The objective of the present work was to study the potential of the permeate obtained by the process of membrane ultrafiltration (MW cut off 10 KDa) of bovine milk whey and lactulose, to improve the growth of two species of probiotic bacteria. The main components (w/w db) of the whey permeate (WP) were lactose (85%), minerals (9.6%) and nitrogenous substances (4.1%). Lactose was isolated from the WP by vacuum evaporation and crystallization. Conversion of lactose to lactulose was done by isomerization (pH 11) using sodium borate as the catalyst. Further purification was performed by ion exchange and molecular exclusion column chromatography. High performance liquid chromatography (HPLC) confirmed the isomerization reaction at a yield of 70%. For the microbiological assay, MRS (De Man, Rogosa and Sharpe) medium was used for \( Lb. \) acidophilus La-5 and modified MRS (MRSm) for \( B. \) lactis Bb-12. Replacement of the water and glucose by WP (75%) in the preparation of the media increased the growth of La-5 and Bb-12 by 97% and 143%, respectively. Fortification of the modified MRS (MRSm) medium with 1% lactulose increased the growth of \( B. \) lactis Bb-12 by 75% while 3% lactulose enrichment of the unmodified MRS medium improved the growth of \( Lb. \) acidophilus La-5 by 36%, compared to the same medium without the addition of lactulose. Both La-5 and Bb-12 were tolerant to bile salts, mainly in the presence of lactulose.
1. INTRODUCTION

The dairy industry has grown both in volume and diversity with the introduction of membrane technologies such as microfiltration, ultrafiltration and nanofiltration. The production of high quality whey protein concentrate (WPC) and protein isolate (WPI) has increased significantly in many countries, which resulted in the generation of large volumes of whey permeate (WP) and a consequent increase in pollution problems, in spite of recent attempts to make use of WP (PAULI; FISTZPATRICK, 2002; VILLAMIEL et al., 2002).

During whey concentration (ultrafiltration), a permeate is generated which contains practically all the lactose, small peptides and amino acids, vitamins, soluble minerals and water (VYAS; TONG, 2003). This whey permeate has been shown to be a good microbial and cell culture medium substrate (SARON et al., 2003; BATISTA, 2003) but it presents high polluting power due to the elevated concentration of lactose (ELLIOTT et al., 2001). By virtue of the low commercial value of lactose, due to its low solubility and intolerance presented by many people, new approaches have been proposed for lactose derivation and novel uses for its derivatives (YANG; SILVA, 1995; ZOKAEE et al., 2002).

Lactose cannot be metabolised by humans or animals due to the lack of an intestinal enzyme capable of breaking the galactose-fructose linkage. It is metabolised in the large intestine preferably by bifidobacteria and lactobacilli (SCHUMANN, 2002), where they promote desirable effects for human health such as balancing the bowel microbial flora, reducing blood ammonia, reducing the carcinogenesis risk and lowering blood triglycerides (SCHOLZ-AHRENS et al., 2001).

The aim of this investigation was to evaluate the efficacy of WP and of lactose as fortifiers of the pre-existing culture media, MRS and modified MRS (DE MAN et al., 1960) for the growth of probiotic bacteria, in this case, Lactobacillus acidophilus La-5 and Bifidobacterium lactis Bb-12. Finding a novel use for the total WP or WP components would contribute to adding value to milk as a raw material for a new industry aimed at producing milk components as functional food ingredients.

2. MATERIAL AND METHODS

2.1 Milk whey permeate

Whey permeate (WP) was obtained as the effluent in a pilot plant process (Borges et al., 2001). The whey was obtained from defatted and pasteurised cow’s milk (72 °C, 15 sec) by treatment with commercial rennet containing chymosin under the conditions of 37 °C, ~40 min (in the presence of CaCl₂). The casein clot was broken into small pieces and centrifuged out (5000 g, 15 min). The whey was then submitted to ultrafiltration (WGM membrane systems, MW cut off 10 KDa) for the production of whey protein concentrate and whey permeate (~5% total solids). A summarized flow diagram of these operations is illustrated in Figure 1.

