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RESEARCH PAPER

Factors stimulating riboflavin produced by *Lactobacillus plantarum* CRL 725 grown in a semi-defined medium

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Riboflavin (vitamin B_2) is one of the B-group water-soluble vitamins and is essential for energy metabolism of the cell. The aim of this study was to determine factors that affect riboflavin production by *Lactobacillus* (*L.*) *plantarum* CRL 725 grown in a semi defined medium and evaluate the expression of its *rib* genes. The factors found to enhance riboflavin production in this medium were incubation at 30 °C, and the addition of specific medium constituents, such as casamino acids (10 g L^{-1}), guanosine (0.04 g L^{-1}), and sucrose as carbon source (20 g L^{-1}). In these conditions, higher riboflavin concentrations were directly associated with significant increases in the expression of *ribA*, *ribB*, and *ribC* genes. The culture conditions defined in this work and its application to a roseoflavin resistant mutant of *L. plantarum* allowed for a sixfold increase in riboflavin concentrations in our semi-defined medium which were also significantly higher than those obtained previously using the same strain to ferment soymilk. These conditions should thus be evaluated to increase vitamin production in fermented foods.

KEYWORDS
chemically defined medium, lactic acid bacteria, *rib* gene expression, riboflavin, vitamin biosynthesis

1 | INTRODUCTION

Riboflavin or vitamin B_2 is a water-soluble vitamin that is important for optimal body growth, red blood cell production, and helps release energy from carbohydrates and fatty acids [1]. This molecule is present in the blood as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), both of which act as oxidation–reduction cofactors involved in a wide range of biological reactions [2–4]. Humans are not able to synthesize riboflavin and therefore, it must be incorporated as part of their diet. According to the Food and Nutrition Board of USA, the recommended daily intake of riboflavin is 1.3 mg for an average adult [5] and this vitamin has to be ingested regularly because the body is unable to store it and excess intake is excreted in the urine. Sub-clinical riboflavin deficiency is frequent in both developing and developed countries [6,7]. In order to prevent this problem, many countries such as the USA, Canada, and Argentina have adopted mandatory fortification programs with different vitamins and minerals that include riboflavin in their formulation.

Riboflavin production in industry can be achieved by chemical manufacturing or by biological processes. Driven by costs, waste, and energy considerations, biological processes are being used more frequently than the former. Moreover, with modern lifestyles, consumers are becoming more health conscious and demand more natural foods that contain little or no chemical additives. In this sense, microbial fermentation constitutes a more natural and economically viable alternative to fortification that could increase vitamin concentrations in foods [8].

Riboflavin biosynthesis has been studied in both gram-positive and gram-negative bacteria, in most detail in *Bacillus subtilis* [9,10] and *Escherichia coli* [11]. The precursors of

riboflavin are GTP and ribulose-5′-phosphate, and the biosynthesis of riboflavin occurs through seven enzymatic steps which is similar in all microorganisms [9]. In recent years, the use of lactic acid bacteria (LAB) was proposed for B-group vitamin synthesis, including riboflavin since they provide a very attractive approach to obtain fermented bio-enriched foods [8,12,13]. Some LAB are capable of riboflavin overproduction which can be achieved either by metabolic engineering or by exposure to purine analogues and/or the toxic riboflavin analogue roseoflavin. Particularly, in some strains of *Lactobacillus* (*L.*) *plantarum*, *Lactobacillus fermentum*, *Lactococcus lactis*, and *Propionibacterium freudenreichii*, the latter approach has been used with success for the manufacture of different vitamin B2-enriched foods [14–17]. It has also been suggested that riboflavin production could enhance the benefits of certain probiotic strains [18]. Previously, it was demonstrated that *L. plantarum* CRL 725 was able to grow and produce high extracellular riboflavin concentration in vitamin B_2-free culture medium [13]. The molecular determinants for riboflavin biosynthesis were determined; it was shown that the *ribH*, *ribA*, *ribB*, *ribC*, and *ribG* genes formed a complete functional *rib* operon [3]. Also, the tolerance of this strain to the toxic roseoflavin resulted in a riboflavin overproducing phenotype in the vitamin B_2-free medium with levels of riboflavin produced higher than those in LAB isolated by others from sourdough [14]. Using soymilk as a fermentation medium, the roseoflavin resistant variant strain was able to increase more than threefold vitamin concentration compared with wild-type strain [15]. Although soymilk medium is relevant to the food industry, the determination of the factors affecting riboflavin production using a complex medium (or foods) is difficult. Furthermore, chemically defined medium is a prerequisite for physiological studies that allows experimentation under reproducible conditions via the control of all nutrient sources.

