Studies of the Microbial and Physical Properties of Oriental Style Dairy Product Kou Woan Lao with Probiotics

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ABSTRACT : The objective of this research was to combine the physiological functionality of probiotics (*Lactobacillus acidophilus* and *Bifidobacterium longum*) and the milk-clotting activity of culture filtrate from lao-chao to develop a new dairy product which was different from the commercial yogurt. *Rhizopus javanicus* and *Saccharomyces cerevisiae* were chosen as a mold and yeast starter for production of culture filtrate. The study results indicated that both probiotic counts increased with incubation time and maintained 10^7-10^8 CFU/ml after 6 h incubation with 10-30% culture filtrates. By contrast, samples with 40% culture filtrates inhibited the growth of *L. acidophilus* and *B. longum*. The more culture filtrates were added, the lower titratable acidities and higher pH values in Kou Woan Lao were detected after 36 h fermentation. No significant differences ($p>0.05$) were found for both *L. acidophilus* and *B. longum*, when grown in differing concentrations of skim milk powders. Storage results showed both *L. acidophilus* and *B. longum* exhibited excellent stability for 14 days at 4°C in the Kou Woan Lao. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 3 : 409-413)***

Key Words : Kou Woan Lao, Lao-chao, Probiotics, *Rhizopus javanicus*, *Saccharomyces cerevisiae*

INTRODUCTION

Kou Woan Lao, a fermented rice product well known in China, is an oriental style dairy product coagulated with culture filtrates from lao-chao (chiu-niang). It has been developed to meet consumer preferences for a low-acid or non-sour tasting yogurt-like product (Kuo et al., 1996; Lin and Chen, 1996). Culture filtrates from lao-chao, which is produced through fermentation by inoculating steamed glutinous rice with commercial starter (chiu-yao) or fungal cultures, have been used as both milk-clotting agents and flavoring agents. The resultant yogurt-like products with soft curd appearance are characterized by a sweet, fruity and slightly alcoholic flavor. It is very difficult to control the microflora of commercial chiu-yao for the fermentation. Therefore, some yeasts and molds were selected to prepare lao-chao from steamed glutinous rice (Wei and Jong, 1983; Lin and Chen, 1996; Chen et al., 1998). The compositions of culture filtrate and the changes of Kou Woan's component during fermentation have been studied by Chen et al. (1998). Pure culture filtrates from *Rhizopus javanicus* and *Saccharomyces cerevisiae* produced more ethanol and less reducing sugars than those with chiu-yao, but exhibited similar aroma components (Chen et al., 1998).

The use of *Lactobacillus acidophilus* and/or *Bifidobacterium spp.* in fermented or culture-containing dairy products became popular by the end of the 1970s as a result of the increase in knowledge encompassing the properties of probiotics (Gomes and Malcata, 1999; Ham et al., 2003). The nutritional benefits of probiotics have been mostly studied in milk-based products fermented with lactobacilli and bifidobacteria. They are characterized by a lower level of residual lactose and higher levels of free amino acids and certain vitamins than non-fermented milks. During the past years, numerous healthy and nutritional benefits have been claimed including maintenance of normal intestinal microflora, alleviation of lactose intolerance, reduction of serum cholesterol levels, potential antitumor activity, and some therapeutic effects on intestinal disturbances and intestinal infections (Fuller, 1989; Gomes and Malcata, 1999; Chen et al., 2004). Experiments performed in animal models showed that a few strains of *L. acidophilus* and *Bifidobacterium spp.* are able to decrease the levels of enzymes responsible for activation of some procarcinogens, and consequently decrease the risk of tumor development (Mital and Garg, 1992). A number of studies indicate that administration of *Bifidobacteria* or *Lactobacilli* alone or with fermentable carbohydrate (defined as a prebiotic) can alter colonic microflora populations and decrease the development of early preneoplastic lesions and tumors. Many of the antitumor activities attributed to lactic cultures have been suggested to involve an enhanced function of the immune response (Hirayama and Rafter, 2000). In order to exert positive health effects, the microorganisms need to be viable, active and abundant in the concentration of at least 10^6 CFU g^{-1} in the product throughout the specified shelf life according to Vinderola et al. (2000).

The purpose of this study was to combine the physiological functionality of probiotics and the unique flavor and milk-clotting activity of culture filtrate from lao-

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chao for developing a new dairy product which was different from the commercial yogurt.