![FIGURE 1. Flow diagram of operations in the fractionation of milk components, purification of lactose and isomerization of lactose to lactulose.](image)

2.2 Production of lactose

For the isolation of lactose, the permeate was concentrated under vacuum at 55 °C to a final concentration of 30% total solids. The concentrated permeate was left under refrigeration (5-8 °C) for about 12 h, when the lactose became insoluble, and was then separated by centrifugation (5000 g, 20 min, 5 °C). Prior to concentration, the permeate pH was adjusted to about 2.5 to avoid forming a protein-mineral-lactose complex (SINGH et al., 1991b).

The lactose was purified by crystallization from an ethanol solution at 73% in a solvent/solute ratio of 10:1 (SINGH et al., 1991a). After mixing the lactose with the solvent the mixture was left to stand for 24 h at 26 °C. After this period a new volume of ethanol solution was added, standing for another 12 h, when the supernatant was withdrawn. The lactose was subsequently dried in an air-circulating oven (FANEM/320SE) at room temperature. The purity of the lactose was verified from the infrared spectrum.

2.3 Characterization of the whey permeate

The protein (N x 6.25), moisture and ash contents were determined by the procedures described in the A.O.A.C. (AOAC,
Lactose was determined by the method of Acton [1977]. Mineral elements were determined in a plasma spectrometer (ICP 2000 Baird), using the simultaneous version with an argon flame. The procedures for the preparation of the samples and quantification of the elements were described by Angelucci and Mantovani [1986] and Imo Industry Inc [1990].

2.4 Isomerization of lactose to lactulose

The conversion (isomerization) of lactose to lactulose was performed using sodium borate in a molar ratio of 1:1 (lactose/borate) at pH 11. The reaction was carried out in a water bath at 70 °C for 3 h. The solution of lactulose was then cooled to room temperature and acidified to pH 2. This sequence of operations for the conversion of lactose to lactulose was based on the work of Hicks et al. [1984], and a summarized flow diagram is illustrated in Figure 1.

2.5 Separation and purification of lactulose

Ion exchange column chromatography was used to remove the boric acid and separate the lactulose from the non-converted lactose after the conversion of lactose to lactulose, in a system using two chromatographic columns connected in series, according to Kozempel et al. [1997]. The first column was packed with an anion exchange resin (Dowex/1X8-50/Sigma), and the second with a molecular exclusion resin (Toyopearl/TSK HW-40/Supelco).

In the lactulose purification process the following analytical procedures were used. The fraction [10 mL of mobile phase] obtained by conjugated ionic exchange and molecular exclusion column chromatography was analysed by thin layer chromatography (TLC) to identify the presence of lactulose and of lactose. The TLC bands containing lactulose were eluted from several runs and pooled together to obtain enough sample for high performance liquid chromatography (HPLC). HPLC was performed in a Shimadzu 10A VP chromatograph with a refraction index detector, using a Merck Lichrocart (100/NH2 5µm) column and a Merck Lichrocart pre-column (100/NH2 5µm). The mobile phase was composed of a mixture of water and acetonitrile (80:20, v/v) and the flow rate was 1 mL/min.

For thin layer chromatography, silica gel 60 F254 Z5 (Merck) was used as the stationary phase, supported on aluminium plates and the mobile phase was a mixture of distilled water (45 mL), ethyl acetate (10 mL) and isopropyl alcohol (55 mL). The staining mixture was prepared according to Walkley and Tillman (1977). The carbohydrate bands on the plate were visualised in an oven at 100-105 °C.

2.6 Probiotic microorganisms

The microorganisms used in this work were freeze-dried Lactobacillus acidophilus La-5 and Bifidobacterium lactis Bb-12. Hydration followed the standard procedure recommended by the manufacturer, using 2% (v/v) probiotic culture for all tests. The MRS medium [De Man Rogosa and Sharpe] and MRS medium supplemented with 0.05 g/100 mL L-cysteine hydrochloride (MRSm) [YAMAMAH et al., 2005] were used as the controls for the growth of Lactobacillus acidophilus La-5 and Bifidobacterium lactis Bb-12, respectively.

