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SYNTHESIS OF α-L-FUCOSIDASE IN DIFFERENT STRAINS OF LACTIC ACID BACTERIA

SÍNTESIS DE α-L-FUCOSIDASA EN DIFERENTES CEPAS DE BACTERIAS ÁCIDO LÁCTICAS

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Abstract

The ability of six lactic acid bacteria to produce α-L-fucosidase is reported here for the first time, opening a wide field of investigation into the metabolism and assimilation of human milk oligosaccharides by lactic acid bacteria. *Lactobacillus casei* IMAU60214, *Lactobacillus casei* Shirota, *Lactobacillus rhamnosus* GG, *Lactobacillus rhamnosus* KLDS, *Lactobacillus helveticus* IMAU70129 and *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB-2772 were all able to produce α-L-fucosidases. Growth kinetics and carbohydrate consumption measurements indicated that the six strains were able to metabolise D-glucose and D-galactose as a carbon source; surprisingly, they did not assimilate L-fucose. However, α-L-fucosidase was a cell-associated enzyme and produced constitutively in different carbon sources. The highest cell-associated α-L-fucosidase activity was observed in *L. rhamnosus* GG (0.16 U mg^{-1}).

Keywords: α-L-fucosidase, lactobacilli, fucose, human milk oligosaccharides.

Resumen

Este es el primer estudio que reporta las síntesis de α-L-fucosidasa en seis bacterias ácido lácticas, lo que abre un amplio campo de investigación sobre el metabolismo y la asimilación de los oligosacáridos de la leche humanos por parte de estos microorganismos. *Lactobacillus casei* IMAU60214, *Lactobacillus casei* Shirota, *Lactobacillus rhamnosus* GG, *Lactobacillus rhamnosus* KLDS, *Lactobacillus helveticus* IMAU70129 y *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB-2772 fueron capaces de producir α-L-fucosidasa. Las cinéticas de crecimiento y el consumo de carbohidratos indican que las seis cepas son capaces de metabolizar D-glucosa y D-galactosa como fuente de carbono; pero no asimilaron L-fucosa. Por otro lado, las α-L-fucosidasas sintetizadas son enzimas asociadas a la célula y se produjeron de manera constitutiva en diferentes fuentes de carbono. La mayor actividad enzimática se observó en *L. rhamnosus* GG (0.16 U mg^{-1}).

Palabras clave: α-L-fucosidasa, lactobacilos, fucosa, oligosacáridos de leche humana.

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1 Introduction

During the last two decades, human milk oligosaccharides (HMOs) have been the subject of intensive research due to their prebiotic properties and other roles beneficial to human health. HMOs contain lactose at their reducing end. Galactose in lactose can be sialylated in (2-3) and/or (2-6) linkages to from 3'sialyl-lactose and 6'sialyl-lactose respectively. Lactose can also be fucosylated in α (1-2) or (1-3) linkages to form 2'fucosyl-lactose and 3'fucosyl-lactose. Complex HMOs are formed by adding 1 to 15 lactosamine molecules to lactose (Bode, 2009). The most abundant HMOs are neutral and contain fucose. Interestingly, fucose is introduced to the so called fucosylated oligosaccharides (FUCOs) through α (1-2) or α (1-3) linkages, and their composition depends on the mother and her lactation stage (Chaturvedi *et al*., 2001).

In particular, FUCOs have been shown to inhibit adhesion of pathogenic microorganisms, such as *Campylobacter jejuni*, *Vibrio cholera*, some strains of *Salmonella* and *Shigella*, and the thermostable toxin of *Escherichia coli*. In a general context, these HMOs are natural prebiotics that promote the growth of probiotics and stimulate the innate immune system (Bode, 2009; Newburg, 2009; Shoaf-Sweeney *et al*., 2009).

The ability of some intestinal bacteria to metabolise HMOs depends on the expression of different carbohydrases, such as α-fucosidase, α-sialidase, β-galactosidase, α-N-acetylgalactosaminidase, as reported by Pokusaeva *et al*. (2011). Studies in bifidobacteria have identified α (1-2)-L-fucosidase, α (1-3/4)-L-fucosidase and endo-α-N-acetylgalactosaminidase respectively; these enzymes are involved in the degradation of mucin and the consumption of HMOs (Ashida *et al*., 2009; Sela, 2011). In contrast, the production of α-L-fucosidase in lactobacilli has received little attention. The genomic analysis conducted by Morita *et al*. (2009) suggested that *L. rhamnosus* ATCC 53103 encodes putative α-L-fucosidase classified in the Carbohydrate-Active Enzymes Database (CAZY). Rodríguez-Díaz *et al*. (2011) reported three putative α-L-fucosidase (AlfA, AlfB and AlfC) encoded in the *Lactobacillus casei* BL23 genome; these α-L-fucosidases are capable of hydrolysing natural fucosyl-oligosaccharides *in vitro*. In 2012, the same authors reported that *Lactobacillus casei* BL23 is able to grow on fucosyl-α (1-3)-N-acetylglucosamine as carbon source; however, the L-fucose moiety accumulates during growth, indicating that only the N-acetyl-glucosamine moiety is metabolised (Rodríguez-Díaz *et al*., 2012).

