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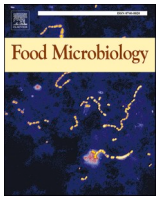

Characterization of riboflavin-overproducing *Bifidobacterium longum* subsp. *infantis* strains selected by roseoflavin treatment

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ABSTRACT

Diet is the primary source of riboflavin (B_2) for humans. It can also be produced by lactic acid bacteria ingested with foods and by gut microbial commensals, including some bifidobacteria. Herein an *in silico* analysis of potential regulatory mechanisms affecting *ribD* transcription and translation in *Bifidobacterium longum* subsp. *infantis* is presented. Riboflavin-overproducing strains were selected by treatment with roseoflavin of *B. longum* susbp. *infantis* CECT4551^T and its spontaneous derivative IPLA60011. Whole genomes of both parental strains and the sequencing of the *rib* clusters of the riboflavin-overproducing ones were conducted. Punctual mutations affecting different stem-loops in the aptamer region of the FMN-riboswitch involved in the regulation of the *rib* expression were detected. Riboflavin overproduction of the derivative strains was confirmed through HPLC quantification in RAMc and MRSc cultures, ranging from 64.9 to 441.2 μg/L. These levels correlated to predicted secondary folding and stability of the aptamer region and/or expression platform of the *rib* FMN riboswitch. Safety and technological properties of the riboflavin-overproducing derivatives, in terms of antibiotic resistance profile and carbohydrate utilization capabilities, were not altered following roseoflavin exposure, thus confirming the potential aptitude of the riboflavin-overproducing derivatives to produce biofortified foods such as those formulated on dairy matrixes.

1. Introduction

Riboflavin, also known as vitamin B_2, is a water-soluble essential vitamin precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), both participating as essential cofactors mediating many processes in living cells. Riboflavin deficiency affects iron absorption, tryptophan metabolism, mitochondrial function and other vitamin metabolism, and has been proposed as a risk factor for cancer and cardiovascular disease, among other conditions (Powers, 2003). However, humans cannot synthesize this vitamin and must exogenously acquire it, mainly through diet. Riboflavin *de novo* synthesis is, however, common in many microorganisms including yeasts, fungi and bacteria. Accordingly, microorganisms ingested with fermented foods (LeBlanc et al., 2013) or those inhabiting the human gut ecosystem (Nysten and Van Dijck, 2023) have been acknowledged as potential sources of riboflavin supply for humans. For these reasons, there has been increasing interest in developing starter cultures with the capacity to produce riboflavin, as a strategy to biofortify fermented foods made from dairy and vegetable matrixes. For this purpose, several species belonging to the lactic acid bacteria group including, among others, *Lactiplantibacillus plantarum*, *Lactococcus lactis, Lactobacillus helveticus Leuconostoc mesenteroides, Weisella cibaria,* or *Lactobacillus bulgaricus* subsp. *delbrueckii*, have been commonly investigated (Capozzi et al., 2011; Juárez del Valle et al., 2014; Russo et al., 2014; Thakur et al., 2015; Yépez et al., 2019; Spacova et al., 2022).

In bacteria, riboflavin is synthesized from GTP and ribulose-5P,

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major intermediary metabolites in the purine and pentose pathways respectively, through the action of up to seven different genes generally organized in a *rib* operon (*ribA/B, ribC, ribD, ribE, ribH, ribR*), although in some bacteria these genes can be scattered throughout the genome (Vitreschak et al., 2002). Since riboflavin acts as a precursor for flavin nucleotide synthesis, FMN and FAD, expression of the *rib* operon is tightly controlled through an FMN riboswitch located in the untranslated region of the *rib* mRNA. Exposure to roseoflavin, a toxic analogous to riboflavin, favors the selection of riboflavin-overproducing strains carrying mutations at the riboswitch region. This approach has generally been employed to increase riboflavin production in several lactic acid bacteria of technological interest for food production (Burgess et al., 2006; Juárez del Valle et al., 2014; Solopova et al., 2020; Hernández-Alcantara et al., 2022).

It is noteworthy, however, that most strains investigated as potential strategies to biofortify fermented foods in riboflavin belong to species naturally associated with foods such as vegetables matrixes, but there are no commensal species in the human gut and hence won't likely contribute to riboflavin production upon ingestion. In this scenario, achieving riboflavin biofortification of foods by using strains with the potential to at least transiently colonize the human gut upon ingestion, would offer an appealing alternative for a dual riboflavin provision to the consumer: through biofortification of foods and *in situ* production in the human gut upon consumption. In this framework, *Bifidobacterium* represents a genus of commensal microorganisms that inhabit the human gut throughout its lifespan, and includes several strains with recognized probiotic properties. Besides, some strains have been traditionally incorporated into various food delivery vectors hence bifidobacteria represent interesting microorganisms that combine adaptive traits to both food matrixes and the human intestinal ecosystem (Hidalgo-Cantabrana et al., 2017; Derrien et al., 2022; Jena and Choudhury, 2023; Lu et al., 2023). Its functional and technological properties have also promoted interest in their genetic engineering or adaptive strategies to further enhance their performance within food matrixes and the gastrointestinal tract (Zuo et al., 2016; Ruiz et al., 2017; Wei et al., 2019).The capacity of some *Bifidobacterium* members to synthesize riboflavin has been investigated in *B. longum* subsp. *infantis* ATCC15697^T through *in silico* identification of a *rib* cluster and semi-quantitative determination of riboflavin production in a roseoflavin-derivative strain that reached riboflavin production levels of 60.8 ng/mL (Solopova et al., 2020).

Herein we report an *in silico* analysis of potential regulatory mechanisms affecting the *rib* cluster transcription and translation in *Bifidobacterium longum* subsp. *infantis*. Besides, riboflavin-overproducing strains were obtained for the parental *B. longum* susbp. *infantis* CECT4551^T and IPLA60011 ones. Their riboflavin production, safety and technological properties were also investigated.

2. Material and methods

2.1. Bacterial strains and culture conditions

The bifidobacterial strains used in this work were routinely cultured in MRS broth (Biokar, France) supplemented with 0.25 % (w/v) L-cysteine hydrochloride monohydrate (Sigma-Merck, Germany), hereafter named MRSc, at 37 °C under anaerobic conditions (80 % N_2, 10 % CO_2 and 10 % H_2) in the anaerobic MG500 chamber (Don Whitley Scientific, UK). For long-term storage at −80 °C, stocks of the strains were made in MRSc with 30 % glycerol. To obtain standardized cultures, bacterial stocks were seeded on the surface of agar-MRSc and incubated for 3 days. Then, a single colony was inoculated in 10 mL MRSc broth and incubated overnight (between 18 and 20 h) before being used to inoculate (2 %) fresh MRSc broth that was cultured for 18 ± 1 h. The type strain *B. longum* subsp. *infantis* CECT4551^T (Colección Española de Cultivos Tipo, Spain) was initially used and a spontaneous mutant, named IPLA60011, was observed during sub-culturing. The mutant had a different macroscopic morphology of the colonies in agar-MRSc, being smaller and less smooth than the parental CECT4551^T, and also slightly different from the parental one when MRSc-broth cultures were observed under an optical microscope (Fig. S1).

