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Original Research Article

Antioxidant profiling and molecular characterization of novel *Lacticaseibacillus rhamnosus* strains isolated from human microbiota

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Abstract

Purpose: *To assess the antioxidant properties of two newly isolated Lacticaseibacillus rhamnosus strains (BVV1 and BBV2) from breastfed infants for potential use in nutritional and probiotic formulations.*
Methods: *Strains were identified using the Basic Local Alignment Search Tool (BLAST) with the National Center for Biotechnology Information (NCBI) 16S database. The in vitro antioxidant activities were assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical scavenging ability, Fe^{2+} chelation, lipid peroxidation inhibition, and superoxide anion reduction compared to L. rhamnosus GG.*
Results: *The BBV2 exhibited the highest DPPH scavenging (82.44 ± 1.87 %) and Fe^{2+} chelation (71.72 ± 2.78 %). Hydroxyl radical scavenging was strongest in GG (83.12 ± 2.59 %) than in BBV2 (79.59 ± 4.49 %). Lipid peroxidation inhibition in GG (63.44 ± 4.66 %) was higher than in BBV2 (58.24 ± 1.38 %).*
Conclusion: *BBV2 shows strong antioxidant potential, making it a promising natural alternative to synthetic antioxidants in probiotic and nutritional applications.*

Keywords: *Lacticaseibacillus rhamnosus, Breastfed infants, Microbiota, Probiotic, Antioxidant*

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INTRODUCTION

Lactic acid bacteria (LAB), a member of the phylum Firmicutes, are gram-positive, non-motile, catalase-negative, and spore-free cocci or bacilli. They primarily metabolize carbohydrates and thrive in acidic environments. Lactic acid bacteria (LAB) encompass over 60 genera, including *Streptococcus*, *Weissella*, *Pediococcus*, *Leuconostoc*, *Lactococcus* and *Lactobacillus*. Recent taxonomic revisions have reclassified *Lactobacillus* into multiple genera [1]. Lactic acid bacteria (LAB) are frequently detected in the human digestive system and are widely employed in food fermentation as GRAS (Generally Regarded as Safe) organisms. They exhibit antioxidant properties and reduce oxidative stress, which arises from an imbalance between the body's antioxidant defenses and reactive species (ROS, RNS). Oxidative stress

contributes to diseases like atherosclerosis, diabetes, and cancer [2]. Several LAB strains, particularly *Lactobacillus* and *Bifidobacterium*, demonstrate antioxidant capabilities, including ROS scavenging, metal ion chelation, and inhibition of lipid peroxidation. Studies report strong antioxidant activity in *L. rhamnosus* and *L. helveticus* [3]. This study investigated the antioxidant properties of two *Lacticaseibacillus rhamnosus* strains (BVV1 and BBV2) isolated from the feces of exclusively breastfed infants.

EXPERIMENTAL

Bacterial strains and media

De Man Rogosa Sharp MRS broth or agar (Oxoid) was used to grow the strains at 30 °C. Strains were stored at - 80 °C in spent Man, Rogosa and Sharpe (MRS) or brain heart infusion (BHI) broth in 15 % v/v glycerol.

Genetic characterization of the strains using polymerase chain reaction

Isolation and identification of selected strains

The strains under study were selected from the faeces of healthy Algerian newborns during their first week of life, who were exclusively breastfed. The strains were characterized by partial 16S rRNA gene sequencing and analyzed through BLAST. The Genomic DNA Mini Kit (Invitrogen) was used to extract genomic DNA from bacterial cultures, following the manufacturer's protocol. The partial 16S rRNA gene was amplified by polymerase chain reaction (PCR) utilizing My Cycler thermal cycler (Bio-Rad) with universal primers PLB (5′-AGAGTTTGATCCTGGCTCAG-3′) and MLB (5′-GGCTGCTGGCACGTAGTTAG-3′) [4].

Phylogenetic analysis

The Maximum Likelihood (ML) approach, which is based on the Tamura 3-parameter model, was used to assess phylogenetic relationships [5]. The phylogenetic tree with the highest log-likelihood score (-5951.69) was presented. The initial topologies of the phylogenetic tree were generated automatically through the BioNJ and Neighbor-Joining (NJ) algorithms, which calculated pairwise distances according to the three-parameter Tamura model. Thereafter, the topology with the highest log-likelihood score was chosen for additional improvement. The analysis incorporated a model of rate variation that accounted for evolutionarily invariable sites ({+*I*}, 32.09 % of sites). The tree was scaled so that branch lengths indicate changes made per site. The proportion of conserved sites containing at least one unambiguous nucleotide across sequences for each descendant clade was indicated at internal nodes.