Since little information is available on how to enhance riboflavin production by lactic acid bacteria, the aim of this study was to determine the influence of specific medium constituents and the incubation temperature on riboflavin production and the expression of the riboflavin biosynthesis genes (*rib*) by a wild-type *L. plantarum* strain. The vitamin production by the roseoflavin resistant strain under these conditions was also evaluated.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains, media, and growth conditions

The riboflavin producing strains *L. plantarum* CRL 725 and CRL 2130 (previously known as CRL 725G) used in this work belong to the Culture Collection (CRL) of Centro de Referencia para Lactobacilos (CERELA-CONICET), Tucumán, Argentina. Cultures were stored at −20 °C in 10% (w/v) sterile reconstituted skim milk containing 0.5% (w/v) yeast extract, 1.0% (w/v) glucose, and 10% (v/v) glycerol.

Commercial riboflavin-free culture medium (Riboflavin Assay Medium (RFM), Difco, Becton, Dickinson, and Co., Sparks, MD, USA) and semi defined medium (SDM), adapted from that described previously [19] were used for experiments. The composition of SDM is shown in Table 1. This medium was prepared from concentrated individual stock solutions which were stored at −4 °C after filtration, except for the cysteine solution, which was freshly prepared. Stock solutions were composed of 100-fold-concentrated solutions of each amino acid, base, and vitamin, 20% glucose, Tween 80, and salts. All amino acids, vitamins, purines, pyrimidines, and inorganic salts were of analytical grade (Sigma Chemical Co., St. Louis, MO, USA). Media and stock solutions were sterilized by filtration through a cellulose acetate membrane (0.22-μm-pore size; Sartorius AG, Göttingen, Germany).

Working cultures of *L. plantarum* strains were prepared by transferring 0.5 ml of the frozen stock culture to 10 ml of MRS broth [20], and incubating for 16 h at 30 °C.

To eliminate carryover nutrients, the cells were harvested by centrifugation at 8000×*g* for 15 min, washed three times with saline solution (0.85% m/v NaCl), and resuspended in this solution to the original culture volume. This cell suspension was used to inoculate the SDM at an initial optical density (determined with Spectronic 2000; Bausch & Lomb, Rochester, NY, USA) at 600 nm (OD_{600}) of 0.2.

2.2 | Influence of carbon source, different concentrations of some SDM nutrients, and incubation temperature on riboflavin production

To evaluate the effect of sucrose and glucose on riboflavin production, each carbohydrate was added individually to SDM at a final concentration of 20 g L^{-1}. All assays were incubated at 30 and 37 °C. Samples were aseptically withdrawn at 0, 4, 8, 12, and 24 h of incubation and used for riboflavin determination and growth parameters.

Similarly, different concentrations of sodium acetate (5–30 g L^{-1}), casamino acids (5, 10, and 20 g L^{-1}), asparagine (0.01, 0.02, and 0.04 g L^{-1}), and guanosine (0.2–1.2 g L^{-1}) on riboflavin production were also evaluated. For these experiments, sucrose was used as carbon source and 30 °C as incubation temperature. After 24 h of incubation, samples were taken and kept on ice until used.

In all the fermentation assays, SDM without sugar (or bacterial inoculum) were used as negative growth controls.

Each growth condition was performed in triplicate and the error in each point of the growth curves was always below 5% with no significant inter-experience variation.