MATERIALS AND METHODS

Preparation of inocula

The two strains of fungal cultures, *Rhizopus javanicus* (BCRC 30288) and *Saccharomyces cerevisiae* (BCRC 21685), from the Bioresources Collection and Research Center, Taiwan, ROC, were selected to inoculate steamed glutinous rice. Before use, *R. javanicus* and *S. cerevisiae* were transferred to slants and incubated at 30±1°C for 6-8 days. In addition, the probiotic strains, *Lactobacillus acidophilus* (BCRC 14079) and *Bifidobacterium longum* (BCRC 14605) were transferred to Lactobacilli MRS (deMan, Rogosa and Sharpe) and MGL (modified Garche's lithium-chloride) media. Fungal spore suspensions and yeast cell suspensions for inoculation were prepared by adding sterilized distilled water containing 0.1 g L^{-1} Tween 80 to slants and shaking the cultures vigorously for 1 min.

Preparation of fermented rice (lao-chao) and culture filtrates

Glutinous rice was purchased from a local market (Taipei, Taiwan). One hundred grams of glutinous rice, which had been washed with distilled water and drained, was soaked in 75 ml distilled water at room temperature (20-25°C) for 12 h, sterilized at 121°C for 15 min, and then cooled to room temperature. The steamed glutinous rice was inoculated with 1 ml of the spore suspensions containing 10^7 cells of *R. javanicus* and *S. cerevisiae*, respectively, followed by static incubation at 30±1°C for 6 days. After fermentation, the culture filtrates were obtained by filtering the fermented rice through four layers of cheesecloth. The sediment in the culture filtrate was eliminated by centrifuging at 1,480×g for 30 min (Kubota, KR-20000T, Tokyo 113, Japan). The culture filtrates were stored at 4°C for further analysis.

Preparation of probiotic Kou Woan Loa

The probiotic Kou Woan Loa was prepared by adding culture filtrates and probiotics (*Lactobacillus acidophilus* and *Bifidobacterium longum*) to pasteurized (at 85°C for 30 min) skim milk containing 12% nonfat milk powder (Anchor, Wellington, New Zealand) and incubated at 37°C for 0-36 h. According our preliminary test, Kou Woan Lao inoculated with 0.3% *L. acidophilus* and 0.7% *B. longum* demonstrated the highest bacteria counts, β-galactosidase activity, and L-(+) lactic acid levels, thus, 0.3% *L. acidophilus* and 0.7% *B. longum* were chosen.

Effect of culture filtrate on probiotic counts

In order to study the effect of culture filtrate on probiotoic counts, various ratios (10-40%) of culture filtrates were used as milk-clotting agents. Bacterial counts, pH value, titratable acidity were evaluated at 0, 6, 12, 18, 24, 30 and 36 h.

Effect of milk solid non fat (SNF) on probiotic counts

Pasteurized skim milk contains various ratios (12-18%) of SNF was inoculated with probiotics and 10% culture filtrate, and then incubated at 37°C for 0-36 h. The viable probiotics were evaluated during the periods of incubation.

The viability of probiotics in Kou Woan Lao during refrigerated storage

Kou Woan Loa were prepared by probiotics inoculation of pasteurized (at 85°C for 30 min) skim milk containing 12% nonfat milk powder and 10% culture filtrate, and then incubated at 37°C for 6 h. The samples were stored at 4°C for further analysis.

Enumeration of probiotics bacteria

The selective enumerations of *L. acidophilus* and *B. longum* were performed on MRS agar and MGL agar, respectivly. The *L. acidophilus* was incubated aerobically at 37°C for 48 h, while, the *B. longum* was incubated anaerobically during 72 h at 37°C.

Determination of pH, titratable acidity, curd firmness, viscosity and microstructure of Kou Woan Lao

The pH measurements were carried out by means of a digital pH-meter (ORION Model 520A, USA). The titratable acidity was determined by titration with 0.1 N NaOH and expressed as percent lactic acid.

The curd firmness, as measured by the breaking force of the Kou Woan Loa product, was determined by using a rheometer (Fudoh NRM-2010J-CW, Tokyo 141, Japan) with a rheoplotter (Rikadenki Kigyo, FR 801, Tokyo 113, Japan). Adaptor No.4 (20 mm dia) of the rheometer was used and table speed was 50 mm/min.

Viscosity was determined by using a Viscometer (Brookfield, LVDV-II+Viscometer, USA) with a No. 3 disc spindle at the speed of 0.3 rpm.