Antioxidant study

Determination of the DPPH radical scavenging effect

The DPPH radical scavenging activity of *L. rhamnosus* strains was assessed following the method of Goto *et al* [6] and Zhang *et al* [7]. Intact cells were suspended in 1 mL phosphate-buffered saline (PBS), mixed with 1 mL 0.05 mM DPPH in ethanol, and incubated in the dark for 1 h. A blank (PBS) and a control (1 mg/mL ethanol) were included. Following centrifugation at 4000 rpm for 10 min, absorbance at 517 nm was measured, and scavenging activity was calculated using Eq 1.

Scavenging Activity (%) = {1-(A sample–A_{blind})/A_{blank}}100 …… (1)

Hydroxyl ion scavenging capacity of the isolated strains

Hydroxyl ion scavenging capacity of *L. rhamnosus* strains was assessed following the method of Wang *et al* [8] using $FeSO_4$-H_2O_2 Fenton reaction. Distilled water (0.5 mL), 0.8 mL H_2O_2 (6 mM) and 0.5 mL $FeSO_4$ (8 mM) were added to the reaction mixture. After adding 0.2 mL sodium salicylate (20 mM) and 1.0 mL bacterial treatment, incubation was done at 37 °C for 1 h. Absorbance was measured at 562 nm, and hydroxyl radical scavenging capacity or inhibition (I) was determined using Eq 2.

I (%) = {1-(A_1–A_2)/A_0}100 …… (2)

where A_0 = absorbance measured using the control, A_1 = absorbance after addition of the sample, and A_2 = absorbance value without sodium salicylate.

Determination of Fe^{2+} ion-chelating activity

The Fe^{2+} chelating activity of *L. rhamnosus* strains was evaluated following the method of Decker and Welch [9]. Intact cells were suspended in 1 mL PBS, mixed with 0.05 mL $FeCl_2$ (2 mM) and 0.2 mL ferrozine (5 mM), and incubated in the dark for 10 min. Ethylenediamine acetic acid (EDTA; 1 mg/mL) served as the positive control. After centrifugation at 13,000 rpm, absorbance was measured at 562 nm, and Fe^{2+} chelating activity (F) was determined using Eq 3.

$F (\%) = \{1–(A_1/A_2)\}100$ ……. (3)

where A_1 is sample absorbance, and A_2 is control absorbance.

Inhibition of lipid peroxidation assay

Inhibition of lipid peroxidation (I) was done following the method of Hsu *et al* [10] and calculated using Eq 4. Fresh egg yolk was homogenized in PBS (0.2 M, pH 7.2) and diluted (1:25, V/V). A reaction mixture containing egg yolk solution (1 mL), 0.5 mL of strain sample (McFarland 5), 1 mL of 25 mM ferrous sulfate, and 2 mL PBS was incubated at 37 °C for 15 min while stirring. Thereafter, 1 mL 20 % w/v trichloroacetic acid was used to halt the process, left for 10 min, and centrifuged (3000 × g, 10 min, 20 °C). After combining 3 mL of the supernatant with 2 mL of 0.8 % w/v thiobarbituric acid, the mixture was heated for 10 min in a boiling water bath, cooled, and centrifuged again (3000 × g, 10 min). Absorbance was taken at 532 nm. A blank was prepared by replacing the sample with PBS.

$I (\%) = \{(A_0-As)/A_0\}100$ …….. (4)

Superoxide anion radicals scavenging activity

The capacity of the *L. rhamnosus* strains to scavenge superoxide anion radical (O_2^-) was assessed by spectrophotometric evaluation utilizing the improved pyrogallol autoxidation method [11]. Pyrogallol (1, 2, 3-benzene-triol) was converted into a superoxide anion radical using autoxidation systems in an alkaline environment. Tris-HCl solution (4.5 mL, 0.05 M, pH 8.2) was mixed with 0.1 mL bacterial sample, adjusted to a 5 McFarland concentration. The reaction mixture was then incubated for 20 min in a water bath at 25 °C. Thereafter, 0.4 mL 0.25 M pyrogallol was added, and the mixture was incubated for 4 min at 25 °C. Hydrochloric acid (0.1 mL, 8 M) was used to stop the reaction, and absorbance was measured at 320 nm. The bacterial sample was substituted with an equivalent volume of 0.05 M Tris-HCl buffer (pH 8.2) in the blank. Brain heart infusion (BHA) and ascorbic acid (1 mg/mL) were positive controls. Percentage superoxide radical scavenging activity (S) was calculated using Eq 5.

