fermented extract of *F. vulgare* was the most potent inhibitor of *S. epidermidis* with inhibition zone diameters 29 mm.

An important activity against *S. aureus* (25 mm) was also observed with the fermented *R. officinalis* infusion. Moreover, *P. aeruginosa* exhibited an interesting sensibility versus *L. citriodora*, followed by *F. vulgare*, black tea and finally *Me. piperita* and *black tea* fermented infusions. *F. vulgare*, *Me. piperita* and *Black tea* fermented then neutralized beverages exhibited an antimicrobial activity against *Mi. luteus* but less important than their corresponding fermented beverages. Such behaviour would explain the contribution of the organic acids of the beverage against the tested bacteria. All the unfermented infusions didn't show any antibacterial activity except *L. citriodora* which had an interesting activity against *P. aeruginosa*.

The neutralisation of the fermented beverages reduced (12%) or suppressed (87%) the antibacterial activity. The same behaviour was noted while acidifying the fermented beverage. Indeed, 39.5%

Table 2
Antibacterial activity of Kombucha.[a]

Plant species	Tested extracts	[a]Inhibition zone diameter (mm)						
		Target microorganisms						
		Staphylococcus epidermidis CIP 106510	*Staphylococcus aureus* ATCC 25923	*Micrococcus luteus* NCIMB 8166	*Salmonella typhimurium* LT2	*Escherichia coli* ATCC 35218	*Listeria monocytogenes* ATCC 19115	*Pseudomonas aeruginosa* ATCC 27853
Camellia sinesis (Black tea)	[b]Fermented infusion	18.5 ± 2.1	14.5 ± 2.1	16.5 ± 0.7	14.0 ± 1.4	11.0 ± 1.4	18.5 ± 2.1	19.0 ± 1.4
	[c]Unfermented infusion	6	6	6	6	6	6	6
	[d]Neutralized	6	9.5 ± 0.7	10.0 ± 0.0	6	6	6	6
	[e]Acidified infusion	6	6	6	6	6	27.0 ± 1.1	15.5 ± 0.7
	[f]Heat-denatured	16.5 ± 0.7	13.5 ± 2.1	13.5 ± 2.1	12.0 ± 0.0	13.0 ± 0.0	6	11.0 ± 0.0
Foeniculum vulgare	Fermented infusion	29.0 ± 0.0	6	21.0 ± 1.4	17.5 ± 0.7	6	26.5 ± 2.1	22.0 ± 1.41
	Unfermented infusion	6	6	6	6	6	6	6
	Neutralized	6	6	10.5 ± 0.7	6	6	6	6
	Acidified infusion	24.5 ± 2.1	26.0 ± 1.4	19.5 ± 0.7	16.0 ± 0.0	19.5 ± 0.7	28.0 ± 0.0	12.5 ± 0.7
	Heat-denatured	23.0 ± 0.0	24.5 ± 2.1	17.5 ± 0.7	12.0 ± 1.4	0	25.0 ± 0.0	9.0 ± 0.0
Lippia citriodora	Fermented infusion	23.5 ± 0.7	24.5 ± 2.1	21.5 ± 0.7	12.5 ± 0.7	17.5 ± 2.1	27.5 ± 0.7	23.5 ± 0.7
	Unfermented infusion	6	6	6	6	6	6	13.5 ± 0.7
	Neutralized	6	6	6	6	6	6	6
	Acidified infusion	17.0 ± 0.0	27.5 ± 0.7	11.0 ± 0.0	6	11.0 ± 0.0	25.0 ± 2.8	09.5 ± 0.7
	Heat-denatured	6	26.5 ± 2.1	16.0 ± 0.0	6	11.5 ± 0.7	26.5 ± 3.5	11.0 ± 1.4
Mentha piperita	Fermented infusion	20.5 ± 2.1	24.5 ± 0.7	19.5 ± 0.7	22.5 ± 2.1	10.0 ± 1.4	28.5 ± 2.1	14.0 ± 2.8
	Unfermented infusion	6	6	6	6	6	6	6
	Neutralized	6	6	11.5 ± 0.7	6	6	6	6
	Acidified infusion	17.5 ± 0.7	6	16.0 ± 1.4	10.5 ± 2.1	12.0 ± 1.4	27.5 ± 3.5	12.0 ± 1.4
	Heat-denatured	24.0 ± 0.0	6	16.0 ± 0.0	17.0 ± 0.0	0	26.0 ± 1.4	10.5 ± 0.7
Rosmarinus officinalis	Fermented infusion	14.5 ± 0.70	25.0 ± 0.0	15.5 ± 2.1	6	13.5 ± 4.9	17.5 ± 0.7	13.5 ± 0.7
	Unfermented infusion	6	6	6	6	6	6	6
	Neutralized	6	6	6	6	6	6	6
	Acidified infusion	6	6	16.5 ± 2.1	6	14.0 ± 0.0	6	14.0 ± 1.4
	Heat-denatured	6	6	12.0 ± 0.0	16.5 ± 0.7	9.0 ± 0.0	6	9.0 ± 0.0
Thymus vulgaris	Fermented infusion	6	6	6	6	6	6	6
	Unfermented infusion	6	6	6	6	6	6	6
	Neutralized	6	6	6	6	6	6	6
	Acidified infusion	6	6	6	6	6	6	6
	Heat-denatured	6	6	6	6	6	6	6