2.2. Genome analysis

The genome of both strains was sequenced after DNA extraction as follows. Bacterial pellets from 3 mL of overnight MRSc-grown cultures, were collected by centrifugation (16,000×*g*, 10 min at room temperature), washed with PBS and lysed for 1 h at 37 °C under stirring after resuspension in freshly prepared lysis buffer composed of 20 mM Tris-HCl pH 8.0, 2 mM Na-EDTA, 1.2 % Triton X-100, 162 kU of lysozyme, 5 U of mutanolysin and 0.15 U of lysostaphin. All reagents and enzymes used were purchased from Sigma-Merck. After the lysis step, samples were transferred to a column of the Blood & Tissue DNA Extraction Kit (Germany) and DNA was purified following the manufacturer's instructions. The DNA concentration was determined using a DNA High Sensitivity Assay in a Qubit fluorimeter (Thermo Fisher, USA). Genome sequencing was performed at GenProbio S.R.L. (Parma, Italy), with an Illumina MiSeq Sequencing system. Genome analysis and comparison were carried out using the PATRIC platform (Bacterial and Viral Bioinformatics Resource Center, USA). Raw sequences generated in this work were deposited in the SRA of the NCBI database (BioProject PRJNA983715).

2.3. Selection of riboflavin-overproducing strains

To isolate riboflavin-overproducing strains, the roseoflavin-resistant strain selection method was followed as previously described (Burgess et al., 2006; Solopova et al., 2020). For this, the Riboflavin Assay Medium (RAM, BD-Difco, Fishers, USA) supplemented with 0.25 % of L-cysteine hydrochloride monohydrate (RAMc), was added with increasing concentrations of roseoflavin (Sigma-Merck), ranging from 5 to 50 mg/L. For the selection, standardized cultures of both CECT4551^T and IPLA60011 were washed twice with PBS and used to inoculate (2 %) RAMc broth supplemented with different roseoflavin concentrations, following incubation under standard conditions. Once turbidity was detected in some of the cultures, they were spread on the surface of agar-RAMc supplemented with the same roseoflavin concentration used in the RAMc broth. Plates were incubated for at least three days under standard conditions, and grown colonies were stored at −80 °C in RAMc-supplemented with 30 % glycerol.

2.4. Analysis of the rib operon of the riboflavin-overproducing strains

The roseoflavin-resistant clones were grown in RAMc to isolate DNA, using the GenElute kit (Sigma) following the manufacturer's instructions, which was used to amplify by PCR the operon of riboflavin synthesis. To achieve this, several primers were designed within this operon (Fig. S2) using as a reference the genome of *B. longum* subsp. *infantis* ATCC15697^T (NCBI reference sequence: NC_011593.1). The whole operon was amplified using these primers, following the next PCR conditions: an initial step of 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 5 min, and a final amplification step of 72 °C for 10 min. Finally, the 5 amplicons obtained for each clone were sequenced in Macrogen (South Korea) and sequences were compared with that of the ATCC15697^T strains using the Clustal Omega tool.

Folding of the secondary structures of the FMN riboswitches and the ρ-independent terminator in the *rib* transcript of the wild-types and riboflavin-overproducing clones were obtained by using the RNAfold web server (The ViennaRNA Web Services, version 2.4.18) and edited with VARNA 3.9 software (Darty et al., 2009).

2.5. Quantification of riboflavin by HPLC

Roseoflavin-resistant clones were grown, under standard conditions for 18 ± 1 h, either in RAMc or MRSc broths to quantify the riboflavin released by the bifidobacteria in the cultures (Juárez del Valle et al., 2014). Cultures of the clones in each broth were made in triplicate. In short, free-cell samples were mixed (1:1) with 1 % acetic acid and centrifuged (5000×*g* for 5 min) to collect the supernatants that were boiled (100 °C) for 5 min and finally centrifuged at 5000×*g* for 5 min. Samples were then filtered, using a 0.45 μm pore-size PTFE filter (VWR-Avantor, USA), and analyzed in an HPLC system as follows. The chromatographic system consisted in a separation module Alliance e2695, a fluorescence detector FLR 2475 and the data acquisition software Empower (Waters, USA). Separation was carried out in a XTerra MS C_{18} 150 x 4,6 mm (5 μm) column, equipped with a Nova-Pak C_{18} 20 × 3.9 mm (4 μm) precolumn (Waters). The mobile phase was composed of an aqueous phase containing ammonium acetate (0.05 M), with pH adjusted to 5.0, and an organic phase of methanol. The methanol gradient, with a flow rate of 1 mL/min, was started from 20 % and programmed: 0–5 min at 28 %, 5–10 min at 50 %, 10–11 min at 100 % and held to 100 % for 3 min before returning to the initial conditions after a re-conditioning for 5 min. The column temperature was maintained at 30 °C during chromatographic runs. The detection of riboflavin was performed with a fluorescence detector with excitation at 440 nm and emission at 510 nm. The sample injection volume was 50 μL for samples obtained from cultures in both broths. The analytical curve was traced by analyses of riboflavin standards and showed good linearity with a correlation coefficient $R^2 = 0.998$.

2.6. Characterization of two selected riboflavin-overproducing strains

The API-Zym and API-50CH (BioMérieux, France) tests were used to determine the enzymatic and carbohydrate utilization profiles, respectively, of two parental and two riboflavin-overproducing strains following the manufacturer's instructions. To evaluate antibiotic resistance patterns, minimum inhibitory concentrations (MICs) to gentamycin, kanamycin, streptomycin, tetracycline, erythromycin, chloramphenicol, ampicillin and vancomycin (Sigma-Merck), and clindamycin (Apollo, USA), were determined using the broth microdilution plate method, according to the protocol previously described with minor modifications (Gueimonde and Arboleya, 2021). The individual colonies grown in agar-MRSc plates were suspended in PBS to a turbidity 2 in the McFarland scale, which corresponded to approximately 3 10^8 CFU/mL as determined in previous experiments (data not shown). This suspension was further diluted 50-fold in 2x LSMc medium, prepared by mixing 2x MRS and 2x Hi-Sensitivity Test Broth (Himedia, India) in a 1:9 proportion, and supplemented with 0.1 % (w/v) of L-cysteine hydrochloride monohydrate. Then, 50 μL of these diluted suspensions were inoculated in each microplate well containing the above-stated antibiotics at the appropriate concentrations. Each plate was covered and incubated at 37 °C in an anaerobic chamber for 48 h. Following incubation, MICs were determined as the lowest antibiotic concentration that inhibited bacterial growth. Interpretation of these values to classify the strains as susceptible/resistant to each of the antibiotics assayed was performed according to the breakpoints identified by the EFSA FEEDAP Panel (2018). *B. longum* ATCC15707^T was used for quality control of the broth microdilution assay, as recommended (Gueimonde and Arboleya, 2021).

2.7. Statistical analysis

The IBM-SPSS statistics for Window version 28.0 (IBM Corp., Armonk NY, USA) was used to determine differences among the four bifidobacterial strains under study, which were assessed by means of an one-way ANOVA test. When needed, the SNK (Student- Newman-Keuls) test was applied to establish the differences ($p < 0.05$) among the means of the data. In the legend of the table and figures the type of comparison carried out is indicated.

