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Genomic characterization and probiotic assessment of *Bifidobacterium breve* JKL2022 with strain-specific CLA-converting properties

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Bifidobacterium breve is a well-recognized probiotic species. *B. breve* JKL2022, a strain isolated from the feces of healthy infants that exhibits superior conjugated linoleic acid (CLA)-converting activity, was functionally characterized for probiotic safety and applicability through genomic and in vitro analyses. The JKL2022 genome comprises a 2,313,948 bp sequence assembled into a single contig, encoding a total of 1,998 genes. In silico predictive analyses confirmed the absence of virulence factors and acquired resistance genes while verifying its intrinsic antimicrobial resistance profile. Several CAZymes were identified, consistent with the strain's fermentation profile. Additionally, the gene encoding the key enzyme for CLA conversion was identified as a 993-bp *lai* gene, underscoring the species-level differences in microbial CLA metabolism. The functionality, stress tolerance, and safety of JKL2022 were further confirmed through experimental assessments. JKL2022 exhibited tolerance to acid and bile salts, auto-aggregation, and cell surface hydrophobicity, indicating its potential to survive gastrointestinal transit. Furthermore, JKL2022 exhibited α -glucosidase inhibitory activity and tested negative for starch hydrolysis, hemolysis, and gelatinase activity. The inherent probiotic properties of *Bifidobacterium*, combined with the strain-specific CLA conversion using growing cells and postbiotic preparations, contribute to the potential health benefits of *B. breve* JKL2022, as verified in this study.

Keywords Probiotics, Whole genome sequence, Antimicrobial resistance, Carbohydrate metabolism, Safety assessment, Conjugated linoleic acid

Bifidobacterium breve is a beneficial bacterial species that colonizes the human gastrointestinal tract and is associated with a variety of health-promoting effects. Members of this species are Gram-positive, anaerobic symbionts of the human intestine^{1,2}. *B. breve* strains have a long history of probiotic use and are included in the list of Qualified Presumption of Safety (QPS) biological agents³ and are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA)⁴. Recent research has focused on supplementing *B. breve* strains capable of producing conjugated linoleic acid (CLA) in various animal models, including pigs, chickens, and mice, to harness the health benefits of CLA, which include anti-atherosclerotic, antidiabetic, anti-obesity, and immunomodulatory properties^{5,6}.

Among natural dietary sources of fatty acids, dairy products (milk, butter, and cheese) and lean meats (beef, lamb, and mutton) contain the highest levels of CLA, reaching up to 3.0–8.0 mg/g fat, whereas fish and vegetables contain negligible CLA levels (<0.1 mg/g fat). The relatively low CLA content in these food products poses a challenge in achieving the recommended daily CLA intake of 3–6 g for potential health benefits^{7,8}. Several strategies have been explored to enhance the CLA content in dairy and meat products, including dietary modifications (e.g., forage type and feeding stage) and processing techniques (e.g., fermentation and storage)^{9–11}. In particular, grass-feeding and oil supplementation (e.g., sunflower, soybean, or flaxseed oil) have been shown to increase CLA levels in dairy milk. For example, dietary supplementation with soybean, rubber seed, or flaxseed oil increased the fatty acid content in cow's milk, with *cis*-9, *trans*-11 CLA levels increasing by 501.85% and *trans*-10, *cis*-12 CLA levels increasing by 125%^{12,13}. Additionally, food-grade, CLA-producing lactic acid bacteria (LAB) have been extensively studied for their potential application in enhancing the CLA content of fermented

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dairy products^{14,15}. Notably, CLA-producing bacteria generate highly specific isomers rather than a mixture of different isomers, which offers advantages in terms of isomer-dependent health effects^{15,16}.

In ruminants, biohydrogenation is the primary process through which linoleic acid (LA) derived from plant materials is converted into stearic acid, with CLA as an intermediate metabolite. This process is facilitated by the metabolic activity of ruminal microorganisms (e.g., *Butyrivibrio fibrisolvens*) and an endogenous enzyme system in the mammary tissue (Δ^9 -desaturase). By contrast, food-grade LAB and CLA-producing bacteria employ distinct enzymatic systems. For instance, *Cutibacterium acnes* (formerly *Propionibacterium acnes*) utilizes the polyunsaturated fatty acid isomerase (PAI) to convert LA exclusively into CLA (*trans*-10, *cis*-12 CLA)¹⁷, whereas other *Cutibacterium* species produce a mixture of isomers (*cis*-9, *trans*-11 CLA: 75–95%; *trans*-10, *cis*-12 CLA: 15–25%)¹⁸. Members of *Lactobacillus* spp. use a multi-enzyme system involving CLA hydrolase (myosin cross-reactive antigen), CLA decarboxylase (acetoacetate decarboxylase), and CLA dehydrogenase (short-chain dehydrogenase/oxidoreductase), resulting in varying CLA isomer compositions^{19,20}. For example, *Lactiplantibacillus plantarum* JCM1551 produces only *cis*-9, *trans*-11 CLA²¹, *Lpb. plantarum* PL62 produces 47% *cis*-9, *trans*-11 CLA and 53% *trans*-10, *cis*-12 CLA²², and *Limosilactobacillus reuteri* ATCC55739 produces 59% *cis*-9, *trans*-11 CLA and 41% *trans*-10, *cis*-12 CLA²³. Similarly, *Bifidobacterium* species, particularly *B. breve*, can convert LA into CLA via a membrane-spanning linoleic acid isomerase (LAI), producing *cis*-9, *trans*-11 CLA as the predominant isomer^{24,25}.

In this study, we characterized the whole genome of the CLA-producing *B. breve* JKL2022 (KACC81214BP), a strain isolated from the feces of healthy infants²⁶, using predictive in silico analyses and in vitro characterization to evaluate its probiotic functionality and safety. The inherent probiotic properties of *B. breve* JKL2022, coupled with its remarkable CLA-converting activity, hold promise for probiotic applications. Current research efforts focus on the development of functional probiotics for both human and animal use, as well as functional compounds derived from JKL2022 to enhance the CLA content of various animal-derived food products.

Materials and methods

Bacterial strains and culture conditions

B. breve JKL2022 (KACC81214BP) and other *Bifidobacterium* strains (*B. breve* JCM7017, JCM7019, JCM1273, JCM1192, and *B. animalis* subsp. *lactis* BB12) were routinely cultured in de Man, Rogosa, and Sharpe (MRS) broth (BD Difco, USA) supplemented with 0.05% (wt/vol) L-cysteine hydrochloride (cys-MRS) (Sigma, USA) at 37 °C under anaerobic conditions (GasPak™ EZ anaerobic chamber, BD Difco). All strains were preserved in 10% skim milk with glycerol (3:1, vol: vol) at -80 °C.

Whole genome analysis

The genomic DNA of *B. breve* JKL2022 was extracted using the QIAamp® PowerFecal® DNA extraction kit (QIAGEN, USA) following the manufacturer's protocol. Whole genome sequencing was performed at CJ Bioscience (Korea) using the Pacific Biosciences (PacBio, USA) Sequel II platform. *De novo* assembly of sequence reads was conducted using the PacBio SMRT analysis program v. 2.3.0. Functional genome annotation was performed using the Rapid Annotation using Subsystem Technology (RAST) pipeline with default parameters²⁷ and KEGG annotation via EzBioCloud. The genome was further annotated using BlastKOALA with the refprok.pep KEGG database²⁸ for pathway classification and dbCAN3 CGC-Finder²⁹ for CAZyme gene cluster identification using default parameters. Transfer RNAs (tRNAs) were identified using tRNAscan-SE v. 1.3.1, and ribosomal and non-coding RNAs (rRNAs) were identified using INFERNAL v. 1.1.3 with the Rfam 12.0 database. Putative genes encoding beneficial enzymes and stress-related proteins were identified through NCBI BLAST searches.

Virulence factors and antimicrobial resistance genes

Identification of virulence factors and antimicrobial resistance genes (ARGs) is essential when characterizing probiotic candidates to ensure safety and efficacy. Genes encoding virulence factors were identified using VirulenceFinder v. 2.0 (Center for Genomic Epidemiology, CGE, <https://cge.food.dtu.dk/services/VirulenceFinder/>) using the genome sequence of JKL2022. Putative enzymes associated with the production of harmful metabolites (e.g., arylsulfatase, β -glucuronidase, nitroreductase, azoreductase, D-lactate dehydrogenase, and amino acid decarboxylase) were identified in the JKL2022 genome using the EzBioCloud BLAST function. Moreover, ARGs were detected using the Resistance Gene Identifier (RGI) from the Comprehensive Antibiotic Resistance Database (CARD, <https://card.mcmaster.ca/analyze/rgi>), and the ResFinder v. 4.4.2 (parameters: 90.0% ID threshold, 60.0% minimum length) and ResFinderFG v. 2.0 (parameters: 98.0% ID threshold, 60.0% minimum length) (<http://genepi.food.dtu.dk/>). The detected ARGs were correlated with the antimicrobial susceptibility profile of strain JKL2022 based on the in vitro antibiotic susceptibility assay.