The microstructure of the product was investigated by scanning electron microscopy (SEM). Samples were taken from the center of the curds, cut into 3×3×2 mm pieces, and fixed for 4 h in 0.7% glutardialdehyde. Specimens were washed twice with deionized water and dehydrated in 20, 40, 60, 80, 95 and 99.5% absolute alcohol at 1 h intervals. Samples were critical-point-dried from CO_2 using a Critical Point Dryer Samdri-PVT-3B (Tousimis, Rockville, USA), mounted on aluminum stubs and coated with gold using an Ion Coater JFC1100E (Jeol, Tokyo, Japan). The preparations were observed with a Scanning Electron Microscopy JSM-6300 (Jeol, Tokyo, Japan).

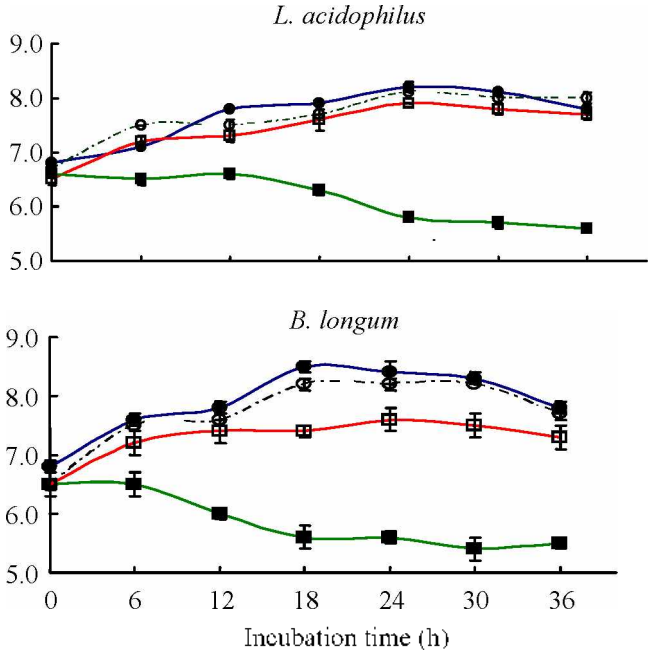

Figure 1. Effects of lao-chao culture filtrate on probiotic counts in Kou Woan Lao during incubation period. ● 10% culture filtrate from lao-cho, ○ 20% culture filtrate from lao-chao, = 30% culture filtrate from lao-chao, ■ 40% culture filtrate from lao-chao. * Values are mean±SD: error bars indicate 95% confidence intervals.

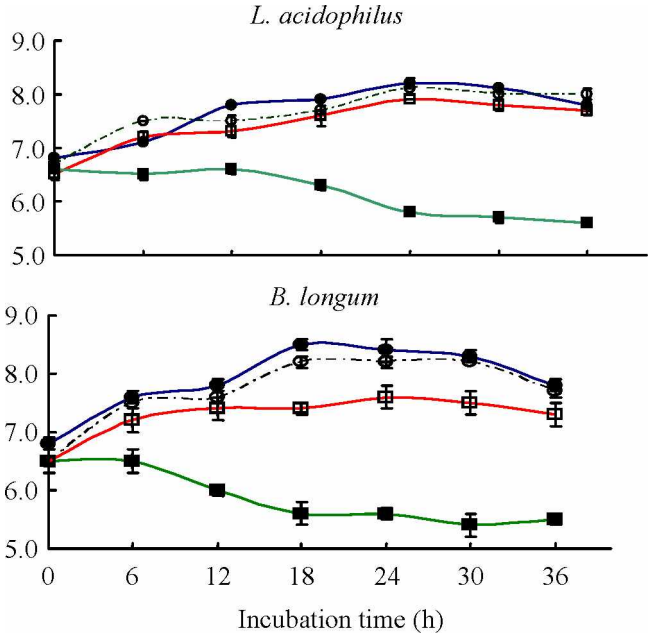

Figure 2. Effects of lao-chao culture filtrate on pH value and titratable acidity in Kou Woan Lao during incubation period. ● 10% culture filtrate from lao-chao, ○ 20% culture filtrate from lao-chao, □ 30% culture from lao-chao, ■ 40% culture filtrate from lao-chao. * Values are mean±SE: error bars indicate 95% confidence intervals.

Statistical analysis

Data were analyzed using the general linear model procedure of the SAS software package (SAS/STAT, 1999), and Duncan's multiple range test (Montgomery, 1991) were used to detect differences between treatment means. Statistical significance was tested at the 5% level. All experiments were replicated three times.