$S (\%) = \{(A_0-A_1)/A_0\}100$ …… (5)

A_1 is the sample's absorbance, and A_0 refers to the blank's absorbance.

Statistical analysis

Data analysis was done using Statistical Packages for Social Sciences (SPSS version 22.0; IBM Inc., Chicago, USA). Results were presented in mean ± standard deviation (SD) and compared using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for pairwise comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Molecular identification of selected strains

Based on multiple sequence alignment, the nucleotide sequences of 16S ribosomal DNA were highly similar (98.63 % for BVV1 and 99.01 % for BBV2) to the sequences of *Lacticaseibacillus rhamnosus* strain TMPC 33831 and *Lacticaseibacillus rhamnosus* strain WEI-30, respectively (Table 1, Figure 1). Accession numbers for the Genbank databases were PP256687.1 and PP256689.1, respectively (Table 2). According to the 16S rRNA gene sequence of the isolated strains and their nearest phylogenetic neighbours, the evolutionary history of the chosen isolates was examined using a maximum likelihood phylogenetic tree (Figure 1).

Evolutionary analysis

A total of 13 nucleotide sequences were analyzed, comprising 1586 positions in the final alignment. All phylogenetic computations and tree visualizations were executed using MEGA11 software [12].

DPPH radical scavenging activity

This technique is based on the principle that antioxidant activity is directly proportional to the reduction in the purple color formed when DPPH radicals are present. The *Lacticaseibacillus rhamnosus* BVV1 and BBV2 strains exhibited DPPH radical scavenging activities of 80.06 % and 82.44 %, respectively, surpassing the *L. rhamnosus* GG strain (68.42 %; Figure 1).

Hydroxyl ion scavenging capacity of the isolated strains

At a test concentration of 10^{10} CFU/mL, *Lacticaseibacillus rhamnosus* BBV2 showed a strong ability to scavenge hydroxyl radicals in a dose-dependent manner (Figure 2).

