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Efficacy of Fermented Teas in Antibacterial Activity

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ABSTRACT

Fermented tea or "*Kombucha*" was prepared by a tea broth (1.7 % w/v) and sucrose (10% w/v) with supplement of commercially available starter culture. Teas used in this study were mulberry tea, Japanese green tea, Jasmine tea, Ulong tea and black tea. The teas were fermented for two weeks as an inoculum, following by inoculation to another tea broth and required further two-week static fermentation. In this study the antibacterial activity of several teas were tested against pathogenic bacteria in human and shrimp (e.g. *Vibrio cholerae*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus* and *Vibrio vulnificus*) by disc agar diffusion assay. The pH of fermented teas decreased from around 5 to 2 and the OD_{600} of tea broth rose significantly from around 0 to 1.5 during fermentation period. Broth from black tea poses the greatest inhibitory activity by measuring diameters of inhibition zones. *V. parahaemolyticus* showed the largest susceptibility to the fermented tea while pathogenic bacteria in human appeared to be less sensitive. Changes in major components of tea broth were also observed by HPLC analysis. The key organic acids such as succinic acid and gluconic acid produced during the period increased with time, proving the major role of these acids in the microorganism's growth inhibition.

Key words: fermented tea, *Kombucha*, tea fungus, pathogenic bacteria, acetobacters

INTRODUCTION

Tea fungus broth comprises of two portions: a floating cellulosic pellicle layer and the sour liquid broth composed mainly of acetic acid, ethanol and gluconic acid (Blanc, 1996). The fermentation is traditionally carried out by inoculating a previously grown culture into a freshly prepared tea decoction and incubated statically under aerobic condition for 7 – 10 days. Eventually, a pleasantly sour and slightly sparkling beverage called "*kombucha*" or teakwass is produced (Reiss, 1994).

Kombucha (tea fungus) is a popular beverage among traditional fermented foods across the world. It is originated in northeast China and later spread to Russia and the rest of the world. It is usually called "tea fungus" although there is actually no fungus involved in the fermentation (Reiss, 1987; 1994; Steinkraus *et al*., 1996). *Kombucha* has been reported to exert a number of medicinal effects, for example, against metabolic diseases, arthritis, psoriasis, constipation, indigestion and hypertension though there is no scientific evidence available yet for its efficacy (O'Neill, 1994; Jacobs, 1995). *Kombucha* is a

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symbiotic growth of bacteria (*Acetobacter xylinoides*, *Bacterium gluconicum*) and yeasts (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Saccharomycodes ludwigii*, etc.) cultured in a sugared tea (Reiss, 1987). The exact microbiological composition also depends on the source of inoculum: the starter culture. Cellulose produced during the fermentation by *A. xylinum* appears as a thin film on the top of the tea where the cell mass of bacteria and yeast is attached. Glucose liberated from sucrose is metabolized for the synthesis of cellulose and gluconic acid by *Acetobacter* strains. Fructose is metabolized to ethanol and carbon dioxide by yeasts. Again, acetobacters oxidize ethanol to acetic acid. Organic acids produced during fermentation shield the symbiotic colony from contamination with unwanted foreign microorganisms that are not composed in the tea fungus (Blanc 1996; Greenwalt *et al.*, 1998). The low rate of contamination from harmful microorganisms causing spoilage and diseases make *Kombucha* safe to prepare at home without pathogenic health risk (Mayser *et al.*, 1995). Recent researches on *Kombucha* have proved that its antimicrobial activity against pathogenic microorganism is largely attributable to acetic acid (Steinkraus *et al.*, 1994; Greenwalt, 1998).Acetic acid is known to inhibit and kill a number of Gram-positive and Gram-negative bacteria (Levine and Fellers, 1940). The physiological changes that occur during the fermentation process of *Kombucha* and the possible relationship of these changes with speculative curative and antibacterial effects are not yet clear and need further systematic investigations.

The focus of this study was to test the antimicrobial activity of *Kombucha* from various sources of tea against some major pathogenic bacteria in human and shrimp with changes in tea concentration. An objective was, thus, to determine whether pathogenic growth can be prevented by the consumption of *Kombucha* and to investigate the changes in major compositions in fermented tea during the fermentation period.

