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Research Note

Raffinose Family of Oligosaccharides from Lupin Seeds as Prebiotics: Application in Dairy Products

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ABSTRACT

The raffinose family of oligosaccharides (RFOs) isolated from lupin seeds (*Lupinus albus* var. Multolupa) was evaluated for bifidogenic effects during the manufacture of probiotic fermented milk. A mixed starter inoculum was composed of *Bifidobacterium lactis* Bb-12 and *Lactobacillus acidophilus* (1:1). Lupins are a rich source of RFOs that can be used as functional food ingredients. The addition of RFOs to milk increased *B. lactis* Bb-12 and *L. acidophilus* populations at the final fermentation time compared with controls. Final fermentation products are positively affected by addition of RFOs, and time of fermentation was reduced from 12 to 10 h. When RFOs were added to milk, they were preferentially used as a carbon source (57.7%) compared with lactose (23.7%) at the end of fermentation. These results suggest that the eventual choice of *B. lactis* Bb-12 and *L. acidophilus* in a mixed culture at a 1:1 ratio and addition of RFOs to produce a fermented milk product would have the advantages of rapid growth and acidification rate and would likely increase the probiotic effect of the final functional product.

The maintenance of the intestinal bacterial population, which mostly contains beneficial microorganisms and minimal putrefactive microbes, is important for maintaining intestinal well-being *(34).* Recent research has focused on the ability of probiotic bacteria to ferment oligosaccharides, which are not hydrolyzed by the intestinal enzymes, and the selective utilization of oligosaccharides by bifidobacteria rather than other intestinal bacteria in the colon *(7, 9, 37).* Because of their prebiotic properties, oligosaccharides have received much attention as functional food ingredients. The most studied prebiotics are inulin and fructo-oligosaccharides, but legumes are a good source of oligosaccharides known as α-galactosides or the raffinose family of oligosaccharides (RFOs), which are utilized by bifidobacteria *(13, 16).* These α-galactosides are soluble low-molecular-weight oligosaccharides represented by raffinose, stachyose, verbascose, and other oligosaccharides formed by α-(1→6)-galactosides linked to C-6 of the glucose moiety of sucrose *(5).* Lupins are legumes with one of the highest α-galactoside contents (7 to 15%) *(29, 31, 33).* The extraction of RFOs from lupins produces a product with much higher protein content than the raw seed. This product can be used for nutritional purposes, and the extracted RFOs can be incorporated into milk to enhance the numbers and acidification activity of bifidobacteria during manufacture of probiotic-fermented milk products.

Bifidobacteria have a number of beneficial health properties. As a result, bifidobacteria are incorporated into fermented dairy foods *(38).* The ultimate aim of this type of fermentation is to provide the gastrointestinal tract of humans with elevated viable populations of bifidobacteria *(30).* In Japan, the Fermented Milks and Lactic Acid Beverages Association has already established a standard that requires $\geq 10^7$ CFU/ml bifidobacteria to be present in dairy products that claim to contain these bacteria. The Swiss Food Regulation and the International Standard of the International Dairy Federation requires that such products contain $\geq 10^6$ CFU/ml bifidobacteria *(20).*

Microorganisms that are used for the production of fermented dairy products are selected for their suitable growth characteristics in milk and for consistently reliable acid production *(26).* The commercially available starter cultures used to manufacture these products should enhance desired organoleptic properties and inhibit the growth of spoilage or potentially pathogenic microorganisms. The incorporation of intestinal species (e.g., *Lactobacillus acidophilus* and *Bifidobacterium* sp.) in milk products has been considered by various researchers *(1, 12, 18).*

The cultivation of bifidobacteria in milk is a difficult task compared with cultivation of conventional starters because milk is an artificial medium for the growth of these fastidious microorganisms *(11).* Klaver et al. *(23)* tried to promote their growth with a coculture of *Bifidobacterium* and *Streptococcus thermophilus.* However, this procedure has the disadvantage of reducing the bifidobacteria counts in the fermented milk, either by competitive inhibition or

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by loss of viability of bifidobacteria due to acid injury *(3)*. To promote the growth of bifidobacteria, it would be necessary to coculture these bacteria with other proteolytic strains such as lactobacilli in milk with specific bifidus factors such as milk hydrolyzates *(11, 32)* and oligosaccharides *(10)*. An appropriate prebiotic would improve the viability of bifidobacteria in milk with a goal of delivering a large number of viable bifidobacteria in the colon after uptake of fermented milk.