Therefore, this work aimed to study the availability of α-L-fucosidase in lactic acid bacteria (LAB) cultures growing in different carbon sources to understand the metabolism and assimilation of HMOs.

2 Materials and methods

2.1 Microorganisms

Six LAB strains were used in this study: *Lactobacilus casei* IMAU60214, *Lactobacillus casei* Shirota, *Lactobacillus rhamnosus* GG, *Lactobacillus rhamnosus* KLDS, *Lactobacillus helveticus* IMAU70129, previously isolated from commercial products by Cruz-Guerrero *et al*. (2014), and *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB-2772 (National Collection of Food Bacteria from United Kingdom).

2.2 Growth conditions

Microorganisms were cultivated in the corresponding culture medium at 37 °C for 24 h, in a New Brunswick incubator (New Brunswick Scientific, Edison NJ, USA). The culture medium contained 5 g L^{-1} yeast extract (B. D. Bioxon, Mexico City, Mexico), 10 g L^{-1} casein peptone (B. D. Bioxon, Mexico City, Mexico) and 10 g L^{-1} of one of the four carbon sources studied: D-glucose, D-galactose, L-fucose or p-nitrophenyl α-L-fucopyranoside (pNP-F) (Sigma-Aldrich, St Louis, MO, USA.). A culture medium without a carbon source was used as a control medium (CM). Growth was measured following the increase in absorbance at 600 nm every 2 h in a spectrophotometer (Shimadzu UV-160A, Tokyo, Japan).

Fermentation broths were centrifuged at 3110 g (Beckman J2-MI, Beckman Instruments, Palo Alto CA, USA) for 20 min at 4 °C. Both, the supernatant and cellular bottom resuspended in phosphate buffer (100 mM, pH 7) were assayed for α-L-fucosidase activity.

2.3 Enzyme activity assay

Microorganisms were cultivated in the corresponding culture medium at 37 °C for extracellular α-L-fucosidase activity was determined in a reaction mixture containing 800 μL of 3.5 mM pNP-F solution in 100 mM phosphate buffer, pH 7, and 200 μL of the cell culture supernatant. The mixture was incubated

for 10 min at 37 °C, and the rate of formation of free p-nitrophenol (pNP) which was recorded directly in a spectrophotometric cell (Shimadzu UV-160A, Tokyo, Japan) at 410 nm and quantified through a standard curve. Cell-associated α-L-fucosidase activity was determined in a reaction mixture containing 800 μL of pNP-F solution and 200 μL of the resuspended cell bottom, and the mixture was incubated for 2 h at 37 °C; 200 μL samples were removed every 30 min and centrifuged at 3110 g for 20 min at 4 °C to eliminate cells. The amount of pNP released was quantified using Elx 808IU microplate reader (BioTeck Instruments, Inc. USA).

One α-L-fucosidase activity unit was defined as the amount of enzyme hydrolyzing one nmol pNP-F per minute at pH 7 and 37 ºC. Enzymatic activity is expressed as U mL^{-1}.

Specific α-L-fucosidase activity was defined as enzymatic activity (U) per mg of biomass, which was measured using a standard curve of dry weight cells.

2.4 Carbohydrate analysis

The consumption of carbohydrates was determined in the fermentation supernatant by HPLC (LabAlliance, State College PA, USA) using a Rezex RHM 7.8 x 300 mm column (Phenomenex, Torrance, CA, USA) for monosaccharides and a light-scattering detector (Polymer Laboratories, Amherst, MA, USA). Samples were eluted with deionized water at a flow rate of 0.3 mL min^{-1}. The column temperature was maintained at 75 °C, and the detector temperature was maintained at 110 °C. The concentrations of each carbohydrate were determined from the corresponding standard curve.