MATERIALS AND METHODS

Mulberry tea, Japanese green tea, Ulong tea and Black teas were purchased from local supermarkets. Four shrimp pathogenic *Vibrio* strains; *V.alginolyticus*, *V.harveyi*, *V.parahaemolyticus* and *V.vulnificus* were kindly provided by The Department of Fisheries, Ministry of Agriculture and Cooperatives, Thailand. Three human pathogenic strains; *Vibrio cholerae*, *Salmonella typhi* and *Pseudomonas aeruginosa* were derived from the Department of Biochemistry, Kasetsart University's laboratory stock. The starter culture was kindly provided by Institute of Food Research and Product Development, Kasetsart University. All bacterial strains were maintained in tryptic-soy agar (TSA) slant (Difco) with additional 0.5% NaCl for the four *Vibrio* strains.

Tea broth preparation

One hundred milliliter distilled water in wide-mouth bottle was boiled and 5% w/v each of sucrose and $(NH_4)_2SO_4$ were added. Tea extracts were obtained from keeping 4.4 to 70 g/l into the boiling solution for 15 min. The leaves were removed by filtration through filter paper and funnel, then the filtrate was further autoclaved at 121°C for 20 min. After the tea decoction was left to cool down at room temperature, 5% v/v of starter culture was added. The bottle was sealed with sterilized gauze and cottons and left in the dark at room temperature for 14 days. At the end of seeding culture, thin cellulosic film formed during this period was used as inoculum for the next main fermentation. The main fermentation was conducted by addition of 5% v/v seeding culture into the sterilized tea filtrate mentioned above and left in the dark at room temperature for another 14 days. The samples were taken aseptically every two days to investigate the changes in pH, OD at 600 nm and for further analysis by HPLC.

Antimicrobial activity

Antimicrobial activity was performed by agar-disc diffusion assay. Twenty milliliter of TSA medium was poured into each Petri dish. Each target strain from stock culture was grown in tryptic-soy broth (TSB, Difco) for 24 h until OD_{600} reached about 0.7 – 0.8. Suspensions (200 μl) of each target strain were spread uniformly on the plate. Tea broth samples, taken every two-day interval, were centrifuged at 5,000 g (Sorvall) for 15 min to remove cell debris. Sterile supernatant was obtained by filtering through 0.45 mm sterile microfilter (Millipore) and 100 μl were subsequently allowed to saturate assay paper discs of diameter 13 mm (Whatman). The discs were then placed on agar plates innoculated with target strains and incubated at 37°C. The diameter of the inhibition zones were measured after 12 – 15 h.

Analyses of organic acids

The major organic acids in fermented tea were determined by high performance liquid chromatography (HPLC). The tea broth samples were filtered through 0.45 μm sterile microfilter and 10 μl of filtrate was injected into Perkins-Elmer HPLC system equipped with reverse phase column (Supelco LC18-DB, 4.6 mm × 250 mm) and 210 nm UV detector. The mobile phase was 20 mM $(NH_4)_2HPO_4$: methanol (97 : 3 v/v) with a flow rate of 0.5 ml/ min and running time of 15 min. 10 g/l of acetic acid, gluconic acid and succinic acid were used as standard.

RESULTS AND DISCUSSION

Changes in pH during the fermentation period of black tea (*Northern tea*) are shown in Figure 1. The increase in acidity is normally due to the formation of organic acids. The pH decreased dramatically within 6 days but remained stable to the end of the period. Except for the lowest tea concentration (4.4 g/l), the increase in acidity of tea broth seems to be independent of tea concentration. Figure 1 also shows changes in OD_{600} suggesting the growth of microbial aggregate in the tea broth. Similar to above result,