TABLE 1 Composition of SDM and SSDM in g L^{-1}

	SDM	SSDM
Sodium acetate	5.0	5.0
Ammonium citrate	1.0	1.0
$MgSO_4 . 7H_2O$	0.4	0.4
$MnSO_4 . 7H_2O$	0.038	0.038
KH_2PO_4	3.0	3.0
K_2HPO4	3.0	3.0
$FeSO_4$	0.02	0.02
Tween 80	1.0	1.0
Sucrose	20.0	20.0
NaCl	0.02	0.02
Casamino acids	0.20	0.10
L-Aspartic acid	0.20	0.2
L-Asparagine	0.60	
L-Phenylalanine	0.1	0.1
L-Tyrosine	0.1	0.1
L-Glutamic acid	0.2	0.2
L-Glutamine	0.2	0.2
L-Tryptophan	0.2	0.2
L-Cysteine	0.2	0.2
Uracil	0.02	0.02
Guanosine	0.02	0.04
Adenine	0.02	0.02
Xantine	0.02	0.02
Orotic acid	0.005	0.005
Biotin	0.01	0.01
p-aminobenzoate	0.01	0.01
Pantotenic acid	0.001	0.001
Nicotinic acid	0.001	0.001
Tiamine	0.001	0.001
Pyridoxal	0.004	0.004
B12	0.001	0.001
Folic acid	0.001	0.001

The bacterial growth was followed by measuring OD_{600} and cell viability was assessed by plating serial dilutions in MRS-agar plate which were incubated at 37 °C for 48 h and the colony-forming units (CFU ml^{-1}) were determined.

The pH of the samples was measured by potentiometric methods.

2.3 | Riboflavin determination

Riboflavin concentration was determined by a modified microbiological assay using *L. rhamnosus* ATCC 7469 as the indicator strain [21]. An aliquot (500 µl) of culture was diluted with same volume of 1% (v/v) acetic acid. Samples were boiled at 100 °C during 5 min, centrifuged 5 min at 5000×*g* and supernatants frozen at −20 °C until B2 quantification.

The samples were diluted with phosphate buffer, and each sample (100 µl) was placed into one well of a 96-well sterile microplate (Deltalab, Buenos Aires, Argentina). The riboflavin concentration of each sample was determined in triplicate. The reference strain (*L. rhamnosus* ATCC 7469) grown in a commercial riboflavin-free medium (RFM) was inoculated at 4% (v/v) in 10 ml of 2 × RFM, and a fraction (100 µl) was added to each well and mixed. Sterile plate covers were placed on the microtiter plates that were then incubated 48 h statically at 37 °C. After this optimized incubation period, $OD_{600\,nm}$ was determined using a microplate reader (VERSAmax tuneable microplate reader, Molecular Devices, Sunnyvale, CA, USA).

The riboflavin concentration of the sample was determined by comparing the OD_{600} with those obtained with the standard curve prepared using different concentrations of commercial riboflavin. These results were confirmed by HPLC analysis as described previously [15].

2.4 | Analysis of transcriptional levels of the *ribA, ribB, ribC, ribH*, and *ribG* genes by RT-*q*PCR

The expression of the following genes was investigated: *ribA* coding for GTP cyclohydrolase II and 3,4-dihydroxy-2-butanone 4-phosphate synthase, *ribB* coding for riboflavin synthase, *ribC* coding flavokinase/flavin adenine dinucleotide synthetase, *ribG* coding for pyrimidine deaminase and pyrimidine reductase, *ribH* coding for lumazine synthase.

