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**Review**

# Towards probiotic lactic acid bacteria strains to remove raffinose-type sugars present in soy-derived products

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**Abstract** – Lactic acid bacteria (LAB), that are widely used in food fermentations and that may survive in the gastrointestinal tract of consumers, are potent candidates as vehicles for delivery of biologically active proteins. To evaluate this potential of LAB, we focus on the degradation of α-galactosides of soy. These sugars, which are not degraded by the pancreatic enzymes of humans, are metabolized by gas-producing bacteria of the large intestine, thus creating intestinal disorders such as flatulence in sensitive individuals. In this study, we aim to demonstrate that α-galactosidase (α-Gal) expressing LAB can efficiently degrade α-galactosides: (i) in soy milk fermentation when they are used as starters; and (ii) in the small intestine when they are administered orally to animals as probiotic preparations. To reach these goals, we characterized the α-Gal structural gene of *Lactobacillus plantarum* ATCC8014. This gene, as well as the structural gene for the α-Gal from guar were expressed in *Lactococcus lactis* so that the enzymes are located in various bacterial compartments. This allows us to take into account the environmental constraints which are specific to each application. Active α-Gals could be produced in *L. lactis* and resulting strains will be evaluated both in soy milk fermentations and in oral administrations in animals. The expected outcomes of this study are discussed.

**Lactic acid bacteria / probiotic / soy product / α-galactoside digestion**

**Résumé – Vers des bactéries lactiques probiotiques pour l'élimination des sucres de type raffinose dans les produits dérivés du soja.** Les bactéries lactiques, déjà largement utilisées comme levain dans les fermentations alimentaires et capables de survivre dans le tube digestif de l'hôte, sont des candidates potentielles comme véhicule pour délivrer des protéines biologiquement actives. Pour évaluer ce potentiel, nous avons choisi comme modèle la dégradation des α-galactosides du soja. Ces sucres qui ne sont pas dégradés par les enzymes pancréatiques chez l'homme, sont métabolisés par la flore du gros intestin et les gaz issus de ces fermentations peuvent engendrer des désordres intestinaux (flatulence, ballonnement, distension…). Dans cette étude, nous visons à démontrer que des bactéries lactiques productrices d'α-galactosidase (α-Gal) sont efficaces dans la dégradation des α-galactosides : (i) dans des fermentations de lait de soja lorsqu'elles sont utilisées comme levain ; et (ii) dans l'intestin grêle lorsqu'elles sont administrées oralement à des animaux comme préparations probiotiques. Pour atteindre ces objectifs, nous avons caractérisé le gène de structure d'une α-Gal de *Lactobacillus plantarum* ATCC8014. Ce gène et

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celui de l'α-Gal de guar ont été exprimés chez *Lactococcus lactis* de façon à ce que les enzymes soient localisées dans différents compartiments cellulaires chez la bactérie. Ceci nous permet de faire face aux contraintes environnementales spécifiques à chaque application. Des α-Gal actives ont pu être produites chez *L. lactis* et les souches obtenues seront testées dans des fermentations de lait de soja et en administration orale chez des animaux. Les possibles retombées de cette étude sont discutées.

**Bactérie lactique / probiotique / soja / digestion des α-galactosides**

## 1. INTRODUCTION

Consumption of soy-derived food products is increasing steadily worldwide due to the distrust of consumers towards animal-derived food products and to the healthy status of soy. Indeed, the nutritional value of this pulse is high. Its major benefit is that it contains high quality proteins whose amino acid balance is comparable with that found in animal proteins. Also, soy contains polyunsaturated fatty acids which lower cholesterol levels in consumers and it is rich in isoflavones which are recognized for their potential health benefits (prevention of cancers, cardio-vascular diseases and osteoporosis) [3, 6, 8]. Soy milk is an interesting substitute for mammal milk since it is rich in calcium, iron, zinc and magnesium and is devoid of lactose, a sugar to which some individuals are intolerant. On the economic side, soy provides low cost proteins compared with animal proteins. However, soy-derived products also have drawbacks, mainly because of their sugar content. Indeed, 40% of the sugars present in soy are α-galactosides (stachyose and raffinose) that cannot be digested by humans due to the lack of pancreatic α-galactosidase. Therefore, α-galactosides pass along the small intestine without being degraded or absorbed and are then taken up by gas-producing bacteria such as *Clostridium* sp. in the large intestine, causing flatulence and intestinal disorders in symptomatic individuals [11].