3. Results and discussion

3.1. Characteristics of the parental B. longum subsp. infantis strains

The species *B. longum* is currently subdivided in four subspecies, *B. longum* subsp. *longum*, *B. longum* subsp. *infantis* and *B. longum* subsp. *suis* and *B. longum* subsp. *suillum*, the two first being present in the human intestinal microbiota, whereas the last two are associated with that of animals (Tarracchini et al., 2021; Yanokura et al., 2015). The subspecies *B. longum* subsp. *infantis* is often present in the intestinal community of breast-fed infants and is less abundant in the adult microbiota, where *B. longum* subsp. *longum* predominates. This fact might be related to the high specialization in the metabolic capabilities of each subspecies given the distinctive glycoside hydrolase (GH) pattern found between both taxa (Calvete-Torre et al., 2023). It was proposed that the GH activities of these two subspecies could allow their taxonomic classification (Blanco et al., 2020). Additionally, in the context of the current work, the search of genes encoding the riboflavin biosynthetic pathway (the *rib* operon) within publicly available genomes also revealed that the *rib* cluster was exclusively present in the *B. longum* subsp. *infantis* (data no shown), which might confer an adaptative advantage in the environment of the infant gut microbiota (Solopova et al., 2020). Therefore, taking into consideration this finding, efforts on characterizing the riboflavin production in *B. longum* subsp. *infantis* were focused.

The genomes of two *B. longum* subsp. *infantis* strains, CECT4551^T and IPLA60011, were initially compared with that of the strain ATCC15697^T (NCIB accession NC_011593.1). The most relevant single nucleotide polymorphism (SNP), found exclusively in the strain IPLA600011, affected the *rfbP* gene, which encodes a priming-glycosyl transferase involved in the synthesis of exopolysaccharides (EPS). This SNP generated a translation stop codon, resulting in a truncated RfbP protein of 549 amino acids instead of 572 of the strain ATCC15697^T (Table 1). Therefore, the polymer production did not take place since this is the enzyme that initiates the EPS synthesis (Hidalgo-Cantabrana et al., 2014). Indeed, this fact could explain the difference in the macroscopic and microscopic morphologies found between IPLA60011 and

Table 1
Single nucleotide polymorphism (SNPs) detected between *B. longum* subsp. *infantis* ATCC15697^T (NCIB accession NC_011593.1) and the parental IPLA60011 strain used in this study. The EPS-cluster of ATCC15697^T strain was described in Hidalgo-Cantabrana et al. (2014). In blue shadow is market the mutation affecting in the priming-GTF in strain IPLA60011, which might be responsible for the change of colony phenotype (non-EPS producing phenotype).

SNP detected	Amino acid modification	Protein function
A443377**G**	Leu - > Pro	RNA binding protein
C1381388**insG**	Frameshift	Hypothetical protein
T1510198**C**	Ser - > Pro	Hypothetical protein
C1672879**delC**	Frameshift	Hypothetical protein
G1689519**A**	Pro - > Leu	Hypothetical protein
GCCGTCGG2106434**ACCATCGT**	Frameshift	3′ downstream ATP binding cassette
T2106450**C**		3′ downstream ATP binding cassette
G2106455ins**TT**	Frameshift	3′ downstream ATP binding cassette
C2140269**T**		5′ upstream phosphoribosyl-formyl-glycinamidine cyclo-ligase
C2174914**T**	Arg - > His	PdxS (pyridoxal-5-phosphate synthase lyase)
C2193552**T**	Ala - > Thr	ABC transporter
G2275318**A**	Pro - > Leu	Galactokinase
G2336343**A**	Trp - > Stop	Sugar transferase (rfbP, priming GTF)

CECT4551^T (Fig. S1), being the first spontaneous mutant obtained from the last. This was confirmed after the observation of liquid cultures obtained in MRSc from both strains before and after a mild centrifugation (Fig. S3). Cultures from strain IPLA60011 lacking EPS-synthesis capability, completely sedimented rending a clear supernatant, whilst those of EPS-producing CECT4551^T strain maintained their turbidity. This is one of the simplest tests to prove the presence of an EPS layer surrounding the bacterial surface (Ruas-Madiedo, 2021), which is a characteristic that can be easily acquired or lost in some bifidobacterial species. A high variability within the *eps* clusters for *B. longum* species has been previously reported (Hidalgo-Cantabrana et al., 2014; Ferrario et al., 2016).

Bioinformatic analysis of the *rib* cluster of the *B. longum* subsp. *infantis* CECT4551^T and IPLA60011 strains revealed that they were identical and constituted by the *ribD ribE ribA/B* and *ribH* genes (Fig. 1A) involved in the riboflavin biosynthetic pathway (Fig. 1B). Upstream of the operon, a putative transcriptional promoter for the binding of the vegetative σ70 factor of the bacterial RNA polymerase was detected. This promoter contains a canonical −35 (TTGACA) region and a −10 (TACAAT) region deviating only in one nucleotide from the consensus sequence (TATAAT). In addition, downstream of the *ribH* gene a characteristic ρ-independent transcriptional terminator was identified, indicating that the *rib* cluster was indeed a *ribDEAH* operon (Solopova et al., 2020). Upstream of the *ribDEAH* operon in both strains is located the *pabB* gene, which encodes a p-aminobenzoic acid synthase (Wegkamp et al., 2007) and, interestingly, its translation stop codon (TAA) is located downstream of the *rib* promoter. Therefore, presumably a transcription of the *pabB* gene could interfere with the binding of the RNA polymerase to the *rib* promoter decreasing its efficiency for the transcription of the *rib* operon. Furthermore, the untranslated region (5′-UTR) located between the *rib* promoter and the *ribD* gene seems to be the main factor responsible for the regulation of the *ribDEAH* operon expression. Prediction of the folding of this region with the RNA fold program revealed the presence of a riboswitch (Fig. 2). The non-coding riboswitch region is an RNA genetic switch, typically found in the 5-untranslated region of the bacterial mRNAs, which can directly bind metabolites without the obligate involvement of proteins, resulting in the formation of alternative RNA secondary structures in this region. These elements, present in many bacterial genomes, act as regulators of different metabolic pathways (Breaker, 2011). Typically, riboswitches are composed of a sensing aptamer for binding to the effector ligand and a downstream expression platform. One common riboswitch-mediated gene regulation mechanism is through a transcription termination anti-termination mechanism, in which the aptamer domain acts as a sensor to detect small-molecule ligands. Thus, the folding of the nascent mRNA changes when the effector-aptamer complex is formed, causing secondary structure changes in the riboswitch that prematurely interrupt transcription (Breaker, 2011).

Riboflavin is the precursor of FAD and FMN, and when an excess of FMN is present in the bacterial cytoplasm a repression of the *ribDEAH* operon expression takes place through an FMN riboswitch. In this case, the effector is the FMN and its binding to the aptamer results, in general, in abortive mRNA synthesis by transcriptional termination. This mechanism has been demonstrated for *Bacillus subtilis* (Winkler et al., 2002) and proposed among others for *B. longum* (Solopova et al., 2020), *Lactiplantibacillus plantarum* (Ripa et al., 2022) and *Weissella cibaria* (Diez-Ozaeta et al., 2023). However, in the case of *B. longum* subsp. *infantis*, a bioinformatic analysis of the folding of the 5′-UTR region

