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Fermentation of a milk–soymilk and *Lycium chinense* Miller mixture using a new isolate of *Lactobacillus paracasei* subsp. *paracasei* NTU101 and *Bifidobacterium longum*

Abstract A milk–soymilk mixture was fermented using *Lactobacillus paracasei* subsp. *paracasei* NTU101 and *Bifidobacterium longum* BCRC11847 at different inoculum ratios (1:1, 1:2, 1:5, 2:1, and 5:1). When the inoculum ratio was 1:2, the cell numbers of both strains were balanced after 12 h of cultivation. The pH and titratable acidity were very similar at the various inoculum ratios of cultivation. The milk–soymilk mixture was supplemented with 5, 10, 15, and 20% *Lycium chinense* Miller juice and fermented with *Lactobacillus paracasei* subsp. *paracasei* NTU101 and *B. longum* BCRC11847. Sensory evaluation results showed that supplementation with 5% *Lycium chinense* Miller juice improved the acceptability of the fermented milk–soymilk. The fermented beverage was stored at 4°C for 14 days; variations in pH and titratable acidity were slight. The cell numbers of *L. paracasei* subsp. *paracasei* NTU101 and *B. longum* BCRC11847 in the fermented beverage were maintained at 1.2×10^9 CFU/ml and 6.3×10^8 CFU/ml, respectively, after 14 days of storage.

Keywords *Lycium chinense* Miller · Soymilk · *Lactobacillus paracasei* subsp. *paracasei* · *Bifidobacterium longum*

Introduction

Soybean products such as soymilk, tofu, soybean cheese and miso are traditional foods in Asia; they are rich in protein, unsaturated fatty acids, lecithin and isoflavones, contain no cholesterol or lactose, and may be consumed by those suffering from lactose intolerance. Soymilk is also a suitable medium for growing lactic acid bacteria, thus improving the quality of fermented products. Similar to yogurt, fermented soymilk is called soyoghurt [1, 29, 35], and soymilk has been recently used to partially replace milk in the production of yogurt-like products [10, 26].

The dried ripe fruit of *Lycium chinense* Miller has been widely used as an ingredient of herbal medicines and functional foods in traditional Chinese families. It contains β-carotene, ascorbic acid and tocopherols. The dried root bark of *L. chinense* Miller has been reported to contain phenolic amides, which have potent antioxidative activity and anti-fungal effects [14, 24]. Its leaves are said to be capable of preventing certain human diseases, such as arteriosclerosis, essential arterial hypertension, diabetes and night blindness [20]. The dried fruit of *L. chinense* Miller has also been reported to improve eyesight and fortify liver and kidney activities. Based on these health effects, and the sweetness and atractive red color it renders to products, *L. chinense* Miller was tested as a supplement to lactic-acid-bacteria-fermented milk–soymilk beverage in this study.

Lactic acid bacteria are currently being used in probiotic preparations, either singly or in combination. *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* are the most commonly used in fermented dairy products [9, 11] since these bacteria comprise a portion of the desirable intestinal microflora [3] and have beneficial effects, including stimulation of the immune response [18, 34], anticarcinogenic activity [16, 25, 37] and inhibition of the growth of pathogens [2, 4, 8, 15, 17, 31]. There is a continuing need to improve existing starter cultures or to select new strains for use in specific fermentation processes when developing new products. The lactic acid bacteria from newborn infant feces were characterized and one of the isolates identified as *Lactobacillus paracasei* subsp. *paracasei* NTU101. This strain showed good survival at low pH, tolerance against high bile concentration, and the ability to reduce serum cholesterol in vitro.

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A drawback to the use of soymilk is its unpleasant bean flavor, which is due to the presence of *n*-hexanal and pentanal, naturally present in soymilk as products of the breakdown of unsaturated acids [27]. The bean flavor and the presence in soymilk of indigestible oligosaccharides (such as raffinose and stachyose), which cause flatulence, have been responsible for low consumer acceptance. *Bifidobacterium* spp. can reduce the off-flavor of *n*-hexanal [30, 32] and ferment sucrose, raffinose and stachyose [7]. Furthermore, a significantly higher reduction in raffinose and stachyose content was found in soymilk fermented with mixed cultures of bifidobacteria and lactic acid bacteria than in those fermented with single lactic acid bacterium culture [36]. Thus, the aim of this work was to combine different percentages of milk, soymilk and *L. chinense* Miller juice with a mixed culture of *Lactobacillus paracasei* subsp. *paracasei* NTU101 and *B. longum* BCRC11847 in order to produce a new fermented lactic acid beverage.

