Riboflavin producing lactic acid bacteria as a biotechnological strategy to obtain bio-enriched soymilk

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ABSTRACT

Riboflavin (vitamin B_2) plays an important role in cellular metabolism participating in numerous oxidation–reduction reactions and energy usage. In this work, lactic acid bacteria that can produce vitamin B_2 in soymilk were identified from 179 strains tested that were previously isolated from a wide range of food products. Only 42 strains were able to grow in a commercial riboflavin-free medium after which the concentration of this vitamin was determined by HPLC. Five of these strains were pre-selected for their capacity to produce elevated concentrations of riboflavin. These were then inoculated in soymilk to evaluate their capacity to grow in this food matrix and increase its low riboflavin concentrations. Only the strain *Lactobacillus plantarum* CRL 725 was able to significantly increase the initial concentration of riboflavin in soy milk from 309 ± 9 ng/mL to 700 ± 20 ng/mL after 12 h of incubation at 37 °C. Roseoflavin resistant variants of this strain were obtained and evaluated in soymilk. One of the variant strains increased 6 times (1860 ± 20 ng/mL) the initial riboflavin levels of soy milk. Roseoflavin-resistant strains capable of synthesizing riboflavin in soymilk constitute an interesting and economically feasible biotechnology strategy that could be easily adapted by the food industry to develop novel vitamin-bioenriched functional foods with enhanced consumer appeal.

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1. Introduction

Riboflavin, also known as vitamin B_2, is a water soluble vitamin which belongs to the B group. Vitamin B_2 is the precursor of two essential coenzymes: flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Fischer & Bacher, 2005) which are necessary for the activity of many flavoenzymes involved in different redox reactions (Fraaije & Mattevi, 2000). This vitamin also plays an important role in energy metabolism of the cell, and in recent years riboflavin was shown to improve the efficiency of conventional therapies in different diseases such as *Staphylococcus aureus* infection and cisplatin-induced intestinal epithelial cell apoptosis (Bodiga et al., 2012; Mal et al., 2013).

Humans are not able to synthesize vitamin B_2 and so it must be incorporated it in their diets. According to the Food and Nutrition Board of the U.S.A., the recommended daily intake of riboflavin is 1.3 mg (Food and Drug Administration, 1996) which has to be ingested regularly because humans cannot store this vitamin and excess intakes are eliminated in urine. Riboflavin deficiency is frequent in many parts of the world, including both industrialized (Bamji, Rameshwar Sarma, & Radhaiah, 1979; Boisvert et al., 1993) and developing countries (Bailey et al., 1997). Severe B_2 deficiency can affect mucocutaneous surfaces of the mouth, with the presence of inflammatory processes in lips (cheilitis) and tongue (glossitis) (Basu & Dickerson, 1996). Riboflavin deficiency is also associated with impaired vision, reduced growth rate, increased levels of homocysteine with consequent cardiac risk (Moat, Ashfield-Watt, Powers, Newcombe, & McDowell, 2003), pre-eclampsia (Wacker et al., 2000), and anemia (Lane & Alfrey, 1965). Countries like USA, Canada and Argentina, have developed different food fortification programs with vitamins to offset nutritional deficiency. Currently, riboflavin is produced industrially by chemical synthesis from ribose (Kurth, Paust, & Hanlein, 1996), but in recent years, the use of lactic acid bacteria (LAB) was proposed since these microorganisms are able to synthesize B-group vitamins to obtain fermented bio-enriched food (Capozzi et al., 2011; Laiño, Juarez del Valle, Savoy de Giori, & LeBlanc, 2012; Samaniego-Vaesken, Alonso-Aperte, & Varela-Moreiras, 2012).

Soy products have an excellent status for their high protein content; high unsaturated fatty acid concentrations (Deckelbaum & Torrejon, 2012) and soy proteins contain enough of all the essential amino acids to meet biological requirements when consumed at the recommended level of protein intake. Many beneficial effects of the consumption of soy products have been described such as the reduction of cardiac risk (Zyriax & Windler, 2000), reducing symptoms of menopause (Nagata, Takatsuka, Kawami, & Shimizu, 2001), and a protective effect against

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breast and prostate cancer (Andres, Abraham, Appel, & Lampen, 2011; Sakamoto, Horiguchi, Oguma, & Kayama, 2010).

