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# Reduction of α-galactooligosaccharides in soyamilk by *Lactobacillus fermentum* CRL 722: *in vitro* and *in vivo* evaluation of fermented soyamilk

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## ABSTRACT

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**Aims:** Consumption of soya-derived products has been hampered by the presence of α-galactooligosaccharides (α-GOS) because mammals lack pancreatic α-galactosidase (α-Gal) which is necessary for their hydrolysis. These sugars thus reach the large intestine causing gastrointestinal disorders in sensitive individuals. The use of lactic acid bacteria (LAB) expressing α-Gal is a promising solution for the degradation of α-GOS in soyamilk.

**Methods and Results:** The capacity of the LAB *Lactobacillus fermentum* CRL 722 to properly degrade α-GOS was studied *in vitro* using controlled fermentation conditions and *in vivo* using a rat model. *Lactobacillus fermentum* CRL 722 was able to grow on commercial soyamilk and completely eliminated stachyose and raffinose during fermentation because of its high α-Gal activity. Rats fed soyamilk fermented by this LAB had smaller caecums compared with rats fed unfermented soyamilk.

**Conclusions:** Soyamilk fermentation by *Lact. fermentum* CRL 722 results in the reduction of α-GOS concentrations in soyamilk, thus eliminating possible undesirable physiological effects normally associated with its consumption.

**Significance and Impact of the Study:** Fermentation with *Lact. fermentum* CRL 722 could prevent gastrointestinal disorders in sensitive individuals normally associated with the consumption of soya-based products. This LAB could thus be used in the elaboration of novel fermented vegetable products which better suit the digestive capacities of consumers.

**Keywords:** α-galactosidase, α-GOS, flatulence, lactic acid bacteria, soyamilk.

## INTRODUCTION

Soya products have an excellent status for high protein contents, and soya protein contains enough of all the essential amino acids to meet biological requirements when consumed at the recommended level of protein intake. However, soyabeans as well as other legumes characteristically contain high concentrations of the α-galactooligosaccharides (α-GOS) stachyose and raffinose (Leske *et al.* 1993). Hydrolytic digestion of α-GOS is relatively weak in mammals because they do not possess pancreatic α-galactosidase (α-Gal) which is necessary to hydrolyse the α-1, 6 linkages found in these sugars (Slominski 1994). The undigestibility of these soluble carbohydrates results in

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their delivery into the colon where they are rapidly fermented by the microflora resulting in the production of large amounts of gas (Steggerda *et al.* 1966). This induced flatulence greatly hampers the acceptability of soya products as a major food source for humans and animals (Suarez *et al.* 1999).

The use of microbial α-Gal is a promising solution for the degradation of α-GOS in soya products. Lactic acid bacteria (LAB) have been consumed in fermented foods by human beings for centuries without any obvious adverse effects (Fuller 1989). LAB such as *Lactobacillus* (*Lact.*) *plantarum, Lact. fermentum, Lact. brevis, Lact. buchneri* and *Lact. reuteri* are able to hydrolyse α-GOS into digestible carbohydrates during vegetable fermentations because of the action of α-Gal. α-Gals from bifidobacteria and lactobacilli have previously been characterized biochemically and physiologically (Garro *et al.* 1993, 1994, 1996). The first genetic characterization of an α-Gal from lactobacilli was also performed in our laboratory (Silvestroni *et al.* 2002) and recently the characterization of genes involved in α-GOS hydrolysis by *Lactococcus (L.) raffinolactis* has been described (Boucher *et al.* 2003).

In our laboratory, two different strategies are being developed to evaluate the efficiency of α-Gal producing LAB to remove α-GOS present in soya products first and later in the gut (Connes *et al.* 2004 and LeBlanc *et al.* 2004b): both make the use of α-Gal producing strains as (i) starters in soyamilk fermentations and (ii) as probiotics to degrade α-GOS *in situ* in the gastrointestinal tract.