Prophage and mobile genetic elements

Phages integrated into the JKL2022 genome (prophages) were identified using sequence similarity analysis with the PhageBoost tool³⁰. Mobile genetic elements (MGEs) associated with antimicrobial resistance genes and virulence factors were detected using MobileElementFinder v. 1.0.3 (<https://cge.food.dtu.dk/services/MobileElementFinder/>) with the MGE database v. 1.0.2³¹. Genes located within prophage and MGE regions were identified based on the annotated genome of JKL2022 and correlated with the identified ARGs.

Prediction of genetic markers

Potential genetic markers were identified to enable rapid identification and differentiation of strain JKL2022 from other *B. breve* strains. Housekeeping genes with at least one base difference from other *Bifidobacterium* strains were selected through sequence analysis.

Probiotic characteristics

Acid and bile salt tolerance

The resistance of *B. breve* JKL2022 to acidic conditions and bile salt exposure was evaluated as follows. JKL2022 was exposed to artificial gastric juice (AGJ) composed of 0.2% pepsin (Roche Diagnostics, USA) and 0.35% NaCl, with the pH adjusted to 2.0 and 3.0 using 3 N HCl. Briefly, cells from an overnight culture (15–18 h) were harvested by centrifugation (8,000 × g, 4 °C, 10 min), washed twice with 1× PBS, and resuspended in an equal volume of PBS to achieve a 1× cell concentration (approximately 1×10^9 CFU/mL). Subsequently, 1% (v/v) of the washed cells was inoculated into 5 mL AGJ (pH 2.0 and 3.0) and incubated at 37 °C. Viability was assessed by spread plating on cys-MRS agar after 0, 15, 30, 60, and 120 min of exposure followed by anaerobic incubation at 37 °C. Viable cell counts were reported as log(CFU mL⁻¹).

Survival in the presence of bile was determined by inoculating 1% (v/v) of washed cells into 5 mL MRS broth supplemented with 0.5% (w/v) porcine bile extract (PBE; Sigma Aldrich, USA). Cultures were incubated anaerobically at 37 °C, and viable cell counts were determined at 6 h intervals over a 24 h period. The survival rate at each time point was calculated using the formula:

$$\text{Survival Rate}_t (\%) = \frac{\log(\text{CFU mL}^{-1}_t)}{\log(\text{CFU mL}^{-1}_{t_0})} \times 100$$

where CFU mL⁻¹ at time t represents the average number of colony-forming units after exposure, and CFU mL⁻¹ at time t_0 represents the average number of colony-forming units of the initial inoculum. Experiments were performed in triplicate, with three replicates per experiment.

Auto-aggregation capacity

The auto-aggregation capacity of JKL2022 was assessed following a previously established protocol. An overnight culture of JKL2022 was harvested, washed with sterile distilled water, and resuspended to an optical density (OD) of 0.3 at 660 nm using an INNO microplate spectrophotometer (LTEK Co., Ltd.). A 1 mL aliquot of the cell suspension was transferred to a 1.75 mL microcentrifuge tube and incubated at ambient temperature (25 °C) for 60 min, followed by centrifugation at 980 × g for 2 min. The supernatant was transferred to a 96-well plate, with each well containing 250 µL. Absorbance at 660 nm was measured, and the degree of auto-aggregation was calculated using the formula:

$$\text{Auto-aggregation } (\%) = \frac{OD_{\text{initial}} - OD_{\text{final}}}{OD_{\text{initial}}} \times 100$$

where OD_{initial} represents the optical density of the cell suspension at 660 nm before incubation, and OD_{final} represents the optical density after incubation.

Cell surface hydrophobicity assay

Bacterial adhesion to hydrocarbons was evaluated using a previously established method³². Briefly, an overnight culture of JKL2022 was harvested, washed, and resuspended in 1× PBS to an OD of 0.7 at 600 nm. A 3 mL aliquot of the JKL2022 cell suspension was mixed with 1 mL of xylene, dichloromethane, or hexane, followed by incubation at 37 °C for 10 min. The mixture was vortexed and incubated for 1 h at 37 °C to allow phase separation. The aqueous phase was collected, and absorbance at 600 nm was measured. Surface hydrophobicity was calculated using the formula:

$$\text{Surface Hydrophobicity } (\%) = \frac{OD_{\text{initial}} - OD_{\text{final}}}{OD_{\text{initial}}} \times 100$$

where OD_{initial} represents the optical density at 600 nm before incubation and OD_{final} represents the optical density of the aqueous layer after incubation.

Functional characterization

Carbohydrate fermentation profile

The carbohydrate fermentation profiles of *B. breve* JKL2022, JCM7017, and JCM7019 were determined using the API 50CH kit (bioMérieux, USA) according to the manufacturer's instructions, with minor modifications. Briefly, cell suspensions were prepared in 0.85% NaCl equivalent to 2 McFarland standard (approximately 6.0×10^8 CFU/mL). A 2% (v/v) inoculum of the cell suspension was introduced into API CHB medium supplemented with 0.05% L-cysteine. The cupules were then filled with the inoculated suspension (~150 µL) and incubated anaerobically at 37 °C. A color change from purple to yellow (or from purple to black for esculin) was indicative of carbohydrate fermentation. The results were denoted as follows: (-) negative fermentation, (+) positive fermentation, and (w) weak fermentation.

Additionally, the carbohydrate-active enzymes of the strains were analyzed using the data from the CAZy database (<https://www.cazy.org/bb.html>). The number of genes associated with glycoside hydrolases, glycosyl transferases, carbohydrate esterases, and carbohydrate-binding module families was assessed. The genome of *B. breve* UCC2003 was used as a reference.

Starch hydrolysis test

The ability of the strains to hydrolyze starch was evaluated by streaking overnight cultures of *Bifidobacterium* spp. onto cys-MRS agar supplemented with 1% (w/v) soluble starch (Duksan, Korea). The plates were incubated anaerobically at 37 °C for 24–48 h, after which they were flooded with iodine solution. The presence of a clearing zone around the bacterial growth indicated a positive result for starch hydrolysis.

α -Glucosidase inhibitory activity

The α -glucosidase inhibitory activity of the *B. breve* strains was assessed following previously established methods³³. Briefly, 25 μ L of JKL2022 cell-free supernatant (CFS), obtained from overnight cultures of JKL2022, was added to a reaction mixture containing 150 μ L of 1× PBS and 75 μ L of 0.02 M p-nitrophenyl- α -D-glucopyranoside (PNPG) solution (Sigma). The mixture was incubated at 37 °C for 10 min. The enzymatic reaction was initiated by adding 50 μ L of α -glucosidase (0.17 U/mL, Sigma), followed by further incubation at 37 °C for 10 min. The reaction was terminated by adding 1 mL of 0.1 M Na₂CO₃ (Sigma). The amount of p-nitrophenol released was measured spectrophotometrically at 405 nm. The inhibition rate was calculated using the formula:

$$\text{Inhibition (\%)} = \left[\frac{1 - (C - D)}{(A - B)} \right] \times 100$$

where A represents the absorbance of the reaction mix containing only α -glucosidase (positive control), B represents the absorbance of the reaction mix without α -glucosidase and CFS (blank control), C represents the absorbance of the reaction mix containing both α -glucosidase and CFS (treatment group), and D represents the absorbance of the reaction mix containing only CFS (negative control).

Conjugated linoleic acid production

CLA production by *Bifidobacterium* spp. was assessed in vitro by culturing the strains in cys-MRS broth supplemented with 1% linoleic acid (LA, 50 mg/mL, Sigma), incubated at 37 °C for 24 h. CLA concentration was quantified following a previously established protocol²⁴. Briefly, 400 μ L of culture was transferred to a sterile 1.75 mL microfuge tube and mixed with 800 μ L of isopropanol and 600 μ L of hexane. The mixture was vortexed briefly and centrifuged at 980 × g for 5 min to facilitate phase separation. The upper phase was collected and diluted in methanol (100:900 μ L), and its absorbance at 233 nm was measured using an INNO spectrophotometer.

Safety assessment

Antibiotic susceptibility

The antibiotic susceptibility of *B. breve* JKL2022 was evaluated using a modified broth microdilution assay³⁴. An overnight culture of *B. breve* JKL2022 grown in cys-MRS was harvested by centrifugation (8,000 × g, 4 °C, 10 min), washed with 0.85% NaCl, and resuspended in 2 mL of 0.85% NaCl equivalent to the 0.5 McFarland standard (approximately 1.5 × 10⁸ CFU/mL).

The antibiotics listed in Table 3 were prepared at an initial concentration of 2,048 μ g/mL in modified cation-adjusted Mueller-Hinton broth (CAMHB; Merck, Germany). Sterile 96-well plates were prepared by adding 100 μ L of sterile modified CAMHB to each well. Then, 100 μ L of each antibiotic solution (2,048 μ g/mL) was added to the first lane, followed by serial two-fold dilutions across the plate until lane 10 (final concentration, 2 μ g/mL). A 10 μ L aliquot of JKL2022 cell suspension was inoculated into wells in lanes 1–11. Lane 11 contained *B. breve* JKL2022 without antibiotics, serving as a negative control, and lane 12 contained only modified CAMHB, serving as a blank control. The plates were incubated anaerobically at 37 °C, and cell growth was assessed at 24 and 48 h by measuring absorbance at 600 nm using an INNO spectrophotometer. Antibiotic resistance was interpreted based on the reported intrinsic resistance profile of *Bifidobacterium* derived from molecular characterization³⁵ and the prevalence of ARGs among human gut-derived bacteria³⁶.