To circumvent this nutritional drawback of soy, lactic acid bacteria (LAB) are excellent potential candidates: (i) they are microorganisms with great ability to convert sugars into lactic acid; (ii) they are present at high levels ($10^9$–$10^{10}$ bacteria per gram) in various fermented products where they are used as starters and this allows them to efficiently exert activities with a strong input into the properties of the final product; and (iii) some LAB that are able to survive in the gastrointestinal tract (GIT) of man are likely to exhibit various activities there [14]. In the present project, we are pursuing two goals. The first is to show the ability of dedicated LAB to degrade the unwanted sugars raffinose and stachyose during the fermentation of soy milk. This goes towards the development of starter preparations for soy fermentation. The other aim is to demonstrate that LAB exhibiting the relevant enzymatic activity could be used as vehicles to deliver this activity in the small intestine of mammals. This goes towards the design of probiotic preparations for improved digestion of antinutritional compounds.

In this project, the chosen approach is to use the best characterized LAB, *Lactococcus lactis*, as a model and to construct relevant genetically modified organisms to demonstrate the desired phenomena. We present here the rationale we followed and the work we performed in the construction of relevant *L. lactis* strains that will be further used in soy milk fermentations and in animal trials.

## 2. WHAT ARE THE α-GALACTOSIDES IN SOY AND HOW TO DEGRADE THEM?

Soybeans contain 1.6% raffinose and 3.3% stachyose (w/w) and soy milk about

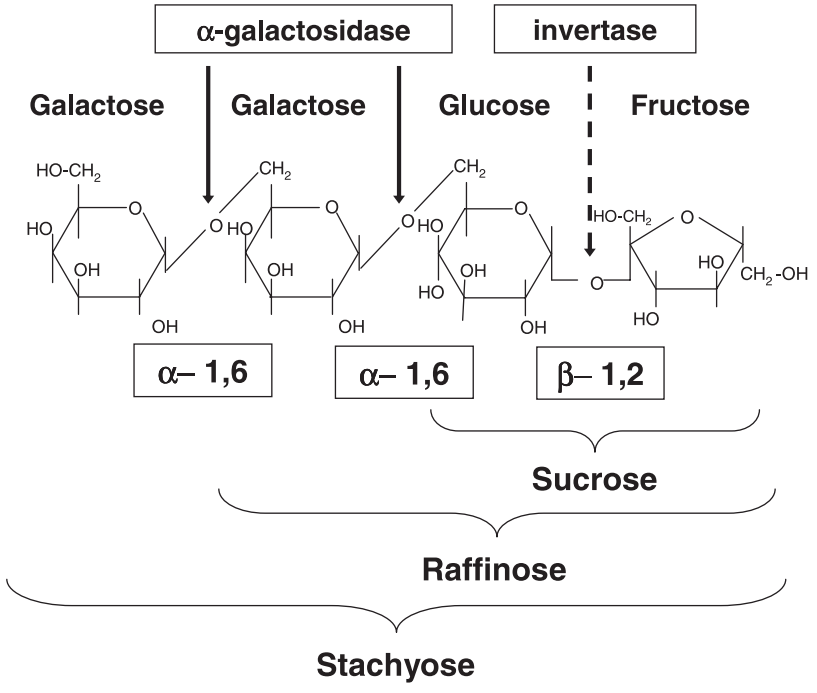


**Figure 1.** The structure of α-galactosides raffinose and stachyose and the enzymes that catalyze their hydrolysis.

3 g·L$^{-1}$ and 8 g·L$^{-1}$, respectively. Raffinose and stachyose both contain one sucrose moiety linked by α-1,6 bonds to one (raffinose) or two (stachyose) units of galactose (Fig. 1). The enzyme able to catalyze the hydrolysis of α-1,6 linkages is α-galactosidase [EC 3.2.1.22] (α-Gal). Although man and monogastric animals are deficient in pancreatic α-Gal, this enzyme can be found in various living organisms including plants, fungi and bacteria such as some LAB (*Lactococcus raffinolactis*, *Lactobacillus* sp., and *Bifidobacterium* sp.). Instead of using these native LAB, our strategy was to select some relevant α-Gals for overexpression in *Lactococcus lactis* in order to increase their potential in vivo activity. The wide distribution in nature of α-Gals of different properties allowed us to choose those that appeared most appropriate to reach our goals within this project.