In terms of α-GOS removal from soya-derived products, various methods (soya germination, bean soaking, and water extraction) exist but are all very laborious. Obviously, the utilization of LAB strains that ferment soya-products and simultaneously degrade α-GOS is economically attractive. In this first strategy, the α-Gal producing strains must be able to grow on the support medium (soyamilk) and to remove or degrade the α-GOS into digestible sugars. Soyamilk is suitable for the growth of some LAB, especially bifidobacteria (Garro *et al.* 1999, 2004; Chou and Hou 2000) because of its content of growth stimulating factors such as oligosaccharides, amino acids and peptides (Bezkorovainy 2001). In fermentation studies, the technological properties of the strains to be used as starters are very important and properly selected strains must be used in order to enhance specific characteristics of the final product. For example, the taste of soyamilk can be improved using some LAB strains that decrease the beany, grassy and soya flavour (Mital *et al.* 1974; Granata and Morr 1996; Liu and Lin 2000).

In the second strategy, α-Gal producing strains are to be used as probiotics to degrade α-GOS *in situ* in the upper gastrointestinal tract thus preventing their delivery in the colon where they would otherwise be fermented. In this probiotic strategy, the strains must not only be able to survive the harsh conditions of the gastrointestinal tract and produce active α-Gal, but they must also be innocuous in order to prevent any danger to the consumer. Also, the α-Gal to be delivered in the gut must be active in the conditions of the small intestine.

The present study fits within the frame of the first strategy outlined above. We have shown that two strains, *Lact. fermentum* CRL 722 and CRL 251, isolated from Argentinean cheeses, were able to degrade raffinose and use this sugar as their sole carbon source in controlled fermentation conditions (LeBlanc *et al.* 2004a). Both strains possess similar growth characteristics and sugar degradation capabilities; however, the α-Gal activity level of *Lact. fermentum* CRL 722 was found to be 1·5 times higher than that of *Lact. fermentum* CRL 251 (LeBlanc *et al.* 2004a). Furthermore, upon screening LAB strains for α-Gal activity, *Lact. fermentum* CRL 722 showed the highest α-Gal activity of all strains tested in our laboratory (Silvestroni 2004). For these reasons, this high α-Gal producing strain was retained in the present study as a potent starter candidate for α-GOS degradation in soyamilk fermentations.

This α-GOS reduction in soyamilk by *Lact. fermentum* CRL 722 was studied *in vitro* using controlled soyamilk fermentation conditions. The resulting fermented product was then evaluated *in vivo* using a rat model to study whether the above mentioned fermentation process of soyamilk allowed to circumvent the physiological effects observed in rats receiving native soyamilk.

## MATERIALS AND METHODS

### Bacterial strains, growth conditions and sample preparation

*Lactobacillus fermentum* CRL 722 used in this study was obtained from the Culture Collection (CRL) of the Centro de Referencia para Lactobacilos (CERELA), Tucumán, Argentina. This strain was selected because of its high production level of α-Gal, resulting in its capacity to degrade raffinose (LeBlanc *et al.* 2004a). Before experimental use, cultures were propagated (2% v/v) twice in MRS medium (de Man *et al.* 1960), then in commercial soyamilk (AdeS Natural, Argentina) and incubated at 37°C for 16 h.

Fermentation experiments were carried out in batch cultures (Erlenmeyer flasks) with 500 ml of soyamilk and incubated statically at 37°C. Samples were taken at 0, 2, 4, 6, 8, 10, 12 and 24 h after inoculation and were tested for pH, cell viability and α-Gal activity. Samples were frozen at −20°C until residual sugars and organic acids were analysed.

Growth was followed by monitoring changes in: (i) conductance using a Bactometer Microbial Monitoring System (Biomérieux, Marcy l'Etoile, France) expressed as micro Siemens ($\mu$S) as described previously (Garro *et al.*

2002) and (ii) pH. Furthermore, serial dilutions of samples were plated out in triplicate on MRS agar and incubated 48 h at 37°C.