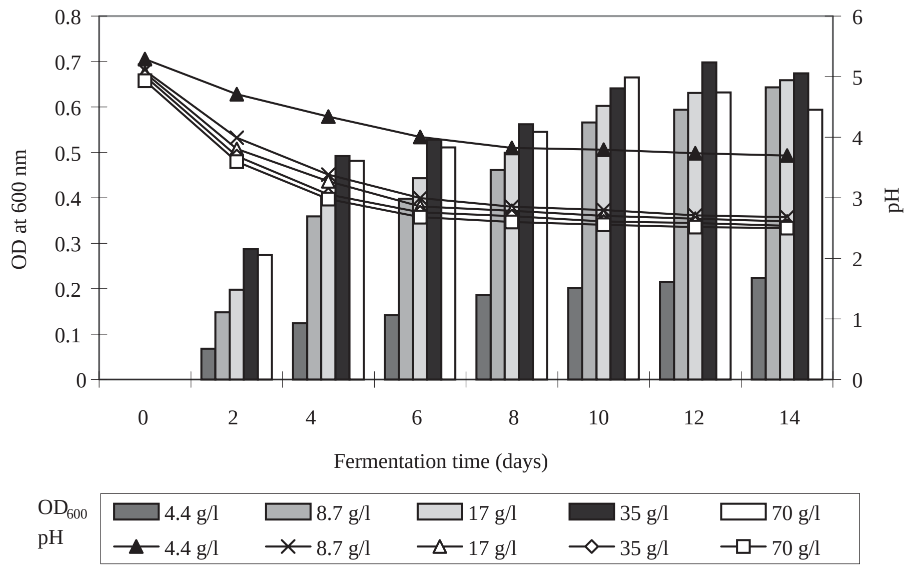

Figure 1 Changes in pH and OD_{600} for various concentrations during fermentation of black tea.

increase in optical density was largely observed in the first four days. After that, the OD increased gradually until the end of fermentation.

The comparison in growth and antibacterial activity of three kinds of tea (mulberry tea, Japanese green tea and *Ulong* tea) was made (Figure 4). The results indicated that broth from mulberry tea yields the highest microbial growth. The comparison among another set of three varieties of tea (*Ulong* tea, *Namtao Kheaw* tea and Jasmine tea) was also made to investigate antibacterial activity but none of them showed the significant inhibitory effects (data not shown). Antibacterial activity by disc-agar diffusion method of black tea against four pathogenic bacteria in shrimp (Vibrio strains) is shown in Table 1. For the purpose of control, each concentration of fermented tea was neutralized to pH 7 with NaOH and sterilized by filtration filtered prior to the assay. From this result, inhibitory effect measured from clear zone diameter increased with increasing concentration of tea extract. At concentration of 4.4 g/l the inhibitory effect was not observed with the exception of *V. parahaemolyticus*. The growth of four Vibrio strains were inhibited and the most sensitive strain was *V. parahaemolyticus*. No inhibition zone was observed neither when the tea broth was neutralized to pH 7 nor in the unfermented sample controls. This explained that the inhibitory property of fermented tea depends primarily on the formation of key organic acids, such as acetic acid and gluconic acid. Another set of tea broths (mulberry tea, Japanese green tea and *Ulong* tea) were also tested for their antibacterial activities against two kinds of pathogenic bacteria in shrimp; *V.harveyi* and *V. parahaemolyticus* (Figures 3, 4). The tea concentration was optimized to 17g/l from previous investigation indicating this concentration showed optimum inhibitory effects to these strains. Among the three kinds of tea, no obvious difference was found in the results and all had less inhibitory effect as compared to the fermented black tea mentioned above.

From above observations, black tea possessed the greatest inhibitory effect against pathogenic bacteria in shrimp. Black tea was then used to investigate antibacterial property against pathogenic bacteria in human; *Vibrio cholerae*, *Salmonella typhi* and *Pseudomonas aeruginosa*. The diameters of inhibition zone against the target bacteria increased gradually from day 0 to day 14 (Figure 5). The most sensitive strain to antibacterial effect of fermented tea was *Vibrio cholerae*, bacterium that causes cholera, the serious disease in tropical countries.