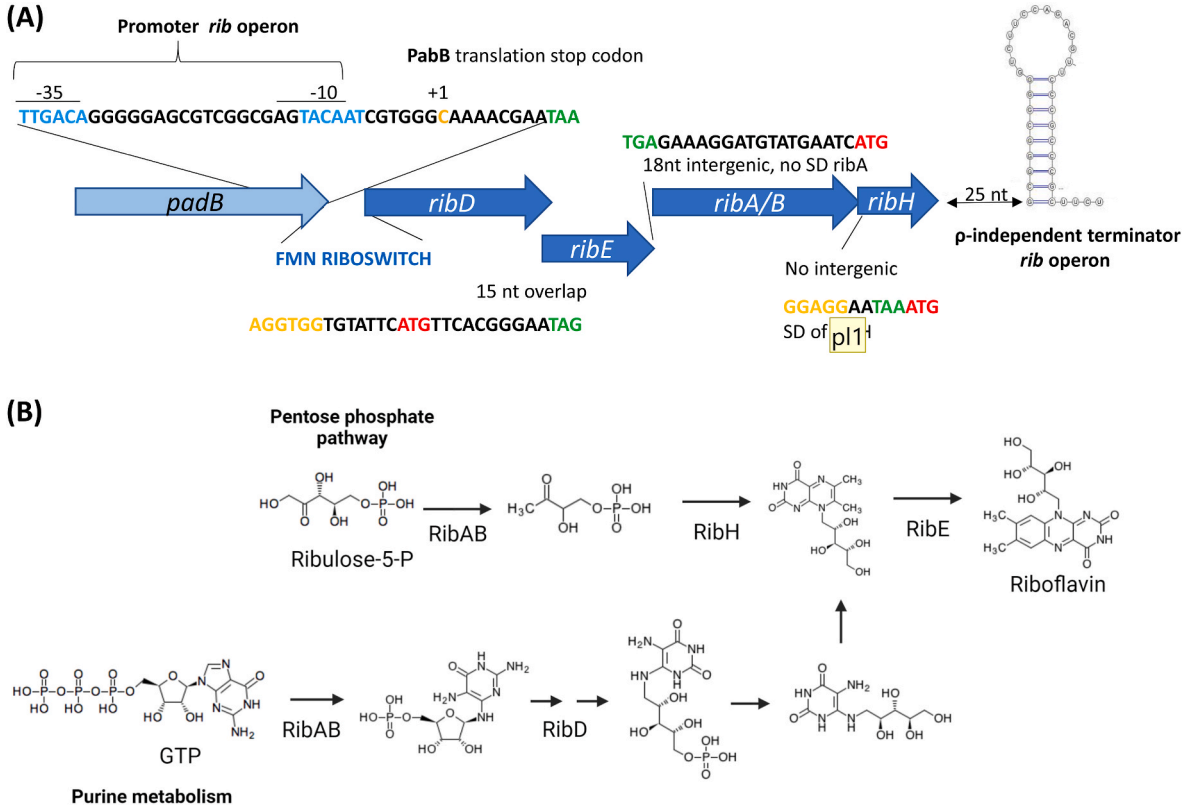

Fig. 1. (**A**) Schematic representation of the *rib* operon in *B. longum* subsp. *infantis*. The location of the *ribD*, *ribE*, *ribAB* and *ribH* genes of the *rib* operon and of *pabB* gene are indicated. The sequence of the putative transcriptional promoter and terminators, as well as the potential start site for transcription (+1) are also depicted. (**B**) Schematic representation of the metabolic routes leading to riboflavin *de novo* synthesis and enzymes involved. Enzymes: **RibD** bifunctional diamino-hydroxy-phospho-ribosyl-aminopyrimidine deaminase [EC:3.5.4.26]/5-amino-6-(5-phosphoribosylamino)uracil reductase [EC:1.1.1.193]; **RibE** riboflavin synthase [EC:2.5.1.9]; **RibAB** bifunctional GTP cyclohydrolase II [EC:3.5.4.25]/3,4-dihydroxy-2-butanone 4-phosphate synthase [EC:4.1.99.12]; **RibH** 6,7-dimethyl-8-ribityl-lumazine synthase [EC:2.5.1.78 (Solopova et al., 2020).

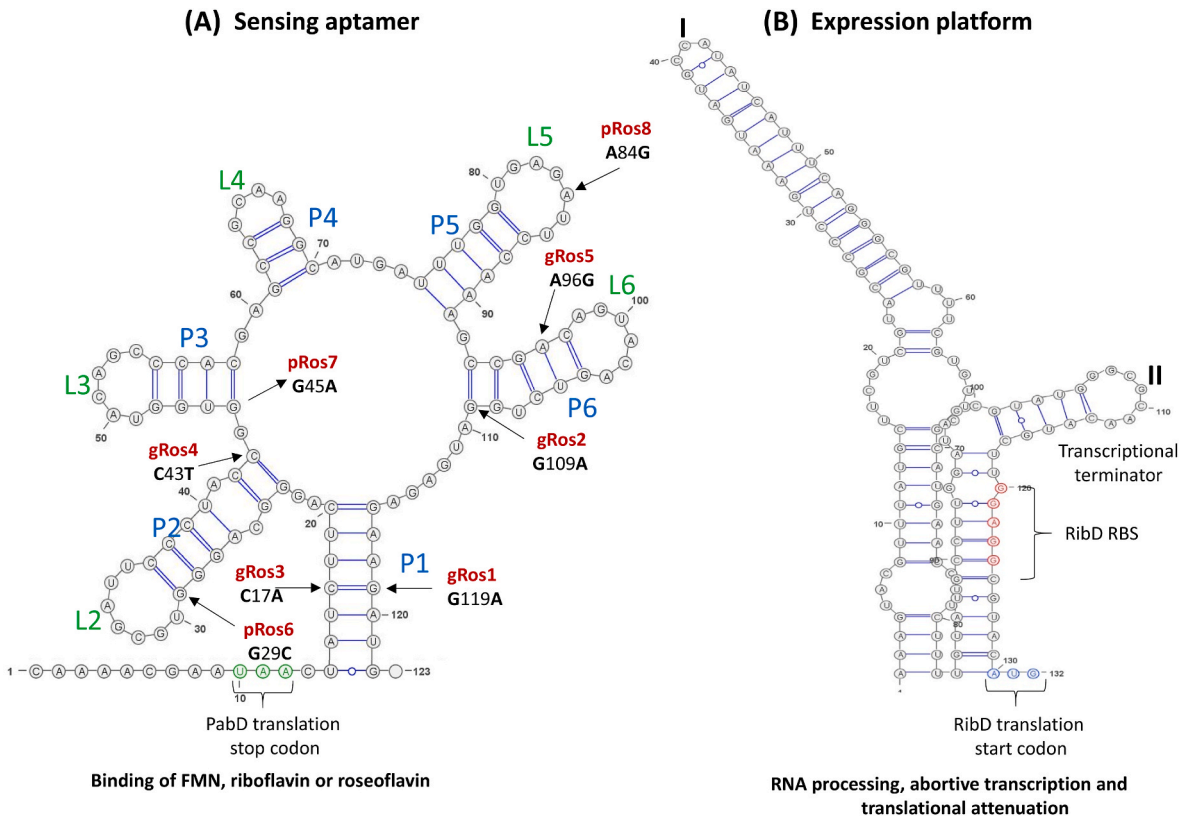

Fig. 2. Folding predicted by the Fold RNA program of the two domains of the FMN riboswitch in *Bifidobacterium longum* subsp. *infantis* CECT4551^T and IPLA60011: (**A**) the FMN sensing domain and (**B**) the adjacent expression platform. In the FMN sensing aptamer the location and changes detected (indicated with arrows) in the riboflavin-overproducing clones are depicted (see Table S1 for additional information). The nucleotides were numbered considering that number 1 is the +1 of the *rib* transcript. The aptamer is composed of a pairing region of its 5′-and 3′-ends (P1) and five stem-loop structures (from P2/L2 to P6/L6).