Materials and methods

Bacterial strains

Lactobacillus paracasei subsp. *paracasei* NTU101 was isolated from infant feces in our laboratory. *B. longum* BCRC 11847, was obtained from the Biosource Collection and Research Center, the Food Industry Research and Development Institute (FIRDI), Hsinchu, Taiwan.

Preparation of milk and soymilk

Milk was 8% (w/v) reconstituted skim milk powder. Soymilk was prepared following the method of Chou and Hou [5]. Whole soybeans were washed and soaked overnight in distilled water. After decanting the water, the soaked soybeans were mixed with ten times their weight in distilled water and placed in a blender for 3 min. The resultant slurry was filtered through double-layered cheesecloth to obtain soymilk. The milk and the soymilk were heat-treated individually in water baths at 90°C for 1 h. After heat treatment and cooling to room temperature, they were mixed at a proportion of 25% milk and 75% soymilk.

Ratio of mix-culture strains

The strains were cultivated in MRS broth (Difco, Detroit, Mich., USA) and then subcultured in skim milk at 30°C for 16 h; this served as the inoculum. The inoculum ratios of *Lactobacillus paracasei* subsp. *paracasei* NTU101 and *B. longum* BCRC11847 were at 1:1, 1:2, 1:5, 2:1, and 5:1 (v/v), respectively, for the fermented 25% milk and 75% soymilk combinations. The final inoculum volume was 3% (v/v).

Ratio of *L. chinense* Miller juice to milk–soymilk

Lycium chinense Miller was washed and mixed with five times its weight in distilled water and blended for 3 min. The resultant slurry was filtered through a sieve (juice contains pulp) and then heated in a water bath at 90°C for 1 h. After heat treatment and cooling to room temperature, *L. chinense* Miller juice was added to the milk–soymilk mixtures at the rate of 5, 10, 15, and 20%, respectively.

Batch fermentation and analytical assays

Lactobacillus paracasei subsp. *paracasei* NTU101 and *B. longum* BCRC11847 were cultivated in 100 ml of sterilized milk–soymilk with different amounts of *L. chinense* Miller juice at 30°C for 12 h. During incubation, samples were taken at 0, 2, 4, 6, 8, 10, and 12 h for testing of pH, culture growth and titratable acidity. The pH of the sample was measured with a pH meter (MP2000, Mettler Toledo, Greifensee, Switzerland). MRS agar (Difco, Detroit, Mich., USA) was used to enumerate *L. paracasei* subsp. *paracasei* NTU101 and *B. longum* BCRC11847. Plates were incubated in an ordinary incubator at 37°C for 2 days. The colonies (the morphologies of two strains differed) that appeared on the plates were then counted. Titratable acidity, expressed as a percentage of lactic acid by weight, was measured by titration with 0.1 N NaOH.

Sensory evaluation

The milk–soymilk mixtures were supplemented with 5, 10, 15, and 20% *L. chinense* Miller juice and fermented with *L. paracasei* subsp. *paracasei* NTU101 and *B. longum* BCRC11847 at 30°C for 8 h. The organoleptic properties of the fermented products were then evaluated. Sensory evaluation was carried out after 24 h storage at 4°C. Samples (ca. 5% sugar) were served at about 4°C and were scored on a 1–9 hedonic scale (nine being considered excellent, five acceptable, and one extremely poor). A panel of 32 untrained students evaluated each sample. The characteristics evaluated included color, flavor, taste, texture and overall acceptability. Fermented milk–soymilk beverage, with no *L. chinense* Miller juice added, was used as the control. The other samples were compared with the control, evaluated and scored. Duncan's multiple range tests were used to determine significant differences.

Shelf-life studies

The shelf-life of the sample with the highest mean score of overall acceptability was assessed. The fermented beverage was stored at 4°C for 14 days, and then evaluated for pH, culture viability and titratable acidity.