The analysis of organic acids by HPLC showed the only two major components in fermented tea; gluconic acid and succinic acid (Figure 6). The concentrations of both organic acid tended to increase slowly at the first stage of fermentation but elevated drastically as it reached day 12 of the batch fermentation. This suggested that bacteria could utilize sucrose as carbon source for growth for the first 12 days. After that, the carbon-containing molecules were metabolically transformed to several kinds of organic acids. If the batch time were further extended, we could have seen the dramatic increase in these acids. However, some literatures have shown that the prolonged fermentation time and so much high dry tea concentration affects directly to the organoleptic quality of *Kombucha* and could make health concern (Farnworth *et al.*, 2000). Although the consumption of *Kombucha* generally presents no adverse side effects, a few cases of health disorders have been reported (Farnworth *et al.*, 2000). The vast consumption of tea fungus could be harmful for those predisposed to acid sensitivities and renal insufficiencies. When *Kombucha* is a home made fermentation, there is the possibility of contamination by potentially pathogenic bacteria and yeasts. Since the fermentation is conducted in non-aseptic conditions the probability of contamination is high. Despite the fact mentioned, *Kombucha*, similar to

Table 1 Inhibitory effects of fermented black tea against *Vibrio* strains. The diameters of clear zone were measured after 2-14 days of fermentation.

Test organisms	Diameters of inhibition zone (cm)										
	Control tests	4.4 g dry tea/l		8.7 g dry tea/l		17.0 g dry tea/l		35.0 g dry tea/l		70.0 g dry tea/l	
	Unfermented	Fermented	Neutralized	Fermented	Neutralized	Fermented	Neutralized	Fermented	Neutralized	Fermented	Neutralized
V.alginolyticus											
2 days	0	0	-	1.5	-	1.65	-	1.8	-	2	-
4 days	0	0	-	1.6	-	1.7	-	1.85	-	2	-
6 days	0	0	-	1.7	-	1.8	-	1.9	-	2.1	-
8 days	0	0	-	1.7	-	1.8	-	1.9	-	2.15	-
10 days	0	0	-	1.75	-	1.85	-	1.95	-	2.15	-
12 days	0	0	-	1.75	-	1.85	-	1.95	-	2.15	-
14 days	0	0	0	1.8	0	1.9	0	1.95	0	2.2	0
V.harveyi											
2 days	0	0	-	1.5	-	1.6	-	1.7	-	1.9	-
4 days	0	0	-	1.6	-	1.65	-	1.8	-	2	-
6 days	0	0	-	1.65	-	1.7	-	1.85	-	2	-
8 days	0	0	-	1.7	-	1.75	-	1.85	-	2	-
10 days	0	0	-	1.7	-	1.8	-	1.9	-	2.05	-
12 days	0	0	-	1.75	-	1.85	-	1.95	-	2.1	-
14 days	0	0	0	1.8	0	1.9	0	1.95	0	2.1	0
V.parahaemolyticus											
2 days	0	1.45	-	1.7	-	1.9	-	2.1	-	2.2	-
4 days	0	1.45	-	1.75	-	2	-	2.1	-	2.25	-
6 days	0	1.55	-	1.75	-	2.05	-	2.15	-	2.3	-
8 days	0	1.55	-	1.8	-	2.1	-	2.2	-	2.3	-
10 days	0	1.6	-	1.85	-	2.1	-	2.2	-	2.3	-
12 days	0	1.65	-	1.9	-	2.15	-	2.25	-	2.35	-
14 days	0	1.65	0	1.9	0	2.2	0	2.3	0	2.35	0
V.vulnificus											
2 days	0	0	-	1.6	-	1.65	-	1.8	-	1.85	-
4 days	0	0	-	1.7	-	1.75	-	1.85	-	1.9	-
6 days	0	0	-	1.75	-	1.8	-	1.85	-	1.9	-
8 days	0	0	-	1.8	-	1.85	-	1.9	-	2.05	-
10 days	0	0	-	1.8	-	1.85	-	2	-	2.15	-
12 days	0	0	-	1.85	-	1.9	-	2.05	-	2.15	-
14 days	0	0	0	1.9	0	2.15	0	2.1	0	2.2	0

other kinds of fermented foods, has gained popularity for a long time as traditional beverage. Theoretically speaking, the fermentation process by itself restricts the growth of foreign microorganisms.

