Lactobacillus fermentum CRL 722 is able to deliver active α-galactosidase activity in the small intestine of rats

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Abstract

α-galactooligosaccharides (α-GOS) found in legumes such as soybeans can cause gastrointestinal disorders since mammals lack α-galactosidase (α-Gal) in the small intestine which is necessary for their hydrolysis. *Lactobacillus fermentum* CRL 722 is a lactic acid bacterium (LAB) capable of degrading α-GOS due to its elevated α-Gal activity. When conventional rats were fed live *L. fermentum* CRL 722 or cell-free extracts of this strain, a short-lived α-Gal activity was detected in the upper gastrointestinal tract. The safety of this LAB was also assessed. *L. fermentum* CRL 722 could thus be used as a vehicle to safely confer α-Gal in the small intestine of monogastric animal.
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1. Introduction

Soy products have an excellent status for their high protein content, and soy proteins contain enough of all the essential amino acids to meet biological requirements when consumed at the recommended level of protein intake. However, soybeans as well as other legumes characteristically contain high concentrations of the α-galactooligosaccharides (α-GOS) such as stachyose and raffinose [1]. Hydrolytic digestion of α-GOS is relatively weak in mammals because they do not possess α-galactosidase (α-Gal) in the upper gastrointestinal tract which

is necessary to hydrolyze the α-1,6 linkages found in these sugars [2]. The indigestibility of these soluble carbohydrates results in their delivery into the colon where they are rapidly fermented by the resident microbiota resulting in the production of large amounts of gas [3]. This induced flatulence greatly hampers the acceptability of soy products as a major food source for humans and animals [4]. The use of microbial α-Gal is a promising solution for the degradation of these undesirable α-GOS.

Lactic acid bacteria (LAB) such as *Lactobacillus* (*L.*) *plantarum*, *L. fermentum*, *L. brevis*, *L. buchneri* and *L. reuteri* are able to hydrolyze α-GOS into digestible carbohydrates during vegetable fermentations due to their α-Gal activity. The first genetic characterization of an α-Gal from lactobacilli was performed in our laboratory

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[5] and recently the characterization of genes involved in α-GOS hydrolysis by *Lactococcus raffinolactis* has been described by another group [6]. Also, we have previously shown that *L. fermentum* is able to grow on soymilk and to remove or degrade the α-GOS into digestible sugars due to its high α-Gal activity [7]. For these reasons, two different strategies are being developed to evaluate the efficiency of LAB to remove or degrade α-GOS present in soy products [8,9] using α-Gal producing strains to degrade these non-digestible sugars: (i) previous to consumption by means of soymilk fermentations and (ii) in situ in the upper gastrointestinal tract thus preventing the delivery of α-GOS in the colon where they would otherwise be fermented. In this second strategy, the strains must not only be able to produce or liberate active α-Gal in the small intestines, but they must also be innocuous in order to prevent any danger to the consumer.

This paper describes the use of *L. fermentum* CRL 722 as a vehicle to liberate active α-Gal in the small intestine of rodents.

2. Materials and methods

2.1. Bacterial strains and growth conditions

L. 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 and consequent capacity to degrade raffinose in soymilk [7,10]. Before experimental use, cultures were propagated (2% v/v) twice in MRS medium (Britania S.A., Buenos Aires, Argentina) and incubated at 37 °C for 16 h. For animal trials, *L. fermentum* CRL 722 was suspended in peptone water (0.1% m/v microbiological grade peptone, Difco Argentina) at the required concentration.

2.2. Cell-free extract preparation

L. fermentum CRL 722 was grown in MRS as described above. This pre-culture was used to inoculate 1 l of MRS (2% v/v) which in turn was incubated at 37 °C during 16 h. This culture was centrifuged (6000g during 10 min at 4 °C) and the pellet was resuspended in 100 ml cold McIlvaine buffer ((Na$_2$HPO$_4$-citric acid, pH 5.8 [11]). This washing step was repeated 3 times and the pellet was resuspended in McIlvaine buffer at a final concentration of 40% (m/v). This suspension was passed three times through a French Pressure Cell Press (Thermo Spectronic, Rochester, NY, USA) at a pressure of 25,000 psi (172 400 kPa) in order to disrupt cell integrity and to liberate the intracellular contents. Unbroken cells and large cellular components were elim-

inated by centrifugation (10,000g for 10 min at 4 °C) and the supernatant was maintained on ice until used.

