LWT - Food Science and Technology 119 (2020) 108871

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Soymilk bio-enrichment by indigenously isolated riboflavin-producing strains of *Lactobacillus plantarum*

Bharat Bhushan[a], Kumkum C.R.[a], Mamta Kumari[a], Jayesh J. Ahire[b], Leon M.T. Dicks[b], Vijendra Mishra[a,*]

[a] Department of Basic and Applied Sciences, National Institute of Food Technology, Entrepreneurship and Management, Kundli, Sonipat, 131028, India
[b] Department of Microbiology, Stellenbosch University, Stellenbosch, 7600, South Africa

ARTICLE INFO

Keywords:
Isolation
Lactobacillus plantarum
Riboflavin-production
Soymilk
Bio-enrichment

ABSTRACT

Lactobacilli (n = 68) isolated from human feces and fermented milk products were screened for the production of riboflavin (vitamin B_2) by culturing into riboflavin assay medium (RAM). Cell-free culture supernatants from positive isolates (BBC32A, BBC32B, BBC33 and BIF43) were transferred onto RAM agar pre-seeded with *Enterococcus faecalis* MTCC2729 (a riboflavin auxotroph). The enhanced growth of B2-auxotroph confirmed the bioavailability of produced vitamin. Isolate BBC32B produced the highest riboflavin (319 ± 36 μg/l), followed by BBC33 (304 ± 91 μg/l), BBC32A (276 ± 8 μg/l) and BIF43 (257 ± 91 μg/l). All four isolates contained riboflavin genes *ribG*, *ribB*, *ribA* and *ribH*. Sequencing of DNA fragments amplified from the 16S–23S rRNA intergenic spacer region and areas flanking the 23S rRNA gene grouped these isolates within the species *Lactobacillus plantarum*. Identifications were confirmed by sequencing 1300-bp of amplified *16S rDNA* fragments. Fermentation of soymilk by single cultures of *L. plantarum* BBC32B, BBC33 and BIF43 yielded 49.05%, 38.97% and 35.94% riboflavin enrichment respectively, which is more than 18.75% recorded in literature for *Lactobacillus fermentum* MTCC8711. Maximum levels of riboflavin were obtained within 12 h of fermentation in soymilk. *Lactobacillus plantarum* BBC32B may be used as starter culture for developing of riboflavin-enriched soymilk.

1. Introduction

Riboflavin (vitamin B2) and its two metabolic derivatives, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), play an essential role in energy production, redox potential and cellular growth (Bhushan et al., 2019; Said & Ross, 2014). Prolonged riboflavin deficiency may lead to the development of ulcers and cataracts, cheilosis, glossitis, angular stomatitis, sore throat, anaemia, hyperhomocysteinemia and dementia (Rivlin & Pinto, 2007). Typical subclinical symptoms are characterized by low anti-oxidative and glutathione reductase (GR) activities (Rivlin & Pinto, 2007).

The recommended dietary allowance (RDA) of riboflavin is 1300 μg/day for adults and 500–900 μg/day for children (Food and Nutrition Board, 2008). However, despite the presence of riboflavin in basic foods such as milk, eggs, fruits and vegetables (Juarez del Valle, Laino, Savoy, & LeBlanc, 2014), vitamin B2 deficiency is a worldwide problem and occurs in all age groups (O'Brien et al., 2001; ENNyS, 2007; Burgess, Smid, & van-Sinderen, 2009; Juarez del Valle et al., 2014). According to a survey conducted in Europe, 15% of adults have a riboflavin intake less than the lower reference nutrient intake value of 0.8 mg/d (Mensink et al., 2013). Statistics for adults in India is worse, with a higher percentage suffering from riboflavin deficiency (O'Brien et al., 2001; Shalini et al., 2018; Siddiqi, Uroo, & D'Souza, 2019). It is expected to affect half of adult population (Sivaprasad et al., 2019). More than 70% of preschool children in India consume less than 50% of the riboflavin RDA (INSA, 2011).

Enrichment of fermented food with riboflavin-producing lactic acid bacteria (LAB) may be the answer to prevent riboflavin deficiencies (Capozzi, Russo, Dueñas, López,& Spano, 2012; Chandrasekar, Chamlagain, Kariluoto, Piironen, & Saris, 2017; Juarez del Valle, Laiño, Savoy de-Giori, & LeBlanc, 2014). Selection of such functional strains of LAB from ecological and human samples has always been a challenging task, owing to such properties being strain-specific (LeBlanc et al., 2011). In recent times, indigenously isolated, autochthonous, and functional LAB strains have been suggested to be used for human/animal consumption (Ganguly et al., 2011; Sirichokchatchawan et al., 2018). Thus far, only a few lactic acid bacteria with the ability to produce riboflavin have been described (Juarez del Valle et al., 2014;

* Corresponding author.
E-mail address: vijendramishra.niftem@gmail.com (V. Mishra).

https://doi.org/10.1016/j.lwt.2019.108871
Received 18 September 2019; Received in revised form 20 November 2019; Accepted 21 November 2019
Available online 22 November 2019
0023-6438/

LeBlanc, Burgess, Sesma, de Giori, & van Sinderen, 2005; Russo et al., 2014; Thakur, Tomar, Brahma, & De, 2016).

The composition of a food-base for development of novel functional food product has to be considered carefully (Bhushan, Tomar, & Chauhan, 2017; LeBlanc et al., 2011). Soymilk is lactose-free, high in protein, folate and polyunsaturated fatty acids and is the ideal product to enrich with riboflavin. Soymilk produced by the traditional process contains, in average, following composition; 3.4% protein, 1.8% of lipids, 1.5% of carbohydrate (sucrose, raffinose and stachyose) and 0.5% of ash. It is the only vegetarian food containing all essential amino acids and economically more viable than cow milk (Singh, Vij, Hati, Singh, & Bhushan, 2016). Although the riboflavin content in soymilk is very low in comparison to bovine milk (Juarez del Valle et al., 2014; Odo, 2003), it is sufficient to sustain the growth of LAB (LeBlanc et al., 2011; Salvetti, Celandroni, Ghelardi, Bggiani, & Senesi, 2003) but an increase in riboflavin content pot-fermentation of soymilk is desirable. In this study, we report on riboflavin-producing strains of *L. plantarum* isolated from human feces and the fermented milk products Dahi and Lassi, and report on their ability to curdle and enrich soymilk.