The present study was designed to investigate the effects of RFOs isolated from *Lupinus albus* var. Multolupa seeds on the growth and acid production of *Bifidobacterium lactis* Bb-12 cocultured with *L. acidophilus* in milk. The ultimate goal was to obtain a large number of viable bifidobacteria in the fermented milk. The final dairy product obtained should contain both probiotics and prebiotics, which would act synergistically to promote health.

MATERIALS AND METHODS

Isolation of RFOs from lupin. RFOs were extracted from *L. albus* var. Multolupa seeds according to the method of Gulewicz et al. *(15)* and purified according to Martínez-Villaluenga et al. *(28)*.

Determination of soluble carbohydrate content. Lactose, sucrose, and α-galactoside content were determined by high-performance liquid chromatography (HPLC) following the procedure described by Granito et al. *(14)*. The HPLC system (Waters Associates, Milford, Conn.) consisted of a Waters model 510 pump, a Rheodyne model 7000 sample injector, and a Waters reflection type differential refractometer detector model R410. The HPLC system was controlled by a PC with Maxima software (Waters). Chromatography was performed on a precolumn (0.32 cm inside diameter by 4.0 cm) packed with C_{18} Porasil B and a Carbohydrate Analysis column (0.39 cm inside diameter by 30 cm) (Waters). Acetonitrile–distilled water (75:25, vol/vol, HPLC grade) was used as the mobile phase at a flow rate of 2.0 ml/min. Solvents were filtered through a Millipore FH (0.45-μm-pore-size) membrane and degassed under helium. Injection volumes were 100 μl.

Different amounts of lactose, sucrose, raffinose, and stachyose standards (Merck, Darmstadt, Germany) and samples were dissolved in distilled water. Acetonitrile was added to each solution to obtain a composition similar to that of the mobile phase (75:25, vol/vol). Both lupin RFOs and standard solutions were filtered through a Millipore FH (0.45-μm-pore-size) membrane before injection. Quantification of each sugar was performed by comparing the peak areas with those of the standard solutions. Commercial verbascose standard was not available, so verbascose was quantified using stachyose as a standard based on previous works *(8)*. Calibration curves were plotted for each sugar and adjusted by using the method of least squares. The regression coefficients of the curves for sucrose, raffinose, and stachyose were always greater than 0.990.

Determination of organic acid content. Lactic and acetic acids in the coculture were quantified by HPLC, using the method described by Dubey and Mistry *(6)*. One hundred microliters of 15.8 M HNO_3 and 14.9 ml of 0.009 M H_2SO_4 were added to 1.5 ml of sample and centrifuged at 4000 × *g* for 10 min. The supernatant was filtered through a 0.22-μm-pore-size Millipore membrane, and 2-ml aliquots were stored in HPLC vials at −20°C until analysis. The HPLC system consisted of a model 1040 quaternary solvent delivery system (Hewlett-Packard, Palo Alto, Calif.) equipped with a 1040 UV/visible diode array detector (Hewlett-Packard) and fitted with an ION-300 column (10 μm, 300 by 6.5 mm; Interaction Chemicals, Mountain View, Calif.) maintained at 65°C. The degassed mobile phase of 0.009 M H_2SO_4, filtered through a Millipore 0.45-μm-pore-size membrane was used at a flow rate of 0.3 ml/min. The wavelength of detection was optimized at 220 nm for the organic acids being quantified. The standard solutions of lactic and acetic acids (Sigma-Aldrich Química S.A., Madrid, Spain) were prepared in water to establish elution times and calibration curves. The standard curve coefficients were greater than 0.990 for both lactic and acetic acids.

Bacterial cultures. Single strains of *B. lactis* Bb-12 and *L. acidophilus* in the form of starter concentrates were obtained from Chr. Hansen Pty. Ltd. (Arpajon, France). Cultures were incubated anaerobically in deMan Rogosa Sharpe (MRS) broth (Oxoid S.A., Madrid, Spain) containing 5% (wt/vol) lactose at 37°C for 24 h using Gas Paks (Anaerobic system BR038B, Oxoid) and stored at −80°C. Prior to use, the frozen starter concentrates were thawed at 20°C for 30 min, centrifuged for 15 min at 1,000 × *g* at 4°C, and resuspended in sterilized 12% (wt/vol) reconstituted skim milk.