2.3. Animal trials

Conventional Wistar rats weighing 200–250 g from the inbred closed colony maintained at INSIBIO-UNT (Tucumán, Argentina) were divided into 2 groups of at least 10 animals. Animals were fed a solid conventional diet (Balanced Rodent Chow, Cargill, Córdoba, Argentina) and water ad libitum and were maintained in a room with a 12 h light/dark cycle at 18 ± 2 °C. Animals were administered 1 ml of the cell-free extract or of live *L. fermentum* cells (1.5×10^{11} CFU/ml) using an intra-esophagic probe. Animals were sacrificed by vertebral dislocation at different intervals post-administration (0, 5, 10, 15, 30 and 60 min). The stomach and the small intestine (separated into 3 sections of equal size corresponding to the duodenum, jejunum and ileum, respectively) were removed and their contents collected adding 1 ml cold McIlvaine buffer. The contents were homogenized then centrifuged (10,000g during 2 min at 4 °C) and the supernatants were maintained on ice until α-Gal activity was determined.

2.4. α-Galactosidase activity

α–Gal activity was determined using a modified technique of Church et al. [12]. To an 85 μl sample, 27.5 μl McIlvaine buffer (4.5×) 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 Tuneable Microplate Reader (Molecular Devices, 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 min under the given assay conditions.

2.5. Effect of gastrointestinal contents on α-Gal activity and L. fermentum survival

The gastrointestinal contents of non-treatment animals were obtained as described above. The contents of 10 animals were pooled, centrifuged (10,000g for 10 min at 4 °C), sterilized by membrane filtration (0.22 μm, Millex-AP, Millipore-Biopore, Buenos Aires, Argentina) and conserved on ice until used. To determine the effect of gastrointestinal contents on α-Gal activity and *L. fermentum* survival, 100 μl of the content of one gastrointestinal compartment was added to the same amount of diluted *L. fermentum* cell-free extract (1/10, v/v in McIlvaine buffer, see above) or live overnight culture of *L. fermentum*. This mixture was incubated during 0, 15, 30, 60 or 180 min at 37 °C and α-Gal activity was determined as described above. Cell

viability was determined by plating serial dilutions on MRS-agar and incubation at 37 °C during 48 h.

2.6. Safety assessment of L. fermentum CRL 722

The general safety of *L. fermentum* CRL 722 was investigated in feeding trials where animals received 5×10^{10} colony forming units (CFU)/kg body weight/day for 4 weeks (concentrated in peptone water, or sterile peptone water in the control group) as described previously [13]. Throughout this time, feed intake, water intake, and live body weight were monitored. At the end of the 4 week observation period, samples of blood, liver and spleen were collected to determine: hematological parameters (red (RBC) and white (WBC) blood cell counts, differential leukocyte counts, hematocrit and hemoglobin concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration(MCHC)); microbial translocation to extra-gut tissues (liver and spleen) as previously described [14]; and relative organ weight (liver and spleen).

2.7. Reproducibility

Unless otherwise indicated, all values are the means of 3 independent trials ± standard deviation (SD) where each data point represents $n = 15$ individuals (3 trials where each sample consisted of 5 individual assays/rodents). Comparisons were done with the software package, SigmaStat (SPSS, Chicago, IL) by one-way ANOVA followed by a Tukey's post-hoc test and $P < 0.05$ was considered significant.

3. Results

3.1. α-Gal in the gut of rats fed cell-free extracts of L. fermentum CRL 722

After giving conventional rats the cell-free extract of *L. fermentum* CRL 722, α-Gal activity was detected in the stomach and duodenum, but not in the jejunum and ileum (Fig. 1), whereas no activity was detected in the control group. This enzymatic activity was short-lived since no significant α-Gal activity was detected after 15 min post-inoculation.

3.2. α-Gal in the gut of rats fed live L. fermentum CRL 722

After administering live *L. fermentum* CRL 722 to conventional rats, a brief α-Gal activity was detected in the stomach and duodenum, but not in the jejunum and ileum (Fig. 2). This enzymatic activity was short-lived since no significant α-Gal activity was detected after 30 min post-inoculation.
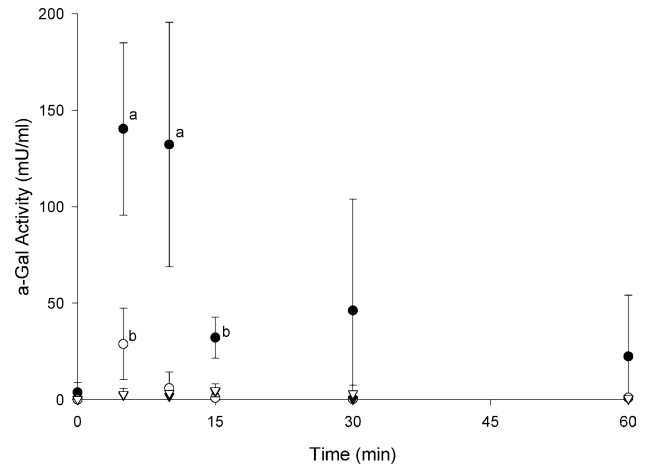