Primers were designed using online softwares such as Primer 3 Plus (www.primer3plus.com), Primer BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/), and the sequences obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg), were: ***ribA*** forward CTGACCATCCTTGGTCGGTA-3′, ***ribB*** (f-) 5′-AGCTTTGGCAGAGGGCTTAC-3′, reverse (r-) 5′- f-5′-GTTCAGGGGCACATCGATTA-3′, r-5′ CAACTGCCAC CGAACCTTTAGTCGGATCGTGGAAATCTA -3′, ***ribC***, f-5′-CGCTACAGGTGGACCGACTA-3′, r-5′-GCATTCA ACCCGACAAGGTA-3′, ***ribG***, f-5′-ACGTACCAAAAT CCCCAGGT-3′, r-5′-ACATCCACCTCAGCATGGTC-3, ***ribH***, f-5′-ACCGGGAGCTTTTGAGATTC-3′, r-5′-AT AGTCAAAGTGCGCGGTTG-3′, ***recA*** (housekeeping), f-5′- −3′, r-5′- GGGTCTAGTGCGTTTTCAGC -3′.

RNA was extracted from samples obtained after 8 h of growth in optimal and reference conditions, using a NucleoSpin® RNA kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's recommendations for RNA extraction from bacteria. Total RNA was quantified using Qubit™ RNA Assay Kit (Invitrogen, Carlsbad, CA, USA) with Qubit™ 2.0 fluorometer (Invitrogen).

*q*RT-PCR reaction was performed using SensiFast™ SYBR & Fluorescein One-Step Kit (Bioline, Tauton, MA,

USA). Changes in gene expression were quantified in a thermocycler with a coupled Bio-Rad iQ5 Multicolor Real Time-PCR Detection System (Bio-Rad, Hercules, CA, USA). The cycling conditions were: 45 °C for 10 min (1×); 95 °C for 2 min (1×); 95 °C for 5 s, 60 °C for 10 s, 72 °C for 5 s (40×); 95 °C for 1 min (1×); 55 °C for 1 min (1×), and 55 °C for 10 s (81×). For each amplification run, the calculated threshold cycle of the 16S rRNA was used for normalization [18]. The formation of nonspecific products was excluded by using the melting curve function of the Bio-Rad iQ5 Optical System Software, Standard Edition v2.0.148.60623.

The fold change of each transcript in each sample relative to the control sample was measured in triplicates, normalized to internal control gene *recA* and calculated according to the $2^{-\Delta\Delta CT}$ method [22]. The relative transcription levels were expressed as means ± standard deviations (SD) fold changes. Genes were significantly down- or upregulated if their relative expression level was found to be at least twofold lower or higher than reference condition as previously described [23].

Gene expression was compared from samples taken under the stimulation condition [early stationary-phase-cells grown in SDM-sucrose (2%) with guanosine (0.04 g L^{-1}) and casamino acids (10 g L^{-1}) medium] and compared to those of the reference condition [early stationary-phase-cells grown in SDM-sucrose (2%) with guanosine (0.01 g L^{-1}) and casamino acids (5 g L^{-1}) medium], at 30 °C during 8 h.

2.5 | Statistical analysis

All values were expressed as means ± 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

The influence of carbohydrates on growth and riboflavin production by *L. plantarum* CRL 725 were evaluated using 30 and 37 °C as the incubation temperatures. Cultures in SDM-glucose displayed similar bacterial growth to those obtained with SDM-sucrose (logs 8.68 and 8.73, respectively) in both incubation temperatures (Fig. 1) and no differences in pH in both conditions were observed (staring at pH 6.5 and reaching pH 4.3 ± 0.2 after 24 h incubation). However, riboflavin production was higher using SDM-sucrose than SDM-glucose; with this difference being more marked at 30 °C (Fig. 1a) than at 37 °C (Fig. 1b) (1569 and 896 ng ml^{-1} of riboflavin, respectively). From the results obtained, the medium containing sucrose instead of glucose (SDM-sucrose) and 30 °C as incubation temperature were chosen for further assays. Riboflavin production by *L. plantarum* CRL 725, under these growth conditions, began at early exponential growth phase (8 h of fermentation) and reached the maximum after 12 h (early stationary phase) (Fig. 1a).