Yeast and bacteria in the tea fungus make use of substrates in different and complementary ways. Yeast cells hydrolyze sucrose to glucose and fructose, and then produce ethanol, with a preference for fructose as a substrate whereas acetic acid bacteria utilize glucose to produce gluconic acid and ethanol to produce acetic acid. In this report, very low level of acetic acid was observed (data not shown). This indicated that

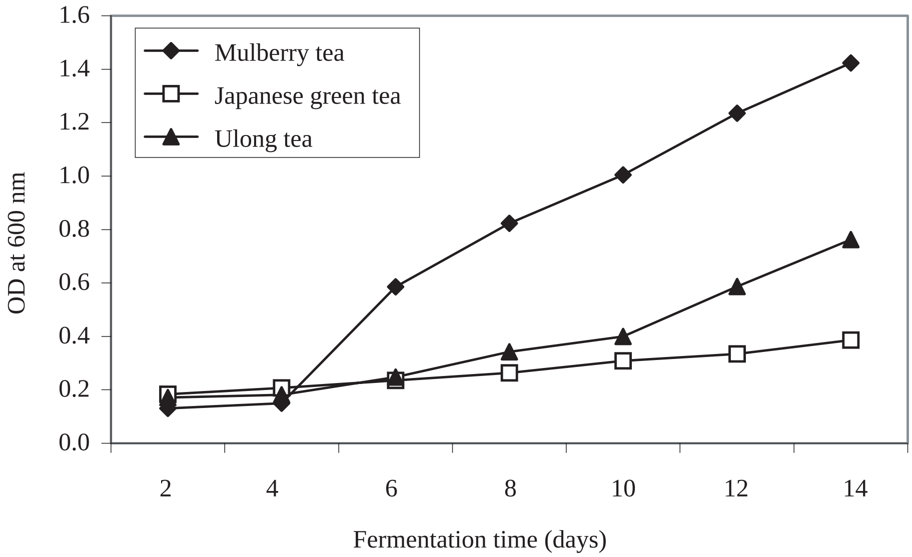

Figure 2 Changes in OD_{600} during fermentation of 17 g/l Mulberry tea, Japanese green tea and *Ulong* tea.

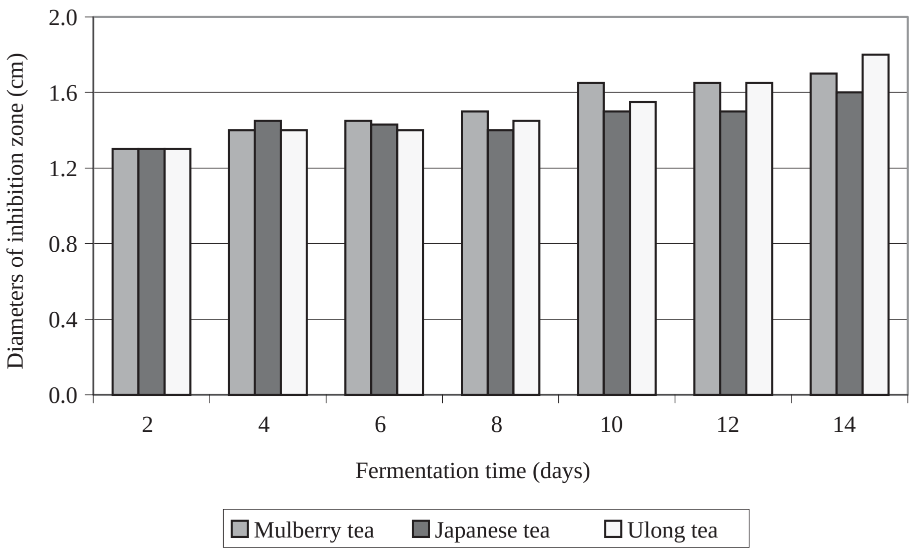

Figure 3 Inhibitory effects of fermented teas (17.0 g/l) against *Vibrio harveyi*.

either acetic acid bacteria in the starter culture were initially low or the fermentation time was not long enough to produce acetic acid.

Although antibacterial property of fermented tea is due primarily to organic acids and ethanol formation, to the lesser extents, it is promoted by polyphenolic compounds within the tea. Black tea, the most popular form around the world, is the result of the oxidation of leaf polyphenols through a multi-stage enzymatic process (Hara *et al*., 1995). New polyphenol molecule complexes are formed during the processing of black tea. For instance, green tea cathechins is converted to proanthocyanidins, theaflavin, caffeine and other molecules (Hara *et al*., 1995).