[a] Inhibition zone diameter (means value and standard deviation including wells diameter of 6 mm). Each experiment was carried out in triplicate.
[b] Fermented infusion (Kombucha) at natural pH value without any adjustment.
[c] Unfermented infusion prepared in the same way as that for making Kombucha, and HCl (1 mol/L) or NaOH (1 mol/L) was used to adjust their pH.
[d] Neutralized Kombucha: pH 7 fermented infusion adjusted with NaOH (1 mol/L).
[e] Acidified infusion with acetic acid according to the acidity of Kombucha pH 2.6 samples.
[f] Heat-denatured fermented infusions were treated at 120 °C for 20 min.

of the activity decreased while 27.5% of the activity disappeared, 18.1% remained the same and 15.1% increased. As a consequence of the thermal treatment, antimicrobial activity against the different tested bacteria varied with a general decrease note in half of the studied cases (56%), followed by a disappearance of the activity (25%), then an increase and finally the same antibacterial activity was held in 6% of the cases.

It should be mentioned that being initially resistant to the fermented product, *S. typhimurium* became sensitive after thermal treatment of the *R. officinalis* Kombucha analogue while *E. coli* became sensitive to the acidified infusion of *F. vulgare*.

3.3. Screening for anti-Candida activity of Kombucha extracts

Table 3 summarizes the resulting antifungal activity of Kombucha drinks against *Candida* strains. In spite of their known resistance, over the seven tested *Candida*, four strains were inhibited by conventional Kombucha. However, *R. officinalis* and *T. vulgaris* fermented beverages were not active against all of the tested yeasts. Relatively larger inhibition zones of the Kombucha analogue beverages were observed compared to the traditional Kombucha produced from black tea. Indeed, *C. albicans*, *C. dubliniensis* and *C. tropicalis* were the most sensible to the four fermented beverages: black tea, *F. vulgare*, *Me. piperita* and *L. citriodora*, followed by *C. glabrata* which was resistant towards the fermented *Me. piperita*. *C. parapsilosis* was shown to be sensitive to fermented *F. vulgare* and *Me. piperita* while *C. sake* was inhibited by the fermented *F. vulgare* and *L. citriodora*. Finally, the fermented *F. vulgare* acted moderately on *C. krusei*.

The heat treatment of the fermented product affected the observed antifungal activity over all the exhibited activities. In 40% of the total fermented beverages studied, the activity disappeared, it was improved in 30%, remained constant in 25% and finally it decreased in 5%. Nevertheless, the unfermented infusions showed no antimicrobial activity against most of the target *Candida*, except for *L. citriodora* where *C. glabrata* and *C. dubliniensis* exhibited an interesting sensibility to this infusion. Neutralised fermented beverage lost its antimicrobial effect. All the seven strains of tested *Candida* were inhibited by fermented infusion of *F. vulgare* with inhibition zones ranging from 9 mm for *C. krusei* to 14.5 mm for *C. albicans*. The *L. citriodora* fermented beverage showed the most important inhibition zones ranged from 12.5 mm to 14.0 mm. However, *C. parapsilosis* and *C. krusei* growth were not affected by the last mentioned Kombucha analogue beverage.

4. Discussion

The tested antimicrobial potency of original Kombucha tea in addition to the five fermented medicinal plant extracts were investigated for the first time against seven pathogenic bacteria and seven *Candida* yeasts. The infusions prepared from the following Tunisian medicinal herbs: *T. vulgaris* L., *L. citriodora*, *R. officinalis*,

Table 3
Antifungal activity of Kombucha beverages.[a]