Preparation of fermented milk. Both *B. lactis* Bb-12 and *L. acidophilus* were cultured in ultraheat-treated skim milk. RFOs were added aseptically at 2% (wt/vol) in skim milk. Inoculation was at 10% according to the method of Varnam and Sutherland *(41)*, and flasks were entirely filled (to exclude oxygen completely). Samples were incubated without agitation at 37°C in 20% CO_2 atmosphere, and fermentation was terminated at pH 4.6.

Bacterial counts. Bacterial growth was estimated using colony plate counts. One gram of sample was diluted with 9 ml of peptone water diluent (Oxoid). Subsequent serial dilutions were prepared, and viable numbers were determined using the pour plate technique. The counts of *B. lactis* Bb-12 were differentially determined on MRS-NPNL (nalidixic acid, paramomycin sulfate, neomycin sulfate, and lithium chloride; Sigma-Aldrich Química) agar, and incubated anaerobically at 37°C for 72 h *(2)*. *L. acidophilus* were differentially enumerated on spread plates of triptone–glucose–meat extract agar supplemented with 20 g/liter NaCl *(11)* and incubated under microaerophilic conditions (20% CO_2) for 72 h. Individual strain identities were confirmed by visual inspection of colony morphology and microscopic observation.

Statistical analysis. Data were subjected to a multivariable analysis of variance using the least-squares difference test with Statgraphic 5.0 (Statistical Graphics, Rockville, Md.) and a multiple correlation analysis using Statistica 5.1 (Statsoft, Tulsa, Okla.) for Windows with a PC Pentium.

RESULTS

Composition of RFOs isolated from lupin seeds. The concentrations of sucrose, raffinose, stachyose, and verbascose extracted from *L. albus* var. Multolupa are shown in Table 1. The isolated RFOs included only a small amount of sucrose (0.8%) and a high amount of stachyose (62.3%), followed by verbascose (24.5%) and raffinose (12.6%).

Effects of inoculum ratio on growth and metabolic activity of *B. lactis* Bb-12 and *L. acidophilus* in mixed culture. To test whether the growth of *B. lactis* Bb-12 was enhanced by addition of a bacterial strain with proteolytic activity in a coculture, a set of growth experiments was

TABLE 1. *Concentrations of raffinose family of oligosaccharides (RFOs) isolated from* Lupinus albus *var. Multolupa*

Soluble carbohydrates	Concentration (g/100 g)[a]
Sucrose	0.82 ± 0.01
Raffinose	12.56 ± 0.91
Stachyose	62.33 ± 0.48
Verbascose	24.47 ± 0.05
Total RFOs	99.36 ± 1.43

[a] Mean ± standard deviation of three determinations.

performed with *B. lactis* Bb-12 and *L. acidophilus* at 10:1, 1:1, and 1:10 inoculum ratios in milk with or without 2% RFOs (Table 2). The addition of RFOs to milk enhanced the growth and the metabolic activity (coagulation time and the ratio of lactic acid/acetic acid) of both bacteria regardless of inoculum ratio used.

In milk without RFOs, no significant differences were observed in numbers of viable *B. lactis* Bb-12 cells at different inoculum ratios assayed (Table 2). In milk with RFOs, significantly higher *B. lactis* Bb-12 counts ($P \leq 0.05$) were found with 1:1 and 10:1 inoculum ratios than with the 1:10 ratio. However, no significant differences were observed between the 1:1 and 10:1 inoculum ratios (Table 2).