RESULTS AND DISCUSSIONS

Effect of concentrations of culture filtrate

Culture filtrates from a fermented rice product (lao-chao) were used as both a milk-clotting agent and a flavoring agent. In order to study the effect of culture filtrate on viability of probiotics, various ratios (10-40%) of culture filtrates were applied and probiotics counts, pH value and titratable acidity of Kou Woan Lao were evaluated. Results indicated both probiotic counts (Figure 1) increased with incubation time and maintained 10^7-10^8 CFU/ml after 6 h incubation with 10-30% culture filtrates. By contrast, samples with 40% culture filtrates inhibited the growth of *L. acidophilus* and *B. longum*, thus both probiotic counts decreased to 10^5 CFU/ml after 36 h fermentation.

Effects of various ratios of culture filtrates with probiotics on pH value and titratable acidity in Kou Woan Lao during incubation are shown in Figure 2. The pH values of Kou Woan Lao decreased and titratable acidity increased gradually with incubation for all ratios of culture filtrates. However, the more culture filtrates were added, the lower titratable acidities and higher pH values in Kou Woan Lao were detected after 36 h fermentation. Since, addition of 40% culture filtrate resulted in the lowest titratable acidity and the highest pH value. The titrable acidity and pH vale of Kou Woan Lao was depended on the viability of probiotics. This result is in line with the finding for probiotic counts (Figure 1). Several research stated that survival of *L. acidophilus* and *B. longum* were affected by low pH of the environment (Shah et al., 1995; Shin et al., 2000; Bruno et al., 2002). A rapid decrease in their numbers has been observed under acid conditions, both *in vitro* and *in vivo*. *B. longum* is significantly retarded below pH 5.0 (Shah et al., 1995). The pH of culture filtrate was 4.5 and the initial pH value of 40% culture filtrate sample was 5.5. Both pH values might retard the growth of both *L. acidophilus* and *B. longum*. *Bifidobacteria* species such as *B. longum* are fastidious organisms that require specific growth factors and prefer a low oxidation-reduction potential for growth (Shah, 2000).

Effect of milk solid non fat (SNF)

Effect of different ratios (12-18%) of milk SNF on probiotic counts in Kou Woan Lao is depicted in Fig. 3. No significant differences ($p>0.05$) were found for both *L. acidophilus* and *B. longum*, when grown in differing

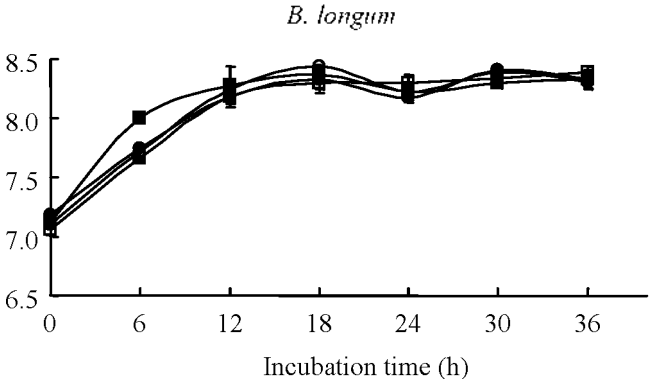

Figure 3. Effects of SNF on probiotic counts in Kou Woan Lao during incubation period. ● 12% SNF, ○ 14% SNF, ▫ 16% SNF, ■ 18% SNF. * Values are mean±SD: error bars indicate 95% confidence intervals.

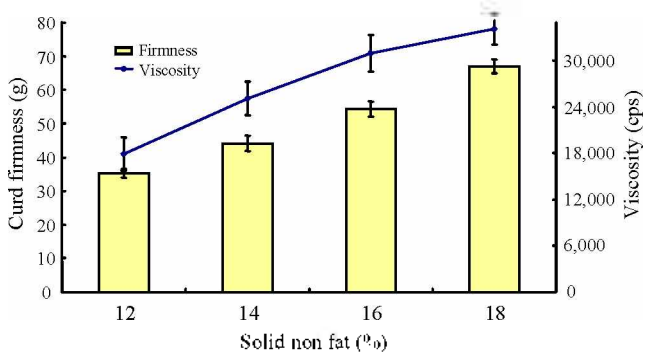

Figure 4. Effects of SNF percentage on curd firmness and viscosity of curd. * Values are mean±SD: error bars indicate 95% confidence intervals.

concentrations of skim milk powders. The counts of two strains for all fermented samples were maintained in the level more than 10^8 cfu/m l after 18 h incubation.

