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Research Article

Whole-Genome Analysis and Tolerance Assessment on *Bifidobacteria*-like Bacteria Isolated from Breast Milk and Infant Fecal Samples in Indonesia

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ABSTRACT

Probiotics are living microorganisms that can provide health benefits to the host when they are present in the gastrointestinal tract in sufficient quantities. Probiotics should be able to survive the harsh conditions in the gastrointestinal tract. This study sequenced whole genomes of *Bifidobacteria*-like isolates (i.e., BS2-PS1, BS2-PS2, BS2-PB5, and BR2-12) and assessed their abilities to resist a low-pH environment and bile salt. Molecular identification through whole-genome sequencing indicated that the bacteria isolated from breast milk were identified as *Bifidobacterium breve* (BS2-PS1 and BS2-PS2) and *Lacticaseibacillus paracasei* subspecies *paracasei* (BS2-PB5), whereas the bacterial isolate isolated from infant feces was identified as *Lactiplantibacillus plantarum* (BR2-12). BS2-PS1 and BS2-PB5 exhibited resilience upon exposure to bile salts at concentrations of 0 %, 0.3 %, and 0.5 %, with survival observed even at the highest concentration (0.5 %) after 3 h of incubation. The ability to withstand bile salt was mediated by various genes (e.g., *bsh*, *cbh*, *dps*, *glf*, *cfa*, or *nagB*) found in genomes of tested isolates. Upon exposure to pH 5 for 3 h, BS2-PS1, BS2-PS2, and BR2-12 exhibited acid resistance as well. The ability to withstand low pH could be mediated by the presence of relevant genes (e.g., *argC*, *argH*, *dapA*, *pyk*, *pyrG*), as well as genes that encode the F_0F_1-ATPase enzyme, such as *atpC*, *atpB*, *atpE*, *atpF*, *atpH*, *atpA*, *atpG*, and *atpD*, in their genomes. These results highlighted the advantages of integrating *in vitro* and whole-genome data to evaluate the potential of bacterial isolates for further development as probiotic candidates.

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INTRODUCTION

Probiotics are well known for the many health benefits they offered. According to the Food and Agriculture Organisation of the United Nations (FAO) and World Health Organisation (WHO), probiotics are living microorganisms that can provide health benefits to their hosts if present in the gastrointestinal tract in sufficient quantities (Hill et al. 2014). Some of the benefits include reducing the risk of diarrhea and acting as an immunomodulator (Virk et al. 2024). The popularity of probiotic products has been on the rise globally due to the growing public awareness of their benefits. In 2018, the sales of probiotic products reached 8,618.9 million euros, and 9,401.6 million euros in 2021. Furthermore, the average annual growth of probiotic products is expected to reach 8.7 % in the period 2021–2030 (Europe International Probiotics Association 2023).

Lactobacillus and *Bifidobacterium* are commonly used probiotics that can be isolated from breast milk and infant fecal samples (Bazireh et al. 2020). Breast milk is crucial for infant growth and development. It contains key immunological elements (e.g., antibodies) and contributes to the development of the infant's immune system. Human Milk Oligosaccharides (HMOs) function as prebiotics, promoting the growth of beneficial bacteria in the digestive tract, such as *Bifidobacterium* species (spp.) (Ojo-Okunola et al. 2018; Yi & Kim 2021). A review on microbial communities in breast milk from 44 studies showed that a core microbiome of seven to nine bacterial genera was mainly found, including *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Pseudomonas*, *Corynebacterium*, *Enterococcus*, *Acinetobacter*, *Rothia*, *Cutibacterium*, *Veillonella*, *Bacteroides*, and *Bifidobacterium* spp. (Zimmermann & Curtis 2020). In line with these findings, *Bifidobacteria* are mainly found in breast-fed infants owing to the HMOs growth-stimulating effect (Lawson et al. 2020). Furthermore, analysing infant feces can provide valuable insights because it is made of metabolic digestive waste from the large intestine. Thus, microorganisms in the feces of infants reflect the bacteria present in the large intestine, of which they normally included *Bifidobacterium* spp., *Bacteroides* spp., *Clostridia* spp., and *Faecalibacterium* spp. (Martinez et al. 2013).

