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Preparation of a Whey-Based Probiotic Product with *Lactobacillus reuteri* and *Bifidobacterium bifidum*

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Summary

Lactobacillus reuteri and *Bifidobacterium bifidum* were inoculated ($2.8 \cdot 10^8$ and $4.7 \cdot 10^8$ CFU/mL, respectively) into reconstituted whey containing sucrose and pectin in order to prepare a fermented probiotic product. Inoculation levels were: 0.5, 1 or 2 % for *Lactobacillus reuteri* and 0.5 or 1 % for *Bifidobacterium bifidum.* The treatment with the highest bacterial counts and sensory scores was selected and stored at 4 °C for 30 days. Microbial counts, changes in pH values, titratable acidity and both triangle test and sensory attributes were monitored on the stored product. The beverage fermented for approx. 11 h and prepared with 2 % *Lactobacillus reuteri* and 0.5 % *Bifidobacterium bifidum* met the probiotic criterion by maintaining both bacterial populations at counts greater than 10^6 CFU/mL for the whole storage period. Titratable acidity and pH values as well as sensory properties did not change appreciably during the first 14 days of storage. At the end of the storage period (30 days), slight acidification was detected, although the beverage still retained an acceptable flavour.

Key words: lactic acid bacteria, probiotics, *Lactobacillus reuteri*, *Bifidobacterium bifidum*, whey, fermented beverage

Introduction

In addition to their role in fermentation processes, some probiotic lactic acid bacteria have been studied as dietary sources of live microorganisms destined to promote a positive impact in the host by improving the properties of the indigenous beneficial microbiota (*1*). Documented benefits of the ingestion of probiotics include: reduction of serum cholesterol, alleviation of lactose intolerance, reduction of cancer risk, antihypertensive effect, and resistance to enteric pathogens, among others (2). Traditionally, probiotics have been added to yoghurt, and it is estimated that currently more than 70 products containing lactobacilli or bifidobacteria are being produced worldwide, including sour cream, buttermilk and frozen desserts. Recently, the key growth sector has been probiotic drinks (*3*). The inclusion of these microorganisms into dairy products induces unique flavour profiles and texture; and the major difference among products, apart from the amount and type of supplementation, is the specific organism used as a probiotic and its health promoting effects (*4*). *Bifidobacterium bifidum* and, in recent years, *Lactobacillus reuteri* have been considered important bacteria for human health; the main probiotic effects attributed to these bacteria include: improvement in lactose utilisation, prevention of diarrhoea, colon cancer, hypercholesterolemia, improvement of vitamin synthesis and calcium absorption (*5*), development of longer villi and significantly deeper crypts in the ileal region of the gut and production of substances of low molecular mass with antimicrobial activity (*6*,*7*). It is therefore understandable that lately there has been an

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increasing interest in the incorporation of these species into fermented milk products.

There are different opinions about the number of microorganisms that should remain viable in a beneficial product to perform their probiotic action, but in general it has been accepted that dairy products should contain $\geq 10^6$ CFU/mL of viable probiotic bacteria to be effective and should be consumed regularly (*8*,*9*). The aim of this work was the formulation of a whey-based fermented product containing populations of *L. reuteri* and *B. bifidum* greater than 10^6 CFU/mL during storage.

Materials and Methods

Probiotic strains

L. reuteri (NRRL14171) was kindly provided in freeze-dried form by the Agricultural Research Service (USDA) in Peoria, IL, USA. The strain was activated in MRS broth (De Man, Rogosa and Sharpe, Difco™) and maintained by routine subculture in slanted tubes containing LBS agar (*Lactobacillus* selection, Difco™) at 4 °C. Three subcultures, with 1 % inoculum, incubated for 14 h at 37 °C in anaerobic jars (Difco, BBL® Gaspak® anaerobic system), were performed consecutively before the use of the strains in experiments.

B. bifidum (NCFB2715) was kindly provided by Prof. Mariano Garcia Garibay from the Universidad Autonoma Metropolitana (Mexico City, Mexico). The strain was maintained in MRS medium containing 1 % lithium chloride, 0.3 % sodium propionate and 0.5 % L-cysteine at 4 °C. Two subcultures (1 % inoculum, incubated for 10 h at 37 °C in anaerobic jars) were performed immediately before the culture was used experimentally.