2.7 Monitoring growth

The growth of either Lactobacillus acidophilus La-5 or Bifidobacterium lactis Bb-12 was monitored by turbidimetric readings of the agitated culture at 620 nm in a spectrophotometer (Micronal Model B390, SP, Brazil). Absorbance readings were expressed as a percentage, taking the absorbance readings of the control as 100%.

2.8 Whey permeate (WP) as complement for MRS and MRSm media

Growth media were prepared removing all the glucose from the composition of the MRS and MRSm media, and substituting 50, 75 and 85% of the water with whole liquid WP. The pH of the media was adjusted to 5.5 for the Lactobacillus acidophilus La-5 and 6.5 for the Bifidobacterium lactis Bb-12. Immediately after formulation and prior to inoculation, the culture media were cold sterilized (0.22 µm membrane filter) in order to preserve all the vitamins and other growth factors. The sterilized media were distributed in sterile tubes in aliquots of 30 mL and inoculated (2% v/v) with stock probiotic cultures, which were then incubated at 37 °C for 8 h.

2.9 Lactulose as a prebiotic

The growth stimulating activity of lactulose on the Lactobacillus acidophilus La-5 and Bifidobacterium lactis Bb-12 was tested using concentrations of 1, 2 and 3% (w/v) in the MRS and MRSm growth media, respectively.

2.10 Evaluation of tolerance to bile salts

The tolerance of the probiotics to bile salts was evaluated according to the methodology of Gilliland and Walker [1990]. Incubations of 2% Lactobacillus acidophilus La-5 or Bifidobacterium lactis Bb-12 on the MRS or MRSm media, respectively, with or without lactulose supplementation [1% for Bb-12 & 2% for La-5], but in the presence of 0.3% bile salts (Oxgall-Diffco) were tested. The MRS and MRSm media without lactulose supplementation and without the addition of bile salts were used as controls. Incubation was at 37 °C and growth was quantified by the absorbance at 620 nm (Micronal Model B 390 spectrophotometer).
2.11 Statistical analysis

All statistical analyses were performed with the Computational Statistic Program: Basic Statistics and Tables. Data are expressed as the mean value ± standard deviation (sd). The statistical analysis was performed using the Student t-test for assessment of the mean differences between two culture media, p values < 0.05 being considered statistically significant.

3. RESULTS AND DISCUSSION

Table 1 presents the lactose, ash, total nitrogenous substances and some essential mineral element contents of the whey permeate. Quantitatively lactose was the main component, 85% (w/w db), ash coming second at 9.6% (w/w db) followed by the nitrogenous compounds at 4.1% (w/w db).

In a previous study, the analyses of the permeate showed that about 76% of the total lactose and about 16% of the total calcium from the milk whey were present in the permeate (VYAS; TONG, 2003).

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macrocomponents</strong> (g/100g)</td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>85.05 ± 0.12</td>
</tr>
<tr>
<td>Ash</td>
<td>9.64 ± 0.08</td>
</tr>
<tr>
<td>Protein (N x 6.25)</td>
<td>4.14 ± 0.01</td>
</tr>
<tr>
<td><strong>Mineral elements</strong> (mg/100g)</td>
<td></td>
</tr>
<tr>
<td>Potassium</td>
<td>132 ± 8</td>
</tr>
<tr>
<td>Calcium</td>
<td>28.3 ± 2.2</td>
</tr>
<tr>
<td>Phosphorous</td>
<td>38.2 ± 2.3</td>
</tr>
<tr>
<td>Sodium</td>
<td>41 ± 4.8</td>
</tr>
<tr>
<td>Magnesium</td>
<td>6.7 ± 0.3</td>
</tr>
<tr>
<td>Chloride</td>
<td>127 ± 17</td>
</tr>
</tbody>
</table>

*Results are the averages of three independent determinations.