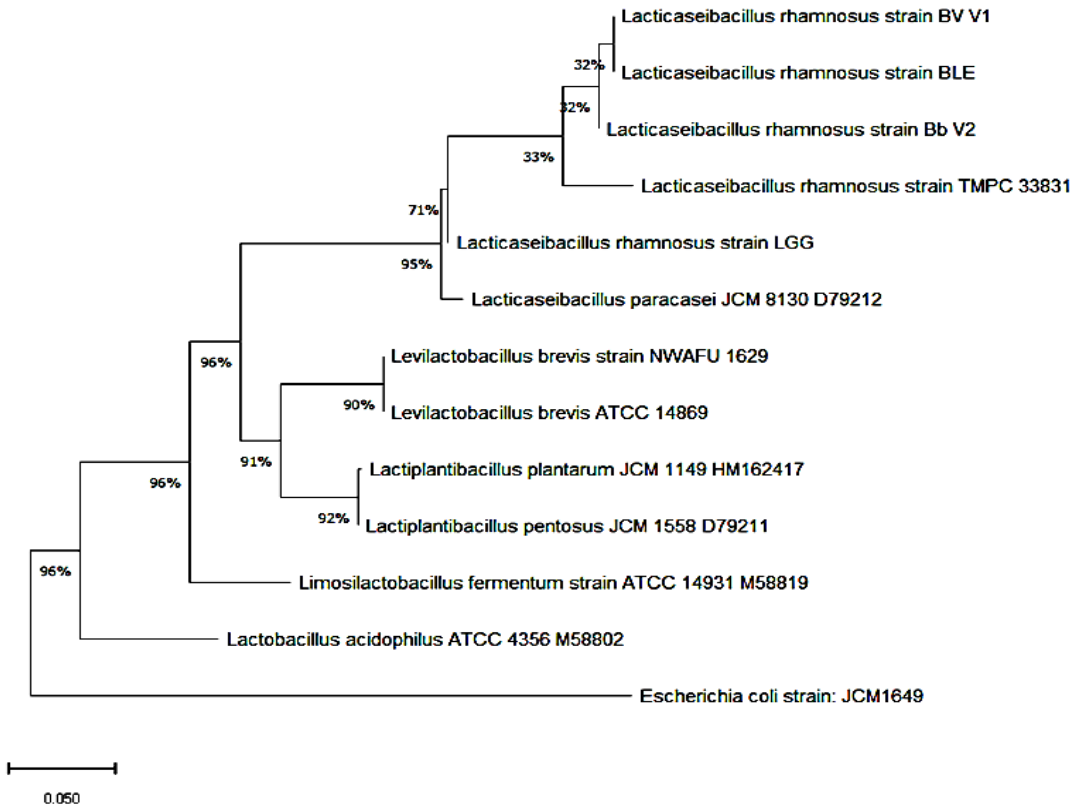

Figure 1: Phylogenetic tree using neighbor-joining based on the isolates' and their nearest neighbors' 16S rRNA gene sequences

Table 1: Molecular identification of selected strains using NCBI (nt_prok database)

Isolate	Top-hit taxon	GenBank accession no	Identity (%)	Query cover (%)
BVV1	*Lacticaseibacillus rhamnosus strain TMPC 33831*	MT512165	98.63	98
BBV2	*Lacticaseibacillus rhamnosus strain WEI-30*	KY041760	99.01	97

Table 2: GenBank accession nos. of the isolated strains

Isolate	GenBank accession no.	NCBI Link
BVV1	PP256687.1	https://www.ncbi.nlm.nih.gov/nuccore/PP256687
BBV2	*PP256689.1*	*https://www.ncbi.nlm.nih.gov/nuccore/PP256689*

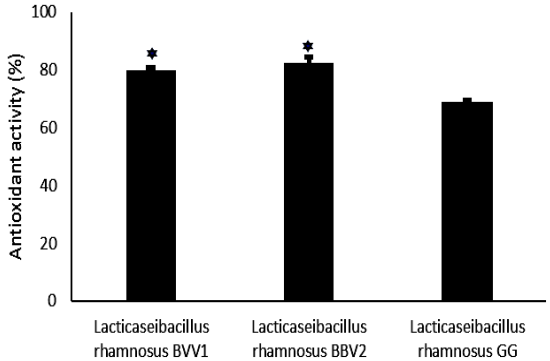

Figure 1: The DPPH radical scavenging activities of the tested strains. *P < 0.05 vs reference strain (*L. rhamnosus* GG)

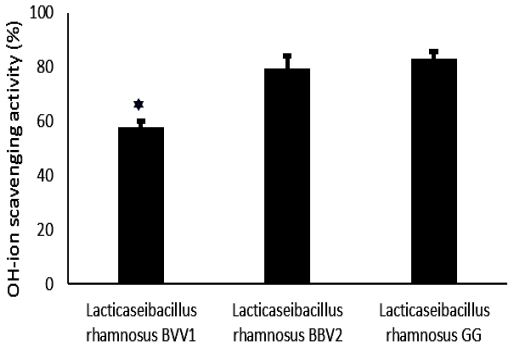

Figure 2: Hydroxyl (OH) ion scavenging activities of selected strains. *P < 0.05 vs reference strain (*L. rhamnosus* GG)

Fe^{2+} ion chelation activity

The results indicated that *Lacticaseibacillus rhamnosus* BBV2 exhibited the highest Fe^{2+} chelating activity (71.72 %), surpassing the reference *L. rhamnosus* GG strain (68.29 %). *L. rhamnosus* BVV1 showed the lowest chelating activity (47.73 %; Figure 3).

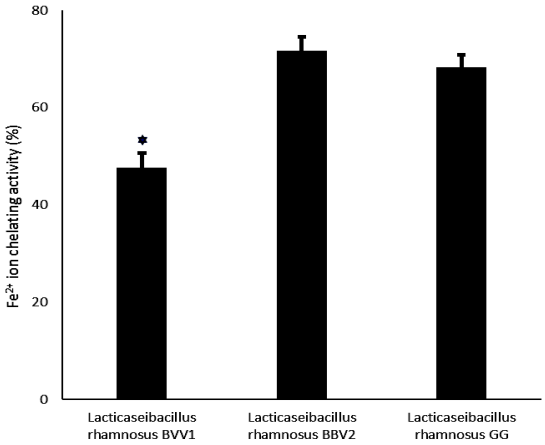

Figure 3: Fe^{2+} ion chelating activity of the tested bacteria. *P < 0.05 vs reference strain (*L. rhamnosus* GG)

Anti-lipid peroxidation

Lacticaseibacillus rhamnosus BBV2 showed a higher lipid peroxidation inhibition activity of 58.24 %, compared to *Lacticaseibacillus rhamnosus* GG, the reference probiotic strain (63.44%). In contrast, *Lacticaseibacillus rhamnosus* BVV1 exhibited a lower inhibition activity (42.86 %; Figure 4).