Probiotic beverage preparation

For the production of the fermented beverage, a base was prepared with reconstituted whey (7 %) (HELM®, Mexico City, Mexico) with added 7 % sucrose and 0.4 % pectin (HELM®). The pH value was adjusted to 6.0 with 0.1 M NaOH and the base was pasteurized at 80 °C for 30 min. Three treatments were performed by inoculating probiotic strains (*L. reuteri* $2.8 \cdot 10^8$ and *B. bifidum* $4.7 \cdot 10^8$ CFU/mL) at different ratios: T_1 (*L. reuteri* 1 %, *B. bifidum* 0.5 %); T_2 (*L. reuteri* 1 %, *B. bifidum* 1 %); and T_3 (*L. reuteri* 2 %, *B. bifidum* 0.5 %). The inoculated beverage was incubated in sterilized glass bottles at 37 °C in anaerobic atmosphere. When maximal bacterial populations were reached, fermentation was stopped by quick chilling. The fermented beverage was stored for 30 days at 4 °C.

Calculation of kinetic parameters

To estimate the appropriate time to stop the fermentation for each treatment, the kinetic parameters from the total cell counts were calculated using a modified form of the Gompertz equation (*10*):

$$F = ae^{-e^{(c-t)be^{(1/a)+1}}} \qquad /1/$$

where F is the $\log_{10}$ CFU/mL, a is the maximum cell population once the stationary phase was reached (log (N/N_0)) either for each bacterium or the sum of both (total cell count), b is the growth rate (h^{-1}), c is lag time (h) and t is time. The parameters were estimated by fitting the experimental data to the model using non-linear regression.

Microbiological analysis

The viable count of the probiotic bacteria was determined using the pour-plate method (*5*) and the results were expressed as CFU/mL. Selective media were used to quantify the two strains. *L. reuteri* was enumerated by plating the appropriate dilutions on modified LBS agar, in which the dextrose acting as carbon source was replaced by arabinose (0.3 %), and the pH of the medium was adjusted to 5.0. Modified MRS medium, with added lithium chloride 0.3 %, nalidixic acid 0.3 %, neomycin sulphate 0.2 % and L-cysteine hydrochloride 0.05 % at pH=7, was used to enumerate *B. bifidum* cells. Total population of viable microorganisms was counted on regular MRS medium (pH=5.5). All plates were incubated anaerobically at 37 °C for 48 h.

Chemical analysis

The pH values were determined using an Orion pH meter (Model 290A). Titratable acidity was measured in 10-mL samples with 0.1 M NaOH using phenolphthalein as an indicator. The results were expressed as percentage of lactic acid (*11*).

Sensory evaluation

Ten judges (8 female and 2 male, age range 22–27) were selected to detect differences among the probiotic beverages, using the triangle test. Twelve sessions were performed with 12 tests per session for each of the comparison pairs (T_1 *vs.* T_2, T_2 *vs.* T_3, T_1 *vs.* T_3). The degree or extent of the difference among treatments was estimated by d' value, which is defined as the difference between the means of the intensity distribution for two products measured in perceptual standard deviations. In other words, the d' value indicates how different the two products are from each other; the higher d' value the more different the two products are. The frequencies of response were used to compute d' values, using triangle tables (*12*). Tests for significant differences between d' values were performed according to Bi *et al.* (*13*). Preference tests were also performed on these formulations. For this, 109 children from eight to twelve years old from a local elementary school took part in the study. According to the sensory results and microbiological analysis obtained so far, the treatment 3 was selected to perform a second round of sensory tests during the storage period in order to determine possible differences between the fresh and the beverage stored for 30 days, and to establish which attribute could be the responsible for such difference (attribute test).

Statistical analysis

The results of triangle and preference tests were analysed by χ^2 ($p \leq 0.05$). ANOVA and Tukey's mean comparison tests ($p \leq 0.05$) were used to evaluate the sensory data obtained from the attribute test using the MINITAB statistical package ver. 10.51. All experiments and analyses were run in duplicate.

Results and Discussion

Prepared probiotic beverages

Three sets of fermented beverages were obtained by inoculation of the probiotic strains in the whey base. The counts of *B. bifidum* showed the same trend in the three sets (see Fig. 1), which suggested that the concentration of inoculum affected neither the highest cellular population reached nor the growth rate. In the case of *L. reuteri*, the growth rate was similar in the three sets (Table 1); however, the final counts reached were dependent on the initial concentration of the inoculum. Neither symbiotic nor antagonistic relationships between the probiotic strains were observed, since the trend of the viable counts for each strain cultured alone in the whey base was similar to that measured for the same strain in the different treatments performed. The growth rate of *L. reuteri* was different from that of the *B. bifidum* ($p \leq 0.05$).