From the mineral elements determined, potassium and chloride were the most abundant followed by sodium, phosphorous and calcium. Most of the calcium ions precipitated with the casein clot in the process of obtaining the “sweet” whey (see flow diagram of fractionation). In cow’s milk about 800 mg L⁻¹ of the total calcium is bound to casein micelles and about 370 mg L⁻¹ is soluble or free calcium (VYAS; TONG, 2003).

The procedures for the recovery and purification of lactose from WP performed in this study using pilot scale equipment were shown to be viable for use on an industrial scale. High yields in the purification process and the low cost of the materials used were some of the positive features of this process (ELLIOTT et al., 2001). In addition to its use as a raw material for lactulose synthesis, the purified lactose can be used for making other products such as lactitol, lacto-oligosaccharides and lactose syrup (VYAS; TONG, 2003).

Following the operations illustrated in Figure 1, the purified lactose was isomerized to lactulose with a 70% yield, and then purified by TLC and analyzed by HPLC. The HPLC chromatogram obtained shows the profile of the pooled TLC sample (Figure 3A). A fairly pure band of lactulose appears with a retention time of 18.52 min (Figure 3A), showing a very small shoulder of lactose. The retention times for the mixture of the standards were 18 min for lactulose and 20 min for lactose (Figure 3B).

The lactose isolated and purified by the procedures of Singh et al. (1991b) presented 0.76% ash and 0.002% crude protein. The crystallization time was very important for the formation of higher purity crystals. Figure 2 compares the spectra of pure lactose, thicker line, with the lactose purified in the present work. The two profiles are very similar, although not identical.

![Infrared spectral comparison between standard lactose and purified lactose.](image)

A microbiological assay (Table 2) showed that the addition of different levels of whey permeate to the MRS (De Man, Rogosa and Sharpe) or modified MRS (MRSm) media for *Lb. acidophilus* La-5 and *B. lactis* Bb-12, respectively, resulted in a significant increase in growth for both probiotic bacteria.
Culture media with 75% of whey permeate showed the highest growth stimulation for both La-5 (97%) and Bb-12 (143%). For all levels of water substitution plus total substitution of glucose in the culture media, Bb-12 responded better to the introduction of WP than La-5.

TABLE 2. Effect of introducing different levels of WP into the MRS or MRSm culture media, on the development of the probiotics *Lb. acidophilus* La-5 and **B. lactis** Bb-12.

<table>
<thead>
<tr>
<th>Culture media</th>
<th>Probiotic development mean ± sd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>La-5</td>
</tr>
<tr>
<td>MRS/MRSm [controls]</td>
<td>100 ± 1.52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MRS/MRSm (50% WP)**</td>
<td>163 ± 2.93&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MRS/MRSm (75% WP)**</td>
<td>197 ± 2.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MRS/MRSm (85% WP)**</td>
<td>168 ± 2.34&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

MRS = basic medium; MRSm = modified MRS (0.05 g/100 mL Cys.HCL); WP = Whey Permeate.

Different superscript letters (columns) indicate statistically different (p < 0.05) results.

* The values for the controls were adjusted to 100, for the purpose of comparison.

** Culture media without glucose.

These results are very important from the operational point of view, because they allow one to find the concentration range of added lactulose that promotes efficient growth stimulation of the bacteria La-5 and particularly of Bb-12. It would be interesting to carry out assays with animals and humans to determine the concentration of lactulose able to promote beneficial changes in the intestinal flora. In a study by Nagendra et al. (1995), the incorporation of 0.5% lactulose into infant foods, promoted changes in the microbial flora, showing a predominance of bifidobacteria in their faeces, whilst children fed on the control formulations showed a predominance of pathogenic bacteria.