Hemolytic activity

The hemolytic activity of *B. breve* strains was assessed by streaking overnight cultures onto tryptic soy agar (TSA) or cys-MRS plates supplemented with 5% (v/v) sheep blood, followed by anaerobic incubation at 37 °C for 24–48 h. Hemolytic activity was classified based on colony morphology: the presence of a clear zone around the colonies indicated β -hemolysis, a greenish discoloration indicated α -hemolysis, and the absence of a clearing zone indicated γ -hemolysis³³.

Gelatinase activity

The gelatinase activity of the strains was evaluated in vitro using nutrient gelatin stab cultures. Overnight cultures of *Bifidobacterium* spp. were inoculated by stabbing into nutrient gelatin and incubated anaerobically at 37 °C for 24–48 h. Liquefaction of gelatin observed following 1 h of refrigeration was indicative of gelatinase activity³⁷.

Statistical analysis

All in vitro experiments were performed in triplicate, and the data are expressed as the means ± standard deviations, analyzed using the GraphPad Prism (v. 9.5.1). The surface adhesion capacity of bifidobacteria was analyzed using the ordinary one-way analysis of variance (ANOVA) (α , 0.05) with Tukey's multiple comparison post-hoc test. The differences in α -glucosidase inhibition were measured using the Brown-Forsythe and Welch ANOVA test (α , 0.05) with Dunnett's T3 multiple comparison post-hoc test.

Results and discussion

The genomic and phenotypic characteristics of *B. breve* JKL2022 related to its probiotic functionality were evaluated through various in silico and in vitro analyses. Consistent with the established probiotic traits of the genus *Bifidobacterium*, JKL2022 exhibited key functional attributes supporting its probiotic potential. Genomic analyses confirmed the presence of genes encoding beneficial enzymes and the absence of virulence factors, reinforcing both the functional and safety aspects of the strain. Moreover, in vitro assessments aligned with the genomic profile, demonstrating a clear correlation between the genotype and phenotype of the strain. Notably, the superior CLA conversion activity of JKL2022 represents a unique functional attribute, further enhancing its probiotic potential.

Whole genome analysis

The genome of *B. breve* JKL2022 consists of a single 2,313,948 bp contig with a guanine and cytosine (G + C) content of 58.7% (Fig. 1A). The genome encodes 1,936 protein-coding genes, 53 tRNA genes, and 9 rRNA genes. The EggNog/COG functional categorization revealed a high abundance of genes involved in carbohydrate transport and metabolism (212), replication, recombination, and repair (172), amino acid transport and metabolism (161), translation, ribosomal structure, and biogenesis (134), transcription (113), and cell wall and membrane envelope biogenesis (89), as shown in Fig. 1B and C. Annotation using BlastKOALA resulted in 1,044 annotated entries out of the 1,930 entries (54.1%), classified into metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, and human diseases (Fig. 2). Among the metabolism-related genes, the most abundant were those associated with global and overview maps (797), carbohydrate metabolism (145), and amino acid metabolism (145).

Table 1 presents a summary of genes associated with probiotic functionalities identified in the JKL2022 genome. Notably, the CLA-converting activity of *B. breve* JKL2022 was attributed to a 993-bp *lai* gene (JKL2022_00014), which encodes a putative linoleic acid isomerase with a membrane-spanning configuration²⁴. Additional genes encoding beneficial functions include carbohydrate-active enzymes (CAZymes) belonging to glycoside hydrolase (1,3- β -galactosyl-N-acetylhexosamine phosphorylase), glycosyl transferase (α -D-xyloside xylohydrolase, maltose α -D-glucosyltransferase, and sucrose phosphorylase), carbohydrate esterase (N-acetylglucosamine-6-phosphate deacetylase), and carbohydrate-binding module families, as identified using the Carbohydrate-Active Enzymes database (<http://www.cazy.org/>)³⁸. The abundance of CAZymes suggests that *B. breve* JKL2022 possesses a diverse carbohydrate metabolism capability, facilitating adaptation to local carbohydrate availability and contributing to the degradation of carbohydrates in the host gut³⁹.

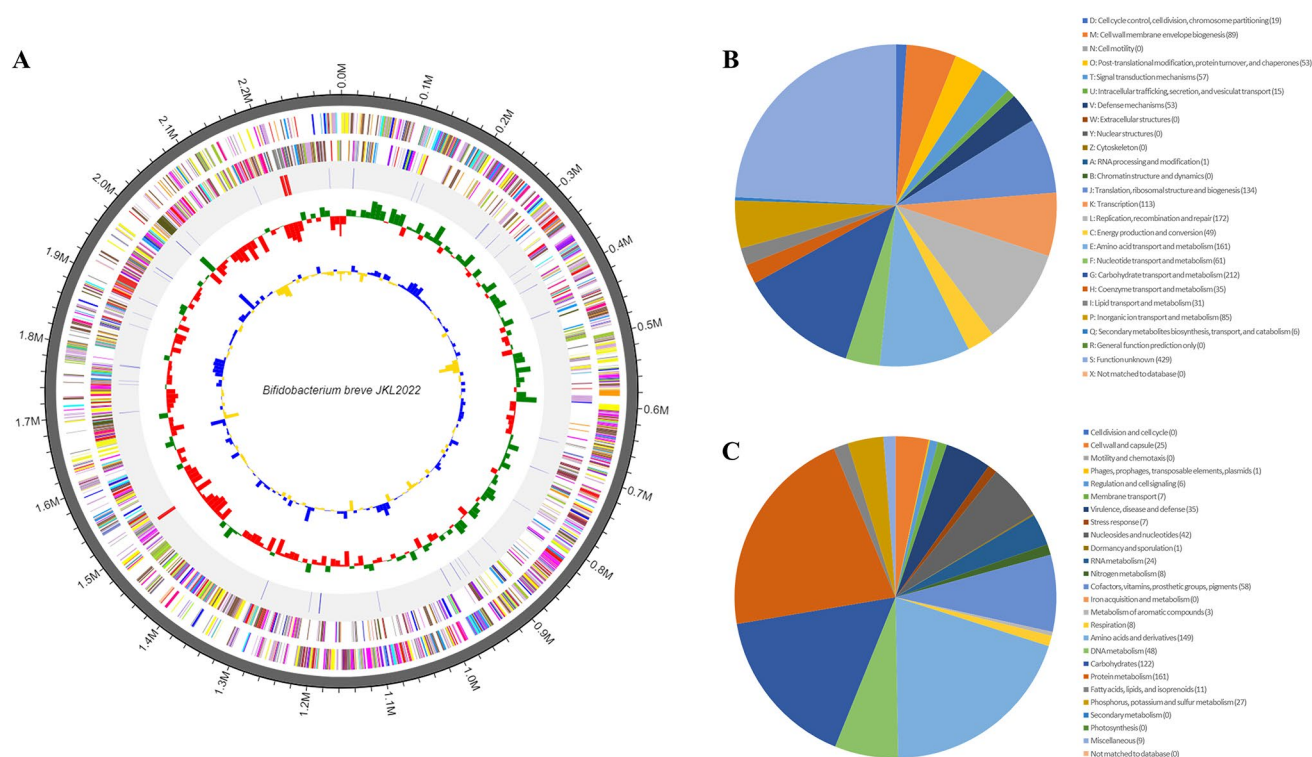


Fig. 1. Genome properties of JKL2022. (A) Circular genome map of *Bifidobacterium breve* JKL2022. Circles represent the following characteristics from the outermost circle to the center: (1) contig information, (2) coding sequences on forward strand, (3) coding sequences on reverse strand, (4) transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), (5) GC skew, and (6) GC ratio. G, guanine; C, cytosine; CDS, coding sequences. (B) Functional annotation based on Kyoto Encyclopedia of Genes and Genomes and (C) Cluster of Orthologous Groups.