However, soybeans possess low levels of some B vitamins, such as thiamine and riboflavin, and these are further reduced during the preparation of soy based foods, such as soymilk, tofu, and cheese (Fernando & Murphy, 1990).

The aim of the present work was to select LAB that are able to synthesize riboflavin for the elaboration of soy product naturally bio-enriched in riboflavin, and thus increase the nutritional and economic value of this substrate.

2. Materials and methods

2.1. Microorganism media and growth conditions

One hundred seventy-nine strains of LAB belonging to the culture collection of CERELA (Centro de Referencia para Lactobacilos, CONICET, San Miguel de Tucumán, Argentina) were used in this study. The species involved were *Lactobacillus* (*L.*) *delbrueckii* subsp. *bulgaricus* (40 strains), *Lactobacillus plantarum* (17 strains), *Lactobacillus helveticus* (5 strains), *Lactobacillus paracasei* (12 strains), *Lactobacillus acidophilus* (7 strains), *Lactobacillus casei* (1 strain), *Lactobacillus rhamnosus* (11 strains), *Lactobacillus curvatus* (3 strains), *Lactobacillus collinoides* (1 strain), *Lactobacillus coryniformis* (1 strain), *Lactobacillus mali* (1 strain), *Lactobacillus fructivorans* (1 strain), *Lactobacillus brevis* (3 strains), *Lactobacillus buchnerii* (1 strain), *Lactobacillus hilgardii* (1 strain), *Lactobacillus reuteri* (5 strains), *Lactobacillus sakei* (3 strains), *Lactobacillus fermentum* (12 strains), *Lactococcus* (*Lc.*) *lactis* (13 strains), and *Streptococcus* (*St*) *thermophilus* (41 strains).

Cultures were maintained by subculturing in MRS (lactobacilli, De Man, Rogosa, & Sharpe, 1960) or LAPTg (lactococci and streptococci), containing (w/v) 1.5% peptone, 1% tryptone, 1% yeast extract, 1% glucose and 0.1% Tween 80 using a 2% inoculum and incubated at 37 °C for 16 h.

2.2. Selection of riboflavin-producing strains

After activation in the above-mentioned conditions, these LAB were washed 3 times with saline solution (0.85% m/v NaCl), resuspended in this solution at the original culture volume, and used to inoculate at 2% (v/v) riboflavin-free culture medium (Riboflavin Assay Medium, Difco, Becton, Dickinson, and Co., Sparks, Maryland) that was then incubated without agitation at 37 °C for 18 h. After growth, this washing–resuspension procedure was repeated, and the resulting LAB solution was used to inoculate at 2% (v/v) fresh B_2-free medium. This last step was repeated 4 times with the cultures showing good growth (observed by increased turbidity); strains that did not grow in B_2-free medium were not used in further studies.

To determine total riboflavin concentration, an aliquot of 500 μL of culture was taken and diluted with same volume of 1% (v/v) acetic acid. Intracellular (sample 1) and extracellular (sample 2) riboflavin concentrations were determined by taking an aliquot of 500 μL of the culture and diluted with 1% (v/v) acetic acid. Samples were centrifuged during 5 min at 5000 ×g, and supernatant was transferred (sample 2) to a clean tube. Cells were re-suspended in 500 μL of 1% (v/v) acetic acid (sample 1). All samples (total, intracellular and extracellular) were then boiled at 100 °C during 5 min, centrifuged during 5 min at 5000 ×g and supernatants frozen at −20 °C until for B_2 quantification.