performed in this work (Fig. 2B) predicted that the regulation of the *rib* operon expression is more complex in this bacterium. The riboswitch contains the sensing aptamer for binding of the FMN at is 3′-end followed by an expression platform composed of two complex secondary structures (I and II). Both structures are constituted by one stem with internal loops that could decrease the efficiency of transcription and even provoke transcription termination as well as they could be the target for endoribonucleases. In addition, the ribosomal binding site (RBS) of the *ribD* gene (the first of the operon) contains a GGAGGA sequence complementary to the 3′-end of the 16S ribosomal RNA, and with a spacing of 5 nt to the translation start codon AUG, optimal for binding of the ribosomes in *B. longum* (Jianlong et al., 2012). This RBS is buried in the stem of the structure II and its formation could impair translation of the *ribD* gene. This location is unusual in the FMN riboswitches. However, in the expression platform of the *Escherichia coli* FMN *ribB* riboswitch there exists a secondary structure similar to the *B. longum* subsp. *infantis* structure II (Fig. 2B), and it has been proposed that it is a hybrid transcription terminator/RBS sequester stem-loop structure (Pedrolli et al., 2015). However, in *E. coli* FMN *ribB* riboswitch the existence of structure I is not predicted. Therefore, it seems that *B. logum rib ribDEAH* FMN riboswitch is an example of a new very complex mechanism of post-transcriptional regulation probably including in addition to abortive transcriptional termination, RNA processing and translational attenuation of *ribD* expression.

3.2. Isolation of riboflavin-overproducing strains

The parental *B. longum* subsp. *infantis* CECT4551^T and IPLA60011 strains were used to select spontaneous riboflavin-overproducing strains by treatment with roseoflavin, a toxic analog of vitamin B_2, which can bind to the aptamer of the FMN riboswitch. This strategy has been previously used among others for the selection of mutants of *B. subtilis* (Perkins et al., 1990) and different lactic acid bacteria (Burgess et al., 2006; Russo et al., 2014; Hernández-Alcántara et al., 2022; Diez-Ozaeta et al., 2023) as well as of bifidobacteria (Solopova et al., 2020). Thus, after exposure to increasing concentrations of roseoflavin using a medium lacking riboflavin (RAMc), a collection of ten clones able to survive this toxic compound was obtained, seven derived from strain CECT4551^T and three from IPLA60011 (Table 2). The *ribDEABH* operon of these clones was sequenced and, in all cases different punctual nucleotide changes were detected in the aptamer of the FMN riboswitch (Fig. 2A–Table S1). It is worth noting that two pairs of clones had the same mutation located at the same position, being the reason why the number of clones with different single mutations was reduced from ten

Table 2
Roseoflavin-resistant clones obtained in this study from the strains *B. longum* subsp. *infantis* CECT4551^T and IPLA60011. The highest roseoflavin concentration tolerated by each clone is indicated. All mutations were placed in the riboswitch region of the operon and the location showed in the table was that of the *B. longum* subsp. *infantis* ATCC15697^T genome (NCIB accession NC_011593.1). Sequences of the *rib* operon can be checked in Table S1.

Parental strains	Clones	Roseoflavin (mg/L)	Mutations
CECT4551^T	gRos1	50	**G446526A**
	gRos21	50	**G446516A**
	gRos22	30	
	gRos3	50	**C446424A**
	gRos41 (IPLA60015)	30	**C446450T**
	gRos42	30	
	gRos5	50	**A446503G**
IPLA60011	pRos6	20	**G446436C**
	pRos7 (IPLA60012)	20	**G446452A**
	pRos8	20	**A446491G**

to eight for further analysis. The aptamer is composed of a pairing region of its 5′-and 3′-ends (P1) and five stem-loop structures (including P2/L2 to P6/L6), and the mutations were located as follows: C17A and G119A of gRos3 and gRos1 mutants in P1; G29C of pRos6 in P2; C43T and G45A of gRos4 and pRos7 in the base of P2 and P3, respectively; A84G of pRos8 at L5; A96G and G109A of gRos5 and gRos2, both in P6.

Upon the same type of selection as performed here, mutations in the FMN aptamer have been detected in the isolated riboflavin-overproducing strains in bacteria belonging to various genera. In addition, in the case of *W. cibaria*, sequencing of the entire genome of the highest riboflavin-overproducing mutant BAL3C-5 C120T and its parental BAL3C-5 revealed that indeed they differ only in a single punctual mutation at the FMN riboswitch (Diez-Ozaeta et al., 2023). Therefore, the single mutations detected in the bifidobacteria should be involved in the deregulation of the riboflavin pathway synthesis in the roseoflavin-derived clones obtained from both *B. longum* subsp. *infantis* parental strains (Fig. 2). Nonetheless, it should be noted that among the roseoflavin-mutants of bacteria able to overproduce riboflavin described in literature other modifications, in addition to those of the riboswitch region, have been described. This is the case of one identical punctual mutation found in the *ribC* gene of the mutants *B. subtilis* RB50 and RB52 (Coquard et al., 1997); however, the mechanism of riboflavin overproduction in these last mutants is not clear.

3.3. *In vivo and in silico characterization of riboflavin production of the overproducing strains*

To demonstrate if the mutations observed in our clones effectively modify the capability of bifidobacteria to produce this vitamin, the concentration of riboflavin in the supernatants of RAMc-grown cultures of the eight selected bifidobacterial clones and their parental strains was quantified by HPLC (Fig. 3A). High variability among clones was observed, but all of them produced more vitamins than the corresponding parental strain, with differences being statistically significant. In general, *B. longum* subsp. *infantis* clones derived from the parental CECT4551^T strain released higher concentrations of the vitamin to the culture medium than those derived from IPLA60011. In order to check whether this overproducing phenotype was conserved in the presence of the vitamin, six clones producing more than 150 μg/L of riboflavin in RAMc were selected to obtain cultures in MRSc, a medium having a high concentration of this vitamin. After quantification by HPLC, no statistical differences were detected in the concentration of produced riboflavin between both media, for each of the six clones analyzed, which could support the stability of the riboflavin-overproducing phenotype (Fig. 3B). Following a similar approach, several riboflavin-overproducing strains, capable of releasing up to 10 times more riboflavin than the parental one, were obtained from different species, such *as Lc. lactis* (Burgess et al., 2004), *L. (Limosilactobacillus) fermentum* (Russo et al., 2014), *W. cibaria* (Diez-Ozaeta et al., 2023) or *L. plantarum* this last species being one of the most successful (Capozzi et al., 2011; Juárez del Valle et al., 2014; Yépez et al., 2019; Kim et al., 2021). Of note is that several of these riboflavin-overproducing strains have been used for the biofortification of different food matrices. Regarding bifidobacteria, Solopova et al. (2020) have described the obtention of a riboflavin-overproducing strain derived from *B. longum* subsp. *infantis* ATCC15697^T that was able to release (on average) 60.8 μg/L of vitamin quantified using a bioassay with *Lacticaseibacillus rhamnosus* ATCC7469 as the test microorganism. In our case, the average concentration released by the eight clones obtained from both parental strains was 248.4 μg/L (range 64.9–441.2 μg/L), as quantified by HPLC.