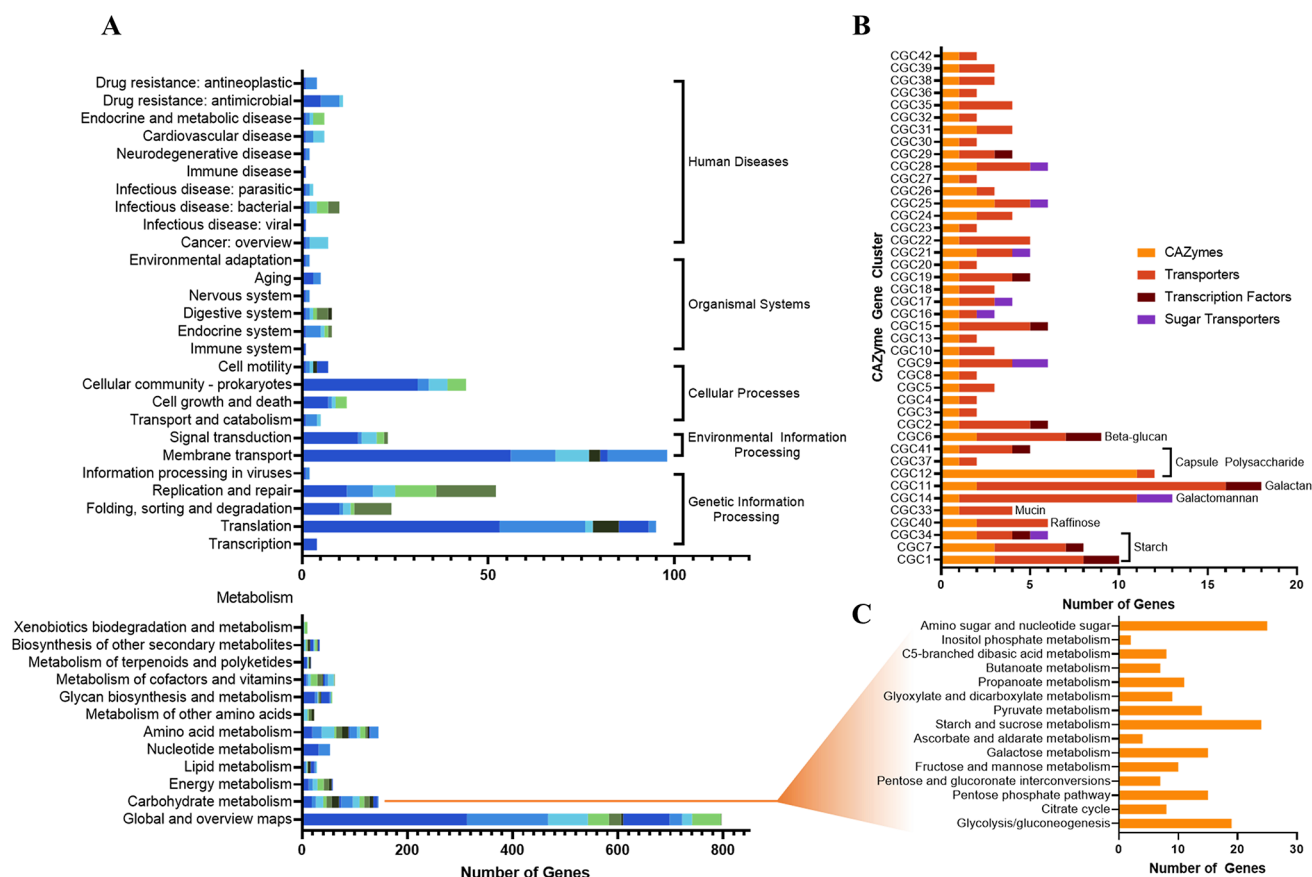


Fig. 2. Genotype analysis of *Bifidobacterium breve* JKL2022 based on (A) KEGG Mapper Reconstruction using the BlastKOALA, (B) predicted CAZyme gene clusters using the dbCAN3 program, and (C) distribution of carbohydrate metabolism genes.

Gene tag(s)	Product	Predicted function	Probiotic function
01410	Alkaline phosphatase	Cell membrane; Membrane; Transmembrane.	Promotes bacterial growth and improves gut barrier maintenance ⁸³
00088	Lysophospholipase	Hydrolase	Lipid transport and metabolism, membrane maintenance, and modulation of immune response ⁸⁴
00732, 01472	Exopolyphosphatase/Phosphoserine phosphatase	Hydrolase	Improves intestinal barrier function ⁸⁵
00812, 00813	Endopeptidase Clp	Cytoplasm; Hydrolase; Protease; Serine protease	Stress survival ⁸⁶ and heat-shock response ⁸⁶
00274, 01946	Alpha-galactosidase	Glycosidase; Hydrolase; Secreted; Signal.	Sugar and oligosaccharide breakdown ⁸⁷
00010, 00389, 01575, 01906	Beta-galactosidase	Glycosidase; Hydrolase; Secreted; Signal.	Hydrolysis of lactose and alleviation of lactose intolerance ³⁷
00126, 01453, 01458, 01689, 01980, 01981, 01985	Beta-glucosidase	Carbohydrate metabolism; Cellulose degradation; Glycoprotein; Glycosidase; Hydrolase; Polysaccharide degradation; Signal.	Hydrolysis of glycosides/carbohydrate metabolism; release of plant secondary metabolites ⁸⁸
01161, 01162	Alpha-mannosidase	Alternative splicing; Glycosidase; Hydrolase; Metal-binding; Polymorphism; Zinc.	Carbohydrate metabolism; promotion of eubiosis and suppression of tumorigenesis ⁸⁹
00180	Alpha-L-fucosidase	Apoplast; Glycoprotein; Glycosidase; Hydrolase; Secreted; Signal.	Breakdown of fucose from glycoconjugates ^{90,91}
00180	Exo-alpha-sialidase	Glycosidase; Hydrolase; Repeat; Secreted; Signal.	Enhances adhesion and carbohydrate assimilation ⁹²

Table 1. List of genes involved in beneficial probiotic functions.

Furthermore, JKL2022 harbors a gene encoding a choloylcholine hydrolase (JKL2022_00976), also known as a bile salt hydrolase. Bile salt hydrolases play a critical role in probiotic function by facilitating bile salt detoxification, leading to the formation of deconjugated bile acids and enhancing the strain's survivability during gastrointestinal transit. Several stress-related genes were also identified, including heat shock protein (HsP) *HsP.16.4* (JKL2022_00089), putative *HspR* (JKL2022_00146), *GroEL* (JKL2022_01010), chaperone

proteins *dnaK1* (JKL2022_00143), *GrpE* (JKL2022_00144), *DnaJ* (JKL2022_00145), and the large-conductance mechanosensitive channel (JKL2022_01468). The presence of these genes suggests that JKL2022 can tolerate temperature, pH, and osmotic stress—critical factors in probiotic viability during production and host consumption.

Additionally, the genome of JKL2022 was screened for potential genetic markers that may facilitate strain identification in mixed *Bifidobacterium* populations. Table 2 lists candidate genetic markers with at least one base-pair difference, many of which correspond to housekeeping genes. These markers could serve as the basis for developing a rapid PCR-based screening method for JKL2022, enabling strain-specific detection and identification independent of sequencing analysis.

Virulence factors

In addition to possessing genes associated with probiotic functionality, it is equally important that a probiotic strain does not harbor virulence factors, as their presence could pose risks to the host and potentially induce disease. The absence of virulence factors enhances the probiotic safety profile by eliminating the possibility of horizontal gene transfer (HGT) to other bacteria and reducing the risk of pathogenicity. Common virulence factors in lactic acid bacteria include adherence factors, toxins, surface-associated proteins, and proteins involved in collagen and fibronectin binding. Predictive analyses using the VirulenceFinder 2.0⁴⁰ confirmed that JKL2022 lacks genes associated with virulence. Furthermore, the PathogenFinder prediction tool (<https://cge.food.dtu.dk/services/PathogenFinder/>) classified JKL2022 as non-pathogenic, with a probability score of 0.241.

Antimicrobial resistance genes

Antimicrobial resistance typically arises through three fundamental mechanisms: (1) enzymatic degradation of antimicrobial compounds, (2) modification of antimicrobial target molecules or proteins, and (3) alterations in membrane permeability to antibiotics⁴¹. Prediction of ARGs using the CARD-RGI⁴² identified the presence of the rifamycin-resistant β -subunit of RNA polymerase (*rpoB*) (92.48% ID). ResFinderFG v. 2.0 identified the *dfr* (98.91% ID) and *van_ligase* (99.92% ID) genes, associated with resistance to trimethoprim and D-cycloserine, respectively. In contrast, no acquired ARGs were detected using ResFinder v.4.4.2.

All three ARGs identified in the JKL2022 genome share a common resistance mechanism, target modification or replacement, attributed to mutations in their nucleotide sequences. The *dfr* gene (JKL2022_01586) encodes dihydrofolate reductase, the target of trimethoprim. *dfrF* was originally identified in *Streptococcus pyogenes*; however, *B. breve* and *B. longum* have been reported to harbor sequence variants of this resistance determinant⁴³. JKL2022_01586 exhibited ten nucleotide differences compared with the *dfr* sequence. The *van_ligase* factor was identified as D-alanine-D-alanine ligase (JKL2022_00307), the target of D-cycloserine⁴⁴. Notably, JKL2022_00307 showed only a single nucleotide difference from the reference sequence, suggesting a high likelihood of susceptibility to D-cycloserine. *rpoB* (JKL2022_01402) encodes the RNA polymerase-binding protein RbpA, a known target of rifamycins. Bifidobacteria exhibit intrinsic resistance to rifamycins due to mutations within the rifampicin-binding pocket and adjacent regions (*rpoB*-mutants)⁴⁵.