From the Gompertz equation it was deduced that the time needed to reach maximal counts was 11.70 h for set T_1, 09.30 h for set T_2 and 10.70 h for set T_3. When the fermentation was stopped in the different treatments, *L. reuteri* counts were at the stationary stage in T_1 and T_2, whereas in T_3 the counts remained in the transition between the exponential and stationary stages. For all the treatments, *B. bifidum* had already begun the stationary stage (Fig. 1).

Even when in the different treatments the inoculum levels of *B. bifidum* were lower than of *L. reuteri*, the maximal cell population reached by *B. bifidum* was always higher than that of *L. reuteri* (Table 1), which suggested that *B. bifidum* had better capacity of adaptation to the base beverage. However, the growth of both probiotic bacteria was limited, increasing just 1/2–1 log cycle of their initial counts. This could be due to the low proteolytic capacity (*14*) of the probiotic bacteria or to the relatively low concentration of nutrients available for the growth of these microorganisms. To this respect, Ravula and Shah (*15*) indicated that probiotic bacteria grow slowly in dairy fluids because they have low proteolytic activity, therefore supplemented milk could improve the viability of these bacteria. Modler and Villa-Garcia (*16*) found that the supplementation of whey with cysteine and yeast extract improved the growth of bifidobacteria and other probiotic bacteria.

The results of the triangle test showed no sensory differences between T_1 and T_2. However, T_3 was differ-

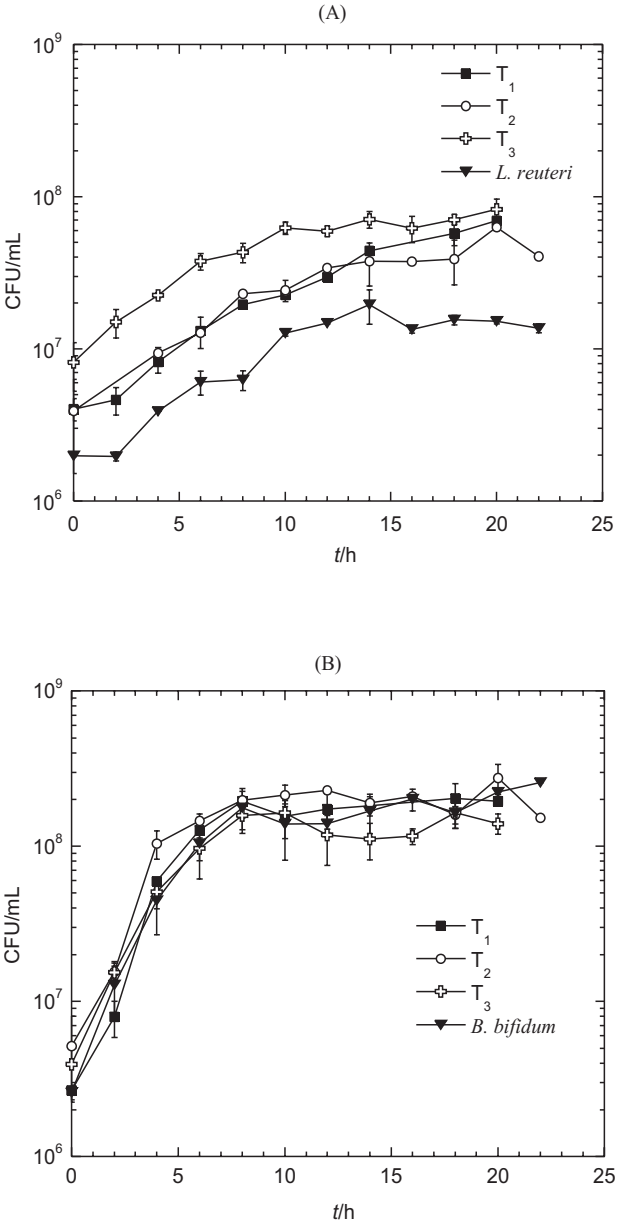

Fig. 1. Growth of *L. reuteri* (A) and *B. bifidum* (B) at 37 °C and pH=6 in the different treatments
■ T_1 (*L. reuteri* 1 %, *B. bifidum* 0.5 %), o T_2 (*L. reuteri* 1 %, *B. bifidum* 1 %), ✞ T_3 (*L. reuteri* 2 %, *B. bifidum* 0.5 %), ▼ probiotic bacterium alone on the whey-based beverage