Plant species	Tested extracts	[a]Inhibition zone diameter (mm)						
		Target microorganisms						
		Candida glabrata	*Candida parapsilosis*	*Candida tropicalis*	*Candida sake*	*Candida dubliniensis*	*Candida krusei*	*Candida albicans*
Camellia sinesis (Black tea)	[b]Fermented infusion	11.0 ± 1.4	6	11.5 ± 0.7	6	12.0 ± 1.4	6	11.0 ± 0.0
	[c]Unfermented infusion	10.5 ± 0.7	6	6	6	6	6	6
	[d]Neutralized	6	6	6	6	6	6	6
	[e]Acidified infusion	17.0 ± 0.0	16.5 ± 2.1	6	14.5 ± 0.7	18.5 ± 0.7	6	6
	[f]Heat-denatured	11.5 ± 0.7	6	6	6	14 ± 0.0	6	6
Foeniculum vulgare	Fermented infusion	12.0 ± 1.4	11.0 ± 0.0	13.5 ± 2.1	10.0 ± 0.0	13.5 ± 0.7	9.0 ± 0.0	14.5 ± 0.7
	Unfermented infusion	6	6	6	6	6	6	6
	Neutralized	6	6	6	6	6	6	6
	Acidified infusion	14.5 ± 0.7	14.0 ± 1.4	15.0 ± 2.8	17.5 ± 2.1	14.5 ± 0.7	6	6
	Heat-denatured	12.5 ± 0.7	11.0 ± 1.4	12.5 ± 0.0	10.5 ± 0.7	16.0 ± 1.4	6	6
Lippia citriodora	Fermented infusion	12.5 ± 0.7	6	14.0 ± 1.1	12.5 ± 0.7	13.5 ± 0.7	6	13.5 ± 2.1
	Unfermented infusion	13.5 ± 0.7	6	6	6	13.5 ± 0.7	6	6
	Neutralized	6	6	6	6	6	6	6
	Acidified infusion	6	6	6	11.0 ± 2.8	17 ± 2.8	6	6
	Heat-denatured	13.5 ± 0.7	14.0 ± 1.1	6	14.0 ± 1.4	14 ± 1.4	6	12.0 ± 1.4
Mentha piperita	Fermented infusion	6	13.0 ± 1.4	12.0 ± 0.0	6	12.0 ± 0.0	6	12.0 ± 1.4
	Unfermented infusion	6	6	6	6	6	6	6
	Neutralized	6	6	6	6	6	6	6
	Acidified infusion	6	14.0 ± 1.4	11.0 ± 1.4	14.5 ± 2.1	13.5 ± 3.5	6	6
	Heat-denatured	6	6	10.0 ± 0.0	6	6	6	6
Rosmarinus officinalis	Fermented infusion	6	6	6	6	6	6	6
	Unfermented infusion	6	6	6	6	6	6	6
	Neutralized	6	6	6	6	6	6	6
	Acidified infusion	14.0 ± 1.4	16.5 ± 0.7	6	6	6	6	6
	Heat-denatured	6	6	6	6	6	6	6
Thymus vulgaris	Fermented infusion	6	6	6	6	6	6	6
	Unfermented infusion	6	6	6	6	6	6	6
	Neutralized	6	6	6	6	6	6	6
	Acidified infusion	6	6	6	6	6	6	6
	Heat-denatured	6	6	6	6	6	6	6

[a] Inhibition zone diameter (means value and standard deviation including wells diameter of 6 mm). Each experiment was carried out in triplicate.
[b] Fermented infusion (Kombucha) at natural pH value without any adjustment.
[c] Unfermented infusion prepared in the same way as that for making Kombucha, and HCl (1 mol/L) or NaOH (1 mol/L) was used to adjust their pH.
[d] Neutralized Kombucha: pH 7 fermented infusion adjusted with NaOH (1 mol/L).
[e] Acidified infusion with acetic acid according to the acidity of Kombucha (pH = 2.6).
[f] Heat-denatured fermented infusions were treated at 120 °C for 20 min.

F. vulgare and *Me. piperita* were fermented with the Kombucha consortium during 21 days and then tested. The results showed that each of the fermented beverages tested (except *T. vulgaris*) have shown specifically antibacterial and antifungal activities. Data have shown that Kombucha beverages at their natural pH exerted the strongest antimicrobial effects; these activities were also exhibited after heating but with a decrease of 56% or an annulation of antifungal activity of 40%. In most of the cases, the highest levels of antimicrobial activity were recorded with *F. vulgare Kombucha*, *Me. piperita Kombucha* and *L. citriodora Kombucha*.

Many previous studies proved that Kombucha of fermented black tea exerts antibacterial activity against a broad range of bacteria (Greenwalt et al., 1998, 2000; Sreeramulu et al., 2001; Steinkraus, 1996), but antifungal effects have been poorly investigated.