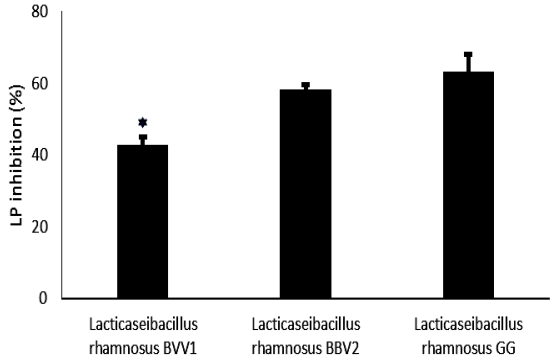

Figure 4: Lipid peroxidation (LP) inhibition activities of the tested bacteria. *P < 0.05 vs reference strain (*L. rhamnosus* GG)

Superoxide anion radical (O_2^-) scavenging activity

The tested *Lacticaseibacillus rhamnosus* strains exhibited varying superoxide radical scavenging activities, ranging from 12.19 to 19.40 %. *L. rhamnosus* BBV2 demonstrated higher hydroxyl radical scavenging activity (16.41 %) than BBV1, while the reference *L. rhamnosus* GG strain showed the highest activity (19.40 %; Figure 5).

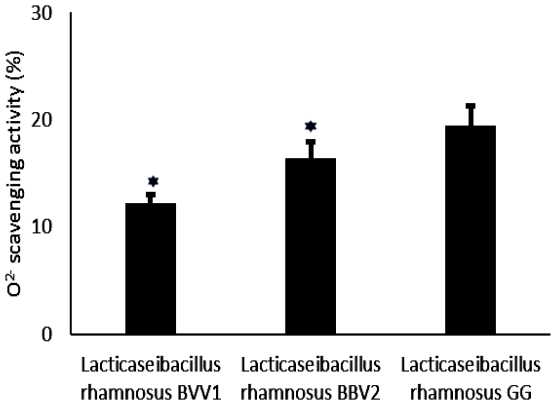

Figure 5: Superoxide anion radical (O^{2-}) scavenging activities of the tested bacteria. *P < 0.05 vs reference strain (*L. rhamnosus* GG)

Correlation analysis of antioxidant activity

There was a strong correlation (0.96) between hydroxyl and DPPH activity, Fe^{2+} chelation (0.85) lipid peroxidation inhibition, lipid peroxidation inhibition (0.97) and superoxide anion inhibition activity (Figure 6).

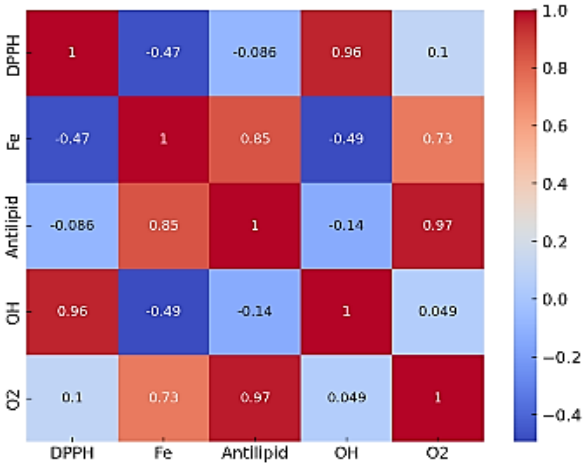

Figure 6: Heatmap of correlation between the studied parameters

DISCUSSION

Recent advancements in molecular characterization and typing have simplified strain identification, enabling the distinction of closely related species. Free radicals generated by incomplete oxygen reduction trigger chain reactions that damage biological molecules, contributing to various diseases [13]. This study

evaluated the antioxidant properties of *Lacticaseibacillus rhamnosus* BVV1 and BBV2, including superoxide radical scavenging, DPPH, hydroxyl, Fe^{2+} chelation and lipid peroxidation inhibition. The DPPH scavenging activities of BVV1 and BBV2 were significantly higher than the reference strain *L. rhamnosus* GG ($p < 0.05$).

