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Manipulation of Probiotics Fermentation of Yogurt by *Cinnamon* and *Licorice*: Effects on Yogurt Formation and Inhibition of *Helicobacter Pylori* Growth *in vitro*

S. Behrad, M.Y. Yusof, K. L. Goh, A.S. Baba

***Abstract*—**Probiotic bacteria especially *Lactobacillus spp*. and *Bifidobacterium* exert suppressive effect on *Helicobacter pylori*. *Cinnamon* and *licorice* have been traditionally used for the treatment of gastric ulcer. The objectives of this study were to determine the effects of herbs on yogurt fermentation, the level of probiotic bacteria in yogurt during 28 days storage and the effect of herbal yogurt on the growth of *H. pylori in vitro*. *Cinnamon* or *licorice* was mixed with milk and the mixture was fermented with probiotic bacteria to form herbal-yogurt. Changes of pH and total titratable acids were monitored and the viability of probiotic bacteria was evaluated during and after refrigerated storage. The *in vitro* inhibition of *H. pylori* growth was determined using agar diffusion and minimum inhibitory concentration (MIC) method. The presence of herbs did not affect the probiotic population during storage. There were no significant differences in pH and TTA between herbal-yogurts and plain-yogurt during fermentation and storage. Water extract of *cinnamon-yogurt* showed the highest inhibition effect (13.5mm) on *H. pylori* growth in comparison with *licorice*-yogurt (11.2mm). The present findings indicate *cinnamon* and *licorice* has bioactive components to decrease the growth of *H. pylori.* [1]

Keywords*—Cinnamon, Helicobacter pylori*, Herbal-Yogurt, *Licorice*, Probiotics

I. INTRODUCTION

LACTIC acid bacteria (LAB) are acid tolerant, Gram-positive microorganism, which produce lactic acid as a main product [1]. The most important genera are *Lactobacillus*, *Lactococcus, Enterococcoccus, Streptococcus, Pediococcus, Leuconostoc, and Bifidobacterium*. There are several health benefits claimed for probiotic bacteria such as inhibition of *Helicobacter pylori* and intestinal pathogens, reduction of the risks associated with mutagenicity and carcinogenicity, prevention of inflammatory bowel disease, and improvement of immune system [2]. The suppression of *H. pylori* growth *in vitro* by *Lactobacillus acidophilus*–and *Bifidobacterium*-containing yogurt (AB-yogurt) can be a direct one [3], [4] and as such, consumption of yogurt may exert therapeutic effects by suppressing the growth of *H. pylori* in infected clinical patients [5], [6].

H. pylori is an important cause of chronic gastritis, peptic ulceration and gastric cancer in humans [7], [8]. It is estimated that one-half of the world's population is infected with *H. pylori* [9]. Numerous clinical evidences show that eradication of *H. pylori* results in improvement of gastritis and decreases the rate of relapse of gastric and duodenal ulcers [10], [11]. *H. pylori* carriage rates are about 80–90% in developing countries [12], with a high risk of gastric cancer and antibiotic resistance [13]. Antibiotics produce undesirable side effects [14] and noncompliance among the patients [15] in the long run. Hence there is a need to develop alternative means to suppress *H. pylori* infection. In this regard the antibacterial activity of several plant extracts have been tested *in vitro* [17], a number of which were effective against *H. pylori* growth. In addition, yogurt bacteria were reported effective in suppressing H. *pylori* infection [18]. The present study investigated the effects of herbs on yogurt fermentation and viability of yogurt bacteria during refrigerated storage, and the effect of yogurts on *H. pylori* growth *in vitro*.

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II. MATERIALS AND METHODS

A. Preparation of extracts

Stem barks of *cinnamon* and roots of *licorice* were obtained from a local Chinese medicinal shop. Both were ground to fine powder. The powdered herbs (10g) were soaked in 100ml of distilled water and left overnight at 70˚C. The suspension was then centrifuged (2000 rpm; 15 min), and the supernatant was sterilized through 0.22 μm filter (Sartorius, Germany).