The presence of 5 g L^{-1} sodium acetate assured the growth and riboflavin production but highest acetate concentrations (up to 30 g L^{-1}) did not improve cell yield or vitamin production. The addition of asparagine (0.01–0.04 g L^{-1}) had no effect neither on growth, pH, nor the vitamin production (data not shown). Therefore, asparagine was removed from SDM-sucrose without leading to a reduction in cell viability. Contrarily, the omission of casamino acids, a mixture of amino acids, and small peptides obtained from the acid hydrolysis of casein, affected both cell growth (data not shown), and vitamin production (Fig. 2a). Therefore, the addition of casamino acids (10 g L^{-1}) to the SDM-sucrose resulted in a ca. fourfold increase of the riboflavin production, whereas no improve in riboflavin levels was observed when 20 g L^{-1} of casamino acids was added in this medium (Fig. 2a).

When SDM-sucrose was supplemented with 0.04 g L^{-1} of GTP, one of the precursors for *de novo* riboflavin biosynthesis, there was a clear increase (1.6-fold) in vitamin production compared to when it was not-supplemented in the medium (Fig. 2b) but had no significant effect on the growth of the strain nor the pH of the culture media (data not shown).

Such an approach made it possible to define the culture conditions that enhance the production of riboflavin by *L. plantarum* CRL 725 using SDM with sucrose 2% (w/v) as carbon source, with guanosine (0.04 g L^{-1}), and casamino acids (10 g L^{-1}) designated as the stimulated SDM or SSDM and incubation temperature of 30 °C.

In order to study the effect of increase in casamino acids and guanosine concentration into SSDM on riboflavin production, relative expression levels of the *ribA*, *ribB*, *ribC*, *ribH*, and *ribG* genes were calculated 8 h after inoculation in comparison with reference condition (30 °C and SDM-sucrose with guanosine [0.01 g L^{-1}] and casamino acids [20 g L^{-1}], used as reference medium). The gene expression profiles for *L. plantarum* cells grown under these conditions were different (Fig. 3).

The *ribG* and *ribH* genes had a steady transcript level (fold change equal to one). This means that expression of these genes was not affected by increase of guanosine and casamino acids concentrations in SDM medium. However, the relative expression levels of the other genes (*ribA*, *ribB*, *ribC*) increased more than twofold in cultures grown in SDM-sucrose supplemented with guanosine (0.04 g L^{-1}) and casamino acids (10 g L^{-1}) in comparison with relative expression levels in reference medium (SDM).

Finally, the growth and the ability to produce riboflavin by roseoflavin resistant variant grown in the SSDM and incubated at 30 °C were examined (Fig. 4). Using these optimized conditions, riboflavin production of 5724 ng ml^{-1}