2.5 Statistical analysis

All experiments were carried out at least in triplicates, and results are expressed as the mean with standard deviations. To assess significant differences in the growth of microorganisms between the different carbon sources, analysis of variance (ANOVA) and Tukey's tests were performed using the NCSS statistical software using $\alpha < 0.05$ as the threshold of statistical significance.

3 Results and discussion

3.1 Lactic acid bacteria growth in different carbon sources

The progression of cell growth of the six LAB strains in different carbon sources is shown in Figure 1, where it may be observed that all LAB cultures reached their highest growth when glucose was used as the carbon source in the culture medium, followed by growth with galactose in the medium (α=0.05). On the other hand, the culture media containing pNP-F or L-fucose did not allow any significant growth when compared to that observed in the CM (α=0.05); the limited growth observed in pNP-F or L-fucose was due to the alternative substrates present in the CM, but not to the two carbohydrates added.

Hickey *et al*. (1986) demonstrated that *L. bulgaricus* is only able to metabolize galactose in a culture medium containing low concentrations of the carbon source, which explains the low growth of *L. delbrueckii* subsp. *bulgaricus* NCFB-2772 observed here (Fig. 1F). *L. bulgaricus* transports galactose by permeases and uses the Leloir pathway to metabolize galactose (Zourari *et al*., 1992). *L. casei* and *L. rhamnosus* have also been reported to metabolize galactose; these bacteria can use both the D-tagatose-6-phosphate pathway and the Leloir pathway (Tsai and Lin, 2006), which explains the observed growth in the other four strains (Fig. 1A, 1B, 1C, and 1D).

Glucose and galactose were completely consumed by the six LAB strains after 24 h of fermentation, whereas L-fucose was not consumed by any of the six studied strains (Table 1). In contrast, in the supernatants of media containing pNP-F, L-fucose was observed for all strains (Table 1), suggesting that none of the LAB metabolized L-fucose. However, because pNP-F was hydrolyzed during the incubation experiments, the presence of α-L-fucosidase activity can be inferred. These results are in agreement with those reported by Rodríguez-Díaz *et al*. (2012), who found that *L. casei* BL23 excretes L-fucose as a product of the metabolism of fucosyl-α-1,3-N-acetyl-glucosamine used as carbon source, consuming only the N-acetyl-glucosamine moiety.

The ability of some microorganisms to metabolize a specific set of carbohydrates is an evolutionary survival advantage, suggesting that HMOs could be a very selective prebiotic for LAB. Ward *et al*. (2007) studied the catabolism and fermentation of HMOs by strains of bifidobacteria. They reported that all studied strains were able to individually ferment the two monosaccharides present in HMOs: glucose and galactose. The ability of *Bifidobacterium longum* subsp. *infantis* and *Bifidobacterium breve* to ferment glucosamine, L-fucose and sialic, is an advantage over other colonic strains. Schwab *et al*. (2011) studied the ability of *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus reuteri*, to digest HMOs components; reported that all