L. acidophilus counts increased significantly ($P \leq 0.05$) with the addition of RFOs to milk irrespective of inoculum ratio used (Table 2). Maximum lactobacilli counts were found at the 1:1 inoculum ratio in milk with and without RFOs. However, the presence of RFOs and the inoculum ratio had a significant effect on the lactic acid/acetic acid ratio produced by mixed culture (Table 2). The addition of RFOs to milk significantly increased ($P \leq 0.05$) the lactic acid/acetic acid ratio at the same inoculum ratio; the highest acid ratios were found in cultures of milk with RFOs at the 1:1 inoculum ratio followed by those with the 1:10 inoculum ratio. Lactic acid was higher than acetic acid in all cases, with the exception of the 10:1 culture in milk without RFOs. The acid production was correlated closely with the time of milk coagulation. A shorter coagulation time (by approximately 2 h) was observed for cocultures in milk with RFOs compared with cultures in milk alone at the same inoculum ratio. Therefore, the 1:1 inoculum ratio was the choice for next experiments.

Growth and acidification rates from mixed culture of *B. lactis* Bb-12 and *L. acidophilus* in the presence of RFOs. Figure 1 shows the effect of RFOs on the growth of *B. lactis* Bb-12 and *L. acidophilus* in a mixed culture at the 1:1 inoculum ratio during milk fermentation at 37°C. Bifidobacteria had significant higher numbers of viable cells in milk with RFOs than in milk without RFOs during fermentation; although the greatest differences ($P \leq 0.05$) were observed at the end of fermentation (0.79 log CFU/ml). Unlike *B. lactis* Bb-12, the response of *L. acidophilus* was not significant until the end of fermentation (Fig. 1).

Figure 2 shows the effect of RFOs on lactic and acetic acid production by *B. lactis* Bb-12 and *L. acidophilus* in a

TABLE 2. *Effects of inoculum ratio on the growth and activity of* Bifidobacterium lactis *Bb-12 and* Lactobacillus acidophilus *in mixed culture at the final fermentation at 37°C*[a]

Medium	Inoculum ratio[b]	Counts (log CFU/ml)		Inoculum activity	
		B. lactis Bb-12	*L. acidophilus*	Lactic acid/acetic acid ratio	Final fermentation (h)
Milk	1:1	8.01 ± 0.08 A	8.33 ± 0.04 B	3.40 ± 0.03 D	12.00 ± 0.14 B
	1:10	8.17 ± 0.24 AB	8.18 ± 0.03 A	3.15 ± 0.04 C	14.25 ± 0.07 D
	10:1	8.04 ± 0.03 A	8.08 ± 0.05 A	0.76 ± 0.01 A	16.55 ± 0.21 F
Milk + RFOs[c]	1:1	8.80 ± 0.04 C	8.49 ± 0.02 C	4.23 ± 0.15 E	10.01 ± 0.01 A
	1:10	8.42 ± 0.08 B	8.32 ± 0.03 B	3.49 ± 0.00 D	13.00 ± 0.21 C
	10:1	8.84 ± 0.01 C	8.35 ± 0.07 B	1.55 ± 0.06 B	15.05 ± 0.21 E

[a] Mean ± standard deviation of three determinations. Values with the same letter within a column are not significantly different ($P \leq 0.05$).
[b] Ratio of *B. lactis* Bb-12 to *L. acidophilus*.
[c] RFOs, raffinose family of oligosaccharides isolated from *Lupinus albus* var. Multolupa.