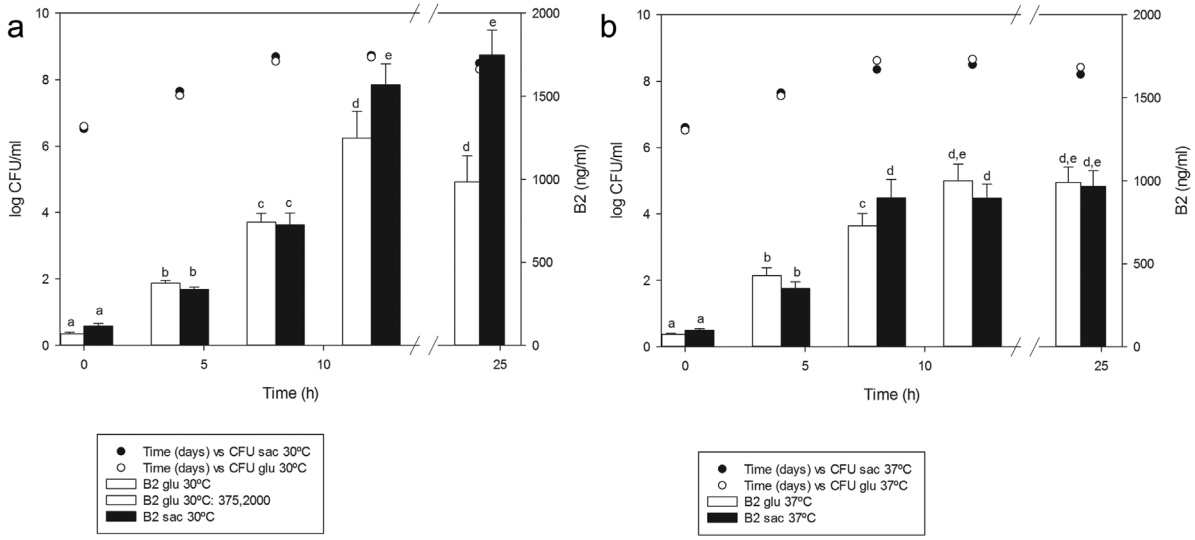

FIGURE 1 Influence of carbohydrates glucose (Glu), and sucrose (Sac) on microbial growth (CFU ml^{-1}), and riboflavin concentration (ng ml^{-1}) produced by *L. plantarum* CRL 725 incubated at 30 °C (a) and at 37 °C (b). Results are expressed as mean ± SD. [a–h]Means with different letter in the same graph differ significantly ($p < 0.05$)

was obtained which is 3.5- and 2.4-fold higher when compared to riboflavin levels formed in reference conditions and stimulation conditions using the wild-type strains, respectively, and almost sixfold higher than when glucose was used as the sole carbon source.

The results clearly showed that these culture conditions, lead to the highest levels of vitamin produced by the variant compared with those obtained with wild-type strain, being more marked this difference at 24 h-incubation than at 12 h whereas the growth was similar for both strains.

4 | DISCUSSION

L. plantarum is a highly versatile LAB and some strains are marketed as starter cultures or probiotics [24] because of their excellent traits, such as the capacity of producing B-group vitamins [14,25,26]. Previously, it was demonstrated that *L. plantarum* CRL 725 was able to produce high extracellular riboflavin concentration in B_2-free culture medium [15]. In this study, culture conditions (incubation temperature and the presence of different concentrations of specific culture media components) that likely affect the sole carbon source for vitamin production compared to glucose. It has been reported previously that there is a strong association between genotypic and phenotypic diversity growth and riboflavin production by *L. plantarum* CRL 725 were analyzed. Sucrose was more efficient, as the carbohydrate metabolism and origin of the strain, so these features would be essential or may confer benefits in different ecological niches exhibiting remarkable ecological adaptability to different environments and growth substrates [27]. *L. plantarum* CRL 725 was

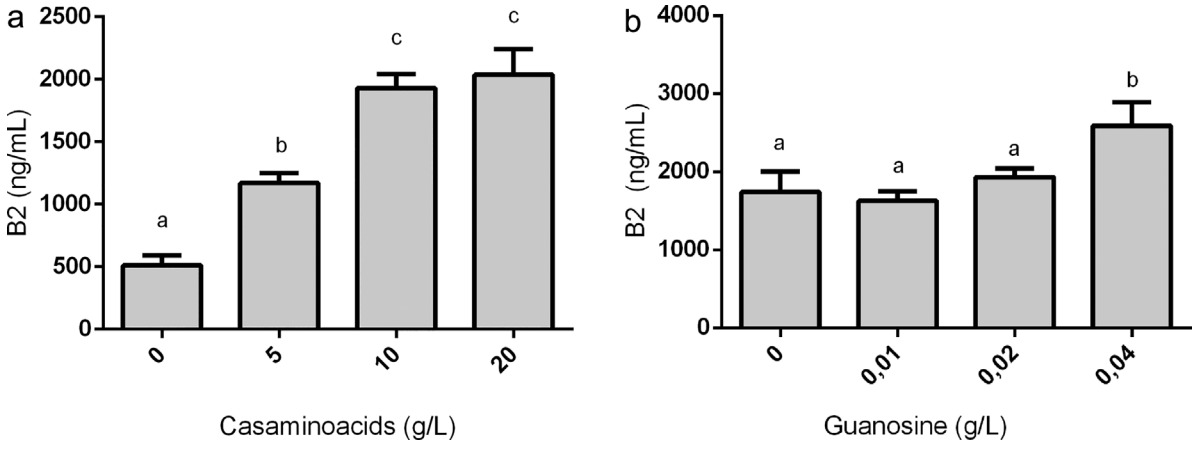

FIGURE 2 Influence of casaminoacids (a) and guanosine (b) concentrations on riboflavin levels (ng ml^{-1}) produced by *L. plantarum* CRL 725. Results are expressed as mean ± SD. [a–c]Means with different letter differ significantly ($p < 0.05$)