Dosan et al. (2024) had previously isolated and characterised 10 *Bifidobacteria*-like bacteria from breast milk (BS2-MB1, BS2-PB3, BS2-PB5, BS2-PS1, and BS2-PS2) and infant fecal samples (BR1-M1, BR1-B1, BR2-5, BR2-6, and BR2-12) collected in Indonesia. Based on their sugar fermentation patterns, while five isolates (BR1-B1, BS2-PB3, BR2-12, BS2-PS1, and BS2-PS2) had a pattern of *B. adolescentis*, two (BR2-5 and BR2-6) and three isolates (BR1-M1, BS2-MB1, and BS2-PB5) exhibited similar fermentation patterns to *B. longum* subspecies *longum* and *B. breve*, respectively (Dosan et al. 2024). However, sugar fermentation patterns have very limited sensitivity and specificity for microbial species identification. Nonetheless, this limitation can be solved through proper molecular identification, that is, via 16S rRNA or whole-genome sequencing. Four isolates (BR2-5, BR2-6, BS2-PB3, and BS2-PS1) were subsequently subjected to 16S rRNA sequencing. Unsurprisingly, the results of molecular identification were different from the carbohydrate fermentation patterns, in which the 16S rRNA sequencing indicated that while BR2-5 and BR2-6 were very similar to *B. animalis* subsp. *lactis*, BS2-PB3 and BS2-PS1 were very similar to *B. breve* (Dosan et al. 2024). Furthermore, the genome of BS2-PB3 had been sequenced and confirmed to be *B. breve*. This isolate was also observed to possess various genes supporting its potential probiotic use (Marbun et al. 2024).

Probiotics must withstand the challenging environment of the gastrointestinal system to affect the health of hosts who consumed them (Skoufou et al. 2024). We had recently investigated the abilities of various bacteria isolated from breast milk samples (*Bifidobacteria*-like bacteria and *Lactiplantibacil-*

lus plantarum) to survive under various stresses encountered in the gastrointestinal tract (e.g., exposure to low pH and bile acids; Sunardi et al. 2023; Dosan et al. 2024). Therefore, we decided to sequence whole genome of four selected *Bifidobacteria*-like isolates (i.e., BS2-PS1, BS2-PS2, BS2-PB5, and BR2-12) and to assess their abilities to resist low pH and bile salt as ones of probiotic characteristics in this study.

MATERIALS AND METHODS

Culture isolation and maintenance

This study was conducted in the Fundamental Biology and the Advanced Biology Laboratories of the Department of Biology, Universitas Pelita Harapan, Indonesia. Isolates BS2-PS1, BS2-PS2, and BS2-PB5, as well as isolate BR2-12, were previously isolated from breast milk and infant fecal samples, respectively (Dosan et al. 2024). The medium used to cultivate the isolates was trypticase phytone yeast (TPY), with the following formulation for 1 L:17.6 g of tryptic soy broth (TSB) (HiMedia Laboratories, India), 1 mL of Tween 80 (Sigma-Aldrich, USA), 5 g of peptone (Liofilchem, Italy), 5 g of glucose (Sigma-Aldrich, USA), 2.5 g of yeast extract (HiMedia Laboratories, India), 0.5 g of L-cysteine (NOWFoods, Canada), and 2 g of KH_2PO_4 (Sigma-Aldrich, USA). When the solid medium was desired, the formulation was supplemented with 15 g of bacteriological agar (HiMedia Laboratories, India). The culture media that were inoculated with each isolate was incubated in anaerobic condition using OxoidTM AnaeroGenTM 2.5 L sachet (Thermo Scientific, USA) at 37 °C for 48 h.

Morphological identification

The morphological identification on the shape, margin, colour, and elevation of bacterial colonies was conducted as described by Dosan et al. (2024). The cellular identification was executed using the Gram staining method.

Molecular identification via whole-genome sequencing

Genomic DNA of BS2-PS1, BS2-PS2, BS2-PB5, and BR2-12 were extracted and sequenced using Oxford Nanopore Technology. Filtering was done using Filtlong v0.2.1 (https://github.com/rrwick/Filtlong/releases/tag/v0.2.1), and the results were transformed using Samtools v1.19.2 (https://github.com/samtools/samtools). Furthermore, quality of reads was evaluated using Nanoplot v1.42.0 (https://github.com/wdecoster/NanoPlot/releases/tag/v1.42.0). Reads correction and assembly were performed using Canu v2.2 (https://github.com/marbl/canu/releases/tag/v2.2) and Flye v2.9.2-b1795 (https://github.com/fenderglass/Flye), respectively. The assembled sequences were polished four times using Racon v1.5.0 (https://github.com/lbcb-sci/racon/releases/tag/1.5.0) and three times using Medaka v1.11.1 (https://github.com/nanoporetech/medaka/releases/tag/v1.11.1). The mapping was performed using Minimap2 2.26-r1175 (https://github.com/lh3/minimap2/releases) and the quality was determined using Quast v5.0.2 (https://github.com/ablab/quast) and Qualimap v2.3 (https://github.com/EagleGenomics-cookbooks/QualiMap). The annotation was performed using PGAP v6.6 (https://github.com/ncbi/pgap/releases).