2. Materials and methods

2.1. Chemicals, growth media and reference strains

Chemicals, enzymes and growth media, except riboflavin assay medium (RAM), were from Sigma-Aldrich (St. Louis, Missouri). RAM was from BD Difco (Franklin Lakes, New Jersey, USA). All PCR reagents were from Thermo Fisher Scientific (Waltham, Massachusetts, USA). *Lactobacillus fermentum* MTCC8711 and *Enterococcus faecalis* MTCC2729 were from the Microbial Type Culture Collection, Institute of Microbial Technology (Chandigarh, India) and served as positive and negative controls, respectively, for riboflavin production. *Lactobacillus plantarum* NCDC772 & NCDC771, and *Lactobacillus rhamnosus* BHM11, received from Dr. Sudhir Kumar Tomar (National Dairy Research Institute, Karnal, India), were used as reference strains in genetic studies.

2.2. Isolation of lactobacilli and presumptive screening for riboflavin production

Fecal and fermented milk samples (Dahi and Lassi), collected from the Kundli and Ganaur villages in the Sonipat district of Haryana (India), were homogenized, serially diluted in sterile saline (0.85%, w/v, NaCl) and plated onto de-Man, Rogosa and Sharpe (MRS) agar. Plates were incubated at 37 °C under anaerobic conditions (Anaerocult A, Merck Millipore) for 24–36 h. Colonies were randomly selected and streaked to purity on MRS agar. Pure cultures were gram-stained, their cell morphology studied, and checked for catalase activity using 3.0% (v/v) H_2O_2.

Gram-positive, rod-shaped and catalase-negative isolates were inoculated into 10 ml MRS broth and incubated at 37 °C for 24 h to reach an optical density (OD_{595}) of 0.9–1.0 (9 log cfu). Seven milliliters from each of these cultures was centrifuged (7168 × *g*, 7 min, 4 °C), washed twice in sterile phosphate buffered saline (PBS, pH 7.0) to remove media compounds and re-suspended in 7.0 ml sterile PBS. From each of these cell suspensions, individual tubes with 7.0 ml RAM were inoculated (1.0%, v/v) and incubated at 37 °C for 24 h. The cells were sub-cultured seven-to ten-times in RAM, unlike three to four sub-culturing steps performed by other researchers (Juarez del Valle et al., 2014; Russo et al., 2014). With each sub-culturing, changes in OD_{595} values were recorded. *L. fermentum* MTCC8711, a riboflavin-producing strain, and *E. faecalis* MTCC2729, an auxotroph for riboflavin, were used as controls. Cell growth was calculated using the following equation:

$\Delta\ OD_{595} = OD_{595}\ (T_x) - OD_{595}\ (T_0)$, where T_x = OD reading recorded after a specific time of incubation and T_0 = OD reading recorded immediately after inoculation.

The morphology (cell length and shape) of cells was determined after each sub-culturing, using a standard light microscope, and compared to the morphology of cells in the original culture (Bhushan, Tomar, & Mandal, 2016). Isolates that maintained their cell morphology and showed a consistent, or slight increase in growth when sub-cultured, were selected for further studies and stored at −80 °C in MRS broth supplemented with 20.0% (v/v) glycerol.

2.3. Confirmation of riboflavin production and bioavailability

A microbiological assay (Salvetti et al., 2003) was used to confirm the production and bio-availbilty of riboflavin. Selected isolates were inoculated (1.0%, v/v) into RAM broth, incubated at 37 °C for 24 h and the cells harvested (7168 × *g*, 7 min, 4 °C). Cell-free supernatants (CFS) were filtered through PVDF membrane filters (0.22 μm pore diameter; Merck, KGaA, Darmstadt, Germany) and 5–10 μl of the filtrate spotted onto RAM agar plates seeded with 50–100 μl *E. faecalis* MTCC2729 (OD_{595} = 0.2). *L. fermentum* MTCC8711 was used as positive control. Cell-free supernatants that supported the growth of *E. faecalis* MTCC2729, judged by increased growth at areas where CFS were spotted, were considered to contain riboflavin. Cells from corresponding isolates were selected for HPLC analysis.

2.4. Detection of riboflavin by HPLC

Selected isolates were inoculated (1.0%, v/v) into RAM broth, incubated at 37 °C for 24 h and sub-cultured seven-to ten-times in the same medium. The last culture of each isolate was used to inoculate (1.0%, v/v) 250 ml RAM broth, tightly sealed in screw-capped 250 ml amber-colored fermentation vessels (Borosil, Ahemedabad, India). Incubation was done at 37 °C until cultures reached late logarithmic growth. Samples of 5 ml were collected after 12 h and 18 h, respectively. To each sample 25 ml 0.1 M HCl was added and the cell suspension autoclaved (121 °C for 15 min). After cooling to room temperature, the pH was adjusted to 4.5 with 4 M Na-acetate. To each cell extract 5 ml of an enzyme solution was added, which consisted of papain (12U), acid phosphatase (22U), α-amylase (420U) and glutathione (0.1%, w/v), left at 25 °C for 1 h and then diluted to 50 ml with 0.01 M HCl. The cell debris were harvested (7426 × *g*, 7 min, 4 °C) and the supernatant filtered through PVDF membrane filters (0.2 μm pore size; Merck). Ten microliters of the filtrate was injected into a Waters HPLC (Model 2707, Waters India Pvt. Ltd), equipped with a reverse-phase C18 column (Kromasil, SIGMA, 5μ 100A, 250 × 4.6 mm), an in-line degasser, auto-sampler, binary 515 pump control (module II) and UV detector. Methanol and water (35:65) was used as mobile phase, according to Russo et al. (2014). The flow rate was 1.0 ml/min. Readings were recorded at 268 nm (Rodriguez, Fernandez-Ruiz, Camara, & Sanchez-Mata, 2012). Pure riboflavin (Sigma, St. Louis, Missouri) was used as control.

2.5. Screening for genes involved in riboflavin production

Genomic DNA was isolated according to the method of Pospiech and Neumann (1995), and screened for the presence of genes encoding ribG (pyrimidine deaminase and pyrimidine reductase), ribB (riboflavin synthase), ribA (3,4-dihydroxy 2-butanone 4-phosphate synthase and GTP cyclohydrolase II) and ribH (lumazine synthase). DNA primers used in PCR (Table 1) were designed based on gene sequences reported for *L. plantarum* SRCM102022, as listed in NCBI. Amplified DNA was separated on a 1.5% (w/v) agarose gel in TAE buffer (1x), at 80 V for 50 min. DNA bands were stained with ethidium bromide (0.5 mg/ml) and visualized under UV light. Purification and sequencing of PCR products were performed by Eurofins Scientific (Bangalore, India). Sequences were compared with those in GenBank, using BLAST (http://blast.ncbi.nlm.nih.gov) with DNA homology threshold > 98%. The evolutionary analysis of genes pertaining to 16S rRNA and riboflavin

Table 1
DNA primers and conditions used in the amplification of riboflavin genes, the 16S–23S rRNA intergenic spacer region, area flanking the 23S rRNA gene and 16S rRNA gene of isolates BBC32A, BBC32B, BBC33 and BIF43.