The antioxidant potential may stem from intracellular enzymes (glutathione peroxidase, catalase, superoxide dismutase) and surface compounds like extracellular polysaccharides and lipoteichoic acids [14,15]. These findings are in tandem with previous studies on the antioxidant activity of lactic acid bacteria isolated from other sources [16,17] and surpass results by Zhang *et al* [18]. The hydroxyl radical scavenging activity of the isolated strains was comparable to *L. rhamnosus* GG, which eliminated hydroxyl radicals up to 83.12 %. Furthermore, BVV1 exhibited the lowest hydroxyl scavenging activity (57.8 %).

Prior studies demonstrated that *L. plantarum* C88 (44.31 %) and *L. plantarum* IH28L (43.6 %) had lower hydroxyl radical scavenging capacities [19,20]. Unlike earlier reports, this study revealed that intact bacterial cells are more effective scavengers, likely due to their Fe^{2+} binding capacity. Among the studied strains, BBV2 demonstrated the highest Fe^{2+} chelation capacity, surpassing BVV1 and *L. rhamnosus* GG. Cellular lipids are extremely susceptible to lipid peroxidation brought on by ROS, resulting in malondialdehyde (MDA) formation and cytotoxic effects [21]. The results of lipid peroxidation inhibition were consistent with Düz *et al* [20] and exceeded those reported by Ural [17].

Lactic acid bacteria employ enzymatic defenses against oxidative stress, including catalase, NADH-peroxidase, NADH-oxidase and superoxide dismutase (SOD). A previous study demonstrated significant superoxide radical scavenging in *Lactobacillus* and *Leuconostoc* strains, with *Lactobacillus* S1 showing the highest activity [16]. Zhang *et al* [18] found *L. rhamnosus* GG and SY13 strains to be effective against superoxide anions. The current study revealed significant differences ($p < 0.05$) in anti-lipid peroxidation, Fe^{2+} chelation, and radical scavenging activities between the isolated and reference strains, highlighting their superior antioxidant potential.

The correlation analysis between different antioxidant activities revealed significant relationships among key parameters. A strong positive correlation (0.96) between hydroxyl and DPPH radical scavenging activities indicated a functional consistency in antioxidant capacity, as an increase in DPPH scavenging is associated with enhanced hydroxyl radical scavenging. Similarly, a strong correlation (0.85) between Fe^{2+} chelation and lipid peroxidation inhibition indicated that strains with superior iron-binding capacity exhibited higher lipid oxidation protection, highlighting a potential synergistic effect. Also, the high correlation (0.97) between lipid peroxidation inhibition and superoxide anion scavenging indicated strong interdependence between limiting lipid oxidation and neutralizing reactive oxygen radicals. Positive correlation (0.73) between Fe^{2+} chelation and superoxide scavenging supported the role of iron sequestration in oxidative stress protection. Furthermore, a moderate negative correlation (-0.47) between DPPH scavenging and Fe^{2+} chelation indicated mechanistic differences, suggesting that DPPH scavenging does not necessarily align with iron ion chelation. These findings highlight the diversity of antioxidant mechanisms and their variable expression across strains.

CONCLUSION

The 16S rRNA sequencing and BLAST analysis successfully identified the isolated strains *Lacticaseibacillus rhamnosus* BVV1 and BBV2. These strains offer a natural alternative with strong antioxidant potential and may serve as functional food ferments and probiotic candidates for bioactive food development. To investigate further health advantages, more *in vitro* and *in vivo* studies would be required.

DECLARATIONS

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Ethical approval

Not required.

Use of Artificial intelligence/Large language models

We also declare that we did not use Generative artificial intelligence (AI) and AI-assisted technologies in writing the manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Conceptualization: Chaalel A. Data curation: Boukezzoula N, Tefiani C. Formal analysis: Benabdelmoumene D. Methodology: Chaalel A, Boukezzoula N. Software: Benabdelmoumene. D, Bentahar MC. Validation: Chaalel A, Writing - original draft: Souna M, Taleb ARIDJ. Review & editing: Chaalel A, Boukezzoula N, Benabdelmoumene D, Tefiani C, Souna M, Bentahar MC, Lamraoui G, Aridj T.

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