Results and discussion

### Effect of different ratios of mixed culture strains

Bifidobacterium spp. can reduce the off-flavor of *n*-hexanal and fermented sucrose, raffinose and stachyose. For this reason, it was used as the starter culture for fermenting soymilk, in order to increase its acceptability. Different inoculum ratios (1:1, 1:2, 1:5, 2:1, and 5:1) of *Lactobacillus paracasei* subsp. *paracasei* NTU101 and *B. longum* were used to ferment the milk–soymilk mixture.

As shown in Fig. 1, *Lactobacillus paracasei* subsp. *paracasei* NTU101 reached higher cell numbers than *B. longum*, except for the inoculum ratio at 1:5, but its presence did not influence the development of *B. longum* in the milk–soymilk. At inoculum ratios of *Lactobacillus paracasei* subsp. *paracasei* NTU101 and *B. longum* were of 1:2 and 1:5, the cell numbers of both strains were about the same. The cell number of *B. longum* was higher than that of *L. paracasei* subsp. *paracasei* NTU101 after 12 h of cultivation at an inoculum ratio at 1:5. At an inoculum ratio of 1:2, the cell numbers of both strains were balanced after 12 h of cultivation. A good balance of these two species is desirable in fermented beverages, since both are associated with beneficial effects [21]. As such, the ratio of 1:2 (*L. paracasei* subsp. *paracasei* NTU101: *B. longum*) was used in subsequent studies. By contrast, at the different inoculum ratios of cultivation, the pH and titratable acidity were very similar (Fig. 2). This result concurs with the report of Macedo et al. [26], indicating that, in spite of the difference in the number of viable cells, the pH and titratable acidity of all the beverages were very similar.
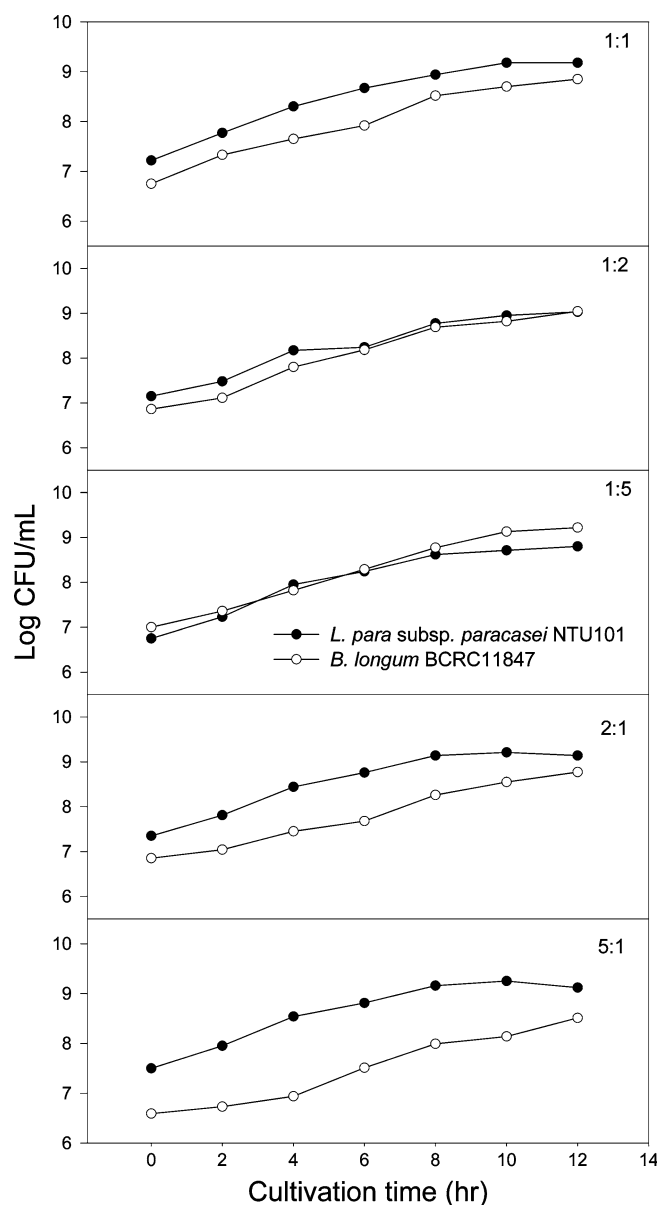
Fig. 1 Cell numbers of different inoculum ratios of *Lactobacillus paracasei* subsp. *paracasei* NTU101 and *Bifidobacterium longum* BCRC11847 in 25% milk and 75% soymilk
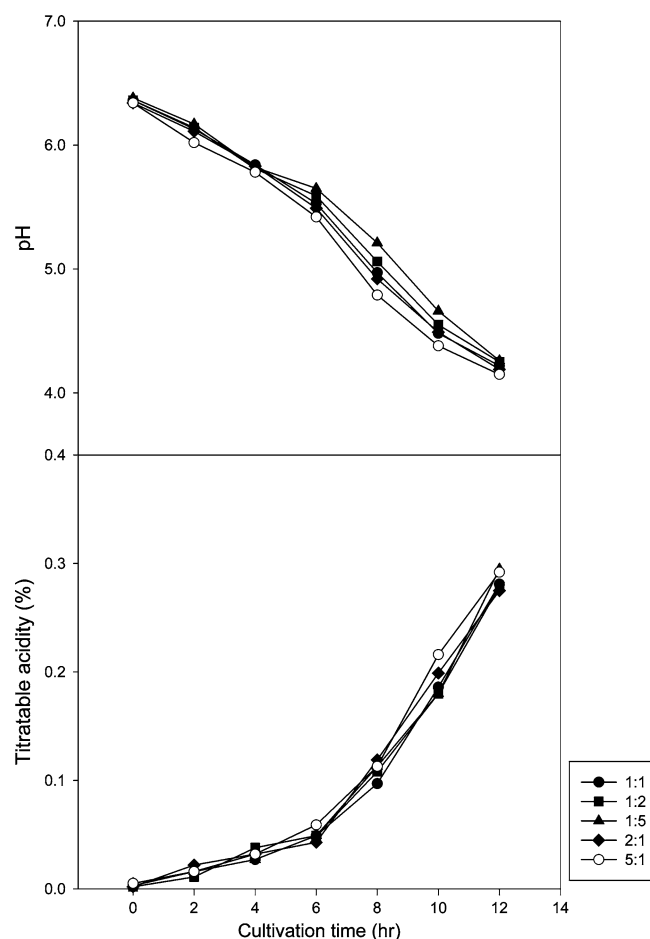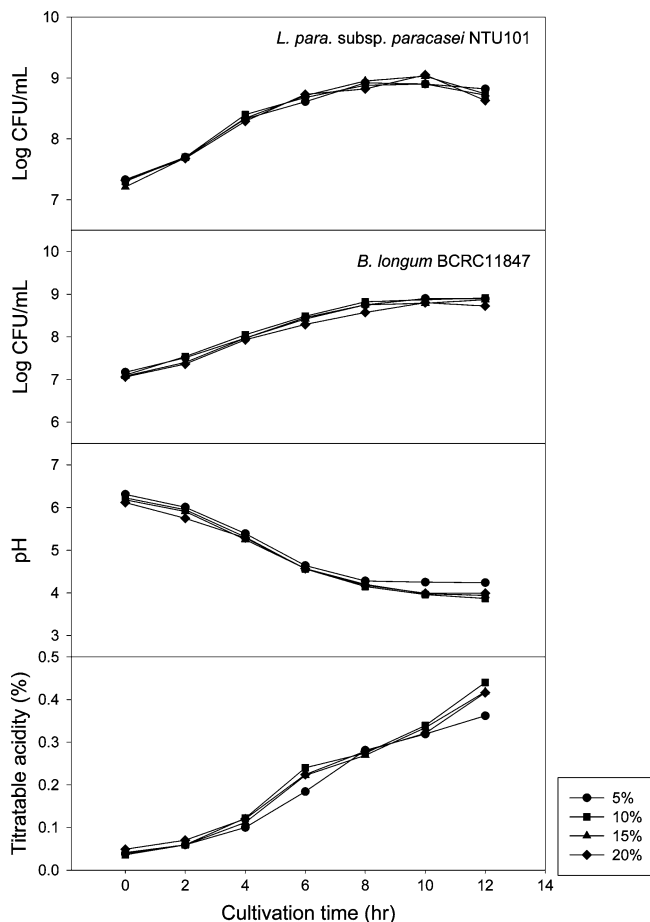