The complete genome sequence of all four isolates had been submitted to GenBank. The assembled whole genomes were subsequently labelled with dFAST v1.6.0 annotation tool (https://dfast.ddbj.nig.ac.jp/dfc/) for genomic prediction and functional characterisation from the isolates (Tanizawa et al. 2016). Furthermore, the visualisation of whole genomes of the isolates was performed with Proksee (https://proksee.ca/) to retrieve circular map of the assembled genome (Grant et al. 2023). Identification of the isolates was subsequently conducted using TYGS webserver (https://tygs.dsmz.de/) and vis-

ualized with iTOL (https:// itol.embl.de/) (Meier-Kolthoff & Göker 2019; Letunic & Bork 2021). To measure similarity between genomes, whole-genome average nucleotide identity (ANI) was calculated with FastANI v1.34 (https://github.com/ParBLiSS/FastANI/releases/tag/v1.34) (Jain et al. 2018).

Identification of genes related to bile salt and acid tolerance

The identification of genes associated with bile salt and acid resistance was conducted as described by Valdez-Baez et al. (2022). Potential genes for bile salt and acid resistance mechanisms in *Bifidobacterium*, *Lacticaseibacillus*, and *Lactiplantibacillus* spp. were identified through a review of publications (Wang et al. 2021; Schöping et al. 2022; Shimizu et al. 2023; Contente et al. 2024) and shown in Table 1. Using the Basic Local Alignment Search Tool (https://www.ncbi.nlm.nih.gov/blast/), the sequences of these potential genes were compared to the genes of the bacterial isolates BS2-PS1, BS2-PS2, BS2-PB5, and BR2-12. For these alignments, a minimum identity percentage of 70 % was used.

Table 1. Acid- and bile-salt-resistance-associated genes in *Bifidobacterium*, *Lacticaseibacillus*, and *Lactiplantibacillus* spp.

Species	Gene	Mechanism	Reference
Bifidobacterium spp. *Lacticaseibacillus* spp. *Lactiplantibacillus* spp.	*bshA*	Taurine-conjugated bile salt hydrolase	O'Flaherty et al. 2018; Engevik et al. 2021; Flórez et al. 2021; Singhal et al. 2022; Kim et al. 2022; Min et al. 2022; Rossi et al. 2022; Schöping et al. 2022; Vestergaard et al. 2022; Li et al. 2024
	bshB	Taurine-conjugated bile salt hydrolase	
	bshC	Glycosyl-conjugated bile salt hydrolase	
	atpB	ATP synthase F0 sector subunit a	
	atpE	ATP synthase F0 sector subunit c	
	atpF	ATP synthase F0 sector subunit b	
	atpH	ATP synthase F1 sector subunit δ	
	atpA	ATP synthase F1 sector subunit α	
	atpG	ATP synthase F1 sector subunit γ	
	atpD	ATP synthase F1 sector subunit β	
	atpC	ATP synthase F1 sector subunit ε	
Bifidobacterium spp.	*cbh*	Breakdown bile salt	Wu et al. 2021; Reyes-Castillo et al. 2023
	argC	Tolerate acid environments	
	argH	Tolerate acid environments	
	dapA	Tolerate acid environments	
	cfa	Cyclopropane-fatty-acyl-phospholipid synthase	
	nagB	Glucosamine-6-phosphate deaminase	
Lacticaseibacillus spp.	*dltB*	D-alanyl-lipoteichoic acid biosynthesis protein DltB	Kim et al. 2022; Vestergaard et al. 2022
	cfa	Cyclopropane-fatty-acyl-phospholipid synthase	
	ppaC	Putative manganese-dependent inorganic pyrophosphatase	
	brpA	Biofilm regulatory protein A	
	ltaS1	Lipoteichoic acid synthase 1	
Lactiplantibacillus spp.	*pyk*	Pyruvate kinases involved in acid resistance	Singhal et al. 2021; Min et al. 2022; Contente et al. 2024
	pyrG	CTP synthase	
	cbh	Choloylglycine hydrolase	
	dps	DNA protection during starvation protein	
	glf	UDP-galactopyranose mutase	
	cfa	Cyclopropane-fatty-acyl-phospholipid synthase	

spp., species (plural).