Table 1. Kinetic parameters of bacterial counts in the different treatments

Parameters	Treatment 1			Treatment 2			Treatment 3		
	Total count	*L. reuteri*	*B. bifidum*	Total count	*L. reuteri*	*B. bifidum*	Total count	*L. reuteri*	*B. bifidum*
a Maximum cell population ($\log(N/N_0)$)	1.45^{a} ±0.09	$1.10^{1,2}$ ±0.17	1.76^{4} ±0.10	1.46^{a} ±0.05	1.19^{1} ±0.15	1.60^{3} ±0.05	1.25^{b} ±0.06	0.97^{2} ±0.06	1.55^{3} ±0.07
b Growth rate/h^{-1}	0.41^{a} ±0.18	0.10^{1} ±0.04	0.53^{2} ±0.24	0.40^{a} ±0.11	0.11^{1} ±0.02	0.52^{2} ±0.19	0.24^{a} ±0.08	0.12^{1} ±0.03	0.34^{2} ±0.13

different letters in each row are significantly different by the total count ($p \leq 0.05$)
different numbers in each row are significantly different for each probiotic bacteria ($p \leq 0.05$)

ent from T_1 and T_2 ($p \leq 0.05$). These results were validated with the calculated d' values (Table 2). d' values either close to or greater than 1 suggest that sensory differences were detected by the judges (*12*). Cell concentration of *L. reuteri* was slightly higher in T_3 than in the other two treatments, whereas *B. bifidum* counts were slightly lower. Sensory differences noted between T_3 and T_1 and T_2 may be attributed to the viable cell concentration of the probiotic bacteria, and therefore to the amount of their fermentation products accumulated in the different treatments. This assumption is based on the fact that

Table 2. Calculated values of d' from the triangle test results

	T_1 *vs.* T_2	T_2 *vs.* T_3	T_1 *vs.* T_3
d'	0.35±0.48	1.09±0.19	0.94±0.21
p	0.308	0.006	0.020

T_1 = treatment 1 (*L. reuteri* 1 %, *B. bifidum* 0.5 %)
T_2 = treatment 2 (*L. reuteri* 1 %, *B. bifidum* 1 %)
T_3 = treatment 3 (*L. reuteri* 2 %, *B. bifidum* 0.5 %)

heterofermentative lactic acid bacteria ferment glucose to produce equimolecular amounts of lactate, carbon dioxide and acetate or ethanol; however, certain modifications in the culture conditions may result in the prevalence of one of these products. According to Ragout *et al.* (*17*), the ratio of acetate to lactate produced by lactobacilli and bifidobacteria can affect the flavour and aroma of the fermented products. In the preference test, T_2 against T_3 were compared. Out of the 109 consumers asked in this test, 61 preferred T_3, which represents 56 %, whereas 38 consumers preferred T_2 (34.86 %) and 10 consumers (9.17 %) did not show preference for any treatment. The χ^2 test ($p \leq 0.05$) showed a clear preference for the set T_3, therefore it was determined that this treatment would be considered as the final product.

Beverage storage

It is recognized that there are some physicochemical factors that might influence the survival of probiotic microbiota in fermented dairy products, being among the most important: acidity, temperature, oxygen concentration, inoculation and storage conditions (*5*). In the set T_3, the viable count of *B. bifidum* remained relatively constant during the 30 days of storage (approx. 10^8 CFU/mL), whereas the population of *L. reuteri* decreased approx. 1 log cycle over the same time period (Fig. 2). This decrease in the viability of *L. reuteri* could be caused by the physiological stage of the strains. Bacteria in the transition between the exponential phase and the stationary phase are more susceptible to the stress of storage conditions than those cells in the stationary phase (*18*). A slight increment in titratable acidity was measured (from 0.315 to 0.378 %) during the whole storage period of set T_3 (Fig. 3), which was also reflected in the decrease of pH values (from 4.85 to 4.50). In this respect, Gorski (*19*) and Ravula and Shah (*15*) reported that according to the Food Standards Code H8, values of pH≤4.5 can affect the viability of probiotic bacteria. This post-acidification may be attributed to *B. bifidum*, since Gobbetti *et al.* (*20*) reported that the production of 70 to 75 % of total acids by bifidobacteria took place in the stationary phase of growth. The viability of the probiotic bacteria could also be influenced by the type of container used during storage. Shah (*21*) reported that the dissolved oxygen content (harmful for anaerobic bacteria) is higher in products stored in plastic containers than glass bottles, and although the acid contents were similar, the survival rate is 30 to 70 % higher in products fermented and stored in glass bottles than plastic. Although the viability of *L. reuteri* population decreased, the viable counts of both probiotic bacteria did not fall below 10^6 CFU/mL. Therefore, we consider that the whey-based beverage T_3 fulfilled the probiotic food criterion acceptably. In related works, Hagen and Narvhus (*22*) prepared ice-cream containing probiotic bacteria. The authors observed that the viable counts did not change significantly during 52 weeks of frozen storage and remained above the recommended