In 1998, Greenwalt et al. have tested the antimicrobial potentials of Kombucha preparation from both black and green tea at 9 days of fermentation against *C. albicans* but didn't reveal any activity, while *A. tumefaciens*, *S. aureus* and *E. coli* were inhibited. This antibacterial activity was attributed to acetic acid content. Later, Sreeramulu, Zhu, and Knol (2000) have demonstrated that Kombucha prepared from black tea was able to inhibit *C. albicans* from the sixth-day to the fourteenth day of fermentation. In the present work, we have used Kombucha beverages at day 21 of fermentation for its antifungal activity. Interestingly, the original Kombucha tea has exhibited inhibitory activity against almost the *Candida* tested. This evidenced activity seems to be elaborated while extending the fermentation duration. However, more interesting antifungal activities were observed with the Kombucha analogue beverages and particularly with the *L. citriodora* Kombucha and the *F. vulgare* Kombucha. Thus, both of these fermented beverages have shown to be a potent inhibitor against almost of the tested *Candida,* with higher inhibition zones noticed compared to the black tea Kombucha. Indeed, recently Bilia, Giomi, Innocenti, Gallori, and Vincieri (2008) evidenced phenyl propanoides with verbascoside as being the most abundant compounds, and iridoids, verbenalin, together with flavonoids, luteolin and apigenin were also identified in *L. citriodora*. These compounds would be partially responsible for the observed antimicrobial effect of the fermented *L. citriodora* extract. Whilst the antimicrobial activity of fermented infusions have disappeared after neutralization, only *S. aureus* and *Mi. luteus* were found to be sensitive to neutralized Kombucha (respectively neutralized black tea Kombucha and neutralized *Me. piperita* Kombucha). These findings suggest that the antimicrobial activity of Kombucha analogue beverages is not only due to the acidity or organic acids in the fermented beverages but also to other biological active compounds (proteins, antibiotics, enzymes…) or metabolites other than acetic acid biosynthesized during the fermentation process. Since such inhibitory compounds are heat sensitive, we used the heat treated Kombucha analogue infusions as an alternative to characterize the nature of the antimicrobial substances. As the data shown the heat treatment process either reduced or improved the initial observed inhibition which implies that the antimicrobial activity was not often due to heat-sensitive molecules. These results are confirming the findings of Sreeramulu et al. (2000) and (2001). Indeed, biological compounds like proteins or enzymes are very sensitive and require specific pH to be active. This may explain the disappearance of the initially observed activity with fermented infusions at neutral pH.

No common rules for these bioactive molecules were evidenced which implies that the optimal effectiveness of a fermented medicinal plant may not be due to one main active constituent, but to the synergic action of different compounds, originally found in the plant infusion and other metabolites produced during the microbial fermentation, including the pH effect.

The heat treatment either reduced or improved inhibition, this fact means that the antimicrobial activity was not often due to heat-sensitive molecules. In some cases we observed a lack of inhibition which changed to a strong inhibition after heat treatment (*R. officinalis* against *S. typhimurium* and *L. citriodora* against *C. parapsilosis*). This increasing of activity might be explained by the epimerizations of the tea catechins under the heating conditions this fact is confirmed by other works (Kim et al., 2007).

Kombucha beverages were fermented aqueous herbal infusions, involving difference in compositions and biological activity. These facts may justify the observed relative efficiency of the fermented extracts against all Gram-positive and negative tested bacteria.

To our knowledge, no comparable study was published dealing with antimicrobial activities of such medicinal herbal infusions, fermented by the tea fungus. In the previous published papers, most of antibacterial studies concerning medicinal plants were undertaken using essential oil or organic extracts of these herbs (Carnat, Carnat, Fraisse, & Lamaison, 1999; Cushnie & Lamb, 2005; Del Bano et al., 2003; Najjaa, Neffati, Zouari, & Ammar, 2006; Santos, Fernandes, & Vicente, 2005; Shan, Cai, Brooks, & Corke, 2007; Sivropoulou et al., 1996; Yadegarinia et al., 2006).

The present results showed that the most potent fermented infusions were those prepared from *L. citriodora*, *Me. piperita* and *F. vulgare* has a long history of folk uses in treating asthma, spasms, fever, flatulence, indigestion, insomnia and anxiety (Carnat et al., 1999; Santos et al., 2005). The leaves essential oil has been shown to exhibit antimicrobial activity (Duarte, Figueira, Sartoratto, Rehder, & Delarmelina, 2005; Duschatzky, Martinez, Almeida, & Bonivardo, 2004; Lopez, Theumer, Zygadlo, & Rubinstein, 2004; Ohno et al., 2003; Sartoratto et al., 2004). A number of publications deal with the analysis and identification of the phenolic compounds (flavonoids and phenolic acids) of the leaves of *L. citriodora* (Argyropoulou, Daferera, Tarantilis, Fasseas, & Polissiou, 2007; Carnat et al., 1999). The antimicrobial activity of flavonoids is being increasingly documented (Cushnie & Lamb, 2005).