For raffinose, saccharose, melibiose, glucose, galactose and fructose determinations, samples were deproteinized as previously described (Mital and Steinkraus 1975). Briefly, 0·4 ml of $Ba(OH)_2$ (1·8% w/v) was added to 0·2 ml samples and mixed. Afterwards, 0·4 ml $ZnSO_4$ (2·0% w/v) was added and the mixture was allowed to stand at room temperature (21°C) for 10 min. Samples were then centrifuged (10 000 *g*, 10 min, 4°C) and the supernatants were stored at −20°C until analysis. Sugars were quantified by HPLC coupled to a differential refractometer (LKB Bromma, Stockholm, Sweden) using a REZEX RSO oligosaccharides column (200 mm × 10 mm; Phenomenex, Torrance, CA, USA) maintained at 70°C. HPLC grade water was used as the eluant at a flow rate of 0·3 ml $min^{-1}$.

For α-Gal determinations, soyamilk samples (1 ml) were centrifuged (8000 *g*, 10 min, 4°C) and the supernatants used directly for extracellular α-Gal determination. The cell pellet was washed three times with 5% McIlvaine buffer ($Na_2HPO_4$–citric acid, pH 5·8; McIlvaine 1921) and resuspended in 0·5 ml of the same buffer. Cells were disrupted using 500 mg glass beads (0·10–0·11 mm; Sigma, Buenos Aires, Argentina) and vortexed at maximum speed during five cycles of 1 min each with 5 minutes pauses on ice in between. Cellular debris were removed by centrifugation (10 000 *g* for 10 min at 4°C) and the supernatant was kept on ice until analysis of intracellular α-Gal activity.

### α-Galactosidase activity determination

α-Gal was determined using a modified technique of Church *et al.* (1980). To a 85-μl sample, 27·5 μl McIlvaine buffer (4·5X) and 12·5 μl of 30 mM *p*-nitrophenyl-α-D-galactopyranoside (*p*NPG) were added and incubated at 37°C for 15 min. The reaction was stopped by adding 125 μl of sodium carbonate (0·5 M). Absorbance at 405 nm was measured using a VersaMax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). One enzyme unit (U) was defined as the amount of enzyme that releases 1·0 μmol of *p*NP from its *p*NPG substrate per minute under the given assay conditions. In animal trials, the results take into account the total weight of samples (mg) obtained from caecum.

### *In vivo* evaluation of fermented soyamilk

Conventional Wistar rats were obtained from the inbred colony maintained in the Nutrition Department of the Universidad Nacional de Tucumán (UNT) and maintained in individual cages. Rats were separated into two experimental groups of 10 animals. Diet and water were removed from the cages during the test period. The first group (fermented soyamilk) received the following product: soyamilk was fermented with *Lact. fermentum* CRL722 at 37°C during 24 h (final pH 5·5), centrifuged (4000 *g* for 10 min at 4°C) and the supernatant was given to each individual animal as a replacement for the drinking water. The second group (control) was given soyamilk which was prepared in the following manner: the pH of 1 L soyamilk was adjusted to 5·5 (the same pH as in the fermented soyamilk group) using lactic acid (Sigma), centrifuged, and the supernatant was given as described for the first group. Each rat received 35 ml of one of the preparations twice a day during two consecutive days. On the third day, they received one dose of 10 ml and were sacrificed four hours later by cervical dislocation. Caeca were removed, immediately weighed and maintained on ice until α-Gal determination. The caecal contents were obtained by washing with 5 ml McIlvaine buffer. This mixture was vortexed for 2 min, centrifuged (8000 *g* for 15 min at 4°C) and the supernatant was tested for extracellular α-Gal activity. To a 0·5-ml sample, 0·5 mg glass beads were added and cells were disrupted as described above for intracellular α-Gal determination.

### Reproducibility

All results presented in this paper are the average of three independent assays. Results are expressed as mean ± standard deviation, and their significance was analysed using the Student's *t*-test.