Bile salt tolerance

The resistance assay to bile salt was conducted as described by González-Vázquez et al. (2022) and Dosan et al. (2024). A 10 % (v v^{-1}) bacteria liquid culture was inoculated into three vials containing TPY broth supplemented with bile salts at concentrations of 0 %, 0.3 %, and 0.5 % (w v^{-1}). Each treatment was incubated for 3 h at 37 °C under obligate anaerobic condition. Subsequently, the cell count for each treatment was determined by inoculating 50 μL of liquid culture with various dilution factors using the spread plate method on TPY agar. The plates were then incubated for 48 h at 37 °C under obligate anaerobic condition. Following incubation, the colony-forming unit (CFU) was counted. The cell count (log CFU mL^{-1}) was calculated as follows:

$$\text{Number of cells} \left(\frac{\log \text{CFU}}{\text{mL}} \right) = \log 10 \left(\frac{\text{CFU X dilution factor}}{\text{volume}} \right)$$

Acid tolerance

The resistance test to low pH was conducted as described by Kanwal et al. (2021) and Dosan et al. (2024). A 10 % (v v^{-1}) liquid culture was inoculated into three vials containing TPY broth with their pH were adjusted using hydrochloric acid (HCl), resulting in pH 2, 5, and 7.5. The pH 7.5 was used as control. Each treatment was incubated for 3 h at 37 °C under obligate anaerobic condition. Subsequently, the cell count for each treatment was calculated by inoculating 50 μL of liquid culture with various dilution factors utilising the spread plate method on TPY agar. The plates were incubated for 48 h at 37 °C under obligate anaerobic condition. After incubation, the CFU was counted using the above-mentioned formula for the bile salt tolerance.

Statistical analysis

Data were analysed and presented with GraphPad Prism version 10.2.3. Statistical significance for results of bile salt and pH testing were calculated using the Kruskal–Wallis test. Upon p-values <0.05, Dunn's multiple comparison test was performed. A p-value <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Morphological identification

To be grouped into taxonomic classifications according to their morphological characteristics, bacteria are identified morphologically, which allow quick distinction and simplify the identification. Additionally, genetic and biochemical assessment are frequently added to morphological identification as a basis for confirmation and in-depth analysis (Khawaldeh et al. 2017). The morphological identification for each isolate is presented in Figure 1. All isolates exhibited similar colony morphology on TPY agar: round shape, white, and convex elevation. Furthermore, all isolates were Gram-positive bacteria, which are evidenced by the purple colour. They are classified as Gram-positive due to the presence of a thick peptidoglycan matrix, which cause the absorption of crystal violet and displaying a purple colour (Harris 2022). The morphological characteristics of all isolates also showed small rod-shaped cells, with branching (bifid) shapes ranging from "V" to "Y." These findings are in compliance with those by Khushboo et al. (2023) which stated that *Bifidobacterium* spp. exhibited distinctive bifid shapes.

Molecular identification

The four genomes were annotated using the dFAST annotation tool (Table 2). Among the four isolates, BR2-12 had the longest genome size, i.e., 3,332,336 base pairs (bp), while BS2-PS1 and BS2-PS2 had the highest GC contents (58.9 %). The tool also predicted that BR2-12 had the highest num-

ber of coding sequences (3,141) and RNA (16 rRNA + 70 tRNA). Of note, *Lb. plantarum* strain BR2-12 was identified to have a clustered regularly interspaced short palindromic repeats. The Proksee webserver (https://proksee.ca/) was utilised to depict the circular genome graph of each isolate (Figure 2). Phylogenomic analysis was conducted to reconstruct the evolutionary history of organisms. The analysis, performed using TYGS (Figure 3) on *B. breve* BS2-PS1 and BS2-PS2, indicated that both strains were closely related to *B. breve* NCTC11815 and JCM 1192. The same analysis also revealed *Lc. paracasei* subsp. *paracasei* BS2-PB5 was closely related to *Lc. paracasei* subsp. *paracasei* JCM 8130, 363.501388 and IBB3423. Of note, *Lb. plantarum* BR2-12 was observed to be closely related to several strains of *Lb. plantarum* (UNQLp11, DSM 20174, and SRCM100442) and *Lacticaseibacillus* spp. This finding was not surprising because both *Lactiplantibacillus* and *Lacticaseibacillus* spp. belong to the same *Lactobacillaceae* family (Zheng et al. 2020).