2.3. Quantitative analysis of riboflavin

The determination of riboflavin concentration was carried out with a HPLC equipped with Fluorescence detection (Shimadzu, Fluorescence detector RF-10 AXL, Prominence Line, Japan) and the excitation and emission wavelengths were 445 and 530 nm, respectively. Riboflavin was eluted in isocratic conditions using as mobile phase consisting of 0.05 M sodium acetate/methanol (30:70, v/v). A C18 column Pursuit (XRs 5 C18, 150 mm × 4.6 mm, Varian) was used. Standard curve was realized with different dilutions of commercial riboflavin (Fluka, Biochemika, Germany).

2.4. Preparation of soymilk and fermentation with LAB

Whole soybeans (100 g of commercial soybeans) were washed and hydrated with distilled water during 16 h, and manually peeled. Slurry was obtained by grinding soybeans with 1 volume of distilled water (300 mL) using a kitchen blender (Home Electric TS-696, China). The slurry was then cooked with 3 volumes of water at 80 °C for 15 min, and 2 volumes of water were added before filtering using a double layered cheese-cloth. The obtained soymilk was autoclaved 15 min at 121 °C.

Selected LAB were activated in MRS broth at 37 °C for 16 h. To eliminate extracellular riboflavin, the cells were harvested by centrifugation at 5000 ×g for 5 min, washed twice with 1 volume of sterile saline solution. Cell suspension was inoculated in soymilk at an initial optical density at 600 nm (OD_{600}) of 0.2. After 0, 4, 8, 12 and 24 h of incubation at 37 °C samples were taken and riboflavin concentrations were determined as described above.

2.5. Identification of genes involved in riboflavin biosynthesis

The presence of genes involved in the biosynthesis of B_2 such as *ribA*, *ribB*, *ribC*, *ribG* and *ribH* in *L. plantarum* CRL 725 was determined by PCR. Primers were designed from known sequences of these genes using DNAMAN software (DNAMAN Version 5. 2. 2 Lynnon BioSoft), PRIMER 3 PLUS (www.primer3plus.com) and PRIMER BLAST (www.ncbi.nlm. nih.gov/tools/primer-blast/) online software (Table 1). Sequences were obtained from KEGG and Genome data bases (http://www. genome.jp/Kegg/pathways.html). The amplification conditions were: 94 °C for 5 min, 94 °C for 30 s, 58 °C for 30 s, 72 °C for 2 min, and 72 °C for 5 min, 35 cycles. The products of amplification were sequenced using an ABI 3130 Hitachi Genetic Analyzer (Applied Biosystems — Life Technologies, Buenos Aires, Argentina), and compared with known sequences using BLAST tool of the U.S. National Center for Biotechnology Information (blast.ncbi.nlm.nih.gov).

2.6. Isolation of roseoflavin-resistant strains

Isolation of roseoflavin-resistant *L. plantarum* CRL725 was performed according to Burgess, O'Connell-Motherway, Sybesma, Hugenholtz, and Van Sinderen (2004); Burgess, Smid, Rutten, and Van Sinderen (2006) by exposition of wild-type train to increasing concentrations of roseoflavin (Santa Cruz Biotechnology, Santa Cruz, CA) in Riboflavin Assay Medium (Becton Dickinson and Company Sparks, MD, USA).

Table 1
Primers used in order to amplify the riboflavin biosynthesis genes in *L. plantarum* CRL 725.

	Primer	Sequence
rib A	Forward	CAAGAACACGGGGTTGATAC
	Reverse	CCCTCCGTTAGTGTGAGTTG
rib B	Forward	TGTAGTGGTGATGCCTGAAA
rib C	Forward	TCTTTGATGGTGTGCATCAG
	Reverse	CATTCAACCCGACAAGGTAA
rib G	Forward	GGTCAATTAGATCCCCATCC
	Reverse	TGAAACTGTGACCGTAAGCA
rib H	Forward	AGATTCCACTGACGGTTCAA
	Reverse	TCTAACAAACTGACCGCACA

2.7. Statistical analysis

All values were expressed as means ± standard deviations (SD). Statistical analyses were performed with the software package SigmaPlot for Windows Version 12.0 (Systat Software Inc., Chicago IL, USA) using ANOVA GLM followed by a Tukey's post-hoc test, and differences were considered statistically significant at $p \leq 0.05$.