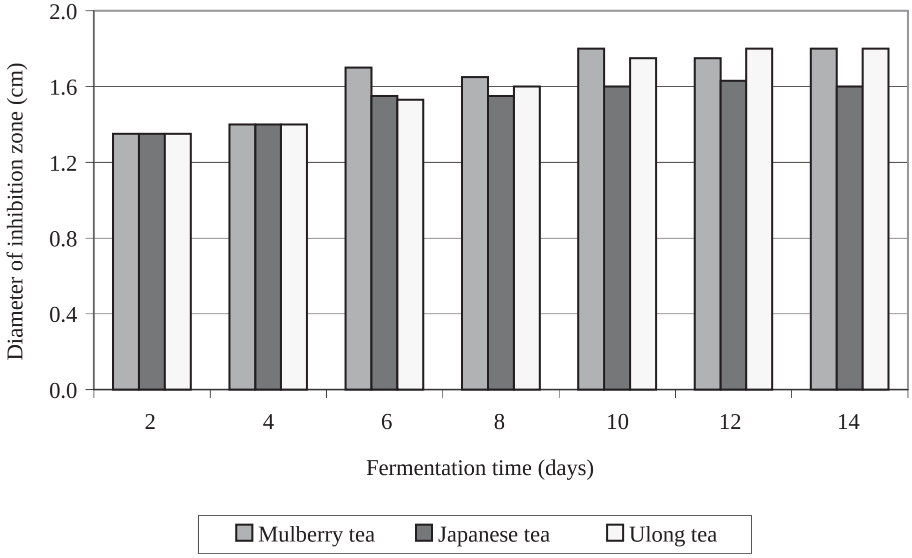

Figure 4 Inhibitory effects of fermented teas (17.0 g/l) against *Vibrio parahaemolyticus*.

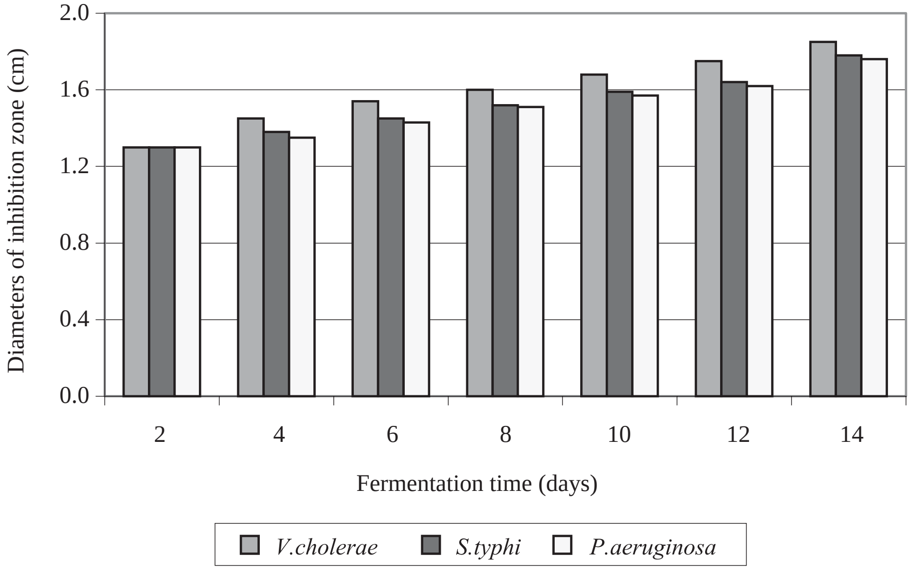

Figure 5 Inhibitory activities of 14-day fermented black tea against three human pathogenic bacteria.