Also, the correlation of riboflavin production with theoretical consequences of FMN riboswitch changes in the mutants was investigated by bioinformatic *in silico* analysis. The RNA fold program was used to predict the folding of the aptamer (Fig. 4A) and the entire FMN riboswitch (Fig. 4B) of the mutants and that of the wild-type parental strains. In addition, provided that the different bifidobacteria cultures grown in

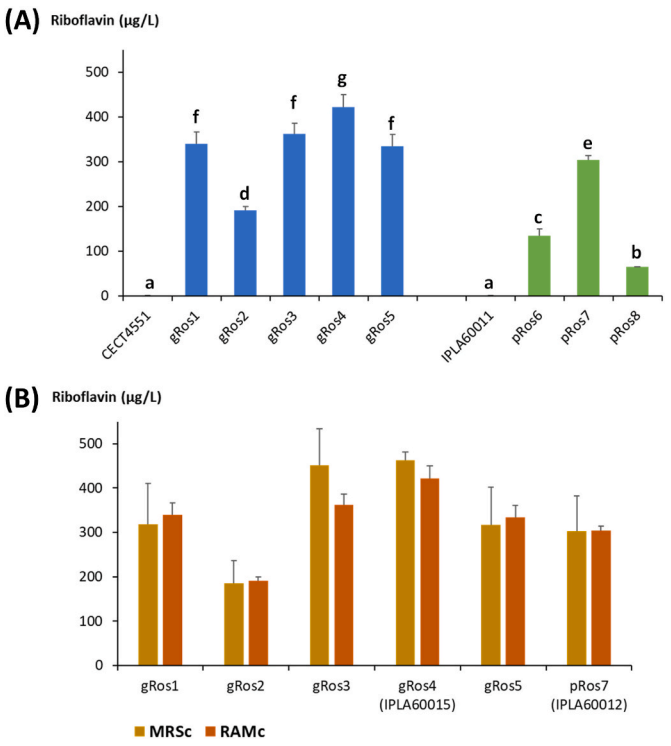

Fig. 3. **(A)** Production of riboflavin (μg/L) in RAMc medium by different clones of *B. longum* subsp. *infantis* obtained from two parental strains (CECT4551^T and IPLA60011) after exposure to increasing concentrations of roseoflavin. Statistical differences among clones and parental bacteria were determined by one-way ANOVA, followed by the SNK (Student-Newman-Keuls) mean comparison test ($p < 0.05$). Clones that does not share a common superscript are statically different. **(B)** Production of riboflavin (μg/L) in MRSc by six clones of *B. longum* subsp. *infantis* obtained from two parental strains (CECT4551^T and IPLA60011) able to produce more than 150 μg/L of the vitamin in RAMc. Concentrations in MRSc were those obtained after subtracting the concentration of vitamin present in the uncultured medium (1.08 ± 0.02 mg/L). For each clone or parental strains, the statistical differences between the two culture-media were determined by one-way ANOVA.

RAMc showed different values of CFU/L (1.5–3.2 x 10^{11}), Fig. 4A also depicts the levels of riboflavin production of the different strains corrected for these variations. These corrections revealed that the levels of riboflavin in the culture supernatants of the mutants varied from 43 μg 10^{-11} CFU, for pRos8, to 150 μg 10^{-11} CFU, for pRos7. The bioinformatic analysis showed that both parental strains presented the same kind of folding in agreement with the detection of similarly low levels of extracellular riboflavin (0.16 μg 10^{-11} CFU and 0.33 μg 10^{-11} CFU for CECT4551^T and IPLA60011 strains, respectively). Changes were observed in the folding of the aptamers of all mutants, besides that of pRos8, which showed the lower overproduction of extracellular riboflavin. In addition, the RNA fold program was also used to calculate the Gibbs free energy (ΔG) required for the aptamer domain formation and the predicted values are depicted in Fig. 4A. Structures are more stable when the thermodynamic energy is lower. All the mutant aptamers showed higher ΔG (from-33.30 to −21.30 kCal/mol) than the wild-type domain (−33.81 kCal/mol), beside the aptamer of pRos8, which presented a slightly lower ΔG (−33.95 kCal/mol) than its parental. Furthermore, the FMN riboswitch of pRos8 retained the structures I and II of the parental expression platform (Fig. 4B). Therefore, the increased overexpression of riboflavin by this mutant seems to be directly connected to its A84G mutation located at L5, since the A residue is conserved among the bacterial FMN riboswitches (Diez-Ozaeta et al., 2023) and presumably involved in the binding of FMN to the aptamer according to the crystallographic study of the FMN riboswitch of

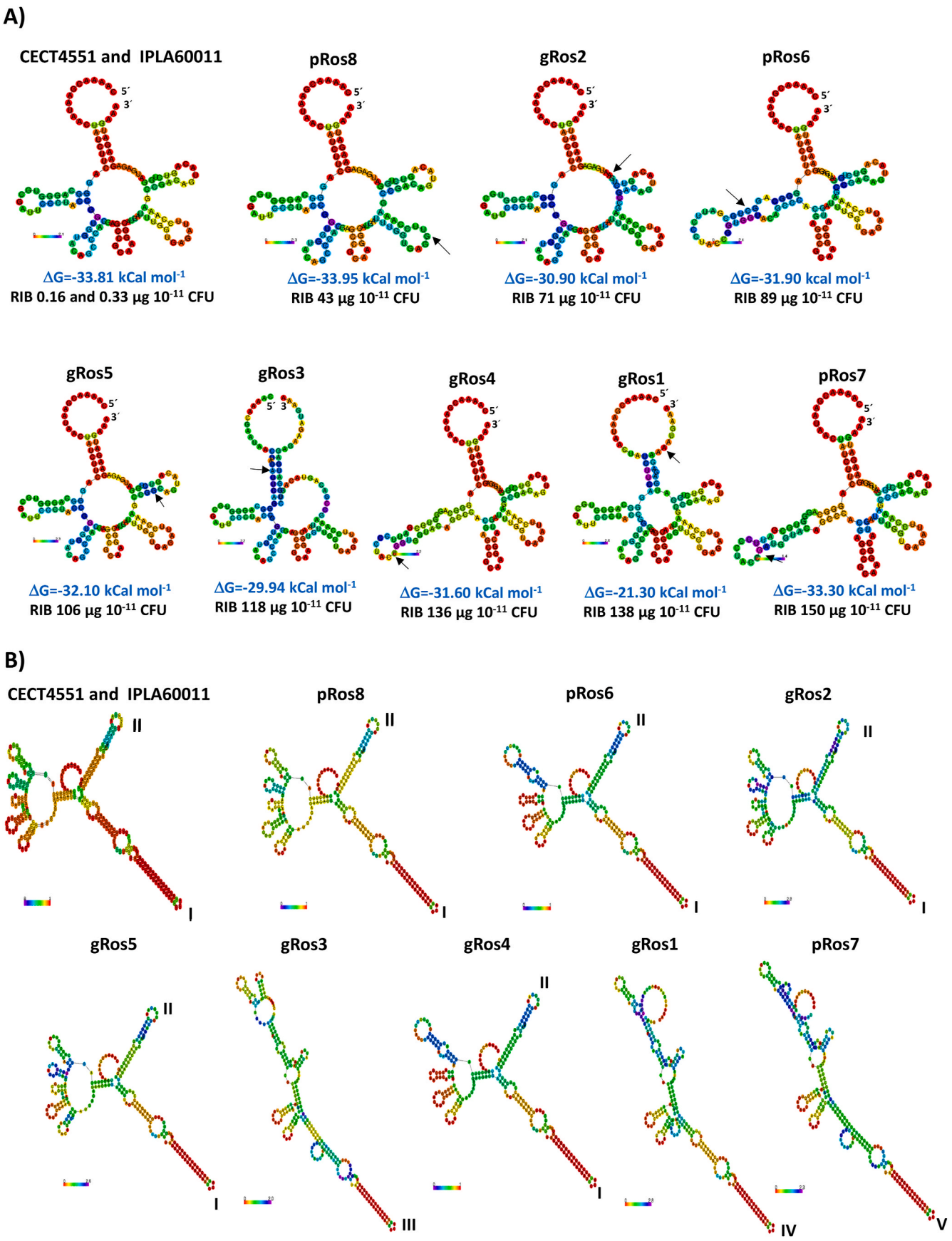

Fig. 4. **(A)** Predicted folding of the FMN riboswitch aptamer of the parental and of the mutant strains. The Gibbs free energy (ΔG) of each secondary structure and the location of each mutation (arrows) are indicated. Also, the riboflavin (RIB) produced by 10^{11} cfu of the parental and mutant strains is depicted. (**B**) Predicted folding of the entire FMN riboswitch of the parental and of all the detected mutant strains.