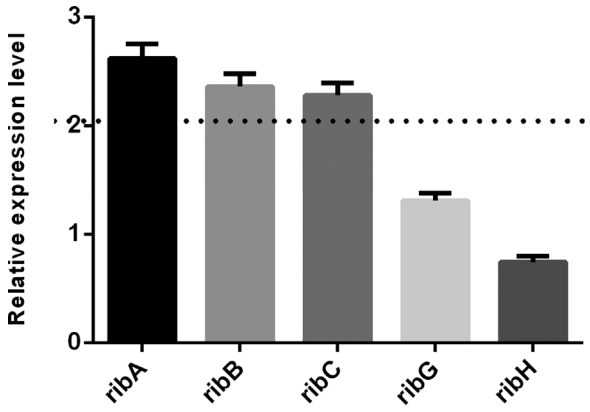

FIGURE 3 Comparison of the relative expression levels of *rib* genes of *L. plantarum* CRL 725 between late-exponential-phase-cells growing in SSDM and late-exponential-phase-cells growing in SSM at 30 °C. Dotted line shows cutoff where significant relative change in *rib* gene expression is present (above line) in stimulated conditions compared to reference conditions

originally isolated from sugar cane residue (bagasse), explaining its preference for sucrose as its principal carbon source. Sucrose is composed by glucose and fructose; this latter is metabolized by pentose phosphate pathway through phosphorylation to fructose 6-phosphate and isomerization to glucose 6-phosphate which is converted into ribose 5-phosphate which in turn can continue until the formation of ribulose-5-phosphate [28]. Ribulose is one of the precursors of riboflavin [9]. Fructose metabolism could thus explain the higher riboflavin production observed in presence of sucrose than that observed with glucose.

Sodium acetate is a normal component in growth media of LAB, for example, MRS broth [20], where it acts as a pH buffer. In our study, the presence of acetate (5 g L^{-1}) in SSDM allowed a good growth of *L. plantarum* CRL 725 while MØretrØ et al. [29] found that acetate removal, from the medium, resulted in good growth of *L. plantarum* strains. However, the addition of acetate at higher concentrations (up to 30 g L^{-1}) produced a reduction in cell viability. Therefore, under these conditions, this compound would act as an inhibitor of cell growth [30].

For certain lactic acid bacteria, the presence of asparagine in a synthetic medium was essential for growth of strains isolated from wines [31]. Our work confirmed some previous observations concerning the amino acids requirements of *L. plantarum* strains isolated from meat [29]. Elimination of asparagine did not affect growth or riboflavin production by *L. plantarum* CRL 725, similar to the previous study where asparagine was not essential for bacterial growth.

Given that riboflavin is synthesized from two precursors: ribose 5-phosphate and guanosine triphosphate (GTP) where guanosine differs by the presence of phosphates which are removed during riboflavin biosynthesis, the effect of guanosine on riboflavin production was evaluated. The addition of this compound (0.04 g L^{-1}) did not effect on strain growth; however, it significantly enhanced riboflavin production.

Based on all of these results, the stimulation conditions for riboflavin production by *L. plantarum* CRL 725 were defined. These conditions that favored riboflavin production did not affect bacterial growth nor caused variations of the media pH (the former was directly related to growth).

In order to understand why these conditions affect riboflavin production, relative expression of *rib* genes were evaluated in these conditions compared to the reference condition. The results show that *ribA*, *ribB*, and *ribC* (coding for GTP cyclohydrolase II and 3,4-dihydroxy-2-butanone 4-phosphate synthase, riboflavin synthase, and flavokinase/flavin adenine dinucleotide synthetase, respectively) expression were increased in SSDM after 8 h-incubation whereas *ribH* and *ribG* (which coding for lumazine synthase and pyrimidine deaminase, and pyrimidine reductase, respectively) were not overexpressed. In these enhanced media conditions, the mRNA levels of these three genes were at least two times higher than in the medium used as reference conditions (SDM-sucrose, guanosine [0.02 g L^{-1}] and casamino acids [20 g L^{-1}]).