However, the presence of these genetic determinants alone does not confirm a resistant phenotype and necessitates experimental validation. The AMR profile of *B. breve* JKL2022 was experimentally assessed using

Gene tag	Size (bp)	Product	Base ^a	Strains ^b	JCM7017	JCM7019	JCM1273	JCM1192
01630	1500	DNA repair protein RadA like protein	6	JTL	11	10	11	11
01260	1602	Dihydrofolate synthase	5	NRBB57	25	48	31	32
01270	489	D-aminoacyl-tRNA deacylase	2	JTL	2	6	2	3
00516	1347	Probable arabinose-binding protein	1	IDCC4401	1	7	3	6
00630	3384	Carbamoyl-phosphate synthase (glutamine-hydrolyzing)	1	215W447a, JCM7017	1	20	12	10
00740	279	Transcriptional regulator WhiB1	1	DRBB30	1	3	2	2
00977	3183	[Glutamine synthetase] adenylyltransferase	1	JCM 7017	1	26	10	12
00996	2418	UvrABC system protein	1	JCM 7017	1	104	90	97
01219	1641	Oligopeptide-binding protein OppA	1	LMC520, JCM7017	1	8	6	7
01262	522	8-oxo-dGTP diphosphatase	1	NRBB51	2	20	8	2
01265	1125	Glucokinase	1	NRBB51	6	17	7	7
01267	1215	Uncharacterized protein	1	JR01	17	34	6	1
01272	1035	Hypothetical protein	1	139W423	4	3	7	5
01436	726	Hypothetical protein	1	JSRL01, JCM7017	1	6	3	n/a
01447	921	Purine nucleosidase	1	082W48, NRBB56, JCM7017	1	5	n/a	3
01467	1857	Long-chain-fatty-acid-CoA ligase	1	DRBB26, NRBB57, JCM7017	1	12	3	2
01175	2721	DNA topoisomerase (ATP-hydrolyzing)	1	JR01	11	17	1	11
01934	1531	16 S rRNA	2	JCM1192	4	5	5	2

Table 2. List of potential genetic markers for *Bifidobacterium breve* JKL2022. ^aSmallest number of base difference with Strains^b. ^b*Bifidobacterium* strain(s) with highest sequence similarity (% ID) based on BLAST search.

the broth microdilution method, revealing concordance between the predicted genotype and the intrinsic resistance to aminoglycosides (Table 3). In 2005, Zhou et al. reported the antimicrobial susceptibility profile of probiotic *Bifidobacterium* strains, highlighting the intrinsic and non-transmissible nature of the genetic factors conferring antimicrobial resistance⁴⁶. The low-level intrinsic resistance of bifidobacteria (e.g., vancomycin, aminoglycosides, and metronidazole) due to their natural cell physiology is considered safe and presents minimal risks for contributing to multi-drug resistance, as they are not acquired via HGT, are non-transferable, and do not pose a public health risk^{46,47}. On the other hand, the presence of mobile ARGs would render the strain unsafe for probiotic use due to the risk of AMR dissemination.

Prophage and mobile genetic elements

Chromosomal genes can be transferred between bacterial cells through various mechanisms, including mobile genetic elements (e.g., plasmids) and phage-mediated lateral transduction⁴⁸. This approach enables bacterial cells to acquire exogenous genetic material beyond the DNA inherited from the parent cell. A single prophage was identified in the genome of JKL2022, located between 506,666 and 522,012 bp. This prophage region harbors 12 genes, including the insertion sequence IS408 (JKL2022_00428), an uncharacterized membrane protein YobI (JKL2022_00430), putative transposases (JKL2022_00431, JKL2022_00433), and several hypothetical proteins. The presence of MGEs associated with antimicrobial resistance was further assessed using the MGE web tool, which predicted a single MGE at positions 35,423–36,691 bp (complement). Additionally, multiple putative transposases associated with the insertion sequence element IS1353 (JKL2022_01155, JKL2022_01431, JKL2022_01852, JKL2022_01863, and JKL2022_01095) were identified in the genome. Notably, none of these MGEs are located in proximity to the previously identified ARGs, indicating a low risk of ARG transmission via HGT.

Stress tolerance

For probiotics to exert beneficial effects on the host, they must survive passage through the gastrointestinal tract (GIT) and establish a stable niche. Previous studies have reported that bifidobacteria exhibit resistance to lysozyme concentrations of up to 200 mg/L, enabling survival in the oral cavity⁴⁹. Following this, they are exposed to the acidic conditions of the stomach and bile salts in the small intestine. Tolerance to these conditions is a critical trait for probiotic development, as it enhances bacterial survival and persistence. Additionally, auto-aggregation and cell surface hydrophobicity may influence bacterial adhesion to epithelial cells, a key factor in successful niche establishment. These characteristics are sought for in probiotic candidates to ensure proper gut colonization⁵⁰. In vitro analyses demonstrated that *B. breve* JKL2022 exhibits acid and bile salt tolerance, further supporting its probiotic potential.

Acid and bile salt tolerance

Acid and bile salt tolerance are critical criteria for probiotic selection, as acid resistance is associated with the ability to maintain intracellular pH homeostasis. The acid tolerance of *Bifidobacterium* spp. was assessed in AGJ at pH 2.0–3.0. At pH 2.0, all *B. breve* strains were rapidly inactivated, with no viable cells detected after 15 min of exposure. At pH 3.0, the *B. breve* strains exhibited moderate acid tolerance, with the JCM strains demonstrating a survival rate of 41.10–45.13% after 120 min (Fig. 3B). Notably, *B. breve* JKL2022 exhibited superior acid tolerance, with a survival rate of 74 ± 5.54% after 120 min. In contrast, the probiotic *B. animalis* subsp. *lactis* remained largely unaffected by the acidic environment, retaining 98.55 ± 0.05% viability after 120 min. JKL2022 harbors the gene encoding the acid tolerance regulatory protein ActR (JKL2022_01381), a

Antimicrobial	MIC ^a (µg/mL)		Phenotype ^b	Genotype	
	24 h	48 h		Related Gene(s)	Product
Ampicillin	1	2	S		
Chloramphenicol	2	2	S		
Ciprofloxacin	8	16	S		
Erythromycin	0	1	S	00854, 00872, 01802	Macrolide efflux protein
Rifampicin	2	2	S	01042	RNA polymerase-binding protein RbpA
Tetracycline	0	1	S		
Vancomycin	1	1	S	01197	Transcriptional regulator ClgR
Clindamycin*	512	1024	R	00854, 00872, 01802	Macrolide efflux protein
Gentamicin*	256	512	R	01610	N-acetylhexosamine 1-kinase
Kanamycin*	512	256	R	01935	Kanamycin kinase
Metronidazole	4	512	R		
Mupirocin	1024	1024	R	01444	Isoleucine-tRNA ligase
Polymyxin B*	> 2048	> 2048	R	01865	Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase
Streptomycin*	> 2048	> 2048	R	00854, 00872, 01802	Macrolide efflux protein

Table 3. Antimicrobial resistance profile of *Bifidobacterium breve* JKL2022. *Resistance is intrinsic. ^aMIC, minimum inhibitory concentration based on broth microdilution assay. ^bS, susceptible phenotype; R, resistant phenotype.

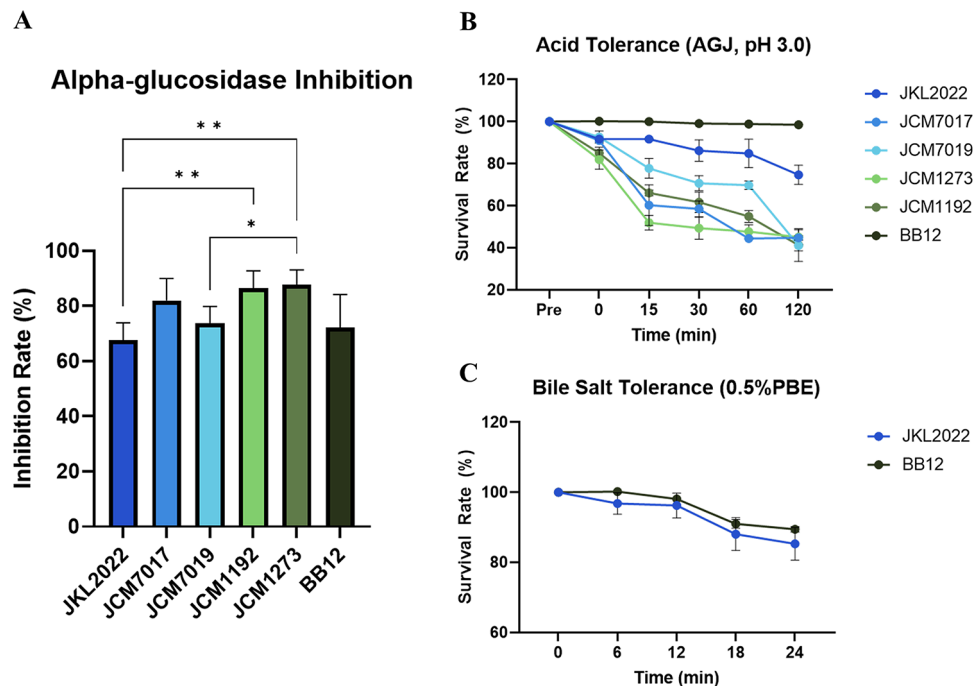


Fig. 3. In vitro assessment of (A) α -glucosidase inhibition and stress tolerance to (B) acid and (C) bile salt of *Bifidobacterium breve* JKL2022. Statistically significant difference in α -glucosidase inhibition is denoted by * based on α , 0.05.

member of the two-component ActS/ActR system involved in pH sensing and response to acid stress⁵¹. ActS is a sensor kinase, which senses changes in external pH environments and phosphorylates ActR under acidic stress. This process leads to activation or repression of downstream genes involved in acid tolerance, including proton transport and metabolism. Furthermore, *B. breve* and *B. longum* have been reported to elicit a stationary phase adaptive acid tolerance response, although not as robust as that of other LAB⁵². *Bifidobacterium* species also possess other two-component systems, such as the PhoR-PhoP system, where PhoR acts as the sensor kinase and PhoP is the response regulator, allowing *Bifidobacterium* to respond to environmental stress (i.e., phosphate starvation)⁵³. These reported mechanisms allow bifidobacterial strains to survive host conditions, particularly during gastrointestinal transit.