B. Herbal yogurt preparation

Homogenized and pasteurized milk was purchased from the local supermarket. Starter culture (5g) consisting of *L. acidophilus LA-5 and NCFM, Bifidobacterium Bb-12, L.casei LC-10, and Streptococcus thermophilus Th-4*, 2% (w/v) skim milk powder and 6% (w/v) herb extract were dissolved in 1L of milk. The mixtures were aliquoted in 100ml plastic cups. Incubation was carried out at 41˚C, and fermentation was terminated at pH 4.5.

C. Determination of pH and TTA

The pH and TTA of yogurts were determined every hour at 17-20˚C during fermentation and storage at 4˚C. Yogurt sample (1g) was mixed with distilled water (1:1), and the pH was measured using a pH meter (Mettler-Toledo 320, Shanghai), calibrated routinely with fresh pH 4.0 and 7.0 standard buffers.

TTA was determined by titrating yogurt sample and distilled water (1:9) mixture with 0.1*N* NaoH using a 0.1% Phenolphetalein as color indicator. The amount of acid produced during fermentation was calculated as follows:

TTA% = Dilution factor (10) x V NaoH x 0.1N x 0.009 x 100%

where V is volume of NaoH required to neutralize the acid.

D. Enumeration of probiotic bacteria

Enumeration of *Lactobacillus spp* was carried out by aseptically mixing yogurt sample (1ml) with 9ml of buffered peptone water (Oxoid,UK). The sample was thoroughly mixed and serial dilutions were performed using peptone water as the diluents. Empty petri dishes were inoculated with 1ml of diluted yogurt, followed by the addition of 15ml melted (45˚C) MRS agar. The petri dishes were covered and the contents mixed thoroughly by gentle tilting and swirling. The petri dishes were inverted and incubated anaerobically (Revco Ultima) at 37˚C for 24-48 hours.

Streptococci was enumerated by initially placing 15ml of melted (45˚C) M17 (Oxoid, UK) into a petri dish followed by cooling of agar to temperature to allow solidification. The agar was then inoculated by spreading the surface evenly with 0.1ml of diluted yogurt. The colonies formed were counted after 24-48 hour incubation at 37˚C. Viable microbial count was calculated as follows:

cfu/ml = cfu/plate x dilution factor

where cfu is colony forming unit

E. Bacterial isolates

Clinical isolates of *H. pylori* were obtained from the University of Malaya Medical Center, Kuala Lumpur, Malaysia. The organisms were identified based on colony morphology, Gram staining, microaerophilic growth (at 37°C), oxidase, catalase, and urease assays. In the present study, two clinical isolates (numbered as UM-1 and UM-2) from hospital patients were used.

The growth of *H. pylori* was maintained under microaerophilic conditions in anaerobic jars with CampyPakPlus (MGC Anaeropack, Microaero) at 37°C for 3–5 days. Bacterial strains were suspended in brain heart infusion broth (BHIB) (Oxoid, UK) containing 15% (v/v) glycerol and stored at -70°C.

F. Bacterial growth inhibition assay

Growth inhibition was evaluated by the filter paper disk diffusion method [21] which conforms to the recommended standards of National Committee for Clinical Laboratory Standards (NCCLS). Each of herbal-yogurt water extracts (25 µl) was aliquoted on standard 6 mm paper disks (Whatman, UK) which were then placed on Columbia agar supplemented with 7% sheep blood (BML, Malaysia), and inoculated with 0.1 ml bacterial suspension (10^8-10^9 cfu/ml) in the brain heart infusion broth (BHIB). The growth of *H. pylori* was maintained under microaerophilic conditions in anaerobic jars with gas pack (to absorb oxygen and generating carbon dioxide) at 37˚C for 3-5 days. The inhibition zone around each disk (average of triplicate) was measured.

The minimal inhibition concentration (MIC) was determined by mixing various volumes (0.25-3 ml) of herbal-yogurt water extract with heated (50˚C) Mueller Hinton blood agar, before inoculation with *H. pylori* suspension [22].