## 3. THE CONSTRAINTS OF THE PROJECT AND THE RETAINED SOLUTIONS

According to the type of application that we considered, different constraints were foreseen.

### 3.1. The pH constraint

In soy fermentation, initial pH is close to neutrality but it decreases during the fermentation due to the conversion of sugars into organic acids. In the small intestine, the pH is also variable from acidic pH at the exit of the stomach to neutral pH in the ileum. To address this constraint, we chose to express in *L. lactis* two α-Gals that exhibited different ranges of optimal activity pH. First, the α-Gal from *Cyamopsis tetragonoloba* (guar) was retained because

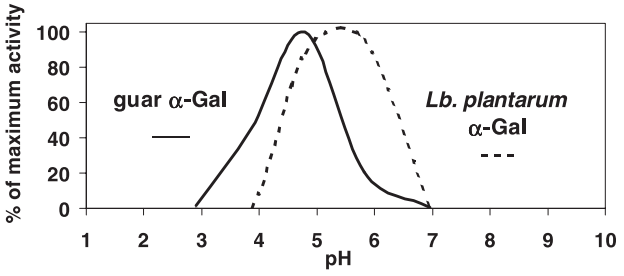


**Figure 2.** The relative activity of guar α-Gal and MelA as a function of pH.

it is most active between pH 4.5 and 5.2 (Fig. 2). Its structural gene has been characterized and it encodes a protein of 40 kg·mol$^{-1}$ that is natively secreted and is active as a monomer [9]. The second retained α-Gal was that of *Lactobacillus plantarum* ATCC8014, a widely studied LAB that may be a commensal of the GIT [5]. This α-Gal is most active between pH 5 and 6.2 (Fig. 2). Since no structural gene for lactobacilli α-Gals had yet been characterized, we first undertook the genetic characterization of this α-Gal.

### 3.2. The gene cluster for *Lb. plantarum* ATCC8014 α-Gal

The α-Gal encoding *melA* gene of *Lb. plantarum* ATCC8014 was probed using PCR with degenerate primers and the gene cluster was characterized using uneven and inverse PCR techniques [10]. This allowed us to unravel the structure of the galactoside metabolism gene cluster shown in Figure 3A. The *melA* gene encodes a 738-amino-acid protein (MelA) with a deduced protein weight of 84 kg·mol$^{-1}$. The *melA* gene is surrounded by genes involved in α- and β-galactoside utilization. The *galM* gene encodes a putative mutarotase, *rafP*, a putative raffinose transporter showing high homology with LacS, the bifunctional lactose and α-galactoside transporter from *Streptococcus thermophilus*, and *lacL* and *lacM*, the putative two subunits of the β-galactosidase. In silico analysis of the *melA* gene product did not show the presence of sorting signals, suggesting that this α-Gal occurs as a soluble enzyme in the cytoplasm of *Lb. plantarum*. This was confirmed by fractionated extraction experiments. Molecular weight assessment of active MelA showed that it occurs as oligomers and that the monomers are inactive.

Northern hybridizations revealed that *melA* is expressed in *Lb. plantarum* ATCC8014 and that it is transcribed from its own promoter. Regulation occurred at the transcriptional level, i.e. *melA* is induced by the α-galactoside melibiose. The *melA* gene could be expressed in *E. coli* yielding active MelA (Fig. 3B) .

### 3.3. Protection of α-Gals against denaturation vs. substrate accessibility

There are two additional constraints to take into account to reach the goals of this project. One is the protection of α-Gals against denaturating agents such as proteinases present in soy fermentations and in the GIT, acids, and/or salts present in intestinal fluids. To address this constraint, a cytoplasmic localization of α-Gals in LAB should best protect the enzymes from denaturation. Another constraint is the substrate accessibility that would be optimal if α-Gals are excreted outside the bacteria. To address these opposite requirements, we undertook to express the chosen α-Gals in expression and export vectors so that α-Gals can be either cytoplasmic, cell wall-anchored or secreted in *L. lactis*. Each of these localizations have their own advantages and drawbacks. The cytoplasmic localization would protect the enzyme but it requires the presence of an efficient α-galactoside