DNA	Target gene	Primers	Amplicon length (bp)	Annealing temperature (°C)	Reference
Genomic	16S–23S rRNA intergenic spacer region (*Lactobacillus* genus identification)	F: CTCAAAACTAAACAAAGTTTC R: CTTGTACACACCGCCCGTCA	250	55	Dubernet, Desmasures, & Gueguen (2002)
Genomic	16S–23S rRNA intergenic spacer region and its flanking 23S rRNAregion (*L. plantarum* specific)	F: ATTCATAGTCTAGTTGGAGGT R: CCTGAACTGAGAGAATTTGA	248	60	Song et al. (2000)
Genomic	16SrRNA gene	F: CAGGCCTAACAC ATGCAA GTC R: GGGCGGWGT GTACAAGGC	1300	55	Marchesi et al. (1998)
Genomic	ribG (Pyrimidine deaminase and Pyrimidine reductase)	F: AGCTGGATCCTACTGGTGAAA R: CAGCGACAGTGCGGTAATC	1261	58	Present study
Genomic	ribB (Riboflavin synthase)	F: GCTGCGATGACACCGTTGAC R: CGCAACAATAATCAATTCGCCCC	781	60	Present study
Genomic	ribA (3,4-dihydroxy 2-butanone 4-phosphate synthase and GTP cyclohydrolase II)	F: ACAAGTTGGAACGCTGGTGA R: CCCAGTTAAAAGTTGCTGCGT	1397	60	Present study
Genomic	ribH (Lumazine synthase)	F: CCTTAGAAATACCAGCCAATCAG R: ACAGAAGCAGTGGCATCAACAA	600	60	Present study

were conducted in comparison to respective published gene sequences of *Lactobacillus* strains in MEGA6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) by using the maximum likelihood (ML) method based on the Tamura-Nei model.

2.6. Identification of riboflavin-producing isolates

Genomic DNA of riboflavin-producing isolates was prepared as described above. The DNA primer-sets used in PCR reactions are listed in Table 1. The *16S rDNA* gene was amplified using the forward and reverse primers of Marchesiet al. (1998). The 16S–23S rRNA intergenic spacer and flanking 23S rRNA regions were amplified using DNA primer sets of Song et al. (2000).

2.7. Soymilk bio-enrichment

Strains that produced riboflavin and contained the genes encoding riboflavin production were cultured in RAM broth, harvested and washed as described above. Cell suspensions were kept in separate test tubes. From each cell suspension, 100 ml freshly prepared soymilk (in duplicate in amber-colored screw-capped vessels) were inoculated with initial cell number of $\sim 10^7$ log cfu per ml. The vessels were incubated at 37 °C for 12 h. Unfermented soymilk and soymilk fermented with *L. fermentum* MTCC8711 were used as negative and as positive controls, respectively. The samples were prepared and analyzed by HPLC as described above. Two fermentations with each strain, performed in separate runs, were subjected to HPLC analysis. All fermentations were performed in triplicate.

2.8. Statistical analysis

Microsoft Office (2007) was used for descriptive representation of data and tabulation. GraphPad Prism (version 5) was used for XY, column and grouped statistics using one and two-way ANOVA equipped with Tukey and Bonferroni's post hoc tests. Differences were considered statistically significant at p-values ($p \leq 0.05$). Phylogenic dendrograms were generated using MEGA-6 software, maximum likelihood (ML) and the Tamura-Nei model with bootstrap (500). Final values were generated from mean values of three individual experiments, unless specified.

3. Results and discussion

3.1. Isolation of lactobacilli and growth in RAM

A total of 150 colonies were randomly selected from MRS agar plates containing processed samples. A total of 68 isolates were Gram-positive, rod-shaped and catalase-negative, and preliminary classified as members of the genus *Lactobacillus*. Only four isolates (BBC32A, BBC32B, BBC33 and BIF43) showed consistent growth, even after seven-to ten-times sub-culturing in RAM broth. This suggested that all four isolates produced riboflavin. Changes in optical density recorded

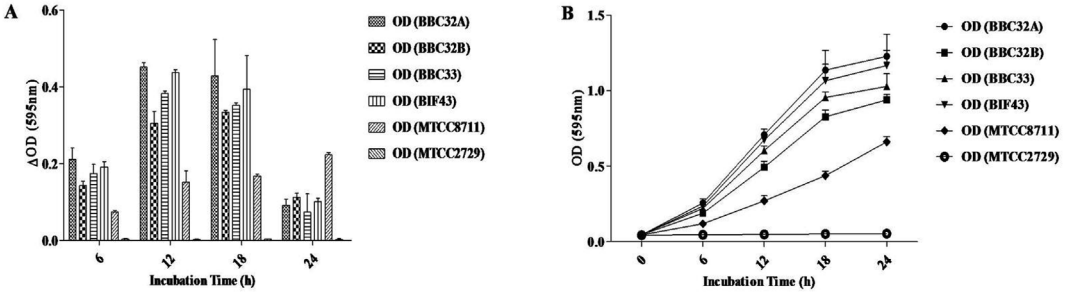

Fig. 1. Growth of isolates BBC32A, BBC32B, BBC33 and BIF43 in RAM broth. (A) Changes in cell density (OD) over 24 h were recorded at 595 nm and (B) resultant generation of growth curves.

for isolates BBC32A, BBC32B, BBC33 and BIF43, grown in RAM broth, is shown in Fig. 1. Highest cell density (OD_{595nm}) readings were recorded for isolate BBC32A, followed by isolates BIF43, BBC33 and BBC32B (Fig. 1). All four isolates grew to cell densities higher than reported for *L. fermentum* MTCC8711 (Fig. 1), suggesting that they were better adapted to grow in riboflavin-deficient medium. Cell morphologies of isolates BBC32A, BBC32B, BBC33 and BIF43 remained unchanged during sub-culturing and during 24 h of growth in RAM broth. Variations in cell shape or size could thus not have contributed to changes in OD readings. *Enterococcus faecalis* MTCC2729 did not grow in RAM broth (Fig. 1), which was expected as the strain requires riboflavin. According to these results, isolates BBC32A, BBC32B, BBC33 and BIF43 were either better adapted to grow in RAM broth than *L. fermentum* MTCC8711, or produced more riboflavin than *L. fermentum* MTCC8711. Similar variations in growth reactions and riboflavin requirements have been reported for strains studied by other researchers (Juarez del Valle et al., 2014; Silva, Biscola, LeBlanc, & de-Melo Franco, 2016). The strains reached early log phase after 6 h and early stationary phase after 18 h in RAM broth, even better than the riboflavin producer *L. fermentum* MTCC8711, which reached early stationary growth after 30 h under the same conditions (Fig. 1).