3. Results and discussion

3.1. Screening of riboflavin producing LAB

In recent times, different biotechnological strategies have been developed in order to obtain more nutritious food without the need of adding chemicals. The use of LAB that are able to produce nutraceuticals and nutrients offers many advantages over chemical synthesis since they first are less expensive, use renewable sources, increase the nutritional value of foods, and show higher acceptance by consumers since they are considered natural foods. Some LAB are able to synthesize B vitamins such as riboflavin (LeBlanc et al., 2013) which is the precursor of FMN and FAD which are involved in the oxidation of succinic acid to fumaric acid in the tricarboxylic acid cycle (Sauer, Cameron, & Bailey, 1998), participates in carbohydrate metabolism, and consequently, in microbial growth.

In this work the selection of riboflavin-producing strains of LAB was performed. From 179 strains, only 42 were able to grow after the fourth passage in a riboflavin free-medium. In these latter strains, intracellular and extracellular concentrations of riboflavin were determined (data not shown). Only five strains (*L. fermentum* CRL 220 and CRL 345, *L. plantarum* CRL 725, *S. thermophilus* CRL 417 and *L. paracasei* subsp. *paracasei* CRL76) were selected due to their high riboflavin producing capabilities in the B₂-free medium, and were then used to inoculate soymilk. The criteria of selection were the highest producers of extracellular riboflavin and optimal growth in riboflavin-free culture medium. Selected strains showed an extracellular concentration of riboflavin above 190 ng/mL reaching high values of 260 ng/mL. The levels of riboflavin produced by these strains were higher than those described by Capozzi et al. (2011) in LAB isolated from sourdough.

3.2. Production of riboflavin in soymilk

With the aim to find new starter cultures, and to produce fermented foods with higher nutritional value, selected strains were inoculated in soymilk. Soy is a nutritionally complete grain because it contains high-quality protein, low concentration of saturated fatty acids and high concentration of unsaturated fatty acids (Scalabrini, Rossi, Spettoli, & Matteuzzi, 1998). During the development of different soy products such as soymilk, many nutrients such as water soluble vitamins are lost. As was described by Garro, de Valdez, Oliver, and de Giori (1998), soymilk is a suitable medium for growth and biochemical activity of some but not all lactic acid bacteria.

The 5 selected strains for the B₂ producing capabilities in a riboflavin-free medium were thus evaluated in order to increase the low concentrations of riboflavin in soymilk. Only the strain *L. plantarum* CRL 725 was able to significantly increase the initial concentration of riboflavin in soy milk from 309 ± 9 ng/mL to 700 ± 20 ng/mL after 12 h of incubation at 37 °C (Fig. 1).

Identification of genes involved in riboflavin biosynthesis was carried only in *L. plantarum* CRL 725 using primers designed based on conserved sequences of previously sequenced LAB. The PCR products were sequenced and in this way it was possible to confirm that *L. plantarum* CRL 725 possess all the genes that encode enzymes involved in different steps in the biosynthesis of riboflavin (Fig. 2).
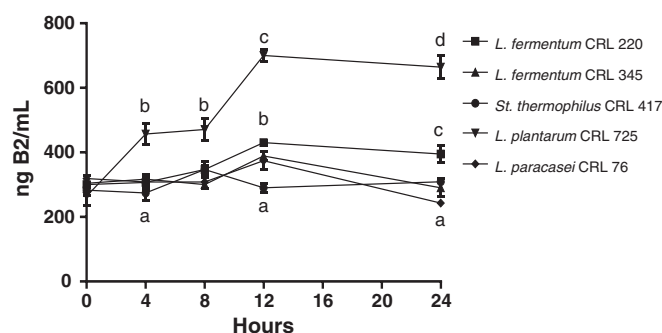

Fig. 1. Production of riboflavin in soymilk by the five pre-selected strains. [a–c]Means with different letters differ significantly ($p < 0.05$).