These results confirm a previous study [32] where it was shown that when an excess of GTP and ribulose-5-phosphate are found in culture media, the *ribA* gene was overexpressed as were the enzymes, GTP cyclohydrolase II, and DHBP synthase, coding by this gene. These same authors also showed that under these conditions, the enzymes coded by

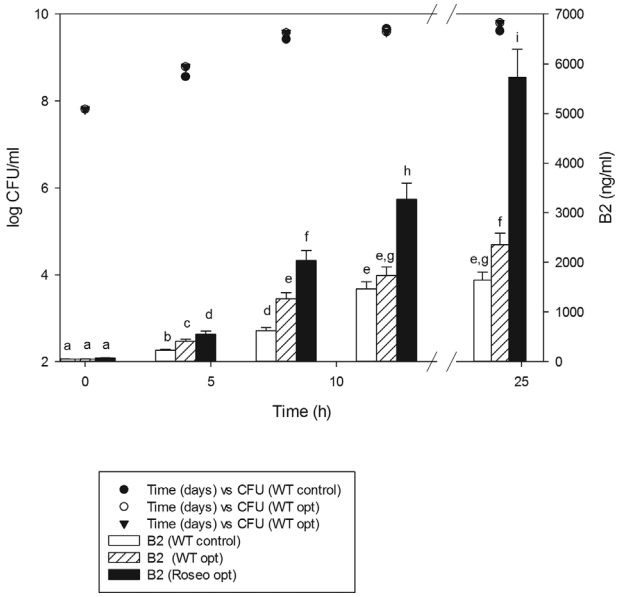

FIGURE 4 Growth (CFU ml^{-1}), and riboflavin concentration (ng ml^{-1}) in control or optimized media at 30 °C for 24 h produced by *L. plantarum* CRL 725 (wild-type strain, black or white circles, white or stripped bars) and CRL 2130 (roseoflavin resistant mutant, black triangle, black bars). [a–g]Means with different letter differ significantly ($p < 0.05$)

ribH (lumazine synthase) and *ribG* (deaminase and reductase) were not modified, suggesting that they are not affected by external nutrients sources and would constitute non-modifiable bottleneck steps in riboflavin biosynthesis.

Moreover, casamino acids contain high concentration of free amino acids, which are precursors of purine metabolism. The role of purine compounds as precursors of riboflavin is well known [33]. Therefore, these observations could explain the increased expression of the genes responsible for riboflavin biosynthesis when casamino acids were added to the medium.

To the best of our knowledge, this is the first report where the riboflavin production and the expression of its biosynthesis genes were evaluated in lactic acid bacteria using a SDM with varying concentrations of key components and incubation temperature. The enhanced culture conditions defined in this work allowed to obtain higher levels (almost sixfold increase) of riboflavin produced by both wild-type *L. plantarum* strain and its roseoflavin resistant mutant than those found when these were grown in soymilk. Using these optimized conditions, riboflavin production of almost $6\,g\,L^{-1}$ were obtained, which is much higher than that obtained by other strains of *L. plantarum* that were selected for their riboflavin producing capability; such as UNIFG roseoflavin derivative isolated from sourdough that produced $0.6\,mg\,L^{-1}$ [14] or roseoflavin-resistant NCDO1752 mutants that also produced $0.6\,mg\,L^{-1}$ [3]. However, one must be cautious that novel strains could be found that produce more elevated concentrations of the vitamin. The addition of guanosine and casamino acids should be tested in a food matrix using different strains of *L. plantarum* to see if they are strain specific and that the results provide a positive effect on riboflavin production in order to evaluate the value added effect of increasing this vitamin in different fermented foods.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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