Fig. 2 Titratable acidity and pH of different inoculum ratios of *L. paracasei* subsp. *paracasei* NTU101 and *B. longum* BCRC 11847 in 25% milk and 75% soymilk

Effect of different ratios of *L. chinense* Miller juice to milk–soymilk

The combination of 25% milk and 75% soymilk was supplemented with 5, 10, 15 and 20% *L. chinense* Miller juice and then fermented. The results are shown in Fig. 3. Supplementation with different amounts of *L. chinense* Miller juice did not affect the growth of *Lactobacillus paracasei* subsp. *paracasei* NTU101 or *B. longum* (in the inoculum ratio of 1:2); the cell numbers for both *Lactobacillus paracasei* subsp. *paracasei* NTU101 and *B. longum* were about the same. The pH levels of the fermented beverages supplemented with 10, 15, and 20% *L. chinense* Miller juice were all below 4.0. The titratable acidity of the milk–soymilk mixture supplemented with *L. chinense* Miller juice was higher than that without added *L. chinense* Miller juice.

Sensory evaluation

The results of the sensory evaluation are shown in Table 1. The mean score for the color of the fermented beverage supplemented with 5% *L. chinense* Miller juice

Table 1 Average scores for the sensory properties of different fermented soymilks. *Control* Milk–soymilk mixture with no added *L. chinense* Miller juice; *5, 10, 15, 20* milk–soymilk mixture supplemented with 5, 10, 15, and 20% *L. chinense* Miller juice, respectively. Numbers with a different letter superscript were significantly different (5% probability) from each other

Characteristics	Sample				
	Control	5	10	15	20
Color	5.0[b]	5.8[a]	5.5[a, b]	5.2[a, b]	3.8[c]
Flavor	5.0[a]	4.9[a]	5.4[a]	5.2[a]	5.2[a]
Taste	5.0[a]	4.9[a]	5.4[a]	5.2[a]	5.3[a]
Texture	5.0[a, b]	5.3[a]	5.4[a]	5.2[a, b]	4.6[b]
Overall acceptability	5.0[b]	5.9[a]	5.0[b]	5.0[b]	4.8[b]

was higher than that of the other samples, and significantly different from both the control and the fermented beverage supplemented with 20% *L. chinense* Miller juice. Supplementation with 5% *L. chinense* Miller juice improved the color of the beverage. Although the flavor and taste scores of the beverage supplemented with 5% *L. chinense* Miller juice were lowest, and highest for the beverage supplemented with 10% *L. chinense* Miller juice, there were no significant differences between any of the samples. The flavor and taste scores for all samples were almost the same. The mean scores for the texture of the fermented beverages supplemented with 5 and 10% *L. chinense* Miller juice were significantly higher than those for samples supplemented with 20% *L. chinense* Miller juice. In overall acceptability, the fermented beverage supplemented with 5% *L. chinense* Miller juice had the highest mean scores; this was significantly different from all other samples. As shown from the results, supplementation with 5% *L. chinense* Miller juice can improve the acceptability of a fermented beverage made from a combination of 25% milk and 75% soymilk.