3.2. Confirmation of riboflavin production and bioavailbility

Verification of riboflavin-production and bioavailability of vitamin produced was confirmed by microbiological assay. Cell-free supernatants of isolates BBC32A, BBC32B, BBC33 and BIF43, and *L. fermentum* MTCC 8711 stimulated the growth of *E. faecalis* MTCC2729 on RAM plates (Fig. 2), whereas cell-free supernatants from one of non-riboflavin producer isolate (isolate no. 68) and the auxotroph itself (MTCC 2729) did not stimulate growth (Fig. 2). According to these findings, isolates BBC32A, BBC32B, BBC33 and BIF43 produced riboflavin in RAM medium. Even though the method has been used in quantitative analyses of riboflavin production (Salvetti, Celandroni, Ghelardi, Baggiani, & Senesi, 2003; Silva et al., 2016), we used it for qualitative confirmation of vitamin B2 production.

3.3. Detection of riboflavin by HPLC

Separation of the CFSs of isolate BBC32A produced peaks corresponding to peaks recorded with pure riboflavin (Fig. 3). Peaks identical to that recorded for isolate BBC32A were obtained for CFSs of isolates BBC32B, BBC33 and BIF43 and are not shown. The linear standard curve with a high R^2 value (0.999), obtained when pure riboflavin was separated under the same conditions, supported the accuracy of the HPLC method. Isolate BBC32B produced the highest level of riboflavin (319 ± 36 μg/l), followed by isolates BBC33 (304 ± 91 μg/l), BBC32A (276 ± 8 μg/l) and BIF43 (257 ± 91 μg/l), as listed in Table 2. These differences in riboflavin production were, however, statistically not significant ($p > 0.05$). Nevertheless, compared to riboflavin production of 190–260 μg/l reported for other *Lactobacillus* strains (Juarez del Valle et al., 2014), isolates BBC32A, BBC32B, BBC33 and BIF43 are more superior, but similar to, *L. fermentum* MTCC8711 that produced 259 ± 80 μg/l riboflavin (Table 2). All four isolates produced maximum levels of riboflavin (257 ± 91 to 319 ± 36 μg/l) during the first 12 h of growth in RAM broth, thus during exponential growth (Table 2). These findings differ from previous studies (Juarez del Valle et al., 2014) that reported maximum

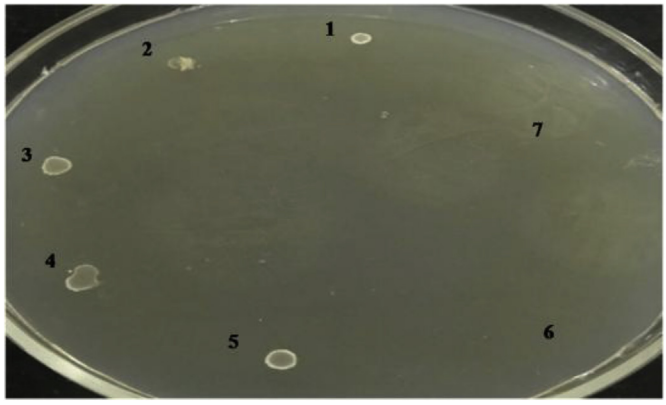

Fig. 2. Growth stimulation of *Enterococcus faecalis* MTCC 2729, a riboflavin auxotroph, on RAM agar by cell-free supernatants of isolates BBC32A (spot 1), BBC32B (spot 2), BBC33 (spot 3) and BIF43 (spot 4), *Lactobacillus fermentum* MTCC8711 (spot 5), isolate 68 – a non-riboflavin producer (spot 6) and *E. faecalis* MTCC 2729 (spot 7).