A recent study showed that α-terpinene (19.7%), isomenthone (10.3%), trans-carveol (14.5%), pipertitinone oxide (19.3%), and β-caryophyllene (7.6%) are the major compounds of the essential oils of *Me. piperita* and demonstrate its antimicrobial activity against *C. albicans*, *E. coli* and *S. aureus* (Yadegarinia et al., 2006).

In the literature, only sparse information on antimicrobial activities of *Rosmarinus* is available, and most of the published work refers to its essential oil (Angioni et al., 2004; Blaschek et al., 2004; Lopez, Sanchez, Batle, & Nerin, 2005; Mangena & Muyima, 1999; Santoyo et al., 2005). In the present study we have used a fermented infusion of *Rosmarinus* leaves that may contain other secondary metabolites different from those of the essential oil (Del Bano et al., 2003).

Santoyo et al. (2005) attributed the antimicrobial property of the essential oil to the presence of alpha-pinene, 1,8-cineole, camphor, verbenone, and borneol, with borneol being the most effective, followed by camphor and verbenone.

Although Shan et al. (2007) demonstrated that essential oil of *T. vulgaris* L. provides antimicrobial activity; none of the preparations using this plant material has shown any antimicrobial potentiality in this study.

Generally, the antimicrobial activity of essential oils is assigned to a number of small terpenoids and phenolic compounds (thymol, carvacrol, eugenol) (Conner, 1993; Didry Dubreuil & Pinkas, 1993; Nevas, Korhonen, Lindstrom, Turkki, & Korkela, 2004). Although essential oils and their components are known to be active against a wide variety of microorganisms including Gram-negative bacteria (Sivropoulou et al., 1996) and Gram-positive bacteria (Kim, Marshall, & Vei, 1995), these were shown to be generally more resistant than Gram-positive bacteria due to the antagonistic

effects of the lipopolysaccharide present in the outer membrane (Russel, 1991). These facts may explain the noticed activity on both Gram-positive and Gram-negative bacteria of the aqueous fermented extracts tested.

5. Conclusions

In the present study, the possibility of using other plant leaves as material in Kombucha fermentation was investigated and the antibacterial as well as the anti-Candida activities of the resulting Kombucha analogues were studied. Based on the data obtained, we may conclude that all of the tested microorganisms were sensitive to the antimicrobial activity of the fermented infusions, except *T. vulgaris* Kombucha which exhibited recalcitrance to all the pathogenic tested microorganisms. Unlike the unfermented infusions, the new Kombucha analogue beverages were in general active with a potential ranged from 11 to 28.5 mm in inhibition assays. These observations prove that the antimicrobial potential was the result of the fermentation process and that the basic composition of the different infusions was improved by the Kombucha's consortium. Thus, the antimicrobial activity observed in the fermented infusions was not only significant against the tested Gram-positive and Gram-negative pathogenic bacteria, but also against all *Candida* strains tested except *C. krusei*. In the addition, our findings suggest that the antimicrobial activity of Kombucha analogue beverages is not only due to the acidity or the organic acids in the fermented beverages but also to other biological active compounds sensitive to the heat treatment (proteins, antibiotics, enzymes…) or metabolites other than acetic acid biosynthesized during the fermentation process. Our results confirm the changing of the chemical composition under the heating conditions which might be explained by epimerizations of the tea catechins, this fact is confirmed by other studies (Kim et al., 2007).

To our knowledge, this is the first report dealing with these new beverages noted as Kombucha-analogue drinks and characterizing theirs antibacterial and antifungal activities compared to the traditional Kombucha tea well known. Our results highlight the improvement of the Kombucha fermentation on the herbal infusions used. In view of its antimicrobial activity demonstrated against a range of pathogenic bacteria and against a number of clinical *Candida* species, Kombucha beverages may be very healthful. Since resistance to antimicrobial agents has become increasingly an important fact pressing the global health problem, these findings would be very promising and could be useful in order to find out an alternative to current synthetic antimicrobial drugs. These results show that the fermentation process can eventually lead to the innovative production of natural antimicrobials as indicated by Mo, Zhu, and Chen (2008). However, more studies are needed to better evidence further related active compounds and understand their relative acting mechanisms.

Acknowledgements

The authors thank Mr. lan Ogino, expert of Japan International Cooperation Agency, for providing the Japanese Kombucha.

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