Fig. 1. α-Gal activity in different compartments of the gastrointestinal tract of rats fed a cell-free extract of *L. fermentum* CRL 722: stomach (filled circles), duodenum (empty circles), jejunum (filled triangles) and ileum (empty triangles). Results are expressed as means with standard deviation. Means without a common letter differ significantly, $P < 0.05$.
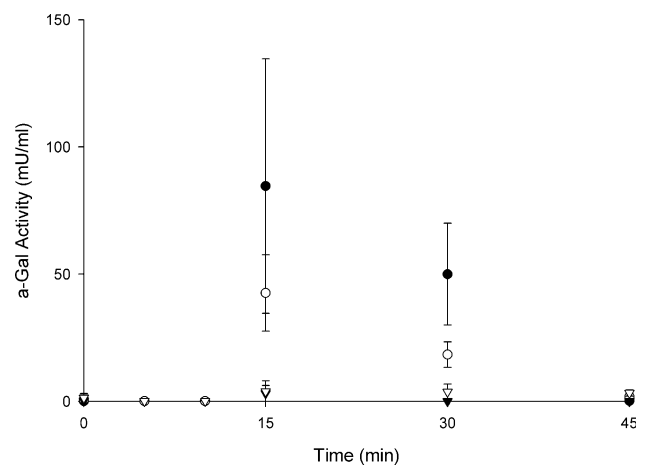

Fig. 2. α-Gal activity in different compartments of the gastrointestinal tract of rats fed a 1.5×10^{11} CFU of *L. fermentum* CRL 722: stomach (filled circles), duodenum (empty circles), jejunum (filled triangles) and ileum (empty triangles). Results are expressed as means with standard deviation. Means without a common letter differ significantly, $P < 0.05$.

3.3. Effect of gastrointestinal contents on α-Gal activity

A rapid decrease in the α-Gal activity of the cell-free extracts of *L. fermentum* CRL 722 was detected when they were incubated together with the contents of the duodenum and jejunum, but not the ileum or in the control group (Fig. 3). After 15 min, more than half of the α-Gal activity was lost in the presence of duodenal or jejunal contents and after 30 min, no more enzymatic activity was detected. Due to the use of buffered solution in the recuperation of the gastrointestinal contents, the pH of the stomach increased to non-physiological levels. For this reason the effect of the stomach contents were
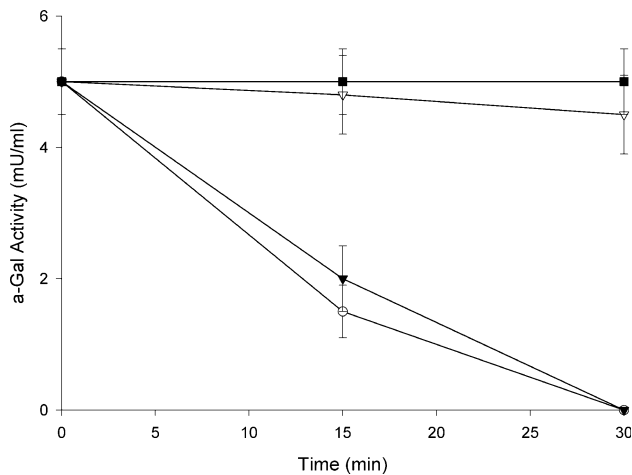
Fig. 3. α-Gal activity of cell-free extracts of *L. fermentum* CRL 722 incubated in the presence of the contents from different gastrointestinal compartments: control (filled squares), duodenum (filled triangles), jejunum (empty circles) and ileum (empty triangles). Results are expressed as means with standard deviation. Means without a common letter differ significantly, *P* < 0.05.

not determined in this portion of the study nor in the following section. However, we have previously shown that no α-Gal activity is detected at pH values lower than 4.0 [7].