Fusobacterium nucleatum (Serganov et al., 2009). A similar situation seems to take place in the gRos5 mutant, as no changes were observed in the folding of the domains of the riboswitch (Fig. 4A), only the mutation A96G at P6 that resulted in a change of the pairing A96 with U105 in the wild type by a pairing G96 with U105 in the mutant. The A residue is also conserved in the FMN riboswitches, but in the case of gRos5 the levels of riboflavin-overproduction were significantly higher than that of pRos8 (106 μg 10^{-11} CFU *vs*. 43 μg 10^{-11} CFU, respectively). Thus, probably the increased ΔG (−32.10 kCal/mol) for aptamer formation, together with the lack of a key nucleotide for formation of the FMN-aptamer complex, are responsible for the higher riboflavin production of gRos5. In the case of gRos2, pRos6 and gRos4 their mutations (G109A, G29C, and C43T) provoked changes in the folding of the aptamer and did not influence the formation of structures I and II in the expression platform (Fig. 4B), but changes in the folding of the aptamer were observed (Fig. 4A). For gRos2 the length of P6 was reduced and the ΔG (−30.90 kCal/mol) of the aptamer domain increased generating a riboflavin production of 71 μg 10^{-11} CFU. For pRos6 and gRos4 with a similar ΔG of (−31.90 and −31.60 kCal/mol, respectively) the P2/L2 and the P3/L3 structures were fused, generating a new longer stem-loop secondary structure and producing different levels of riboflavin (89 μg 10^{-11} CFU *vs*. 136 μg 10^{-11} CFU). Considering that in the tertiary structure of the FMN-aptamer complex described for *F. nucleatum*, the P2/L2 and P3/L3 interact respectively with P6/L6 and P5/L5 (Serganov, 2009), the structural changes produced by the punctual mutations in the gRos2, pRos6 and gRos4 probably should impair the formation of the complex at various extends. Finally, in the case of gRos3, gRos1 and pRos7 their mutations C17A, G119A and G45A are located at P1, in the two first, and at P3 in the third one (Fig. 4A). In these mutants, the structure II, which is a sequester of the SD of *ribD* gene, is not formed and alternative structures were detected that should not interfere drastically with the initiation of translation of *ribD* (Fig. 4B). Thus, gRos3 and gRos1 (whose mutations abolish the pairing C17 to G119) synthesize 118 μg 10^{-11} CFU and 138 μg 10^{-11} CFU of riboflavin, pRos7 reaching values of 150 μg 10^{-11} CFU. In these three mutants the high levels of riboflavin could be connected with increased translation of *ribD*. In addition, the slightly higher value for pRos7 could be connected with the formation in its aptamer of a fused P2/L2 and P3/L3, which should drastically affect the formation of the FMN-aptamer complex like in pRos6 and gRos4. Finally, it should be stated that in the folding of the expression platform of all mutants the terminal stem-loop secondary structure I remains present, which could be the target of RNase III type endoribonucleases and which could be involved in post-transcriptional regulation by RNA processing affecting the half-life of the *rib* mRNA.

3.4. Metabolic potential and antibiotic resistance of riboflavin-overproducing mutants

As far as we know, until now, there are no references about the use of riboflavin-overproducer bifidobacterial strains for the biofortification of food matrices. Therefore, two of the riboflavin-overproducing strains generated were selected for further characterization in order to determine some characteristics of interest before their inclusion into food matrixes, As selection *criterium* the best producers from each parental strain were chosen (Fig. 3A): gRos4 (designated hereafter as IPLA60015) was able to release 1000-fold more vitamin B_2 than its parental CECT4551^T strain (441.3 ± 27.8 *vs*. 0.4 ± 0.1 μg/L, respectively), and pRos7 (designated hereafter as IPLA60012) was able to release almost 400-fold more than its parental IPLA60011 strain (295.5 ± 9.7 *vs*. 0.8 ± 0.1 μg/L, respectively). However, the levels of vitamin produced by our bifidobacteria are about 10-times lower than some lactic acid bacteria strains reported in literature.