The ANI values of BS2-PS1 and BS2-PS2 indicated that they belong to *B. breve* with 99 % identity. Of note, BS2-PS1 and BS2-PS2 shared a similarity of 99 %. The ANI values of BS2-PB5 indicated that it belongs to *Lc. paracasei*

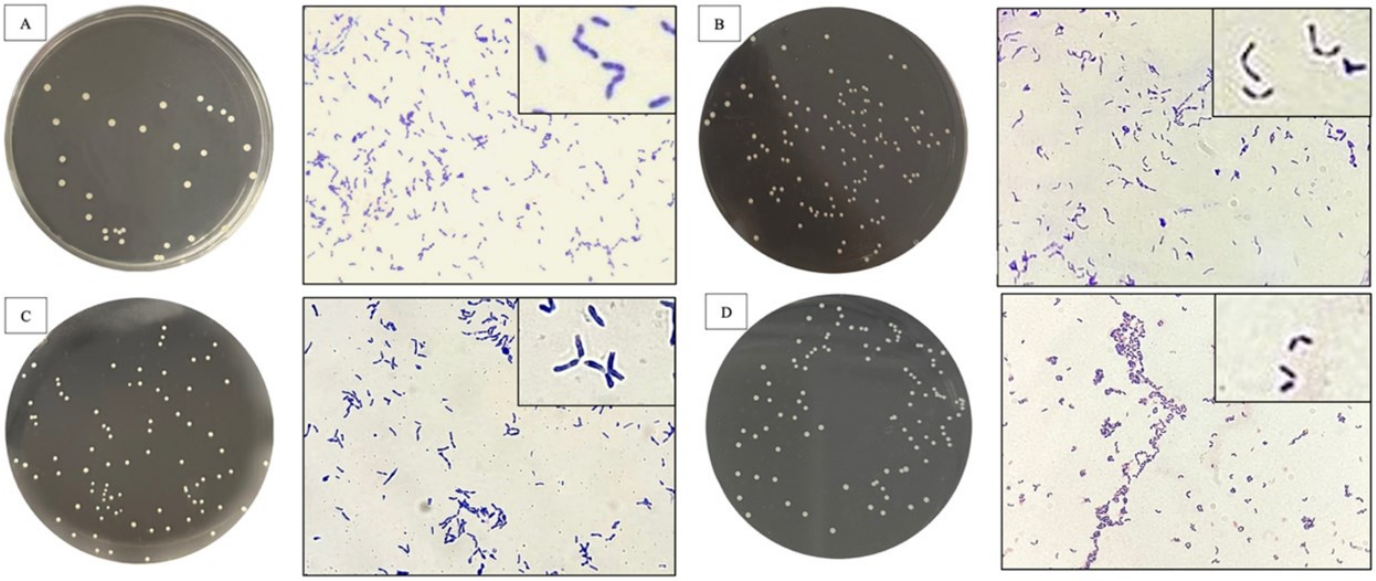


Figure 1. Visualisation of colony and cellular morphologies of the selected four isolates of *Bifidobacteria*-like bacteria. The left column shows the colony morphologies of *(A)* BS2-PS, *(B)* BS2-PS2, *(C)* BS2-PB5, and *(D)* BR2-12. The right column shows the cellular morphology of each corresponding isolate upon Gram staining using a light microscope with a magnifying power of 1000x.

Table 2. Genome annotation statistics of BS2-PS1, BS2-PS2, BS2-PB5, and BR2-12 isolates using the dFAST annotation tool.