## RESULTS

### *Lactobacillus fermentum* CRL 722 growth on soyamilk

*Lactobacillus fermentum* CRL722 was able to grow in soyamilk as suggested by significant changes (more than 25%) in conductance, and in pH (Fig. 1a). This was checked by monitoring microbial growth whose kinetics was similar to those obtained using the bactometer and in pH measurements (Fig. 1b). At end of the soyamilk fermentation, *Lact. fermentum* CRL722 population reached $5 \times 10^8$ CFU $ml^{-1}$.

### α-GOS removal and α-galactosidase activity during fermentation

Within 10 h of growth, *Lact. fermentum* CRL 722 was able to reduce the initial concentration of stachyose found in soyamilk by almost 90% (Fig. 2a). No glucose, galactose, melibiose or fructose accumulated in the medium during soyamilk fermentation (data not shown). *Lactobacillus fermentum* CRL 722 exhibits high α-Gal activity which

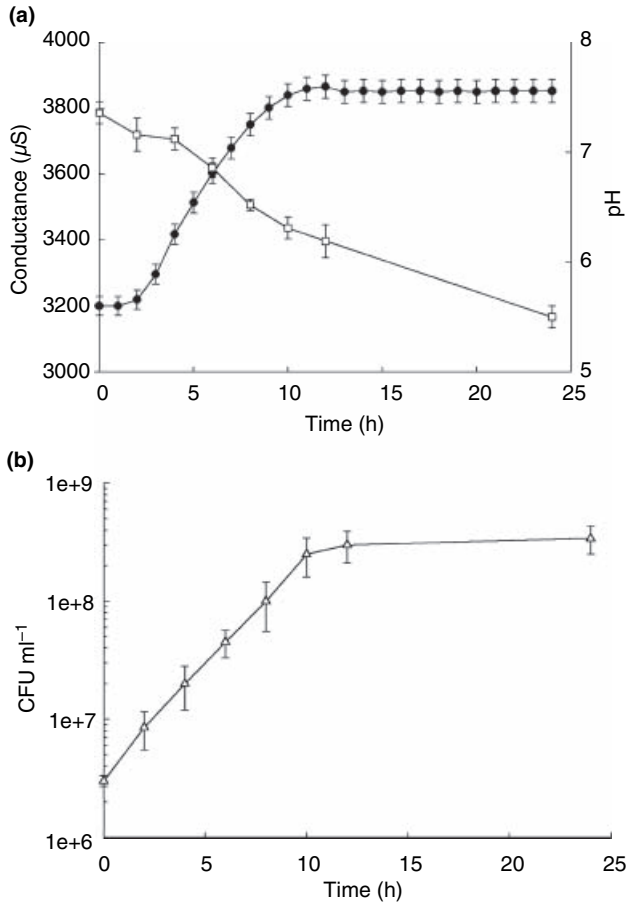


**Fig. 1** Growth of *Lactobacillus fermentum* CRL 722 in soyamilk as determined by: (a) conductance using a bactometer (filled circles) and pH (empty squares) and (b) colony-forming units determination on MRS agar (empty triangles). Results are expressed as mean ± standard deviation