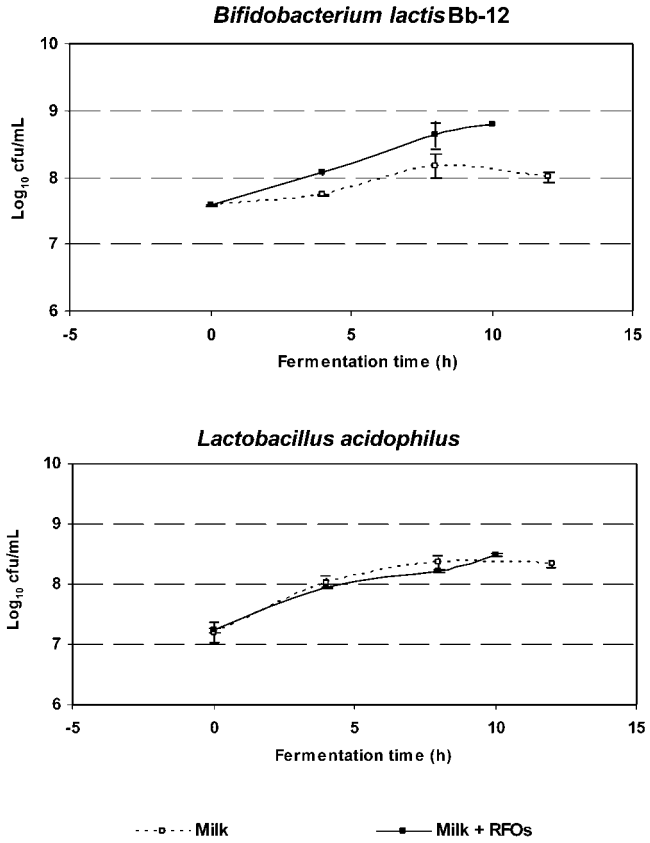

FIGURE 1. *Influence of lupin raffinose family of oligosaccharides (RFOs) addition on the* Bifidobacterium lactis *Bb-12 and* Lactobacillus acidophilus *counts in a mixed culture at a 1:1 inoculum ratio during milk fermentation at 37°C.*

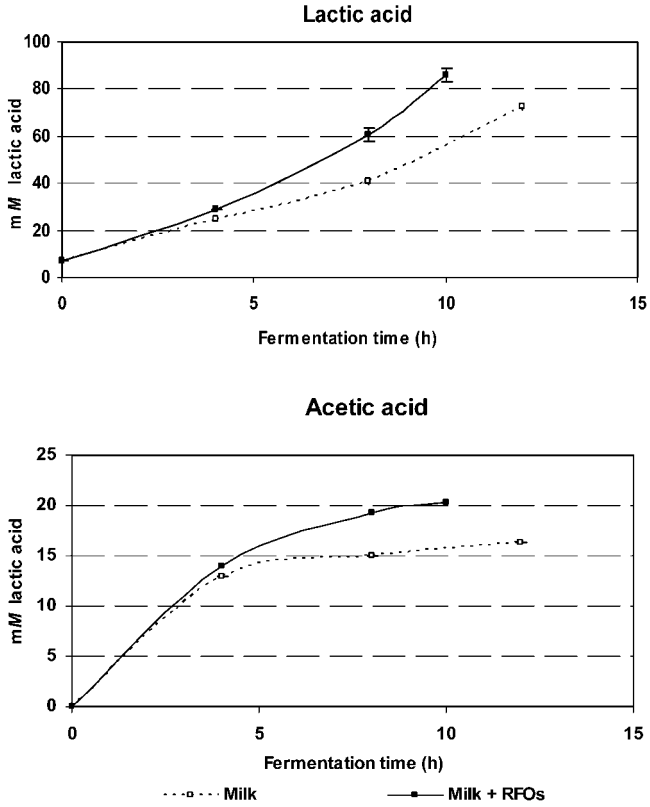

FIGURE 2. *Kinetics of lactic and acetic acid production by a mixed culture of* Bifidobacterium lactis *Bb-12 and* Lactobacillus acidophilus *at a 1:1 inoculum ratio during milk fermentation at 37°C with or without added raffinose family of oligosaccharides (RFOs).*

mixed culture at the 1:1 inoculum ratio during milk fermentation at 37°C. Increased lactic and acetic acid concentrations were detected after the first 4 h of fermentation in milk cultures with RFOs and steadily increased until the end of fermentation. Lactic acid production was higher than that of acetic acid at all fermentation times in milk with or without RFOs. At the end of fermentation, lactic acid was four times higher than acetic acid in milk with galactooligosaccharides and three times higher in milk without these growth promoters. The multiple correlation analyses revealed that viable numbers of *B. lactis* Bb-12 were correlated positively with concentrations of lactic and acetic acids in milk alone ($r = 0.80$ and 0.78, respectively) and in milk with RFOs ($r = 0.95$ for both acids). The same trend was observed for *L. acidophilus* in milk alone ($r = 0.83$ and 0.98, respectively) and in milk with RFOs ($r = 0.96$ and 0.98, respectively). The growth of *L. acidophilus* was correlated with growth of *B. lactis* Bb-12 in milk alone ($r = 0.86$) and in milk with RFOs ($r = 0.92$).