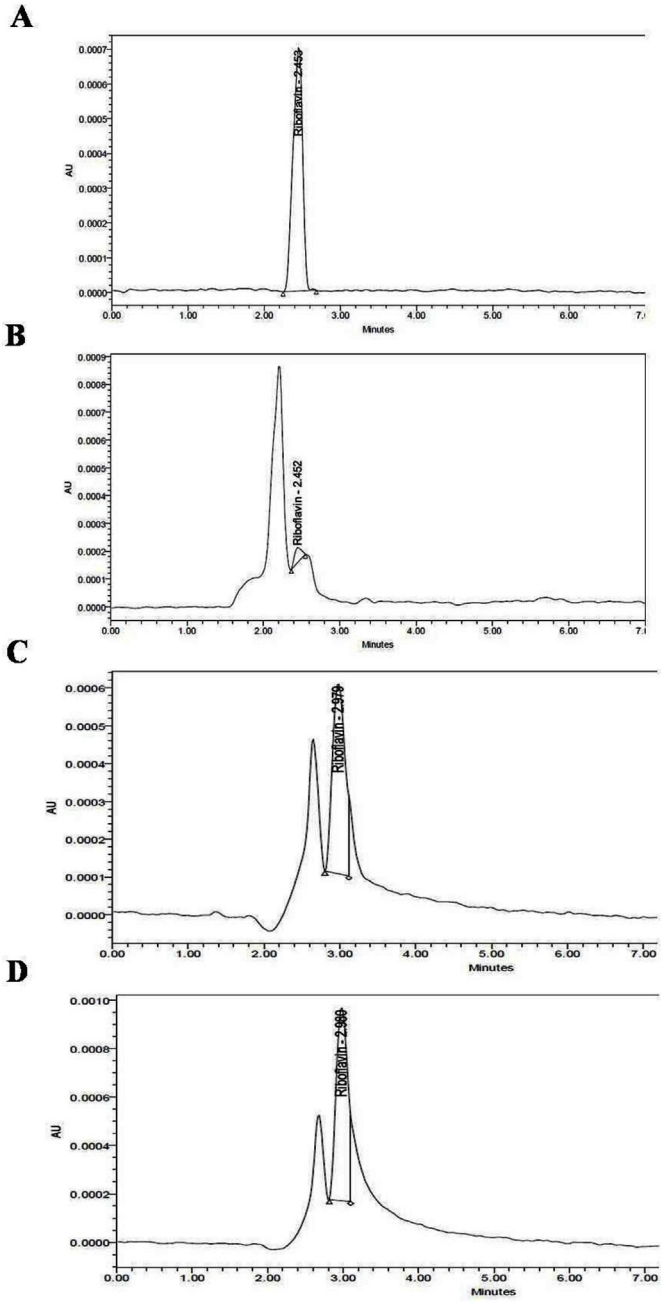

Fig. 3. HPLC separation of (A) commercially pure riboflavin, (B) culture/cell extract of isolate BBC32A, (C) unfermented soymilk (D) and fermented soymilk extract of strain 32B (E). Peaks identical to that shown in (B) were obtained for culture/cell extracts of isolates BBC32B, BBC33 and BIF43 and are not shown.

Table 2
Riboflavin production in RAM and soymilk by *Lactobacillus plantarum* BBC32A, BBC32B, BBC33 and BIF43, and *Lactobacillus fermentum* MTCC8711.

Strain	Isolation source	Riboflavin production (μg/l) in RAM		Riboflavin production (μg/l) in soymilk	
		After 12 h	After 18 h	Final B2 level in soymilk (after 12 h)	Bio-enrichment level (%)
BBC32A	Dahi from buffalo milk	^{a}276 ± 8	^{b}195 ± 0.4	^{A}473 ± 12	1
BBC32B	Dahi from buffalo milk	^{a}319 ± 36	^{b}180 ± 28	^{B}700 ± 31	49
BBC33	Dahi from buffalo milk	^{a}304 ± 91	^{b}186 ± 12	^{BC}652 ± 21	39
BIF43	Feces from 1 year old infant	^{a}257 ± 91	^{b}161 ± 42	^{BC}638 ± 47	36
L. fermentum MTCC8711 (positive std. for B2 production)	Fermented milk	^{a}258 ± 80	^{b}175 ± 36	^{AB}557 ± 46	19

The comparative analysis of riboflavin production (mean ± SD) was analyzed by two-way Anova (Bonferroni's post test) and by one-way Anova (Tukey's post hoc test) in RAM and soymilk samples, respectively. $^{a, b}$ each value without a common letter differ significantly ($P < 0.05$).$^{A, B, C}$ each value without a common letter differ significantly ($P < 0.05$). *BBC*, Bharat Buffalo Curd;*BIF*, Bharat Infant Fecal; *MTCC*, The Microbial Type Culture Collection and Gene Bank; *RAM*, Riboflavin Assay Medium.

riboflavin production during stationary growth. Juarez del Valle et al. (2014) and Yépez et al. (2019) reported riboflavin production as high as 2800 μg/l when mutant strains of *L. plantarum* were grown in RAM broth supplemented with roseoflavin. Aim of present study was to testify the natural fermentation process and did not supplement RAM broth with roseoflavin.

3.4. Detection of genes involved in riboflavin production

Genomic DNA of isolates BBC32A, BBC32B, BBC33 and BIF43, each amplified with primers ribG, ribB, ribA and ribH (Table 1) produced DNA fragments corresponding in size to genes *ribG*, *ribB*, *ribA* and *ribH* (Fig. 4), suggesting that a riboflavin operon is present in each of the four isolates. Strains with an incomplete riboflavin operon do not grow in the absence of riboflavin (Burgess, Smid, Rutten,& van-Sinderen, 2006). It is thus safe to assume that all four of the riboflavin genes were expressed by isolates BBC32A, BBC32B, BBC33 and BIF43. High DNA sequence similarities (99–100%) were recorded between *ribG*, *ribB*, *ribA* and *ribH* from isolates BBC32A, BBC32B, BBC33 and BIF43, respectively, and the riboflavin genes listed for *L. plantarum* in GenBank (Fig. 5). This suggested that the riboflavin genes are most probably conserved.

3.5. Identification of riboflavin-producing isolates

Amplification of the 16S–23S rRNA intergenic spacer region and the area flanking the 23S rRNA gene with species-specific primers generated a 248-bp fragment for each of the isolates (BBC32A, BBC32B, BBC33 and BIF43). These fragments were identical in size to fragments amplified for *L. plantarum* NCDC772 and NCDC771 (Fig. 6A), suggesting that the four isolates are members of *L. plantarum*. No DNA was amplified from the genomic DNA of *L. rhamnosus* BHM11 (Fig. 6A). The identity of the four isolates was confirmed by sequencing 1300 bp of the 16S rRNAgene (Fig. 6B). The phylogenetic relatedness of strains BBC32A, BBC32B, BBC33 and BIF43 to *Lactobacillus* species already reported in NCBI and shown in Fig. S1.

3.6. Bio-enrichment of riboflavin in soymilk

Interestingly, all of the tested lactobacilli could grow in soymilk and curdle it. The peptide- and oligo & disaccharide-utilizing genetic assemblies have been considered responsible for soymilk fermentation potential of lactobacilli. The riboflavin content recorded in unfermented soymilk was 469 ± 8 μg/l (Table 2). After 12 h of fermentation with *L. plantarum* BBC32B, BBC33 and BIF43, riboflavin levels increased to 700 ± 31 μg/l, 652 ± 21 μg/l and 638 ± 47 μg/l, respectively, and were significantly higher ($p < 0.05$ to $p < 0.01$) than in unfermented soymilk or soymilk fermented with *L. fermentum* MTCC8711 (Table 2). Of all strains studied, *L. plantarum* BBC32B produced the most riboflavin, as shown by 49% enrichment (Table 2). Russo et al. (2014) reported a 50% increase in riboflavin production when bread was produced using *L. fermentum*as starter culture. In another study (Juarez del Valle et al., 2014), a strain of *L. plantarum* increased riboflavin levels in fermented soymilk to 660 ± 35 μg/l, which is lower than values obtained in the present study.The riboflavin content in soymilk fermented with *L. plantarum* BBC32A increased only slightly (from 469 ± 8 μg/l to 473 ± 12 μg/l), representing only a 0.74% increase in production (Table 2). The combined extraction procedure (acid and enzymatic digestion), in accordance with previously published reports (Rodriguez, Fernández-Ruiz, Cámara, & Sánchez-Mata, 2012), was used to extract riboflavin. It may be that riboflavin, which is naturally present in soy milk, may repress gene expression which could be strain dependent as observed in case of strain BBC32A, where no *in situ* enrichment in soymilk was recorded. However, further research is required to substantiate this hypothesis.