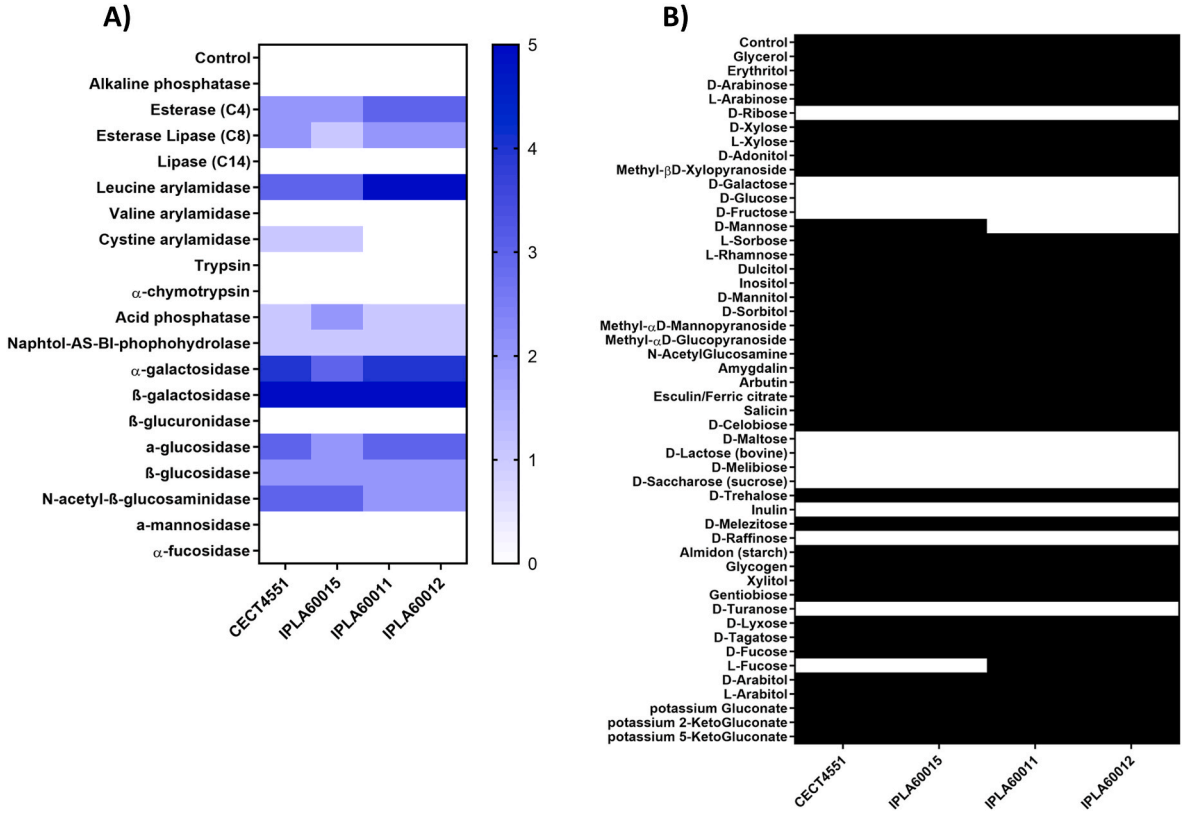

Fig. 5. Characterization of the two riboflavin-overproducing *B. longum* subsp. *infantis* strains, IPLA60012 and IPLA60015, obtained in this study. (**A**) Enzymatic profile determined by API-zym tests; blue color grading indicates variation in the substrate to product conversion from 0 (no conversion, white) to 5 (maximum, dark-blue). (**B**) Carbohydrate fermentation capabilities analyzed by API-CH50; black means no carbohydrate utilization and white indicates acid production related to a positive carbohydrate fermentation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The enzymatic profile and carbohydrate fermentation capabilities of the selected clones, in comparison with the respective parental strains, were analyzed using standardized colorimetric API-zym and API-CH50 tests (Fig. 5). Regarding enzymatic activities, the most noticeable feature is the strong β-galactosidase activity displayed by the four strains suggesting that milk is a good substrate for their growth and, therefore, a candidate matrix for the production of riboflavin-fortified products (Fig. 5A). This could be related to the fact that *B. longum* subsp. *infantis* is highly specialized in the consumption of human milk oligosaccharides present in breast milk (Blanco et al., 2020), where lactose is also highly abundant (around 7 %; Andreas et al., 2015; Cheema et al., 2021) in comparison with other species such as cow's milk (around 5 %; Ivory et al., 2021). On the contrary, other bifidobacterial species showed a different enzymatic profile suggesting a better adaptation to other environments, such as the case of *Bifidobacterium adolescentis* which is associated with human adult gut microbiota (Tamés et al., 2023). Nonetheless, the four *B. longum* subsp. *infantis* strains also presented high activity for other carbohydrate hydrolases (Fig. 5A), as could be expected for a bifidobacterial species, and, in general, no strong differences were detected among strains, suggesting that the generation of the riboflavin-overproducing phenotype did not affect other phenotypic traits of these strains. In agreement with the enzymatic profile, lactose was one of the carbohydrates that was metabolized by the four stains (Fig. 5B). The only difference detected for each parental-clone couple was the inability to use L-mannose, in the case of CECT4551^T and IPLA60015 couple, or L-fucose in the couple IPLA60011 and IPLA60012, which seems not to be related to the riboflavin-overproducing phenotype.

Finally, the antibiotic susceptibility pattern of the four strains intended for food applications was also tested (Table 3). All strains were sensitive to most of the antibiotics evaluated, without remarkable differences among strains. The only exception was streptomycin for which all strains showed resistance at the highest concentration tested (256 μg/mL). In this regard, it has been previously reported that some bifidobacterial species, including *B. longum*, have high resistance to this antibiotic (Ammor et al., 2007; Wei et al., 2012). This resistance seems to be linked to a single mutation (A128G) in the *rpsL* gene coding for ribosomal protein S12 that is present in some strains, such as the case of *Bifidobacterium breve* Yakult widely used for human consumption (Kiwaki and Sato, 2009). In our case, the presence of the mutation was *in silico* confirmed in the ATCC15697^T strain. Since no SNP was detected in the parental CECT4551^T and IPLA60011 strains in this locus, after comparison with this type-strain, we assume that the intrinsic streptomycin resistance in the four strains was due to this mutation and therefore there is no risk of resistance transference. Altogether, these results show that the riboflavin-overproducing *B. longum* subsp. *infantis* obtained in this work can be used as probiotics. In this regard, another potential concern could be related to the impact of these strains on the intestinal microbiota. A study has demonstrated that no major shifts in the intestinal microbiome were observed after 2 weeks of riboflavin supplementation, but an increase in the production of the beneficial short-chain fatty acid butyrate (Liu et al., 2023). Besides, no major effects on the microbiome structure following the administration of a riboflavin-overproducing *Limosilactobacillus reuteri* strain were observed in an *in vitro* model (Spacova et al., 2022). Hence, in principle, a negative impact on the microbiome due to riboflavin overproduction to the levels reached by the strains reported in this work would not be expected.

4. Conclusions

A collection of riboflavin-overproducing *B. longum* subsp. *infantis* strains was selected from the parental CECT4551^T and IPLA60011 ones following adaptation to increasing concentrations of roseoflavin. The *in silico* analysis of potential regulatory mechanisms involved in the synthesis of the vitamin showed that single nucleotide mutations affected different stem-loop structures in the aptamer region of the FMN riboswitch. Additionally, conformational modifications in the *rib* expression platform in some of the clones were also observed, suggesting different molecular mechanisms involved in riboflavin overproduction. The further characterisation of the riboflavin-overproducing *B. longum* subsp. *infantis* IPLA60012 and IPLA60015 strains showed an absence of transmissible antibiotic resistance which allows their application for human consumption as potential probiotics. Besides, their carbohydrate fermentation profile and enzymatic capabilities point to milk as the most suitable matrix for the production of natural, vitamin B_2-enriched, fermented products. In fact, milk and dairy products are already one of the main sources of this vitamin for consumers with a Western dietary pattern. Future work is required to investigate whether the riboflavin-producing strains can be used for the biofortification of foods as well as to explore the *in vivo* probiotic potential towards, among other parameters, evaluating their ability to modulate the gut microbiota and host health.

Table 3
Antibiotic susceptibility pattern of two riboflavin-overproducing *B. longum* subsp. *infantis* strains, IPLA60012 and IPLA60015, obtained in this study, and their parental strains, IPLA60011 and CECT4551^T, respectively. Numbers indicate minimum inhibitory concentration (MIC) of each strain to each antibiotic according to EFSA cut-off values for genus *Bifidobacterium* (EFSA Panel on Additives and Products or Substances used in Animal Feed, 2018). Superindex "S" is shown when strain is sensitive and "R" when strain is resistant.

MIC (μg/mL)	CECT4551^T	IPLA60015	IPLA60011	IPLA60012
Gentamycin	16^S	8^S	32^S	8^S
Streptomycin	$>256^R$	$>256^R$	$>256^R$	$>256^R$
Tetracycline	2^S	2^S	4^S	4^S
Erythromycin	0.25^S	0.25^S	0.25^S	0.25^S
Clindamycin	0.063^S	0.063^S	0.25^S	0.032^S
Chloramphenicol	1^S	1^S	1^S	1^S
Ampicillin	0.063^S	0.032^S	0.125^S	0.5^S
Vancomycin	0.25^S	0.25^S	0.5^S	0.5^S

CRediT authorship contribution statement

Héctor Tamés: Writing – review & editing, Writing – original draft, Investigation. **Isabel Cuesta:** Writing – review & editing, Writing – original draft, Methodology. **Xenia Vázquez:** Formal analysis. **Paloma López:** Writing – review & editing, Writing – original draft, Conceptualization. **Lorena Ruiz:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Patricia Ruas-Madiedo:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2025.104799.

Data availability

Data will be made available on request.

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