Shelf-life studies

Changes in viable cells and pH levels of fermented products have been found to be related to storage time, temperature, hydrogen peroxide content, oxygen content, initial number of strains and the kinds of strains used [6, 28]. For example, Chou and Hou [5] reported that the cell numbers and pH level of *Bifidobacterium infantis*, in fermented soymilk were lower when stored at 25°C than at 5°C. On the other hand, when Garro et al. [12] fermented soymilk with several single strains, it was found that, during storage at 4°C, the stability of the microflora differed markedly among the starter cultures. Therefore, both the starter used for fermentation and the storage conditions are very important to the quality of fermented beverages. As shown in Fig. 4, after 6 days of storage, the cell numbers of *L. paracasei* subsp. *paracasei* NTU101 and *B. longum* had increased slightly. The reason may be that the fermented beverage was sweetened with 5% sugar, which had been used as a
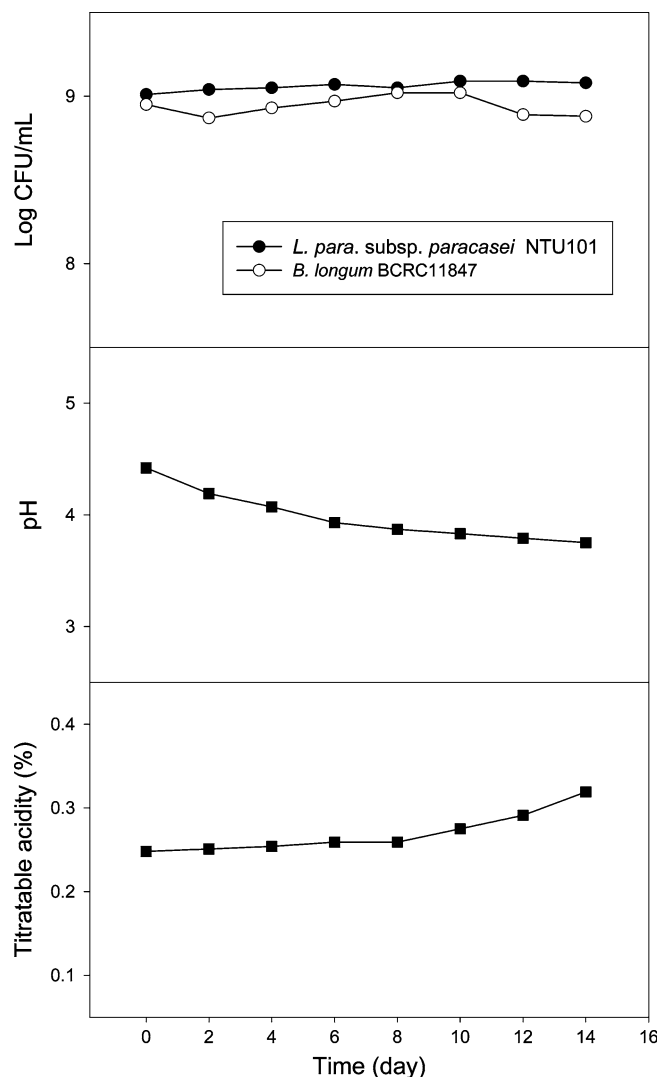

Fig. 3 Growth of culture mix in 25% milk and 75% soymilk supplemented with different amounts of *L. chinense* Miller juice

Fig. 4 Variation in cell numbers, pH and titratable acidity in the fermented beverage during storage

source of carbon for strain growth. After 10 days of storage, the cell numbers of *B. longum* had decreased, reaching 6.3×10^8 CFU/ml on the 14th day. A variation in cell numbers of *L. paracasei* subsp. *paracasei* NTU101 was not apparent. After 12 days of storage, the cell numbers of *L. paracasei* subsp. *paracasei* NTU101 had slightly decreased and remained at 1.2×10^9 CFU/ml at the end of the storage period. The cell numbers of the yogurt organism initially increased after manufacture, decreasing during a long storage period [13, 22, 26]. In this study, the decrease in the cell numbers of *B. longum* was more significant than that of *L. paracasei* subsp. *paracasei* NTU101. Lankaputhra et al. [23] reported that at a pH below 4.3, bifidobacteria survival was greatly affected. It has been suggested that, in order to have a therapeutic effect, the minimum number of probiotics in a product should be above 10^5 or 10^6 CFU/g [19, 33]. Medina and Jordano [28] also reported that a high-quality fermented beverage should contain 1×10^6–1×10^8 CFU probiotics/ml. After 14 days of storage, the

cell numbers of *L. paracasei* subsp. *paracasei* NTU101 and *B. longum* in the fermented beverage in this study were 1.2×10^9 and 6.3×10^8 CFU/ml, respectively. The variation in pH and titratable acidity were slight and may have been due to the buffering effect of solids (soy proteins, sugar) in the fermented beverage [26]. After 14 days of storage, the titratable acidity had increased slightly. During the storage period, syneresis did not occur.

Conclusion

In this study, a fermented beverage was produced with a combination of milk, soymilk and *L. chinense* Miller juice. It was fermented using a culture mix of *Lactobacillus paracasei*. subsp. *paracasei* NTU101 and *B. longum*. According to the sensory evaluation results, supplementation with 5% *L. chinense* Miller juice was found to improve the acceptability of the fermented milk and soymilk combinations. The variations in cell numbers, pH levels and titratable acidity of the products were slight and syneresis was not observed. The quality of this product would, therefore, be retained during storage and could be applied to dietary adjuncts.

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