Figure 4 shows the viscosity and curd firmness of various SNF of Kou Woan Lao. The increase in viscosity and curd firmness corresponded with the increase of solid content. Oliveira et al. (2001) indicated that increase of fermented milk curd firmness depends on the total solids as well as on protein content.

Microstructure of Kou Woan Lao

The scanning electron micrographs of Kou Woan Lao inoculated with and without probiotics are presented in Figure 5. Kou Woan Lao consisted of a protein matrix composed of a branched network of casein particles forming chains and clusters, immobilizing the continuous phase into pores of small sizes (Figure 5a). Cavities were noticeable in Kou Woan Lao. SEM showed that Kou Woan Lao containing probiotics presented the most dense structure and tightest network due to the synergistic action of acid and enzyme in the milk (Figure 5b). Plus, the acidity in the treatment of 0.3% *L. acidophilus* and 0.7% *B. longum* made the curding more prominent. The casein micelles in the milk will connect tightly during the curding process.

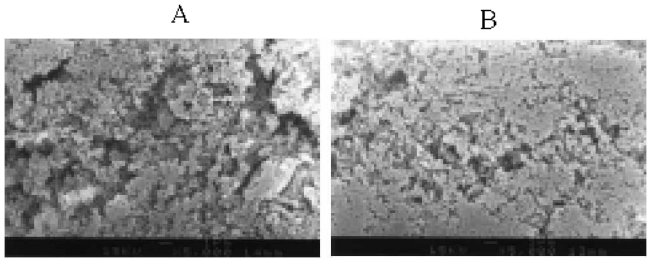

Figure 5. Scanning electron micrographs (×5,000) of Kou Waon Lao (A):(B) inoculated with 0.3% *L. acidophilus* and 0.7% *B. longum*.

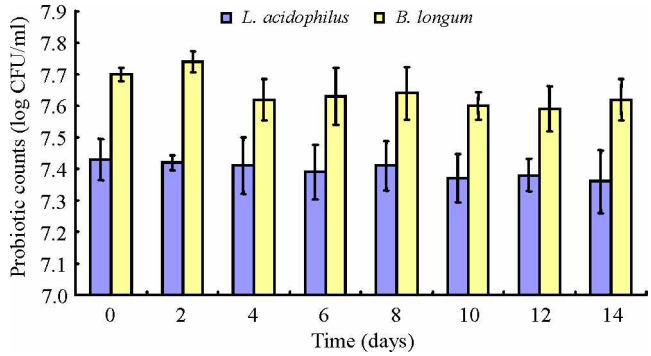

Figure 6. Probiotic counts in Kou Woan Lao during refrigerated storge. * 10% culture filtrate from lao-chao, ** Values are mean±SD.

The viability of probiotics in Kou Woan Lao during refrigerated storage

Figure 6 shows probiotic counts in Kou Woan Lao during refrigerated storage. The study results revealed that both *L. acidophilus* and *B. longum* exhibited excellent stability for 14 days of storage at 4°C in the Kou Woan Lao. The counts of *L. acidophilus* and *B. longum* were maintained above 7.36 and 7.59 log CFU ml^{-1} in all fermented samples, respectively. Several factors affected the survival of the probiotic bacteria. Dave and Shah (1998) reported stability of *Bifidobaceria* and *L. acidophilus* varied in yogurt at refrigeration temperature, depending on the supplementation of the yogurt mix with cystein or protein hydorlysates. Gilliland et al. (2002) reported that pH of the products influenced survival of the probiotic bacteria during storage. Stopping the fermentation at a higher pH might have been beneficial in this regard.

CONCLUSION

In summary, both probiotic counts increased with incubation time and maintained 10^7-10^8 CFU/ml after 6 h incubation with 10-30% culture filtrates for milk-clotting agent. By contrast, sample with 40% culture filtrates

inhibited the growth of *L. acidophilus* and *B. longum*. No significant differences ($p>0.05$) were found for both *L. acidophilus* and *B. longum*, when grown in differing concentrations of skim milk powders. According to the results, the probiotic Kou Woan Loa would be prepared optimally by adding 10% culture filtrate and 0.3% *L. acidophilus* and 0.7% *B. longum* to pasteurized (at 85°C for 30 min) skim milk containing 12% nonfat milk powder (Anchor, Wellington, New Zealand) and incubated at 37°C for 6 h.

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