3.3. Riboflavin production by roseoflavin-resistant L. plantarum CRL 725 in soy milk

Following the procedure described by Burgess et al. (2006) *L. plantarum* CRL 725 was exposed to roseoflavin, a structural analog of riboflavin, which induces mutations in riboflavin-producing strains leading to a novel producer phenotype of the vitamin. Roseoflavin-resistant variants were isolated (Fig. 3). These variants were inoculated in the riboflavin-free medium and incubated for 16 h at 30 °C. The concentration of riboflavin in the culture supernatant was determined. From 7 isolated mutants, only *L. plantarum* CRL 725 variant (G) was able to increase more than 3 times (1100 ± 20 ng B₂/mL) the riboflavin production compared with wild type strain in a culture medium without riboflavin (Fig. 4). To assess if the riboflavin overproducing phenotype in the roseoflavin variant is stable, the strain was grown for 60 consecutive cultures in roseoflavin-free medium. It was observed that the riboflavin overproducing mutant was stable, and no reversion towards the original parental trait was observed (data not shown). Both strains were inoculated in soymilk, and incubated at 37 °C, 24 h. It was observed that the roseoflavin-resistant strain increased the initial concentration of riboflavin in soymilk 6 times (1860 ± 20 ng/mL) while the riboflavin production was 2.8 times higher compared with wild type strain (660 ± 35 ng/mL) (Fig. 5). It was also observed that the storage of soymilk fermented with the roseoflavin-resistant strain during 30 days at 4 °C did not modify the concentrations of riboflavin (data not shown).
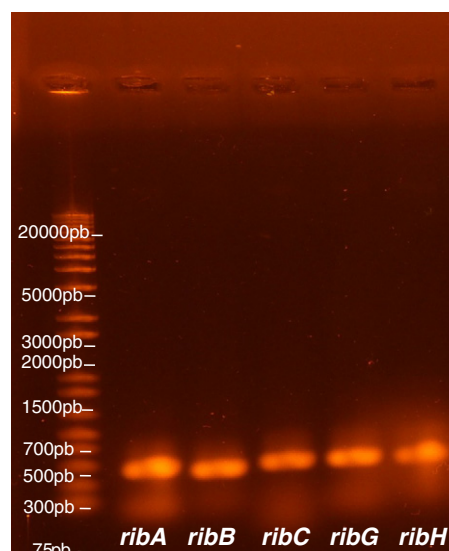

Fig. 2. PCR amplification products of genes involved in riboflavin biosynthesis.
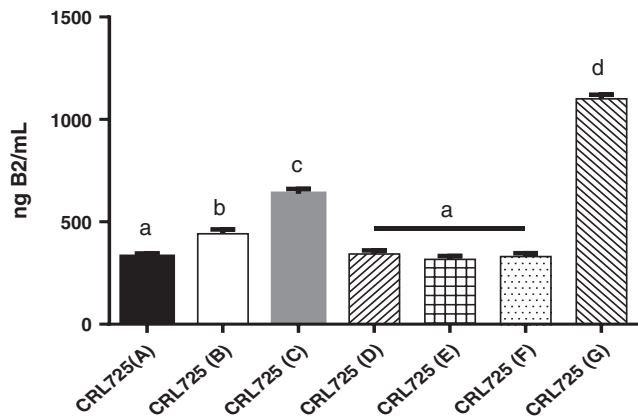
Fig. 3. Riboflavin produced by roseoflavin-resistant *L. plantarum* CRL 725 mutants in the riboflavin-free medium. The various patterns indicate different isolated mutants. [a–d]Means with different letters differ significantly (p < 0.05).

Using this strain, we were able to produce a fermented soymilk with elevated concentrations of riboflavin. A 200 mL portion of the soy milk fermented with the roseoflavin-resistant strain would provide 28% of the Recommended Daily Intake (RDI), whereas the product resulting from the fermentation with the wild-type strains would provide 11% of the RDI, both are significantly superior to the unfermented soymilk which would provide only 4.6% of the RDI. Previously, it was shown that it is possible to increase B_2 production by LAB in soymilk by supplementing the latter with vitamins (Ewe, Wan-Abdullah, & Liong, 2010); however here, we were able to bio-enrich soy milk without the need of added vitamins by using only roseoflavin-resistant LAB.