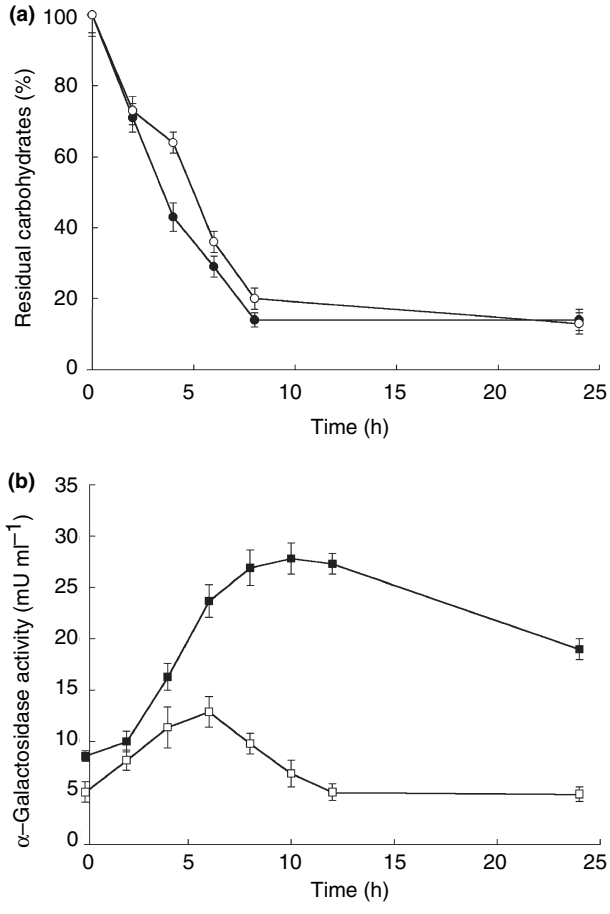


**Fig. 2** (a) Residual stachyose and raffinose (filled circles, same profile for both) and saccharose (empty circles) during soyamilk fermentation by *Lactobacillus fermentum* CRL 722. (b) Intracellular (filled squares) and extracellular (empty squares) α-galactosidase activity during soyamilk fermentation. Results are expressed as mean ± standard deviation

could explain its ability to use stachyose and raffinose for growth (LeBlanc *et al.* 2004a). This important enzymatic activity was monitored during soyamilk fermentations. α-Gal activity was detected in all of the samples taken at different times of the fermentations, showing a maximum level after 10 h of growth (Fig. 2b) which is the start of the stationary growth phase (Fig. 1). Most α-Gal activity appeared to be intracellular (maximum of 30 mU $ml^{-1}$ after 10 h of growth) but significant levels of α-Gal were released in soyamilk (peak of 14 mU $ml^{-1}$ after 6 h of growth).

### *In vivo* evaluation of the fermented product

Unfermented soyamilk (control) and soyamilk fermented with *Lact. fermentum* CRL 722 were compared for their *in vivo* effects in rats (caecal development, caecal α-Gal activity, and body weight) (Table 1). The whole caecal weight and caecal contents were about two-fold lower in rats fed the fermented soyamilk than in the control group (7·9 g vs 121·4 g and 0·9 g vs 1·7 g, respectively). Also, caecal α-Gal activity was about two times lower in the group receiving fermented soyamilk than in the group fed unfermented soyamilk (86 mU $ml^{-1}$ $mg^{-1}$ vs 203 mU $ml^{-1}$ $mg^{-1}$ in extracellular contents). In terms of animal growth, soyamilk consumption, and animal behavior, no significant difference was observed between the two animal groups during the study.

## DISCUSSION

*Lactobacillus fermentum* CRL 722 was able to grow in soyamilk and growth kinetics was comparable with those observed in MRS medium (data not shown) suggesting that soyamilk provided all nutritional requirements for this strain.

**Table 1** Effects of soyamilk administration [unfermented (control) vs fermented with *Lactobacillus fermentum* CRL 722 (fermented)] on body weight, caecal development and caecal α-galactosidase activity

| | Control | Fermented |
|---|---|---|
| Whole caecal weight (g) | 12·4 ± 1·0* | 7·9 ± 0·9* |
| % (g $g^{-1}$) of body weight | 5·9 ± 0·5* | 3·7 ± 0·5* |
| Caecal contents (g) | 1·7 ± 0·2* | 0·9 ± 0·3* |
| % (g $g^{-1}$) of body weight | 0·81 ± 0·09* | 0·42 ± 0·14* |
| Caecal α-galactosidase activity | | |
| Intracellular (mU $ml^{-1}$ $g^{-1}$ caecal contents) | 346·0 ± 45·3* | 201·1 ± 51·4* |
| Extracellular (mU $ml^{-1}$ $g^{-1}$ caecal contents) | 203·6 ± 39·0* | 86·1 ± 29·4* |
| Body weight | | |
| Initial (g) | 200 ± 7 | 200 ± 7 |
| Final (g) | 211 ± 8 | 210 ± 6 |
| Weight gain (g) | 3·6 ± 1·1 | 3·3 ± 0·9 |

Values represent the mean and the standard deviations.
*Significant difference between groups ($P < 0·01$).