Final riboflavin level (638 ± 47 μg/l) in BIF43fermented soymilk was lower than recorded for *L. plantarum* BBC32B (700 ± 31 μg/l) and BBC33 (652 ± 21 μg/l). In a similar pattern, BIF43 produced comparatively least riboflavin (257 ± 91 μg/l) in RAM broth. Kneifel, Kaufmann, Fleischer, and Ulberth (1992) have shown a decrease in

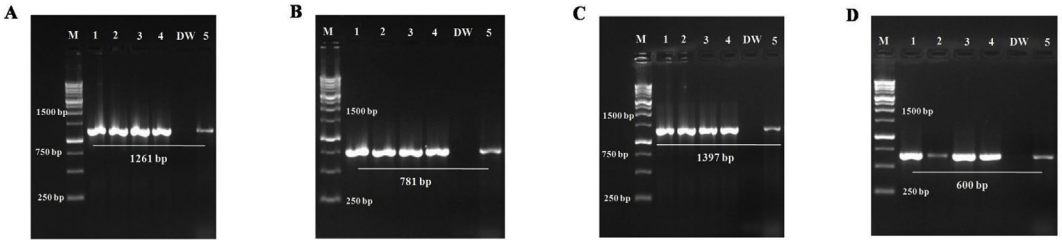

Fig. 4. Molecular characterization of riboflavin-production potential of lactobacilli.
Agarose gel electrophoresis of riboflavin genes ribG (A), ribB (B), ribA (C) and ribH (D) amplified by PCR. Lanes in gels: M = molecular marker, 1 = isolate BBC32A, 2 = BBCF32B, 3 = BBC33, 4 = BIF43, DW = distilled water, 5 = L. fermentum MTCC 8711.

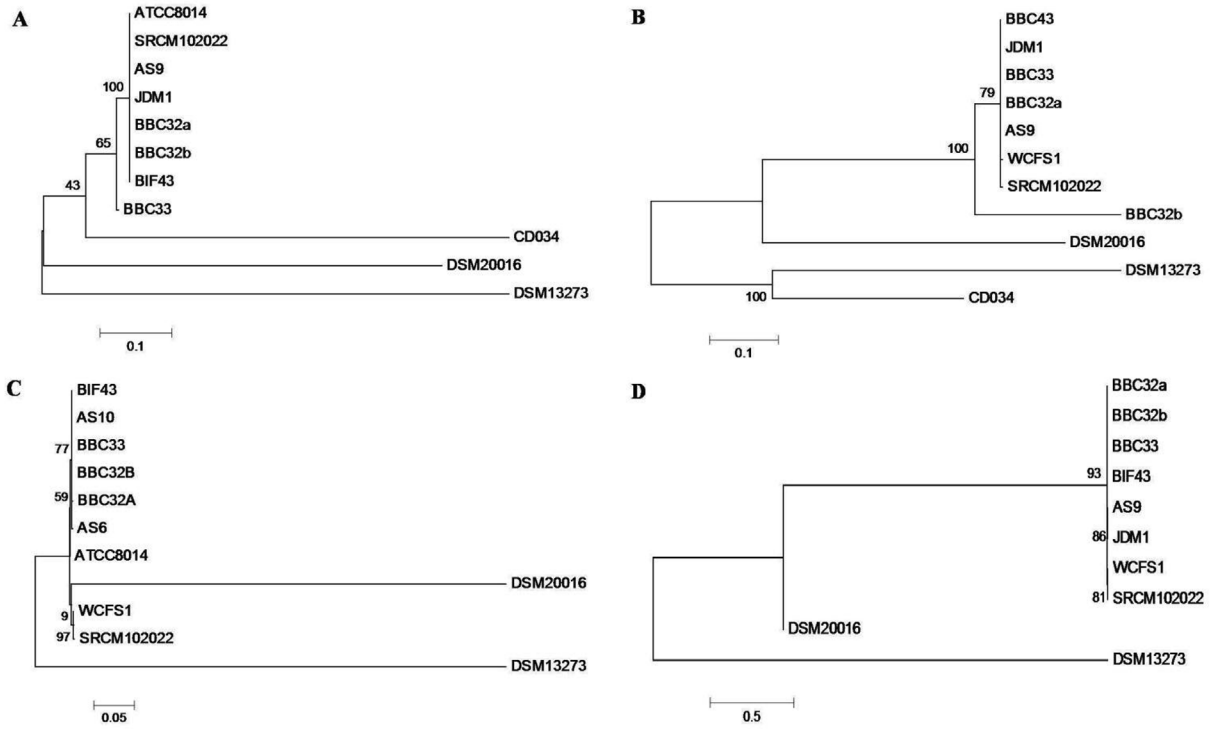

Fig. 5. Phylogenetic relatedness amongst *rib* gene sequences of lactobacilli, as constructed using the maximum likelihood (ML) approach on MEGA software. A: *ribG*, B:*ribB*, C:*ribA* and (D):*ribH*. Trees are arbitrarily rooted with bars (—) showing sequence divergence. Bootstrap values (based on 500 samplings expressed in percentage) are at branch points.

riboflavin production if yogurt is produced with some strains, whilst other strains led to a 60% increase in riboflavin production. Concluded from these findings, nutrients have a major effect on riboflavin production and warrants further research. The product serving size of 200 ml may partially fulfill the daily RDA of children (~19%) and healthy adults (~11%), considering the comparatively higher bioavailability of riboflavin produced post bacterial-fermentation (Hugenholtz & Smid, 2002). This figure is higher than reported by Chandrasekar et al. (2017).

Although not testified in present work, LAB fermentation of legume-based foods (like soymilk) using novel starters has also been known to increase safety (chemical and microbiological) and acceptability (less no. of anti-nutrients) of final product in comparison to traditionally fermented foods (Licandro et al., 2019).