Özer et al. (2005) studied the effect of inulin and lactulose on the survival of *Lb. acidophilus* La-5 and *B. bifidum* BB-02 in acidophilus-bifidus yoghurt. They showed that inulin and lactulose did not affect the growth of the yoghurt starter bacteria, but stimulated the growth of BB-02 to a great extent. Lactulose was found to be more effective on the growth of both probiotic strains than inulin. Inulin did not stimulate the growth of La-5. The cell counts of BB-02 and of La-5 were dependent on the concentrations of lactulose and inulin used. Although obtained in different systems and under different conditions, these results show some similarity with the results presented here with respect to the higher efficacy of lactulose in promoting the growth of bifidobacteria.

As clearly shown in Figures 4 and 5, bile salts (0.3% Oxgall) negatively affected the reproduction and growth of potential for its application in culture medium for lactic culture propagation. However, the use of these components alone is not sufficient for adequate growth of the culture, requiring the addition of complementary factors.

Table 3 illustrates the effects of adding lactulose to the MRS and MRSm media on the growth of La-5 and Bb-12. Compared to the MRS medium, the growth of La-5 increased with increase in lactulose up to 3%, reaching a 36% increase in growth (p < 0.05). Bb-12 behaved differently, reaching a maximum increase in growth (75%) with a 1% addition of lactulose, and then decreasing to the control level with further increases in the addition of lactulose up to 3%. Thus the growth of Bb-12 responds to lower lactulose concentrations than La-5.

TABLE 3. Effect of fortified MRS and MRSm culture media with three levels of lactulose on development of the probiotics *Lb. acidophilus* La-5 and *B. lactis* Bb-12

<table>
<thead>
<tr>
<th>Culture media</th>
<th>Probiotic development mean ± sd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>La-5</td>
</tr>
<tr>
<td>MRS/MRSm [controls]*</td>
<td>100 ± 1.52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MRS/MRSm (1% lactulose)</td>
<td>112 ± 3.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MRS/MRSm (2% lactulose)</td>
<td>131 ± 1.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MRS/MRSm (3% lactulose)</td>
<td>136 ± 0.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

MRS = basic medium; MRSm = modified MRS (0.05 g/100 mL Cys.HCL).

Different superscript letters (columns) indicate statistically different (p < 0.05) results.

* The values for the controls were adjusted to 100, for the purpose of comparison.
both La-5 and Bb-12. In the case of La-5 the presence of 2% lactulose improved growth but did not compensate for the loss imposed by the presence of the bile salts, when compared with the growth curve in the MRS (control) medium. On the other hand, for Bb-12, 1% lactulose in the MRSm (control) medium more than compensated for the retardation in growth imposed by the bile salts. The results again suggest that Bb-12 is more responsive to low concentrations of lactulose in the culture medium than La-5.

**FIGURE 4.** Effect of lactulose 2% (w/v) and bile salts [0.3% Oxgall] on the growth curve of *Lactobacillus acidophilus* La-5.

**FIGURE 5.** Effect of lactulose 1% (w/v) and bile salts [0.3% Oxgall] on the growth curve of *Bifidobacterium lactis* Bb-12.

### 4. CONCLUSION

The results presented in this article allow the conclusion that the permeate from the ultrafiltration of bovine milk whey (MW cut off 10Da) contained predominantly lactose (85%), minerals (9.6%) and a fairly low content of nitrogenous substances (4.1%). The lactose obtained from whey permeate could be purified and converted to lactulose by isomerization under alkaline conditions using sodium borate as the catalyst.

The results of the microbiological assays for the supplementation of the MRS and MRSm media with 75% WP in the absence of glucose, promoted a significant increase in the growth of *B. lactis* Bb-12 and *Lb. acidophilus* La-5. It was demonstrated that the enrichment of MRS and MRSm with 1% and 2% lactulose produced an important enhancement of growth in *B. lactis* Bb-12 and *Lb. acidophilus* La-5, respectively. Both La-5 and Bb-12 were tolerant of bile salts, mainly in the presence of lactulose.

### ACKNOWLEDGEMENTS

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