Monitoring the sugar content of the fermented soyamilk along the fermentation process indicated that the carbon source used by *Lact. fermentum* CRL722 for its growth does not only include sucrose but also and to the same extent, the α-GOS stachyose and raffinose (Fig. 2a). This is consistent with the profile of α-Gal activity measured intra- and extracellularly during the fermentation; the increase in α-Gal activity followed the same time course of α-GOS degradation (Fig. 2b). Maximum α-Gal activity was observed at the start of the stationary phase of growth of *Lact. fermentum* CRL 722 (Fig. 1a), similar to previous results where *Lact. fermentum* was grown in MRS-raffinose (LeBlanc *et al.* 2004a). Interestingly, α-Gal activity was detected intra- and extracellularly (Fig. 2b) with a major portion of α-Gal that remained intracellular. Although this suggests partial secretion of the α-Gal by *Lact. fermentum* CRL722, we cannot rule out at this step that the α-Gal released in the medium was released by partial bacterial lysis. Indeed, bacterial α-Gal so far characterized all occur as intracellular enzymes. The ongoing genetic characterization of this α-Gal should tell us whether it contains a sorting signal possibly involved in secretion. However, whatever the explanation for the presence of extracellular α-Gal might be, this is an interesting point for application as it allows α-GOS to be degraded outside the cells and does not make the α-Gal system rely on a transport system for α-GOS internalization into the cells. This could be particularly important for the development of mixed cultures in soyamilk fermented products as extracellular α-GOS degradation would facilitate the growth of non-α-Gal producing strains.

Although nondigestible oligosaccharides, including α-GOS, are often used to stimulate the growth of beneficial micro-organisms such as bifidobacteria in the gut, this stimulation is not bacteria specific and a common side-effect is the production of large amounts of hydrogen, carbon dioxide and/or methane which are inevitable products of microbial fermentation in the large intestine (Cummings *et al.* 2001). As the undesirable effects of α-GOS such as flatulence are predominantly induced in the large intestine (caecum and colon), we decided to focus on the physiological effects of α-GOS in this portion of the gut. Two markers were selected in this study. One is the caecal weight as it has been shown that animals fed GOS-supplemented diets had significantly bigger caeca than those fed nonsupplemented diets (Chonan *et al.* 1995, 2001). The second marker used is α-Gal activity as it has been shown in rats that supplementation of food with α-GOS increases the metabolic α-Gal activity of the microbiota of the caecum (Gliko-Kabir *et al.* 2000). In our study, both these markers differed according to the type of diet administered to the rats. The relative caecum weight was significantly lower when rats were fed soyamilk fermented with *Lact. fermentum* CRL 722 than when fed unfermented soyamilk (Table 1). Also, α-Gal activity in caecum (intra and extracellular) was significantly lower when rats were fed soyamilk fermented with *Lact. fermentum* CRL 722 than when fed unfermented soyamilk (Table 1). These *in vivo* results (reduction in caecum weight and caecal microbial metabolism) confirm that fermenting soyamilk with *Lact. fermentum* CRL 722 lowers the amount of α-GOS which reach the large intestine thus reducing the fermentation and the possible gas production by the microbiota. These observations prove that the α-GOS reduction by *Lact. fermentum* CRL 722 during fermentation have the expected beneficial physiological effects upon soyamilk consumption. This is an important step in the future development of soya-derived products in food.

This study focused on the potential of *Lact. fermentum* CRL 722 used as a starter strain to remove stachyose and raffinose from soyamilk during fermentation. Another perspective would be to use this strain as a model organism in a probiotic preparation aimed at decreasing gas production associated with the consumption of food products such as various legumes including soya. The rationale down this line would be to administer high α-Gal producers that would release this enzyme in the upper gut thus allowing α-GOS degradation before they reach the large intestine. These studies are currently being performed in our laboratory.

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