3.7. Conclusions

Although the four strains of *L. plantarum* we have studied are phenotypically closely related and their *rib* genes share a high similarity, riboflavin production recorded for the strains differed significantly. The reason for this is not clear. It may be that the *rib* genes are induced, or even repressed, in some strains of *L. plantarum*. The differences in riboflavin production recorded in RAM broth and soymilk suggests that nutrient composition plays a major role, at least for strains of *L. plantarum*. The significant increase in riboflavin production (49%) when soymilk was fermented with *L. plantarum* BBC32B suggests that the strain could be used as a starter culture. The 700 μg/l riboflavin, that obtained in BBC32B fermented soy-curd, is approximately half of the RDA for adults (1300 μg), but falls within the RDA for children

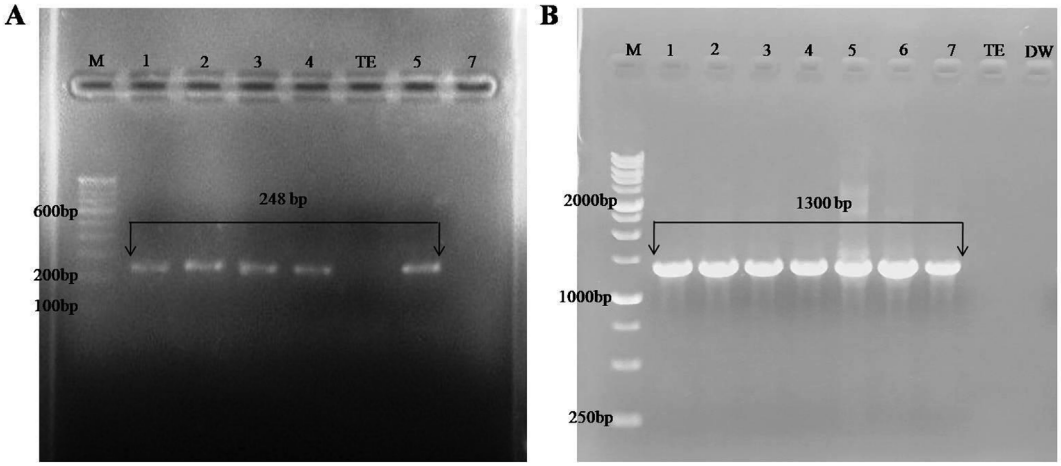

Fig. 6. Agarose gel electrophoresis of DNA amplified from (A) the 16S–23S rRNA intergenic spacer region and area flanking the 23S rRNA gene and (B) 16 S rRNA. Lanes: 1, BBC32A; 2, BBC32B; 3, BBC33; 4, BIF43; 5, NCDC772; 6, NCDC771; 7, BHM11; M, Marker DNA; TE, Tris-EDTA buffer; DW, Distilled Water.

(500–900 μg).

Author contribution statement

Bharat Bhushan: Study conception and design, Acquisition of data, Analysis and interpretation of data, Drafting of manuscript, Critical revision. Kumkum CR: Acquisition of data, Analysis and interpretation of data. Mamta Kumari: Acquisition of data. Jayesh Ahire: Study conception and design, Analysis and interpretation of data, Critical revision. Leon.M.T. Dicks: Analysis and interpretation of data, Critical revision. Vijendra Mishra: Study conception and design, Analysis and interpretation of data, Drafting of manuscript, Critical revision.

Declaration of competing interest

No conflict of interest declared by authors.

Acknowledgements

This work was supported by the Department of Science and Technology, India [grant # DST/INT/South Africa/P-15/2016] under Indo-South African collaboration. Ankur Kumar from the Central Instrumentation Laboratory, NIFTEM, assisted with the HPLC separations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2019.108871.

References

Bhushan, B., Singh, B. P., Saini, K., Kumari, M., Tomar, S. K., & Mishra, V. (2019). Role of microbes, metabolites and effector compounds in host–microbiota interaction: A pharmacological outlook. *Environmental Chemistry Letters,* 1–20.

Bhushan, B., Tomar, S. K., & Chauhan, A. (2017). Techno-functional differentiation of two vitamin B12 producing *Lactobacillus plantarum* strains: An elucidation for diverse future use. *Applied Microbiology and Biotechnology, 101*(2), 697–709.

Bhushan, B., Tomar, S. K., & Mandal, S. (2016). Phenotypic and genotypic screening of human-originated lactobacilli for vitamin B12 production potential: Process validation by micro-assay and UFLC. *Applied Microbiology and Biotechnology, 100*(15), 6791–6803.

Burgess, C., Smid, E., Rutten, G., & van-Sinderen, D. (2006). A general method for selection of riboflavin-overproducing food grade micro-organisms. *Microbial Cell Factories, 5*(1), 24.

Burgess, C. M., Smid, E. J., & van-Sinderen, D. (2009). Bacterial vitamin B2, B11 and B12 overproduction: An overview. *International Journal of Food Microbiology, 133*(1–2), 1–7.

Capozzi, V., Russo, P., Dueñas, M. T., López, P., & Spano, G. (2012). Lactic acid bacteria producing B-group vitamins: A great potential for functional cereals products. *Applied Microbiology and Biotechnology, 96*(6), 1383–1394.

Chandrasekar, R. S. C., Chamlagain, B., Kariluoto, S., Piironen, V., & Saris, P. E. J. (2017). Biofortification of riboflavin and folate in idli batter, based on fermented cereal and pulse, by *Lactococcuslactis* N8 and *Saccharomyces boulardii* SAA655. *Journal of Applied Microbiology, 122*(6), 1663–1671.

Dubernet, S., Desmasures, N., & Guéguen, M. (2002). A PCR-based method for identification of lactobacilli at the genus level. *FEMS Microbiology Letters, 214*(2), 271–275.

ENNyS (2007). *Encuesta nacional de Nutrici_on y Salud. Direcci_on nacional de SaludMaternoInfantil.* Argentina: Ministerio de Salud.

Food and Nutrition Board (2008). Institute of Medicine (US) Standing committee on the scientific evaluation of dietary reference intake and its panel on folate, other B vitamins and choline. *Dietary reference intakes: Thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin and choline* (pp. 87–122). [Washington (DC): National Academic Press (US)].

Ganguly, N. K., Bhattacharya, S. K., Sesikeran, B., Nair, G. B., Ramakrishna, B. S., Sachdev, H. P., et al. (2011). ICMR-DBT guidelines for evaluation of probiotics in food. *Indian Journal of Medical Research, 134*(1), 22.

Hugenholtz, J., & Smid, E. J. (2002). Nutraceutical production with food-grade micro-organisms. *Current Opinion in Biotechnology, 13*(5), 497–507.

INSA. (2011). *Micro-nutrient security for India – priorities for research and action.* Indian National Science Academy. Available at: www.insa.ac.in.

Juarez del Valle, M., Laiño, J. E., Savoy de-Giori, G., & LeBlanc, J. G. (2014). Riboflavin producing lactic acid bacteria as a biotechnological strategy to obtain bio-enriched soymilk. *Food Research International, 62*, 1015–1019.