The residual carbohydrates during fermentation of milk at 37°C are shown in Table 3. Concentration of total RFOs decreased more rapidly than did that of lactose in milk containing RFOs. Among them, verbascose and raffinose were more rapidly utilized than was stachyose. Total RFOs de-

TABLE 3. *Residual carbohydrate content in milk with and without RFOs during fermentation time at 37°C*

		Soluble carbohydrates (g/100 g)[a]				
Medium	Fermentation time (h)	Lactose	Raffinose	Stachyose	Verbascose	Total RFOs
Milk	0	4.46 ± 0.01 E				
	4	3.66 ± 0.01 C				
	8	3.53 ± 0.01 B				
	Final fermentation	2.51 ± 0.01 A				
Milk + RFOs	0	4.52 ± 0.01 E	0.30 ± 0.01 D	0.97 ± 0.01 D	0.67 ± 0.02 D	1.94 ± 0.01 D
	4	3.84 ± 0.01 D	0.22 ± 0.02 C	0.90 ± 0.02 C	0.49 ± 0.01 C	1.60 ± 0.01 C
	8	3.80 ± 0.01 D	0.17 ± 0.00 B	0.77 ± 0.01 B	0.31 ± 0.01 B	1.26 ± 0.02 B
	Final fermentation	3.45 ± 0.01 B	0.15 ± 0.01 A	0.60 ± 0.01 A	0.18 ± 0.01 A	0.82 ± 0.02 A

[a] Mean ± standard deviation of three determinations. Values with different letters within a column are significantly different ($P \leq 0.05$). RFOs, raffinose family of oligosaccharides isolated from *Lupinus albus* var. Multolupa.

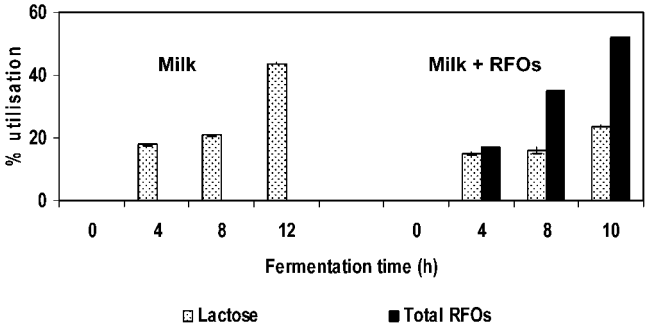

FIGURE 3. *Utilization of lactose and raffinose family oligosaccharides (RFOs) by a mixed culture of* Bifidobacterium lactis *Bb-12 and* Lactobacillus acidophilus *during fermentation at 37°C of milk with or without added RFOs.*

creased from approximately 2% to 0.82% at the end of fermentation, whereas lactose decreased from 4.5% to 3.45%. These results were significantly different ($P \leq 0.05$) from those for milk without RFOs, where lactose decreased from 4.46% to 2.51%.

Figure 3 compares lactose and total RFOs utilized by the mixed culture during fermentation of milk with and without RFOs at 37°C. The microbial utilization of lactose was higher in the fermentation of milk without RFOs than in milk containing these oligosaccharides (43.7% and 23.7%, respectively). There was a faster utilization of total RFOs (57.7%) compared with that of lactose (23.7%) at the end of fermentation of milk containing oligosaccharides.

DISCUSSION

Several researchers have reported poor growth of bifidobacteria in whole milk, which was attributed to a lack of essential vitamins or a lack of essential amino acids in free form or in the form of peptides *(11, 23).* However, *Bifidobacterium* sp. seems to grow better in milk containing *L. acidophilus* than in pure culture *(42),* indicating that *L. acidophilus,* via its proteolytic activity, is important in supplying utilizable nitrogen. *L. acidophilus* can use peptides and free amino acids for its own growth and acidification activity and can hydrolyze milk caseins using proteinases near to the cell wall surface *(40)* to produce low-molecular-mass nitrogen (especially peptides). Therefore, *L. acidophilus* can continue growth and stimulate the growth of *Bifidobacterium* sp. and acidification by the mixed culture of both bacteria. Stimulatory interactions involving nitrogen requirements have previously been observed among several bifidobacterial strains and proteolytic lactobacilli *(17, 23, 24).* However, the results of this study indicate that coculture of *B. lactis* Bb-12 with *L. acidophilus* may not be the sole reason for the growth enhancement of bifidobacteria; RFOs supplementation provided an additional stimulatory growth effect on *L. acidophilus (16, 25).*