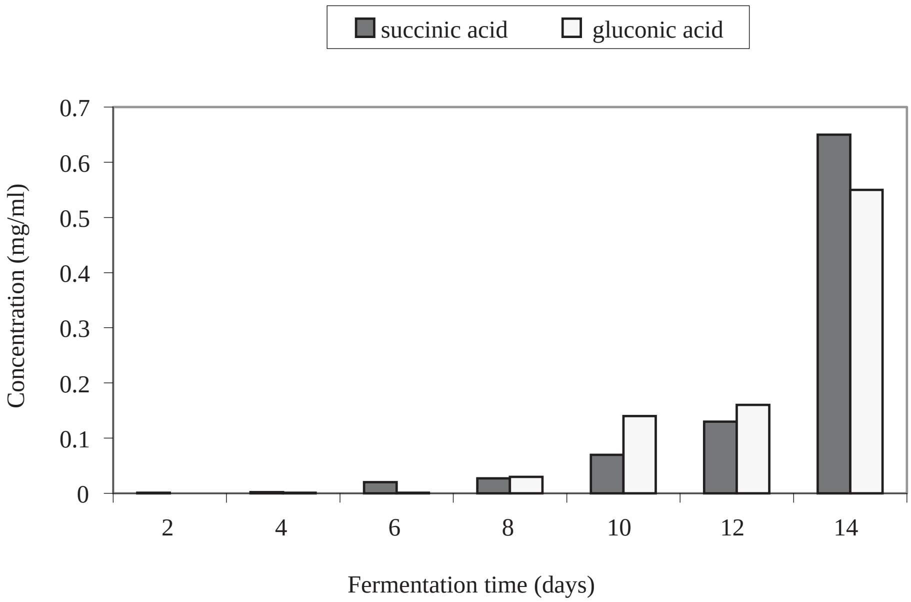

Figure 6 Changes in organic acid concentration in fermented black tea.

CONCLUSION

The efficacies of fermented tea or *Kombucha* were tested against three pathogenic bacteria in human and four in shrimp. Several kinds of tea leaf were used in this study with inclusion of black tea. All four Vibrio strains showed susceptibility to tea fungus particularly the strain *V.parahaemolyticus* while three pathogenic bacteria in human indicated less sensitivity. Major organic acid formations were initially slow but showed rapid increase as the batches resumed. Black tea was found to have the greatest antibacterial activity to the target strains. The inhibitory effects of fermented tea against pathogenic bacteria in shrimps indicated the possibility for the production of shrimp meal with *Kombucha* as an efficient additive.

LITERATURE CITED

Blanc, P.J. 1996. Characterization of the tea fungus metabolites. **Biotechnol. Lett.** 18: 139-142.

Chen, C. and B.Y. Liu. 2000. Changes in major components of tea fungus metabolites during prolonged fermentation. **J. Appli. Microbiol.** 89: 834-839.

Farnworth, E. and C. Dufresne. 2000. Tea, Kombucha, and a health: a review. **Food Res. Int.** 33: 409-421.

Greenwalt, C.J., R.A. Ledford and K.H. Steinkraus 1998. Determination and characterization of the antibacterial activity of the fermented tea *Kombucha*. **Leben.-Wiss. Technol.** 31: 291-296.

Hara, Y., Luo ShaoJun, R.L. Wickremasinghe and T. Yamanishi. 1995. Biochemistry of processing black tea. **Food Rev. Int.** 11: 477-525.

Jacobs, S. 1995. Kombucha used as miracle cure spreading like mushrooms. **The Miami Herald**. 2nd January. 1F-3F.

Kozaki, M. A. Koizumi and K. Kitahara. 1972. Microorganisms of zoogloeal mats formed in tea decoction. **J. of Food and Hyg. Soc. Japan** 13: 89-96.

Levine, A.S. and C.R. Fellers. 1940. Action of acetic acid on food spoilage microorganisms.

J. Bacteriol. 39: 499-515.

Liu, C.H., W.H. Hsu, F.L. Lee and C.C. Liao. 1996. The isolation and identification of microbes from a fermented tea beverage, haipao, and their interaction during haipao fermentation. **Food Microbiol.** 13: 407-415.

Mayser, P., S. Frome, C. Leutzmann and K. Grunder. 1995. The yeast spectrum of the ‘tea fungus Kombucha’. **Mycoses** 38: 289-295.

O’Neill, M. 1994. A magic mushroom or a toxin fad? **New York Times**, 28 Dec, C-1, C-4, C-6, C-8.

Reiss, J. 1994. Influence of different sugars on the metabolism of the tea fungus. **Zeitsch. Lebens. Unters. Forsch.** 198: 258-261.

Steinkraus, K.H. 1996. Tea fungus/Kombucha; 493-496. **Handbook of Indigenous Fermented Food**, 2nd ed. Marcel Dekker Inc., New York.