Kneifel, W., Kaufmann, M., Fleischer, A., & Ulberth, F. (1992). Screening of commercially available mesophilic dairy starter cultures: Biochemical, sensory, and microbiological properties. *Journal of Dairy Science, 75*, 3158–3166.

LeBlanc, J. G., Burgess, C., Sesma, F., de Giori, G. S., & van Sinderen, D. (2005). *Lactococcus lactis* is capable of improving the riboflavin status in deficient rats. *British Journal of Nutrition, 94*(2), 262–267.

LeBlanc, J. G., Laino, J. E., Juarez del Valle, M., Vannini, V., van Sinderen, D., Taranto, M. P., et al. (2011). B-Group vitamin production by lactic acid bacteria - current knowledge and potential applications. *Journal of Applied Microbiology, 111*(6), 1297–1309.

Licandro, H., Ho, P. H., Nguyen, T. K. C., Petchkongkaew, A., Van Nguyen, H., Chu-Ky, S., et al. (2019). How fermentation by lactic acid bacteria can address safety issues in legumes food products? *Food Control*106957.

Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J., et al. (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Applied and Environmental Microbiology, 64*(2), 795–799.

Mensink, G. B. M., Fletcher, R., Gurinovic, M., Huybrechts, I., Lafay, L., Serra-Majem, L., et al. (2013). Mapping low intake of micronutrients across Europe. *British Journal of Nutrition, 110*(4), 755–773.

O'Brien, M. M., Kiely, M., Harrington, K. E., Robson, P. J., Strain, J. J., & Flynn, A. (2001). The north/south Ireland food consumption survey: Vitamin intakes in 18-64-year-old adults. *Public Health Nutrition, 4*(5A), 1069–1079.

Odo, T. (2003). Soy (soya) milk. In B. Caballero, L. C. Trugo, & P. M. Finglas (Eds.). *Encyclopedia of food science and nutrition* (pp. 5403–5405). Academic Press.

Pospiech, A., & Neumann, B. (1995). A versatile quick-prep of genomic DNA from gram-positive bacteria. *Trends in Genetics, 11*(6), 217–218.

Rivlin, R. S., & Pinto, J. T. (2007). Riboflavin (vitamin B2). In J. Zempleni, R. B. Rucker, D. B. McCormick, & J. W. Suttie (Eds.). *Handbook of vitamins* (pp. 233–253). (4th ed.). Taylor & Francis Group.

Rodriguez, R. S. J., Fernández-Ruiz, V., Cámara, M., & Sánchez-Mata, M. C. (2012). Simultaneous determination of vitamin B1 and B2 in complex cereal foods, by reverse phase isocratic HPLC-UV. *Journal of Cereal Science, 55*(3), 293–299.

Russo, P., Capozzi, V., Arena, M. P., Spadaccino, G., Dueñas, M. T., López, P., et al. (2014). Riboflavin-overproducing strains of *Lactobacillus fermentum* for riboflavin-enriched bread. *Applied Microbiology and Biotechnology, 98*(8), 3691–3700.

Said, H. M., & Ross, A. C. (2014). Riboflavin. In A. C. Ross, B. Caballaro, R. J. Cousins, K. L. Tucker, & T. R. Ziegler (Eds.). *Modern nutrition in health and disease* (pp. 325–330). (11th ed.). Lippincott, Williams & Wilkins.

Salvetti, S., Celandroni, F., Ghelardi, E., Baggiani, A., & Senesi, S. (2003). Rapid determination of vitamin B2 secretion by bacteria growing on solid media. *Journal of Applied Microbiology and Biotechnology, 95*(6), 1255–1260.

Shalini, T., Sivaprasad, M., Balakrishna, N., Madhavi, G., Radhika, M. S., Kumar, B. N., et al. (2018). Micronutrient intakes and status assessed by probability approach among the urban adult population of Hyderabad city in South India. *European Journal of Nutrition,* 1–13.

Siddiqi, S., Urooj, A., & D'Souza, M. J. (2019). Dietary patterns and anthropometric measures of Indian children with autism spectrum disorder. *Journal of Autism and Developmental Disorders, 49*(4), 1586–1598.

Silva, F. F. P., Biscola, V., LeBlanc, J. G., & de-Melo Franco, B. D. G. (2016). Effect of indigenous lactic acid bacteria isolated from goat milk and cheeses on folate and riboflavin content of fermented goat milk. *LWT-Food Science and Technology, 71*, 155–161.

Singh, B. P., Vij, S., Hati, S., Singh, D., & Bhushan, B. (2016). Bio-functional significance of soybean based food products. In B. K. Mishra, & S. Hati (Eds.). *Dairy and food product Technology* (pp. 49–59). Biotech Books.

Sirichokchatchawan, W., Pupa, P., Praechansri, P., Am-in, N., Tanasupawat, S., Sonthayanon, P., et al. (2018). Autochthonous lactic acid bacteria isolated from pig faeces in Thailand show probiotic properties and antibacterial activity against enteric pathogenic bacteria. *Microbial Pathogenesis, 119*, 208–215.

Sivaprasad, M., Shalini, T., Reddy, P. Y., Seshacharyulu, M., Madhavi, G., Kumar, B. N., et al. (2019). Prevalence of vitamin deficiencies in the apparently healthy urban adult population: Assessed by sub-clinical status and dietary intakes. *Nutrition, 63*, 106–113.

Song, Y. L., Kato, N., Liu, C. X., Matsumiya, Y., Kato, H., & Watanabe, K. (2000). Rapid identification of 11 human intestinal *Lactobacillus* species by multiplex PCR assays using group-and species-specific primers derived from the 16S–23S rRNA intergenic spacer region and its flanking 23S rRNA. *FEMS Microbiology Letters, 187*(2), 167–173.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular revolutionary genetics analysis version 6.0. *Molecular Biology and Evolution, 30*, 2725–2729.

Thakur, K., Tomar, S. K., Brahma, B., & De, S. (2016). Screening of riboflavin-producing lactobacilli by a polymerase-chain-reaction-based approach and microbiological assay. *Journal of Agricultural and Food Chemistry, 64*(9), 1950–1956.

Yépez, A., Russo, P., Spano, G., Khomenko, I., Biasioli, F., Capozzi, V., et al. (2019). *In situ* riboflavin fortification of different kefir-like cereal-based beverages using selected Andean LAB strains. *Food Microbiology, 77*, 61–68.