3.4. Effect of gastrointestinal contents on L. fermentum survival

A significant decrease in the number of colony forming units of *L. fermentum* CRL 722 was seen when this LAB was incubated in the presence of the contents of the duodenum and ileum (Fig. 4); this decrease was not observed in the presence of ileum contents or in
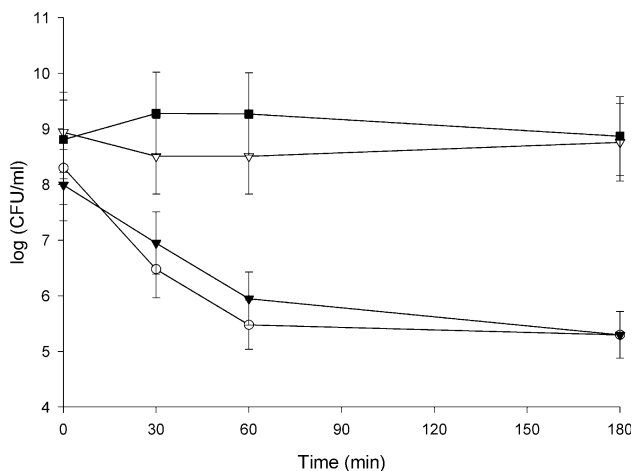

Fig. 4. Cell viability of *L. fermentum* CRL 722 incubated in the presence of the contents from different gastrointestinal compartments: control (filled squares), duodenum (empty circles), jejunum (filled triangles) and ileum (empty triangles). Results are expressed as means with standard deviation. Means without a common letter differ significantly, *P* < 0.05.

Table 1
Safety assessment of animals fed 5×10^{10} CFU *L. fermentum* CRL 722/kg body weight/day for 4 weeks (*L. fermentum*) compared to animals which did not receive this bacterial supplementation (control)

	Control	*L. fermentum*
Animal weight		
Weekly weight gain (g)	4.13 ± 0.38	4.10 ± 0.43
Relative organ weight		
Liver (g/g × 100)	5.02 ± 0.09	5.15 ± 0.28
Spleen (g/g × 100)	0.48 ± 0.05	0.52 ± 0.09
Hematology		
RBC count ($\times 10^6$ mm^3)	6.53 ± 0.22	6.67 ± 0.34
WBC count ($\times 10^3$ mm^3)	4.57 ± 0.49	4.90 ± 0.51
Neutrophils (%)	13.67 ± 4.04	14.33 ± 5.03
Eosinophils (%)	0.00 ± 0.00	0.00 ± 0.00
Basophils (%)	0.00 ± 0.00	0.00 ± 0.00
Lymphocytes (%)	86.00 ± 4.36	85.33 ± 5.51
Monocytes (%)	0.33 ± 0.58	0.35 ± 0.55
Hematocrit (%)	59.67 ± 1.53	60.67 ± 3.06
Hemoglobin (g/dl)	20.30 ± 0.46	20.63 ± 1.04
MCV (fl)	91.39 ± 0.84	90.91 ± 0.09
MCH (pg)	31.10 ± 0.38	30.92 ± 0.14
MCHC (g/100 ml)	34.02 ± 0.10	34.01 ± 0.16
Translocation[a]		
Liver	0/20	0/20
Spleen	0/20	0/20

[a] Animals with any tissue(s) where viable bacterial cell(s) were recovered were defined as microbial translocation positive animals (MT$^+$). The numbers included in this table are MT$^+$ animals/total examined animals. No significant differences were observed between both groups of animals using ANOVA, where *P < 0.05.*

the control group. After 30 min of incubation, all cell viability was lost in the presence of duodenal or jejunal contents, but in the presence of the ileum contents and in the control group, no significant decreases in cell numbers were observed even after 180 min incubation.

3.5. Safety assessment of L. fermentum CRL 722

Four week consumption of *L. fermentum* CRL 722 had no adverse effects on animal's general health status, hematology, or in the incidence of microbial translocation to extra-gut tissues since all values were similar to the control group (Table 1). No noticeable abnormal behavior, change in activity, or decline in hair luster was detected in animals after 4 weeks feeding with *L. fermentum* CRL 722.

4. Discussion

In order for a specific strain to be efficient in preventing the digestive disorders associated to α-GOS consumption, it must be able to degrade these undesirable sugars before they reach the large intestine where they are fermented by the endogenous microbiota. In order to reach this goal, the selected strain must be able to

produce or liberate active α-Gal in the upper digestive tract (duodenum, jejunum or ileum) and thus degrade the α-GOS into digestible sugars capable of being absorbed directly in the small intestine, preventing its arrival to the large intestine.