According to Sasaki et al. *(35),* a ratio of 10:1 of *Lactobacillus bifidus* (now known as *Bifidobacterium bifidum*) to *L. acidophilus* is considered adequate and essential for the growth of *B. bifidum* in mixed culture. Our results agree with those of these authors, although the 1:1 ratio also produced maximum viable bifidobacterial counts. Similar results were reported by Gomes et al. *(11)* for a mixed culture of *B. lactis* and *L. acidophilus.*

In the present study, acetic acid concentrations were maximum at the 10:1 inoculum ratio, which is justified in view of the heterofermentative nature of bifidobacteria, as previously described by Scardovi *(36).* Although lactic acid production is desirable in fermented dairy foods, a high concentration of acetic acid can result in a distinct vinegar flavor in products. Thus, a high acetic/lactic acid ratio is typically undesirable in fermented dairy products. Because the maximum lactic acid/acetic acid ratio was obtained with *B. lactis* Bb-12 and *L. acidophilus* at the 1:1 inoculum ratio, this ratio was selected to prepare fermented milk.

The growth of *L. acidophilus* in coculture with *Bifidobacterium* sp. can be enhanced *(42)* by the buffering effect of acetate (pK_a, 4.8) produced by the bifidobacteria, which prevents sharp declines in milk pH *(22, 27),* and may also suggest a certain degree of symbiosis between the two species. However, galacto-oligosaccharide preparations produce rapid growth rates compared with those obtained with lactose in most of bifidobacteria strains tested, as previously described *(19).* Results from the present study support these findings for bifidobacterial growth and acidification in mixed culture. Although most bifidobacteria strains of human intestinal origin can readily use galacto-oligosaccharides, only a few strains from other origins and genera, including lactobacilli, possess this ability *(21, 39, 43).* Gopal et al. *(13)* demonstrated that two probiotic strains of food origin, *B. lactis* DR10 and *Lactobacillus rhamnosus* DR20, can utilize galacto-oligosaccharides from dairy products (commercial milk powder) to support their growth in vitro. According to these authors, when a mixture of galacto-oligosaccharides is used as growth substrate, each strain has very different preferences; *B. lactis* preferentially utilized oligosaccharides with higher degrees of polymerization (tri- and tetrasaccharides) and *L. rhamnosus* DR20 preferred monosaccharides and disaccharides. These results suggest that these strains possess different uptake systems for the utilization of galacto-oligosaccharides.

Although little is known about the mechanism of carbohydrate uptake in bifidobacteria *(4),* it appears to be more efficient with oligomeric carbon sources. Higher growth rates result in major acid production (lactic and acetic acid) and shorter milk fermentation times. Possible mechanisms for galacto-oligosaccharide utilization by bifidobacteria and some strains of lactobacilli were reported by Gopal et al. *(13).* In most of the strains of lactic acid bacteria, galactosyl-lactose is thought to be transported across the plasma membrane either by direct uptake by lactose permease or after its enzymatic hydrolysis to monosaccharides, in which transport into the cell is by glucose or galactose permease. The same research group suggested that in some strains, especially of *Bifidobacterium,* another permease may be induced by galacto-oligosaccharides that is specific for trisaccharides and tetrasaccharides. The presence of this permease would explain the increase in acid production from RFOs due to the growth of bifidobacteria in milk containing these oligosaccharides.

In the present work, we observed faster utilization of

verbascose (a pentasaccharide) compared with stachyose (a tetrasaccharide) and raffinose (a trisaccharide), which could be the result of the presence of a permease induced by galacto-oligosaccharides that is specific for trisaccharides and tetrasaccharides *(13).* It appears that verbascose is first hydrolyzed by α-galactosidase to stachyose and raffinose, which are utilized by *B. lactis* Bb-12 but nevertheless remains in the fermented milk.

Our results suggest that the use of *B. lactis* Bb-12 and *L. acidophilus* in a mixed culture at a 1:1 inoculum ratio and addition of RFOs to obtain a fermented milk product would have the advantages of rapid microbial growth and acidification rate, consequently accelerated milk coagulation, and an increased probiotic effect of the final product. A combination of probiotic and prebiotic factors in dairy products could result in a synergistic action that can contribute to the promotion of health.

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