Bile salt tolerance was further assessed by evaluating bile salt hydrolase (BSH) activity in vitro. All *B. breve* strains exhibited BSH activity, with JKL2022 displaying intermediate BSH activity relative to the JCM strains. Among the five strains, JCM1273 exhibited the highest BSH activity, followed by JCM119, JCM7019, JKL2022, and JCM7017. In addition, JKL2022 demonstrated moderate bile salt resistance, maintaining a survival rate > 85% after 24 h of exposure to 0.5% bile salt, comparable to *B. animalis* BB-12 (Fig. 3C). Bile salt hydrolysis, a key factor in bile resistance, is attributed to the presence of a functional bile salt hydrolase^{54,55}. JKL2022 harbors a single copy of the gene encoding choloylglycine hydrolase (JKL2022_01482), a member of the C59 peptidase family, which catalyzes the degradation of bile salts in mammalian gut⁵⁶. The process of breaking down conjugated bile salts into free bile acids and amino acids reduces the toxicity of bile salts and potentially alters the bile acid pool⁵⁷. Ruiz et al. emphasized that the expression of the BSH gene resulted in bile salt stress resistance⁵⁸. In addition, the F_0F_1 ATPase, functioning for ATP generation, has been described as the molecular link connecting both acid and bile stress responses in *B. animalis*⁵⁹. The presence of these genetic determinants indicates the potential mechanism employed by *B. breve* JKL2022 for acid and bile salt tolerance, consistent with those previously reported in other probiotic LAB.

Auto-aggregation capacity

All strains exhibited moderate to high auto-aggregation capacity (Fig. 4A). Strains JCM7019, JCM1192, JCM1273, and BB12 demonstrated similarly high auto-aggregation levels (59.38–64.55%, $p > 0.05$), while JKL2022 and JCM7017 exhibited moderate auto-aggregation (40.90–42.88%, $p > 0.05$). Strong auto-aggregation is a desirable characteristic in probiotic strains, as it enhances the likelihood of successful gut colonization by promoting adherence to intestinal epithelial cells. Additionally, bacterial aggregation facilitates the formation of a protective barrier that may prevent pathogen adhesion, thereby reducing the ability of pathogenic bacteria to establish a niche^{60,61}.

Cell surface hydrophobicity

Cell surface hydrophobicity is an important trait of probiotics that reflects their affinity for hydrophobic environments and plays a crucial role in epithelial cell adhesion⁶¹. The hydrophobicity of the bacterial cell

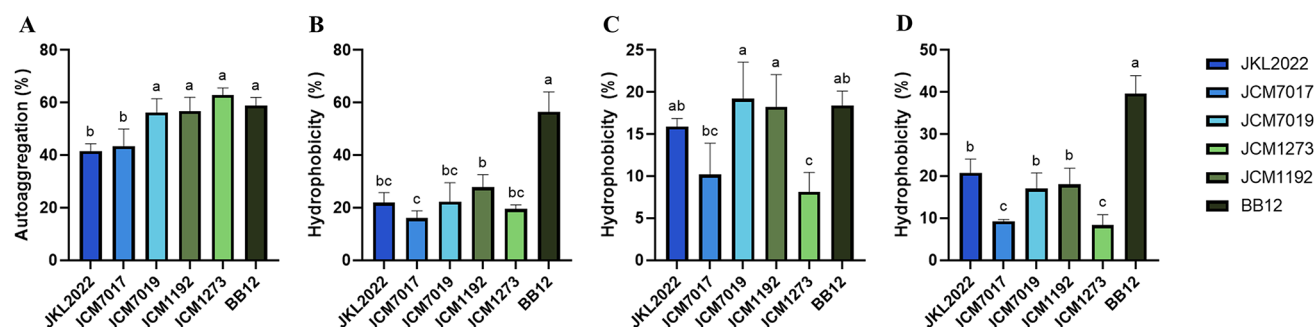


Fig. 4. Surface adhesion capacity of *Bifidobacterium* spp. cells based on (A) auto-aggregation and cell surface hydrophobicity to hydrocarbons: (B) dichloromethane, (C) hexane, and (D) xylene. a-c Different letters denote significantly different results.

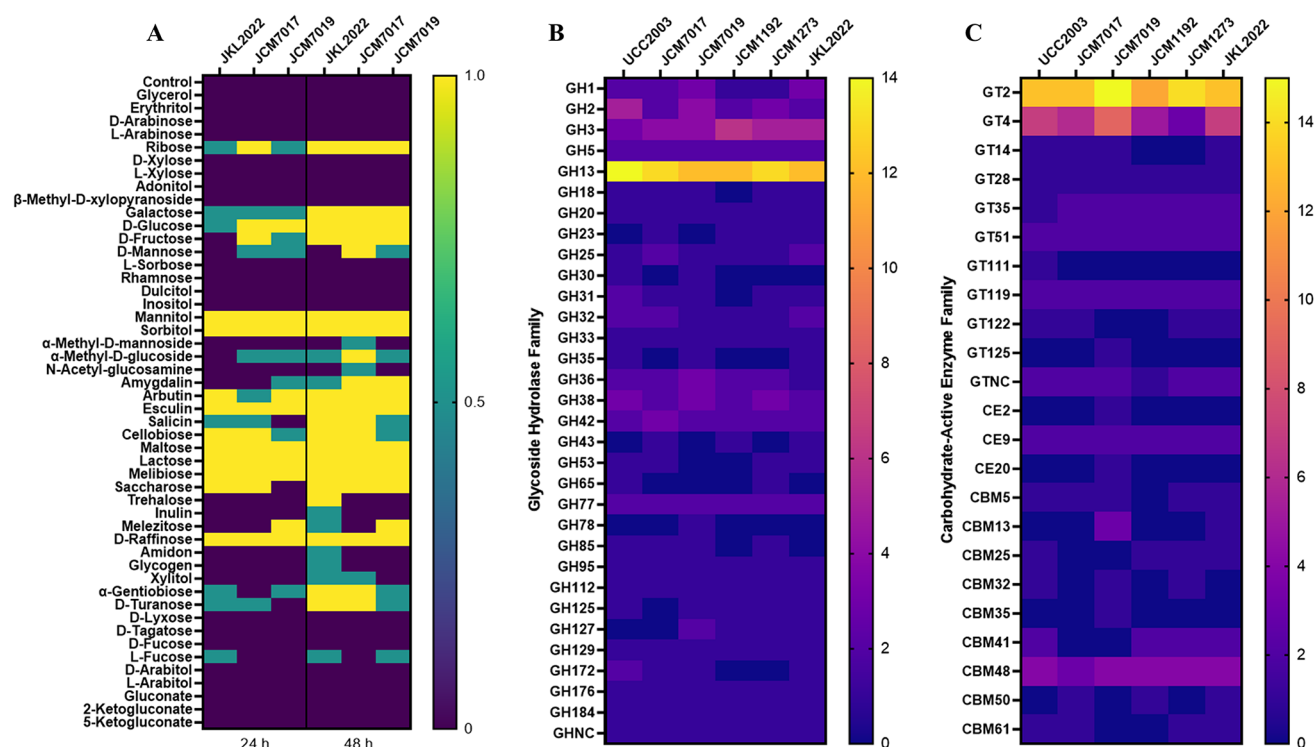


Fig. 5. Carbohydrate profiling of *Bifidobacterium breve* JKL2022 based on: (A) API 50 CH fermentation profile (scale bar, degree of fermentation); (B) in silico prediction of glycoside hydrolase family members based on CaZy classification (scale bar, number of hits); and (C) in silico prediction of glycosyl transferase (GT), carbohydrate esterases (CE), and carbohydrate-binding module families (scale bar, number of hits).

surface, which is associated with adhesion capacity, was assessed using hexane, xylene, and dichloromethane (Fig. 4B and D). The tested strains exhibited 20–50% adhesion to hydrocarbons, classifying them as moderately hydrophobic³². The adhesion ability of the bacterial cells to the host could result in transient colonization, promoting immunomodulatory effects and stimulating gut barrier and metabolism⁶².

Functional characterization

Carbohydrate fermentation profile

The carbohydrate utilization profile of *B. breve* JKL2022 was assessed and compared with the reference strains JCM7017 and JCM7019 using the API 50CH kit (Fig. 5). Notably, all three strains exhibited a common sugar fermentation pattern (glucose, lactose, maltose, and raffinose), consistent with literature. However, JKL2022 demonstrated the ability to ferment trehalose and, to a lesser extent, inulin, suggesting its potential applicability in synbiotic formulations.