Previously, we have shown that *L. fermentum* CRL 722 produces α-Gal which is capable of degrading raffinose and stachyose found in soy and is able to use these α-GOS as energy sources for growth in soymilk [7,10]. This sugar degradation was able to prevent some gastrointestinal disorders associated to α-GOS consumption such as the abnormal growth of the cecum in conventional mice [10]. All of these previous results were obtained when this LAB was used as a starter strain and in these conditions it was not important to know if the α-Gal activity, responsible for these beneficial effects, was capable of exerting a biological effect in the hostile conditions of the gastrointestinal tract.

Here, a statistically significant α-Gal activity could be detected in the stomach and in the duodenum when conventional rats were given cell-free extracts or live *L. fermentum* CRL 722, (Figs. 1 and 2, respectively). This activity was not present in the control group (fed peptone water instead of the *L. fermentum*), an expected result since it is known that monoxenic animals (including rodents and humans) do not produce pancreatic α-Gal [15]. The novel α-Gal activity in the small intestine was short-lived since it was not detected after 30 min post-administration. (Figs. 1 and 2). However, this short-lived α-Gal activity could be sufficient to degrade α-GOS since we have previously shown that it takes less than 2 min for this enzyme to degrade raffinose and stachyose in controlled conditions (unpublished data). Also, preliminary results have shown that this LAB is capable of decreasing hydrogen production in gnotobiotic animals fed soymilk (unpublished data), confirming that α-Gal produced by *L. fermentum* is able prevent gastrointestinal disorders associated to α-GOS consumption as previously demonstrated in other animal models [10].

The digestive tract contains various components which can inhibit and/or decrease exogenous enzymatic activities such as α-Gal. These factors, which include digestive enzymes and bile salts, can deactivate α-Gal or decrease the survival of strains which produce it. To determine if the α-Gal activity of *L. fermentum* CRL 722 or the survival of this LAB strain was affected by components of the small intestine, where this enzyme is required, they were placed in the presence of germ-free intestinal contents. The contents of the duodenum and jejunum significantly decreased the α-Gal activity of the cell-free extracts of *Lb. fermentum* CRL 722 (Fig. 3). These same contents also decreased cell viability of this LAB (Fig. 4). In the small intestine, more precisely in the duodenum and in the jejunum, there are various digestive enzymes and bile salts which are necessary

for the proper functionality of the digestive apparatus. These compounds play an important role in the degradation of foods, which include exogenous digestive enzymes such as α-Gal in this study.

Before proposing that a specific strain be feasible to be inserted into the food chain, it must be shown that it is innocuous to the host/consumer. Feeding rodents with *L. fermentum* CRL 722 at a dose of 5×10^{10} CFU/kg body weight/day for 4 weeks had no adverse effects on general health status, growth, hematology and other physiological parameters examined in this study (Table 1). The strain did not cause infection and did not translocate (or cause microbial translocation) from the original colonization site (gut) after feeding for 4 weeks (Table 1). Therefore, the oral LD_{50} (the dose predicted to cause 50% mortality) for *L. fermentum* CRL 722 would be greater than 20 g/kg/day, i.e., 1.4 kg/day dry bacteria for a 70 kg person assuming that 1 g dry bacterial preparation contains 10^{11} bacterial cells [13]. The acceptable daily intake (ADI) for this same person would be 14 g dry bacteria per day (100-fold of the LD_{50}), which is several hundred times the amount of LAB normally recommended for human consumption [16]. From this it can be implied that *L. fermentum* CRL 722 is non-pathogenic and safe for human consumption.

In conclusion, to our knowledge, this is the first study which has shown that a microorganism is capable of conferring α-Gal activity in the small intestine of a monogastric animal (a rodent in this particular case). This activity, even though short-lived, could significantly reduce the physiological effects associated with the consumption of non-digestible sugars found in legumes such as soy. *L. fermentum* CRL 722 is a very promising LAB strain since it can degrade α-GOS from soy-products, can confer α-Gal activity in the upper digestive tract and does not seem to cause any adverse effect when given to rodents during long periods of time. Currently, we are studying the hydrogen reducing properties of *L. fermentum* CRL 722 in animals fed α-GOS rich diets. The genetic and biochemical analysis of α-Gal from *L. fermentum* is also currently underway.

Federation of European Laboratory Science Associations (FELASA). All experiments comply with the current laws of Argentina.

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