G. Antioxidant activity by 1,1-Diphenyl-2-Picrylhydrazyl (DPPH)

To 3ml of 60µM DPPH in ethanol, 250µl of each herbal-yogurt water extracts was added and the decrease in absorbance was measured at 517 nm. The readings were compared with the controls, which contained 250µl of dH_2O instead of the water extracts. The % of antioxidant activity inhibition was calculated as follows:

$$\%\text{inhibition} = \frac{A_{517}\text{control} - A_{517}\text{extract}}{A_{517}\text{control}} \times 100$$

III. Results and Discussion

A. pH changes in yogurts during storage

The pH for plain yogurt was approximately the same as pH of herbal yogurts. An overall decline of pH of yogurts occurred during refrigerated storage. The pH for all yogurts reduced ($p<0.05$) from the initial values of 4.5 to between 4.09 and 4.12 by day 28 of storage. The presence of herbs did not make herbal-yogurts any different ($p>0.05$) from plain-yogurt.

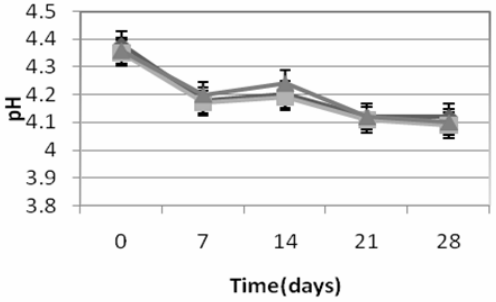

Fig. 1 Changes in pH of plain, *cinnamon*- and *licorice*-yogurts during refrigerated (4˚C) storage. *Cinnamon* (♦); *Licorice* (■); Plain (▲)

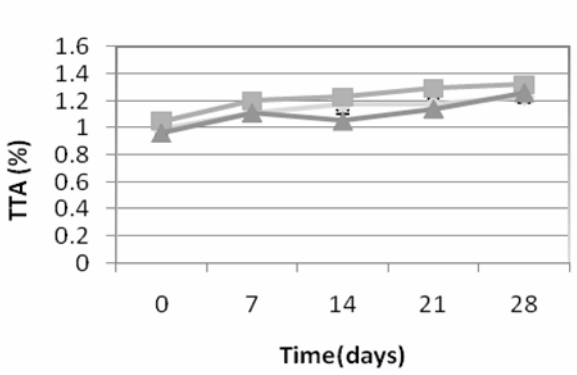

Fig. 2 Changes of total titratable acid (TTA) of plain, *cinnamon*- and *licorice*-yogurts during refrigerated (4˚C) storage. *Cinnamon* (♦); *Licorice* (■); Plain (▲)

B. Changes in Total titraable acid of yogurts during storage

The TTA of all yogurts increased ($p<0.05$) from the initial values of 1% to 1.27% by day 28 of storage. The increase in acids can be attributed to continued production of organic acids by LAB during refrigerated storage [20].

C. Survival of probiotic bacteria

The presence of *cinnamon* or *licorice* resulted in lower *Lactobacillus spp* counts in *cinnamon*- (9.46 x 10^6cfu/ml) and *licorice*- (12.3 x 10^6cfu/ml) *yogurts* on 0 day of storage compared to plain-yogurt (12.96 x 10^6cfu/ml; $p>0.05$). Refrigeration increased ($p>0.05$) viable *Lactobacillus spp.* counts to 15.8 x 10^6cfu/ml in the plain-yogurt but the presence of *cinnamon* or *licorice* inhibited this increase in herbal-yogurts. Viable *Lactobacillus spp* counts reduced from day 7 to day 28 of storage for all yogurts with the fastest rate occurred in plain-yogurt. Viable *Lactobacillus spp* counts on day 28 of storage for *licorice*-yogurt (6.4 x 10^6cfu/ml) was higher than plain-yogurt (4 x 10^6cfu/ml).

Refrigeration also increased ($p>0.05$) viable *S. thermophillus* counts in all yogurts by day 7 of refrigeration (Fig .4) but the effect was significant ($p<0.05$) only in the presence of *cinnamon* and *licorice* (140 x 10^6 cfu/ml) compared to plain-yogurt (115 x 10^6 cfu/ml). The increase in the viable cell counts for both yogurt bacteria during the first 7 days coincided with the increase in TTA and marked reduction ($p<0.05$) in pH recorded on day 7 of storage.