The fermentation profile of JKL2022 correlated with the presence of carbohydrate-active enzymes. Genes involved in sugar metabolism are organized into gene clusters containing one or more associated glycoside

hydrolases and associated regulatory transport proteins. Bottacini et al. (2014) reported the clustering of genes corresponding to the carbohydrate fermentation profile of *B. breve* strains. For instance, ribose metabolism is associated with the gene cluster 45, which includes *rbsD* and *rbsK*, encoding a putative ribose transport system and ribokinase, respectively. Similarly, strain-specific melezitose metabolism corresponds to cluster 10, which contains an α -galactosidase-encoding gene⁶³.

The organization of carbohydrate-active enzymes presented in Fig. 5B and C. indicates the abundance of enzymes belonging to Glycoside Hydrolase Family 13 (GH13) and Glycosyl Transferase Family 2 (GT2)⁶⁴. Family GH13 is the major glycoside hydrolase family acting on substrates containing α -glucoside linkages (e.g., maltose and trehalose) and, to a lesser extent, monosaccharides (e.g., glucose, fructose, and galactose), β -linked sugars (e.g., cellobiose, sucrose, and lactose), and sugar alcohols (e.g., mannitol and sorbitol)⁶⁵. On the other hand, family GT2 includes enzymes that synthesize glycosidic bonds by transferring sugar moieties from donor molecules (i.e., UDP-glucose) to acceptor molecules, resulting in the formation of glycosidic linkages⁶⁶. Some key GT2 enzymes include glucosyltransferases such as glycerol phosphate polymerase (JKL2022_01530), monoglucosyldiacylglycerol synthase (JKL2022_01528), chondroitin synthase (JKL2022_01845), undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase (JKL2022_01865), and putative glycosyltransferase EpsH (JKL2022_00410). GH13 and GT2 enzymes play key roles in the degradation and utilization of dietary and host-derived carbohydrates and prebiotics and exopolysaccharide, cell wall, and cell membrane biosynthesis, respectively⁶⁷. Thus, they enhance nutrient utilization and support gut colonization by enhancing stress tolerance and adhesion, making them important markers of probiotic potential.

Starch hydrolysis test

None of the tested *Bifidobacterium* strains exhibited clear zones around their growth, indicating a negative result for starch hydrolysis. Additionally, these strains failed to grow in media containing starch as the sole carbohydrate source. The enzyme α -amylase catalyzes the breakdown of starch into glucose, a process linked to glycemic control, as rapid starch hydrolysis can cause a spike in blood glucose levels⁶⁸. Several studies indicated the role of inhibiting microbial carbohydrate hydrolyzing enzymes in reducing hyperglycemia^{69,70}. Specifically, the inhibition of α -amylase and α -glucosidase has been the most effectively used mechanism for anti-diabetic drugs⁷⁰. In this context, the lack of α -amylase activity of JKL2022 indicates that the strain does not contribute to starch-induced increases in blood glucose levels. In conjunction with α -glucosidase inhibition, these observations point to possible compatibility with diabetes management treatments.

α -Glucosidase inhibitory activity

Probiotics have been implicated in regulating blood glucose levels through several mechanisms, including inhibition of α -glucosidase activity, regulation of insulin-related signaling molecules, regulation of gut microflora, and appetite modulation, among others⁷¹. In addition, Li et al.⁷² reported that probiotics play a key role in the regulation of several factors (e.g., glycated hemoglobin A1c (HbA1c), quantitative insulin sensitivity check index (QUICKI), total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C)) in prediabetes patients. The α -glucosidase inhibitory activity of certain probiotic strains is beneficial in managing type II diabetes. α -Glucosidase is an enzyme that hydrolyzes starches and disaccharides into glucose, facilitating energy production. Inhibiting α -glucosidase in the intestine can slow down glucose absorption in the bloodstream, thereby regulating postprandial blood sugar levels⁷³. All tested strains demonstrated strong anti- α -glucosidase activity (Fig. 3A). Among them, strain JCM1273 exhibited the highest inhibitory activity ($87.68 \pm 5.34\%$), followed by JCM1192 ($86.68 \pm 6.28\%$), JCM7017 ($91.95 \pm 7.97\%$), JCM7019 ($73.81 \pm 5.94\%$), BB-12 ($72.14 \pm 11.95\%$), and JKL2022 ($67.54 \pm 6.33\%$). In 2023, Kumari et al. reported the antidiabetic activity of potential probiotics belonging to *Limosilactobacillus*, *Levilactobacillus*, and *Lactocaseibacillus* genera, inhibiting α -glucosidase and α -amylase activity by up to 85.88% and 75.89%, respectively⁷⁴. The observed inhibition of α -amylase activity, along with the absence of starch hydrolysis, suggests that JKL2022 may have potential applications in mitigating hyperglycemic conditions³³.

Conjugated linoleic acid production

CLA is associated with several health benefits, including anti-obesity, anti-inflammatory, and anti-cancer effects⁷⁵. In the presence of suitable substrates, such as free LA or α -linolenic acid (LNA), bifidobacteria can convert these compounds into conjugated forms as part of their detoxification mechanisms⁷⁶. During this process, the bifidobacterial LAI converts toxic LA into CLA²⁴.

Among the tested strains, JKL2022 (0.336 ± 0.025 mg/mL) and JCM7017 (0.295 ± 0.034 mg/mL) exhibited the highest CLA conversion rates, followed by JCM7019 (0.192 ± 0.023 mg/mL), JCM1192 (0.185 ± 0.015 mg/mL), and JCM1273 (0.064 ± 0.010 mg/mL). Other *B. breve* strains also exhibit strong CLA conversion, reaching up to 57.20–90.0% conversion⁷⁷. However, these strains were only reported to exhibit CLA conversion during growth and lack evidence of CLA conversion using washed cells or crude protein extracts. Additionally, experimental evidence confirmed that only JKL2022 can mediate the CLA conversion using washed or partially lysed cells and crude cell-free extracts (Supplementary Data), suggesting that this trait may be strain-specific.

The LAI enzyme of *B. breve* JKL2022 was previously characterized and heterologously expressed in *E. coli* BL21²⁴ and confirmed as the key enzyme for bifidobacterial CLA conversion. Figure 6 shows the amino acid sequence alignment of JKL2022 LAI with other bifidobacterial species, showing high sequence homology and the presence of nine transmembrane helices. Furthermore, sequence analysis confirmed that JKL2022 LAI is distinct from the PAI enzyme of *Cutibacterium* or the MCRA of lactobacilli. Figure 7 illustrates the distinct CLA conversion mechanisms employed by various bacterial genera, including *Cutibacterium*, *Lactobacillus*, and *Bifidobacterium*, emphasizing species-specific enzymatic systems.

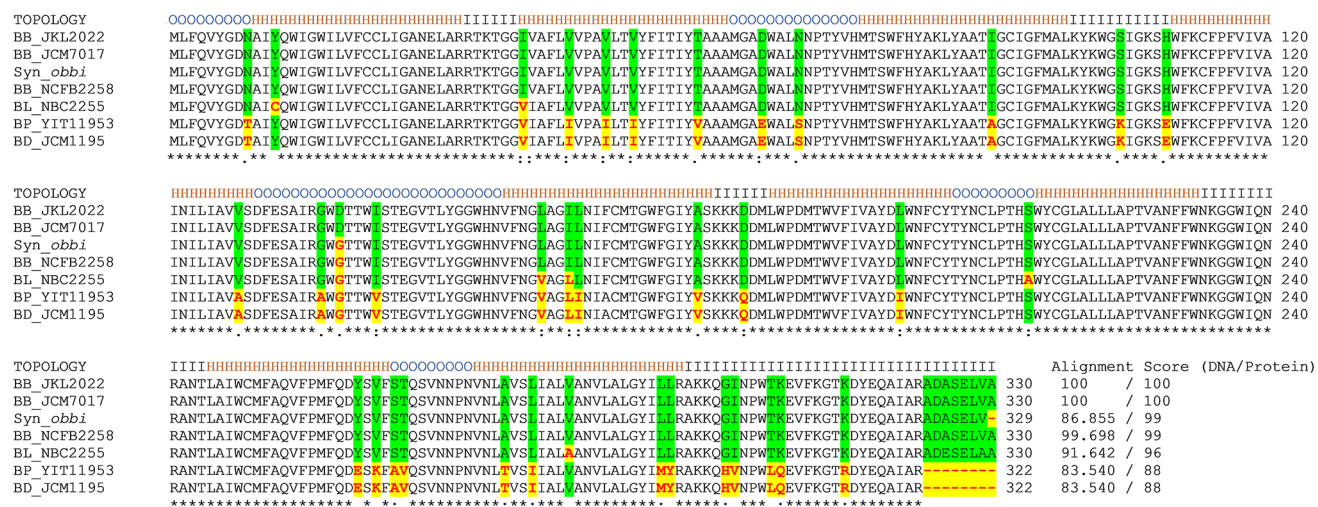


Fig. 6. Amino acid sequence alignment of bifidobacterial linoleic acid isomerase. BB – *Bifidobacterium breve* (JCM7017, CP006712.1; NCFB2258, CP006714.1); BL – *Bifidobacterium longum* (CP176538.1); BP – *Bifidobacterium pseudocatenulatum* (CP079231.1); BD – *Bifidobacterium dentium* (AP012326.1); Syn – synthetic construct of LAI (OM158463.1). Predicted protein topology is indicated as outside (O), transmembrane helix (H), or inside (I). Alignment score is based on multiple sequence alignment of nucleotide and amino acid sequences (ClustalW) using *B. breve* JKL2022 as reference.