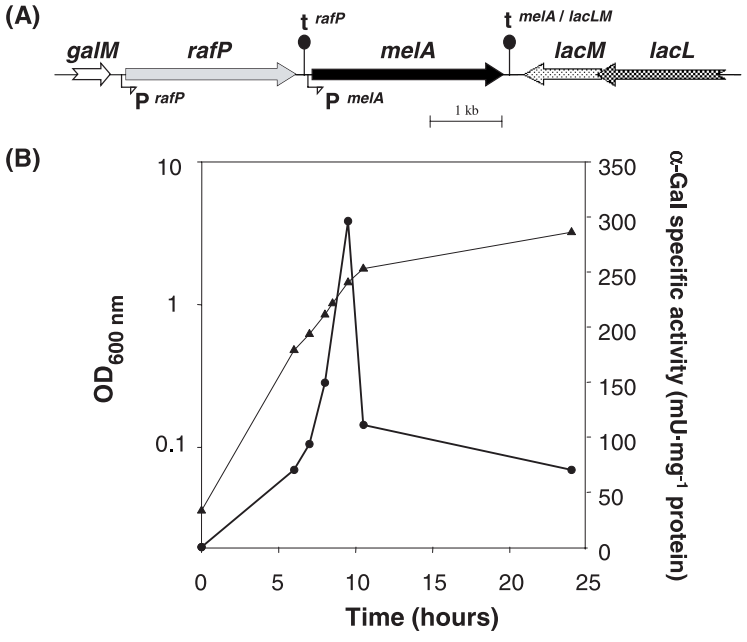


**Figure 3. (A)** Structure of the galactoside gene cluster in *Lb. plantarum* ATCC8014. Putative promoters and terminators are indicated [10]; and **(B)** Growth of *melA* expressing *E. coli* (triangles) and α-Gal activity (dots) in cell extracts.

transporter. The secretion of α-Gals would abolish such a requirement, but it would greatly expose the enzyme to denaturating agents. Halfway between the two previous localizations, cell wall anchoring of α-Gals could favor substrate-enzyme interactions while the cell wall microenvironment would have a partial protective role on the enzyme stability.

Both the guar α-Gal structural gene and *melA* were cloned in the three expression and export vectors shown in Figure 4. These vectors contain the strong constitutive P59 promoter or the nisin inducible Pnis promoter of *L. lactis* [1, 13], the signal sequence of Usp45 for membrane translocation of the secreted and cell wall-anchored α-Gals [12], and the cell wall anchor domain of the M6 protein for cell surface display of α-Gals by the sortase machinery [4, 7]. These vectors have already been shown to be efficient at targeting another enzyme, the staphylococcal nuclease, to various localizations in various LAB including lactococci [2].

Both α-Gals could be expressed in *L. lactis* using these vectors and their activities were assessed on agar plates containing the chromogenic substrate X-α-Gal (Fig. 5). To confirm that the expected bacterial localizations of α-Gals are correct, specific antibodies against the two α-Gals studied are being raised and they will be used in Western analyses of fractionated protein extracts.

## 4. EVALUATION OF THE CONSTRUCTED STRAINS AND EXPECTED OUTCOMES OF THE PROJECT

The lactococcal strains constructed above will be tested in soy milk fermentations and