The reduction in viable cell counts which occurred in consistent manner in all yogurts can be attributed to the organic acids accumulation as a result of growth and fermentation (Figs. 1 & 2), [19], and [20]. Nevertheless, all yogurts contained acceptable level of probiotic bacteria (10^6 – 10^7 cfu/ml) by the end of 28 day of refrigerated storage.

D. DPPH inhibition assay

The addition of *cinnamon* or *licorice* increased the antioxidant activity of yogurts compared to plain-yogurt at all storage periods (Fig. 5). The highest antioxidant activity was recorded on day 7 for *cinnamon*-yogurt (31.8%) followed by *licorice*-yogurt (23.87%) and plain-yogurt (21.8%). *Cinnamon*-yogurt showed the highest antioxidant activity on day 0 ($p>0.05$) and 14 ($p<0.05$) of storage.

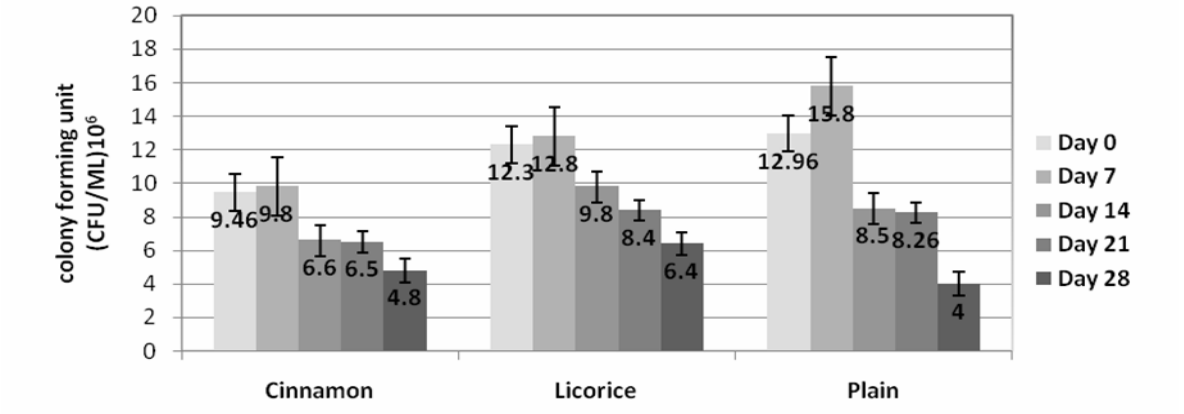

Fig. 3 Effect Viable *Lactobacillus spp* .in *cinnamon*- or *licorice*-yogurts during refrigerated storage.

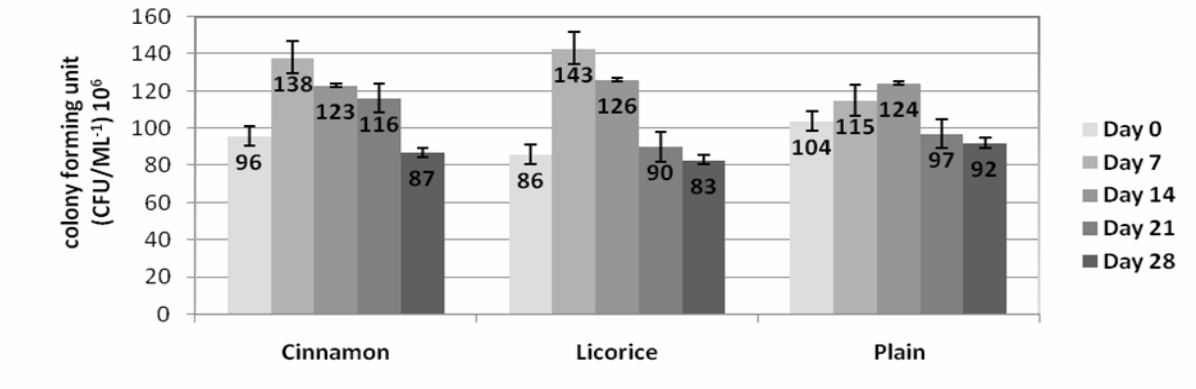

Fig. 4 Viable *S. thermophilus* in *cinnamon*- or *licorice*-yogurts during refrigerated storage.