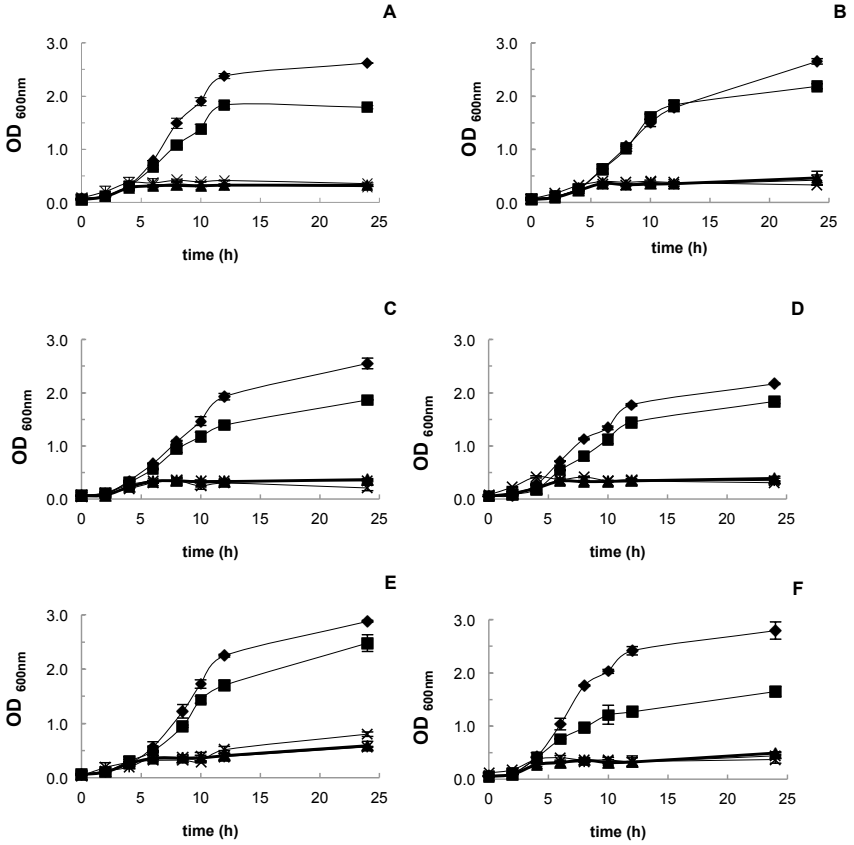

Fig. 1. Growth of *L. casei* IMAU 60214 (A), *L. casei* Shirota (B), *L. rhamnosus* GG (C), *L. rhamnosus* KLDS (D), *L. helveticus* IMAU70129 (E), *L. delbruekii* subsp. *bulgaricus* (F) in media containing D-glucose (♦), D-galactose (■), L-fucose (▲), pNP-F (×), CM (∗). Data represent average values from three independent experiments.

strains grew on lactose and glucose and fucose utilization was absent.

3.2 Determining extracellular α-L-fucosidase activity

Extracellular α-L-fucosidase activity was determined in the supernatants obtained from the fermentations of the six strains in all of the studied carbon sources; interestingly, only the supernatants of media containing pNP-F presented α-L-fucosidase activity (Fig. 2). In the supernatants of the six strains, α-L-fucosidase activity were observed, and *L. casei* Shirota exhibited the highest activity ($\alpha = 0.05$). Nevertheless, later studies showed that the α-L-fucosidase was liberated as part of the natural process of cell death lysis; indicating that the enzymatic activity was

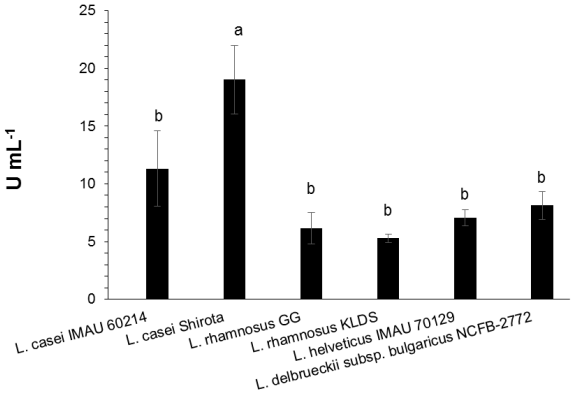

Fig. 2. Extracellular α-L-fucosidase activity in media containing pNP-F with different lactic acid bacteria. The cell culture supernatant was used for extracellular activity. The reaction was carried out for 120 min at 37 °C, employing 3.5 mM pNP-F solution in 100 mM phosphate buffer, pH 7. Different letters indicate significant differences.

Table 1. Consumption of glucose, galactose, fucose (all at 10 g L^{-1}) and pNP-F (5 g L^{-1}) during growth at 37 °C for 24 h

BAL	Glucose (g L^{-1})	Galactose (g L^{-1})	Fucose (g L^{-1})	pNP-F* (g L^{-1})
L.casei IMAU 60214	10 ±0.01	10 ±0.02	0	0.22 ±0.01
L. casei Shirota	10 ±0.03	10 ±0.05	0	0.15 ±0.01
L. rhamnosus GG	10 ±0.03	10 ±0.01	0	0.17 ±0.02
L. rhamnosus KLDS	10 ±0.01	10 ±0.01	0	0
L. helveticus IMAU70129	10 ±0.02	10 ±0.03	0	0.41 ±0.01
L. delbrueckii subsp. *bulgaricus* NCFB-2772	10 ±0.01	10 ±0.04	0	NC

(*) fucose released during fermentation; NC: unquantified

cell-associated. Notably, no previous studies have reported the extracellular α-L-fucosidases activity of lactobacilli.