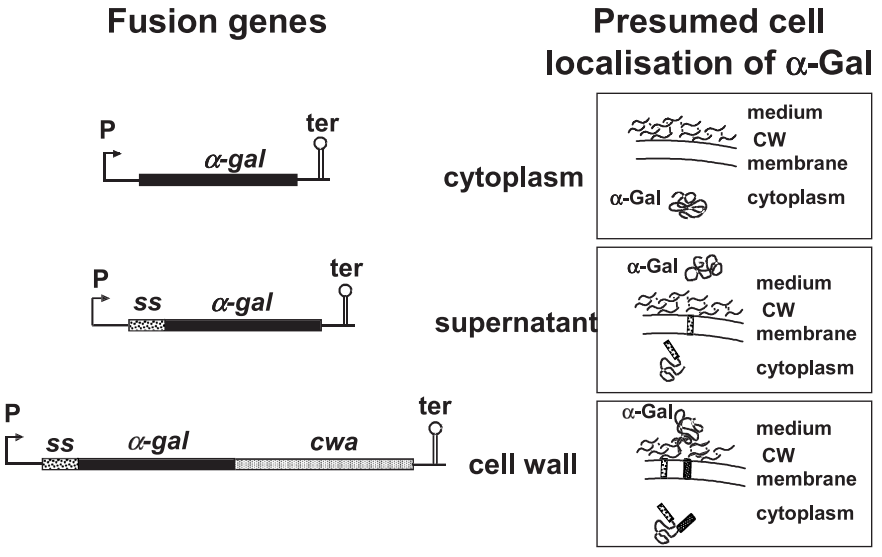


**Figure 4.** The constructs for α-Gal expression and targeting in *L. lactis*. P = promoter; *ss* = signal sequence of Usp45 [12]; *α-gal* = *melA* or guar α-Gal structural gene; *cwa* = cell wall anchor region of the M6 protein [2]; and ter = transcription terminators. The presumed cell localization of α-Gals is shown.

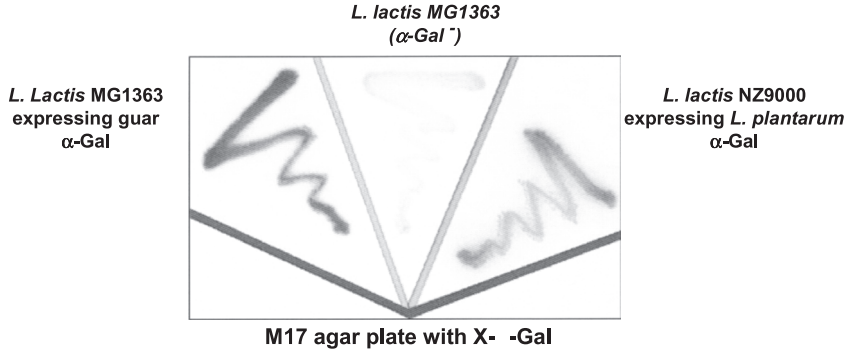


**Figure 5.** Detection of α-Gal activity in strains of *L. lactis* expressing guar (MG1363) and *Lb. plantarum* (NZ9000) α-Gals. Upon cleavage of the chromogenic substrate X-α-Gal by α-Gals, the color of the substrate turns to blue.

in oral administration to an animal model. In soy milk fermentations, the degradation of α-galactosides raffinose and stachyose will be monitored during the fermentation. In in vivo analyses, the strains will be administered to rodents fed with a soy-based diet and α-galactosidase activity as well as residual α-galactosides levels in the small intestine will be measured. In all those trials, different strains with different localizations for α-Gals will be tested comparatively. This should allow us to determine

the required level of α-Gal activity and the preferred α-Gal localization in the bacteria allowing α-galactosides removal from soy fermentations and from the small intestine of consumers. In a second step, we plan to search for natural LAB exhibiting the required phenotypes in terms of level of α-Gal activity and of bacterial localization of α-Gal. If such bacteria cannot be isolated, another solution will be to construct food grade mutants by modifying some properties of native α-Gal-producing LAB (α-Gal expression level and cell localization of α-Gal).

The present study should allow us to reach two main issues. One is to improve the function of LAB in existing food manufacture processes so that the final product suits the digestive capacities of consumers better. During the last decades, much work has been performed to improve the technological output of LAB. We are now on the road to improving their performances with a nutritional aspect. In terms of α-galactosides removal from soy-derived products, various methods (soy germination, bean soaking and water extraction) exist but are all laborious. Obviously, the utilization of LAB strains that perform both fermentation and α-galactosides removal will be economically attractive. Another issue is to demonstrate unambiguously the probiotic activity of LAB. To achieve this, we chose to overexpress an enzymatic activity (α-Gal) which is deficient in man and in *L. lactis*, and to show that this activity is observable in vivo using an animal model.

Both of these issues may contribute to the entry of LAB into the field of nutraceuticals. Other issues pursued by the European Nutra Cells consortium to which this project belongs are vitamin production by LAB, production of the non-metabolized sugars mannose and trehalose by LAB, removal of lactose and galactose from dairy products, and oligosaccharide production using LAB. These achievements should open doors to many applications in the development of both new food products with enhanced nutritional value and probiotic preparations with well-demonstrated in vivo activity. The final use of such bacteria may rely on the still controversial decision on the acceptability of genetically modified organisms (GMOs) in nutrition and nutraceutical preparations. Undoubtedly, consumers will be the major actors in this decision and their position should be greatly influenced by the scientifically proven health benefits that can be gained by consumption of these GMOs.

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