In our previous study, *B. breve* JKL2022 was successfully applied as an adjunct culture in cream cheese and yogurt, and exhibited moderate CLA conversion when cultured in modified skimmed-milk media under aerobic and anaerobic conditions¹⁴. In this regard, the CLA conversion of JKL2022 using washed cells is promising in enhancing the CLA content of milk and dairy products. Overall, the CLA conversion of strain JKL2022 is expected to compound the inherent beneficial health effects associated with *B. breve* species.

Safety assessment

Antibiotic susceptibility

The antimicrobial resistance profile of *B. breve* JKL2022 was assessed using the broth microdilution method (Table 3). *B. breve* JKL2022 was susceptible to most antibiotics outside the known resistance profile of *B. breve*, which includes vancomycin, ampicillin, erythromycin, tetracycline, chloramphenicol, and ciprofloxacin. Notably, JKL2022 exhibited susceptibility to rifampicin despite harboring the rifamycin-resistant β -subunit of RNA polymerase (*rpoB*). Consistently, JKL2022 displayed a resistant phenotype to clindamycin, gentamicin, kanamycin, streptomycin, mupirocin, metronidazole, and polymyxin B, consistent with its intrinsic resistance and Gram-positive classification.

The antimicrobial resistance phenotype of JKL2022 aligns with AMR genes detected in its genome. The AMR profile of JKL2022 is consistent with previously reported resistance patterns in *B. breve* strains. Resistance to aminoglycosides is attributed to the absence of specific transport systems required for antibiotic entry into the cell⁷⁸. Similarly, resistance to polymyxin B, an antibiotic used to treat meningitis, pneumonia, and urinary tract infections, is intrinsic to most bifidobacteria due to their cell membrane structure. Moreover, resistance to polymyxin B often involves modification of the phosphate groups of lipid A with 4-amino-4-deoxy-L-arabinose; in this case, mutations in the UDP 4-deoxy-4-formamido-L-arabinose transferase (JKL2022_01865) may contribute to resistance⁷⁹. Lastly, as a Gram-positive anaerobe, JKL2022 lacks the specific enzymes required for metronidazole activation, rendering it inherently resistant⁸⁰.

Hemolytic activity

The ability of the bacterial strains to lyse red blood cells is a critical safety parameter, as hemolytic activity can have severe health implications⁸¹. All *Bifidobacterium* strains exhibited γ -hemolysis (no hemolytic activity) on blood agar, confirming their safety. Additionally, JKL2022 lacks hemolysin-encoding genes, further supporting its non-hemolytic nature.

Gelatinase activity

Gelatinase is recognized as a virulence factor in lactic acid bacteria. Genomic analysis confirmed that none of the tested strains, including JKL2022, harbored genes encoding gelatinase. Consistently, in vitro assessments showed no gelatinase activity. Lactic acid bacteria strains that do not produce gelatinase are considered safer candidates for probiotic development⁸².

Conclusions

The comprehensive genomic analysis of *B. breve* JKL2022 identified key probiotic traits, including genes encoding various carbohydrate-active enzymes, the absence of critical virulence factors, and a limited repertoire

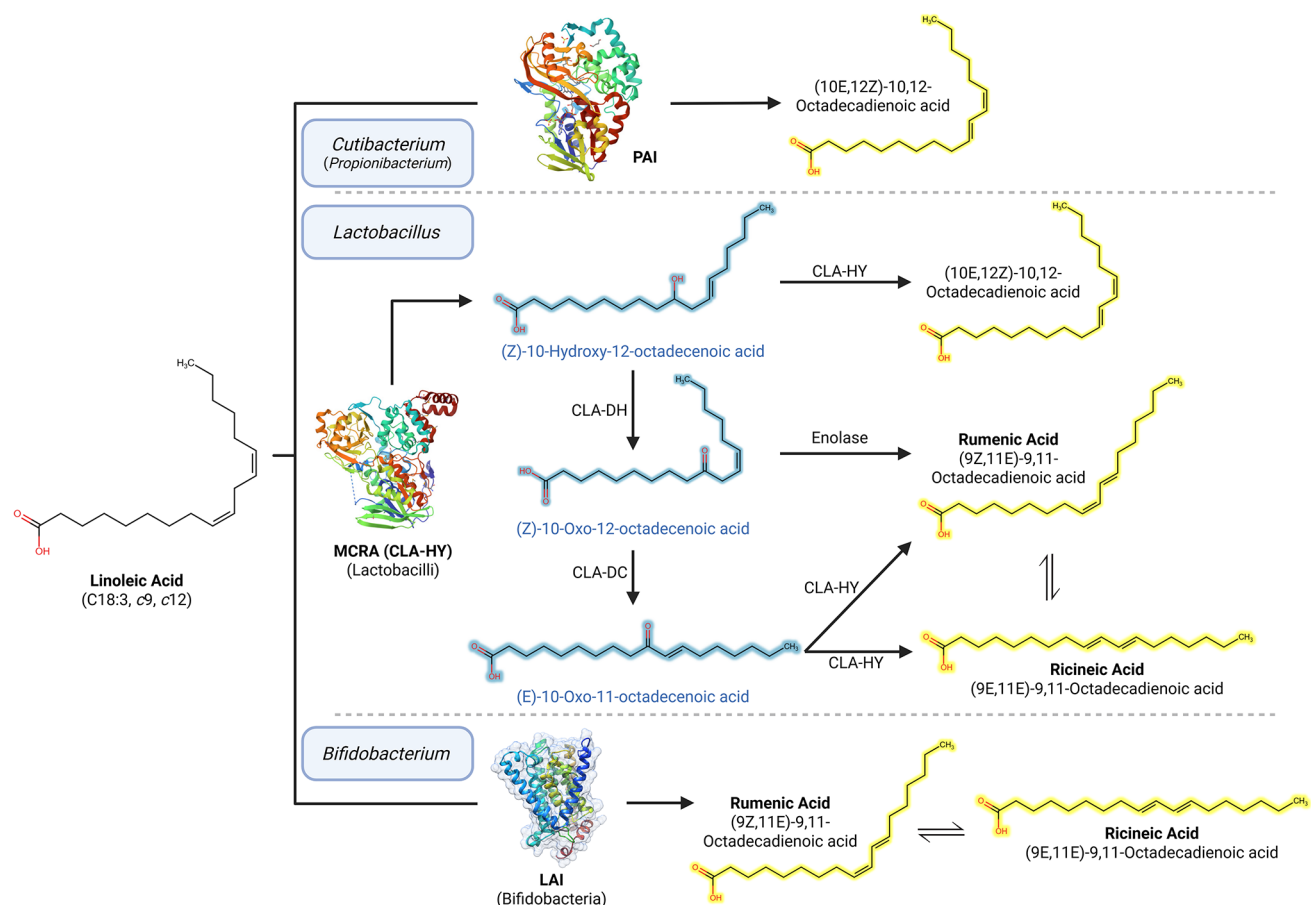


Fig. 7. Proposed microbial-CLA synthesis for *Cutibacterium* (PAI), *Lactobacillus* (CLA-HY, CLA-DH, CLA-DC), and *Bifidobacterium* (LAI). The tertiary structure of the key enzymes is shown; intermediate metabolites are shown in blue and CLA isomers are shown in yellow.

of antibiotic resistance genes. With respect to probiotic safety, JKL2022 is predicted to be non-pathogenic to human hosts. Consistently, in vitro assessments corroborated the genotypic characteristics of the strain, demonstrating acid and bile salt tolerance, cell surface hydrophobicity, broad carbohydrate metabolism, and the absence of harmful traits, including hemolytic activity, gelatinase activity, and α -amylase activity. Notably, JKL2022 exhibited superior CLA conversion compared to JCM reference strains, suggesting its potential to enhance dietary CLA levels. These findings underscore the probiotic potential of *B. breve* JKL2022, highlighting the strain's inherent functional properties and its strain-specific capacity for CLA conversion, which may contribute to additional health benefits.

Data availability

The sequence obtained in this Whole Genome Shotgun project has been deposited in DDBJ/ENA/GenBank under the accession number CP103292. The BioProject accession number is PRJNA871061 and the Biosample accession number is SAMN30403323. Upon a reasonable request, the datasets of this study can be requested from the corresponding author.

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AGE: Writing – original draft, review & editing, Conceptualization, Methodology, Investigation, Data curation. BGE: Writing – review & editing. G-BK: Writing – review & editing, Conceptualization, Methodology, Supervision, Funding acquisition.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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