3.3 Determining cell-associated α-L-fucosidase activity

The cell-associated α-L-fucosidase activity of the six LAB grown in media with glucose or galactose as carbon sources is shown in Figure 3. All LAB produced cell-associated α-L-fucosidase. However, when LAB were grown in a glucose medium (Fig. 3A), no significant differences in the amount of α-L-fucosidase activity for the six LAB (α=0.05) were observed, and *L. casei* Shirota exhibited the lowest enzymatic activity. LAB grown in medium containing galactose (Fig. 3B) did not exhibit significant differences in α-L-fucosidase activity compared with the α-L-fucosidase activities of the six strains (α=0.05). However, compared with the α-L-fucosidase activities for each bacterium in both culture media, *L. casei* Shirota and *L. rhamnosus* KLDS exhibited the highest activity when grown in galactose as a carbon source. Because α-L-fucosidase was produced in both media (glucose and galactose), the enzyme was produced constitutively.

These results agree with those reported by Rodríguez-Díaz *et al*. (2011), who observed that the α-L-fucosidase of *L. casei* BL23 was intracellular because it lacks an N-terminal sequence that allows the enzyme to be secreted. Thus far, only three α-L-fucosidases (AlfA, AlfB y AlfC) encoded in the genome of *L. casei* BL23 have been cloned and characterized. These enzymes have different abilities to hydrolyse fucosyl-HMOs: AlfA only hydrolysed 6-fucosil-glucosamine, whereas AlfB hydrolysed antigen H disaccharide (Fucα1-2Gal), H antigen type II trisaccharide (Fucα1-2Galβ1-4GlcNAc), 2'fucosyl-lactose, 3'fucosyl-glucosamine and 4'fucosyl-glucosamine. AlfC hydrolysed 6'fucosyl-glucosamine, 3'fucosyl-glucosamine and 4'fucosyl-glucosamine.

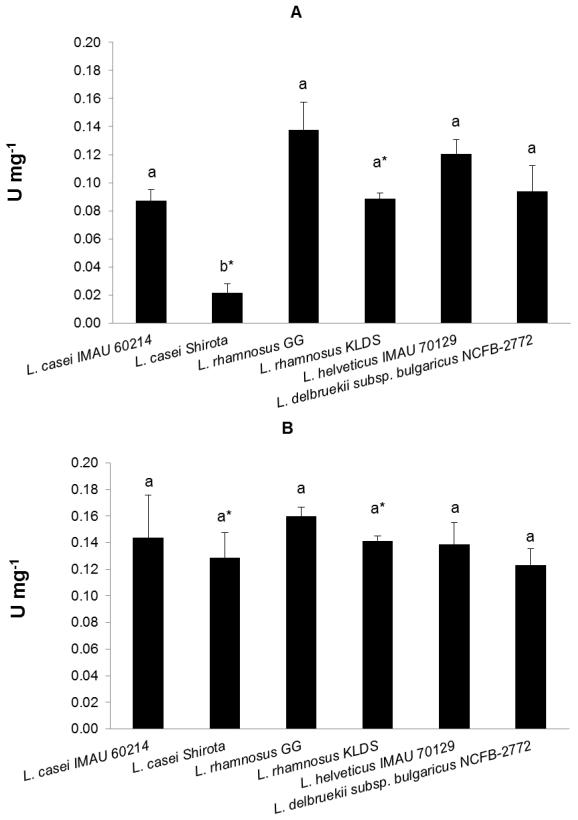

Fig. 3. Cell-associated α-L-fucosidase activity produced by *Lactobacillus* using glucose (A) and galactose (B) as a carbon source. Cellular bottoms of the different fermentations were resuspended in phosphate buffer for cell-associated activity. The reaction was carried out for 120 min at 37 °C, employing 3.5 mM pNP-F solution. Different letters indicate significant differences within the same carbon source. (*) Significant difference between specific activities produced in different carbon sources.

The results obtained here and in the previous reports of Rodríguez-Díaz *et al*. (2011, 2012) suggest that LAB produce α-L-fucosidases to hydrolyse HMOs containing fucose in order to use only the non-fucose moiety. This action confers advantages to LAB over other microorganisms in the gut, making FUCOs highly selective prebiotics. This same advantage occurs in bifidobacteria (Sela, 2011).

The genus Lactobacillus is an important part of the healthy microbiota installed in the human gastrointestinal tract. A healthy gut in infants is largely due to the presence of these microorganisms. Human milk consumption during lactation promotes the proliferation of *lactobacilli* in the gastrointestinal tract because it provides nutrients as prebiotic HMOs. The metabolism of HMOs indicates that colonic bacteria must be able to synthesize enzymes such as fucosidases that assist in the assimilation of these complex sugars.

In summary, the six Lactobacillus strains studied produce α-L-fucosidase constitutively as a cell-associated enzyme, and all strains grew on lactose and glucose whereas that fucose utilization was absent.

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