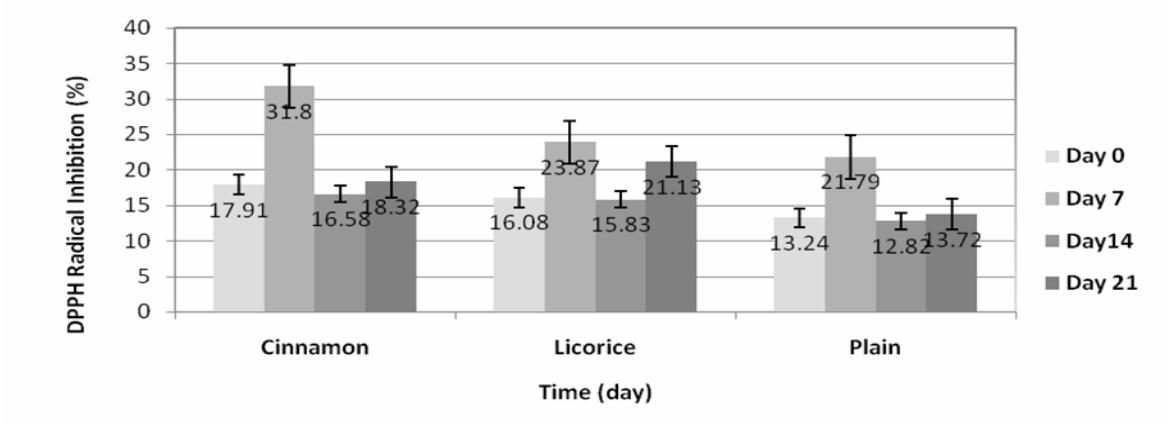

Fig. 5 DPPH radical inhibition capacity of water extracts from plain and herbal-yogurts

E. Bacterial inhibition assay

Water extract of *cinnamon*-yogurt exhibited the strongest inhibitory effect on *H. pylori* growth *in vitro* (13.5mm) in comparison with *licorice*-yogurt (11.2mm) and control-yogurt (10.5mm) for both strains tested. *Licorice*-yogurt extract at volume of 1ml had an inhibitory effect on *H. pylori* growth for both strain UM-1 and UM-2. However *cinnamon*-yogurt can only inhibit *H. pylori* growth at a volume of 3 and 2 ml for UM-1 and UM-2 strains respectively.

It can be concluded that the addition of *cinnamon* or *licorice* did not change yogurt fermentation but sustain the growth of *Lactobacillus spp* during refrigerated storage. C*innamon*-yogurt or *licorice*-yogurt containing probiotic bacteria inhibited the growth of *H. pylori in vitro*. The effectiveness of these herbal-yogurts to halt the growth of *H. pylori* needs to be further investigated under extremely acidic environment of the stomach.

TABLE I GROWTH INHIBITION OF *H. PYLORI* BY YOGURT WATER EXTRACT

	Inhibition Zone (mm)		
	Herbal-Yogurt Water Extract (mg/ml)		
Isolate Number	*Cinnamon-yogurt*	*Licorice-yogurt*	*Control-yogurt*
UM-1	14	11.5	10.7
UM-2	13	11	10.3

TABLE II MINIMUM INHIBITORY CONCENTRATION (MIC) OF YOGURT WATER EXTRACTS ON THE GROWTH OF *H. PYLORI* ISOLATES [a]

Isolates number	Antimicrobial agent	Volume of yogurt or herbal yogurt water extract (ml)					
		0 (blank)	0.25	0.5	1	2	3
UM-1	*Cinnamon-yogurt*	+ + + +	+ + +	+ + +	+ +	+	–
	Licorice-yogurt	+ + + +	+ + +	+ +	–	–	–
	Plain-yogurt	+ + + +	+ + + +	+ + +	++	++	+
UM-2	*Cinnamon-yogurt*	+ + + +	+ + +	+ +	+	–	–
	Licorice-Yogurt	+ + + +	+ + +	+ +	–	–	–
	Plain-yogurt	+ + + +	+ + +	+ + +	+ +	+ +	+

[a] Note: the following notations estimated H. pylori growth as follows: –, no growth; +, scant growth; + +, moderate growth; + + +, extensive growth; + + + +, very extensive growth.

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