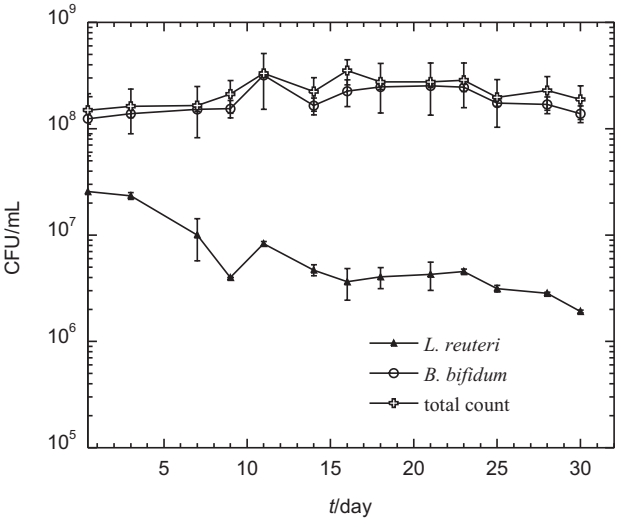

Fig. 2. Viable counts of *L. reuteri*, *B. bifidum* and total count of the fermented beverage during storage at 4 °C

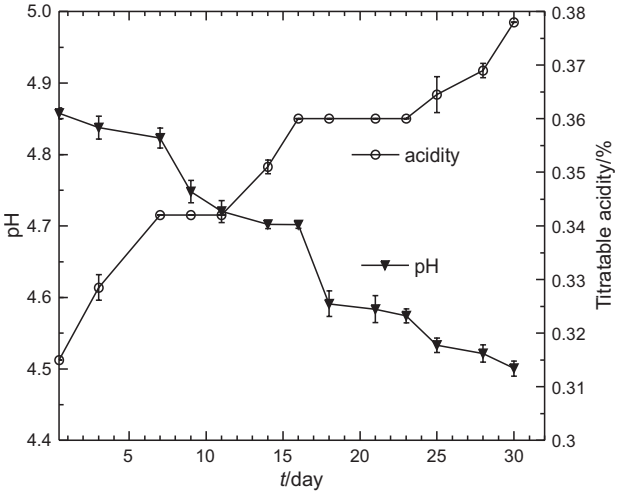

Fig. 3. Titratable acidity and pH values during storage at 4 °C of the beverage fermented by *L. reuteri* and *B. bifidum* (treatment T_3)

minimum limit of 10^6 CFU/g. In a report by Roy *et al.* (*23*), five species of bifidobacteria were used to produce fresh cheese; the microorganisms remained for up to 15 days at population levels higher than 10^6 CFU/g when the cheese was stored at 4 or 12 °C.

Our whey-based beverage did not show sensory differences during the first 14 days of storage, as assessed by the triangle test, but after the second week, fresh and stored products showed sensory differences ($p \leq 0.05$). According to a consensus made with the panellists during the attribute test, it was determined that the main descriptors that characterize the product were acidity, sweetness and texture, with acidity being the attribute responsible for the sensory differences perceived by the panellists. Even though a slight acidification was detected by the judges, they agreed that the beverage had an acceptable flavour. In related works, Davidson *et al.* (*24*) reported that in a fermented frozen yogurt, the balance of flavouring systems may be significantly affected by varying levels of organic compounds. Additionally, they reported that acidity was the most important attribute, in terms of perceived flavours. Rodas *et al.* (*25*) prepared a probiotic buttermilk containing *L. reuteri*. The authors reported that the sensory evaluation indicated that the judges were able to perceive differences after 12 days of storage. Gobbetti *et al.* (*20*) found that the incorporation of bifidobacteria into Crescenza cheese, which contained a higher amount of acid with respect to the control cheese, slightly affected the sensory evaluation.

Conclusion

The beverage fermented for approx. 11 h and prepared with 2 % *L. reuteri* and 0.5 % *B. bifidum* met the probiotic criterion by maintaining both bacterial populations greater than 10^6 CFU/mL during the whole storage period. Acidity and pH values did not change appreciably and no sensory changes were found during the first 14 days of storage, after which a slight acidification was detected. The final product preserved an acceptable flavour. Previous studies that assessed the correct release of the probiotic bacteria in a live system and the result of this work suggest that this beverage may be attractive for entering the growing market of probiotics.

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