4. Conclusions

Although yogurts and other fermented milks are very popular because of their demonstrated health-promoting benefits, there is also an increase in consumer demand for non-dairy beverages. Drawbacks for fermented milk derived products are mainly the ongoing trend of vegetarianism, the increasing prevalence of lactose intolerance and the high concentrations of cholesterol in these products in addition to the fact that in many countries the dairy consumption does not form part of conventional diets. Consequently, the current focus is on the manufacture of non-dairy beverages with new ingredients, having high functionality and acceptability. Considering the increased interest in the consumption of soy derived products and of LAB, the design of a
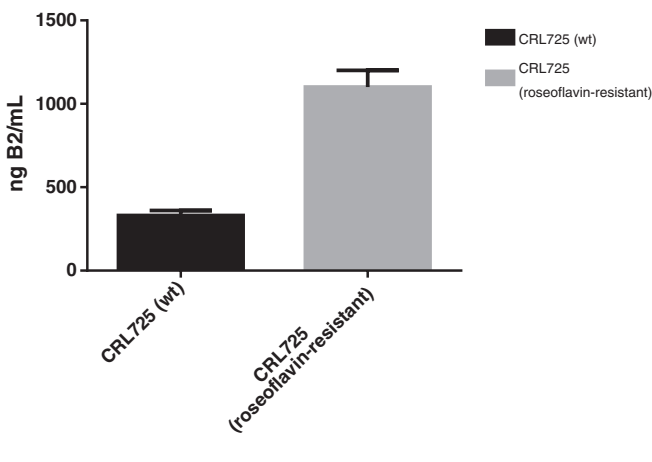

Fig. 4. Riboflavin production by roseoflavin-resistant *L. plantarum* CRL 725 (G) and wild strain in B_2-free culture medium. The line above the bar represents the SD of the mean.
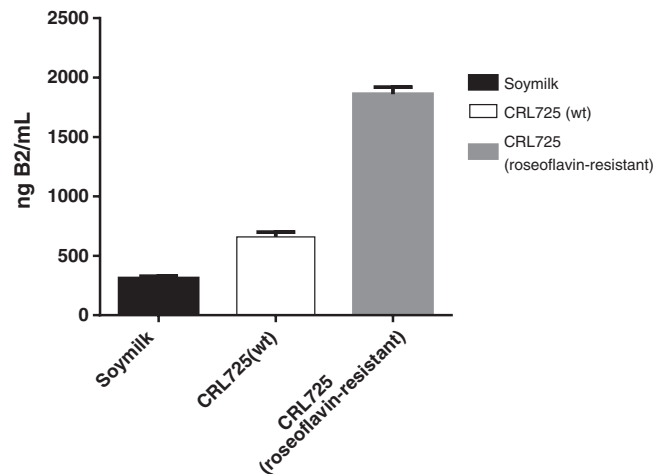

Fig. 5. Riboflavin production by roseoflavin-resistant *L. plantarum* CRL 725 and wild strain in soy milk. The line above the bar represents the SD of the mean.

soybean product that is naturally bio-fortified with riboflavin will contribute to modify the nutritional habits of the population and to diversify local diets. The use of a vegetal food matrix with high protein quality such as hydrosoluble extract of soybeans (soymilk) instead of a milk base is also important from an economic point of view because it is cheaper to produce probiotic beverages using this substrate which could be more affordable to poorer communities where soybean production occurs.

To the best of our knowledge, this is the first to report that enhancement of the riboflavin content in soymilk can occur by the use of roseoflavin-resistant riboflavin producing LAB strains. The variant of *L. plantarum* CRL 725 was able to increase the concentration of riboflavin in soymilk and could be used for the development of new soy products (such as being used as an adjunct culture in soy-based yogurts) specifically designed to possess optimal organoleptic and sensorial qualities in addition to the enhancement of their nutritional value (riboflavin content). These studies are currently being carried out in our laboratory. Such products could be introduced as a part of normal diets in order to prevent or treat riboflavin deficiencies that are still present throughout the world.

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