Features	BS2-PS1	BS2-PS2	BS2-PB5	BR2-12
Species	*B. breve*	*B. breve*	*Lc. paracasei* subsp. *paracasei*	*Lb. plantarum*
Genome size (bp)	2,269,504	2,269,407	2,943,595	3,332,336
Contig	1	1	2	4
GC content (%)	58.9	58.9	46.5	44.5
N50 (bp)	2,269,504	2,269,407	2,905,540	3,218,310
L50	1	1	1	1
CDS	1,986	1,997	2,835	3,141
Total RNA	58 (4 rRNA + 54 tRNA)	58 (4 rRNA + 54 tRNA)	74 (15 rRNA + 59 tRNA)	86 (16 rRNA + 70 tRNA)
CRISPR	0	0	0	1

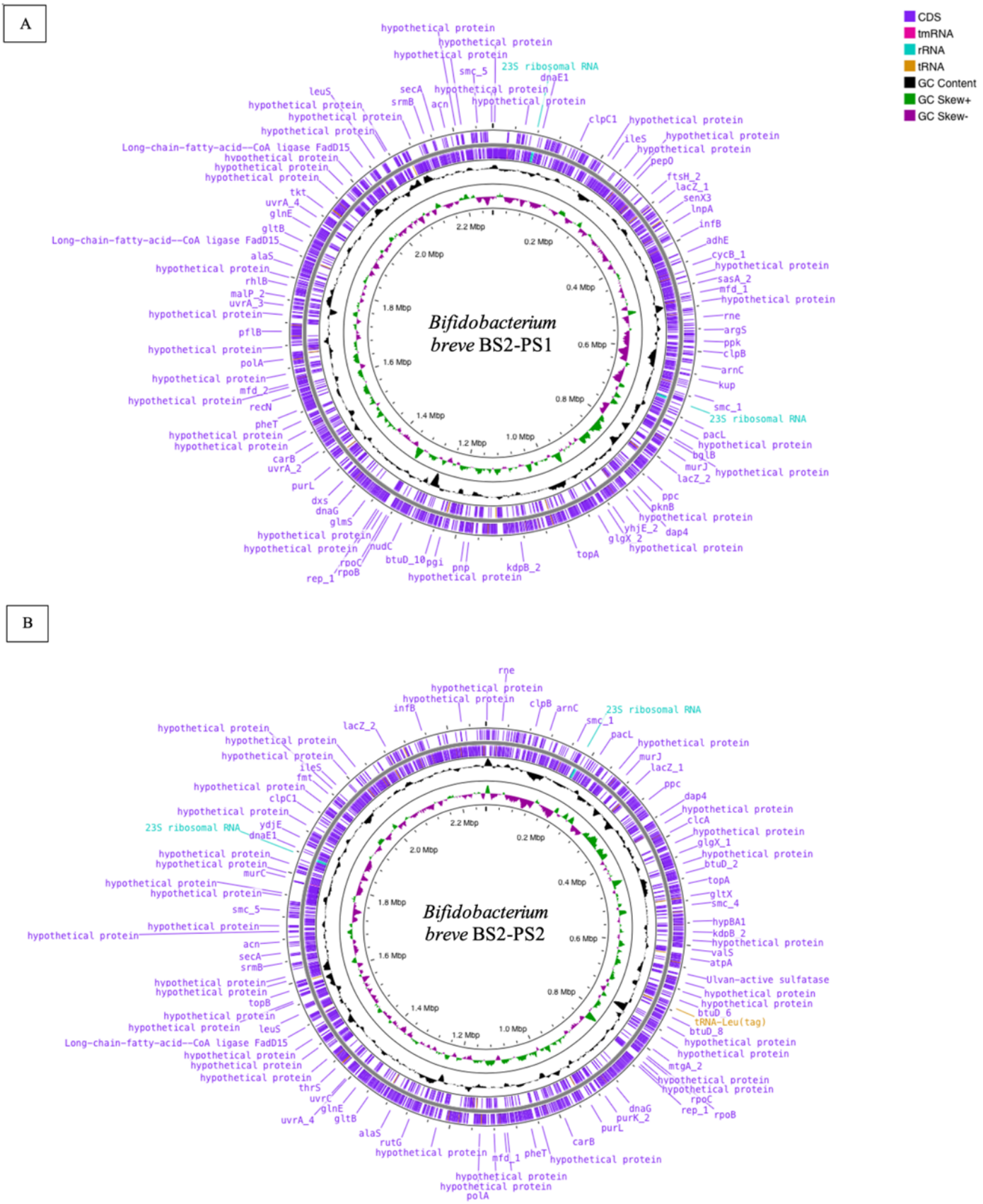
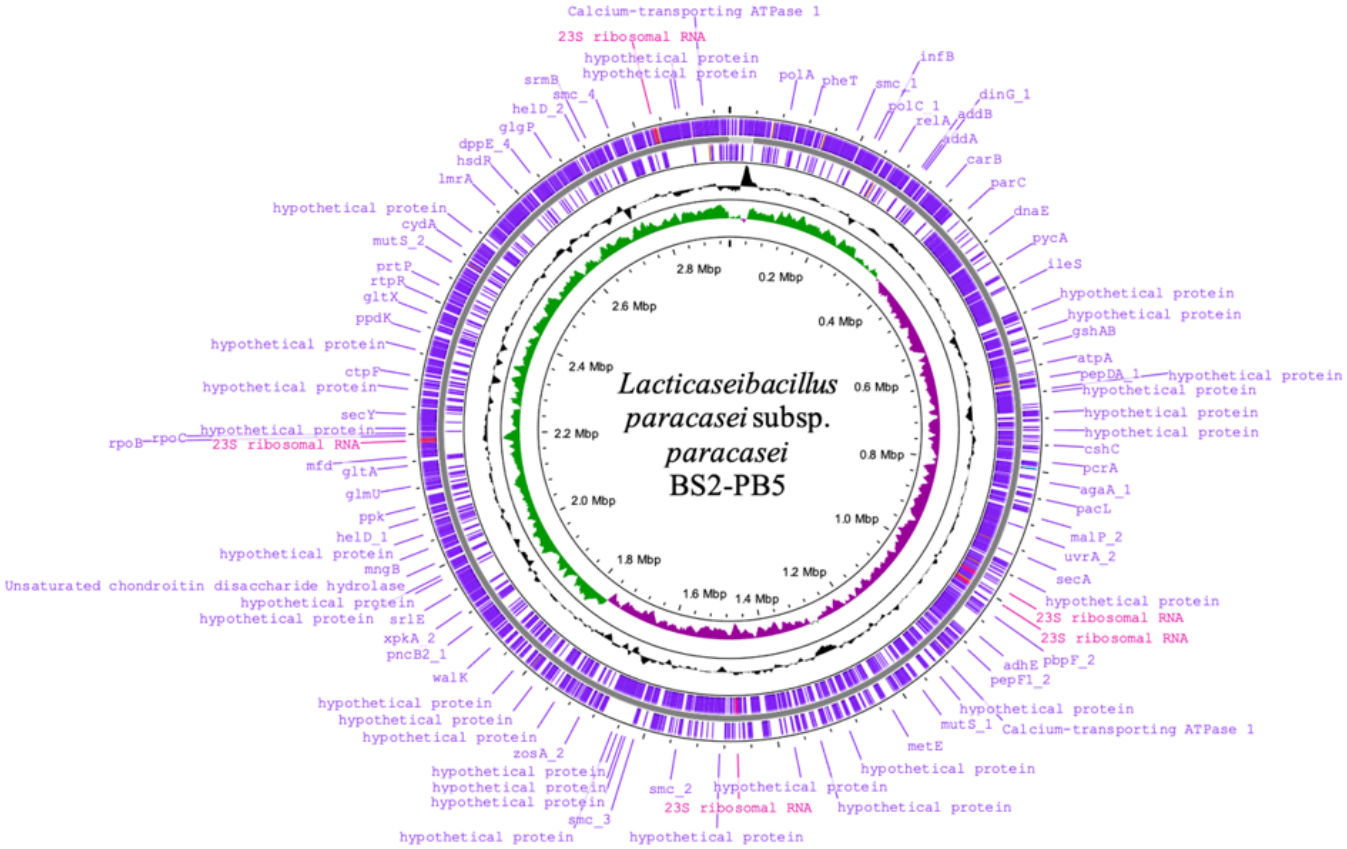


Figure 2. Visualisation of *B. breve* strain BS2-PS1 and BS2-PS2, *Lc. paracasei* subsp. *paracasei* strain BS2-PB5 and *Lb. plantarum* strain BR2-12 whole genomes. The visualization was performed using Proksee (https://proksee.ca/). Various genes, with hypothetical genes labeled by locus location, are depicted on the outermost violet circle. The first two outermost circles illustrated forward and reverse coding sequences (CDS), with hypothetical genes labelled as the locus location. The CDS was supplemented with tRNAs (brown), rRNAs (blue), and tmRNAs (pink). The black circle represents the GC content, whereas the green and dark purple circles represent the GC skew. The fifth innermost circle shows the genome size of *(A) B. breve* strain BS2-PS1, *(B) B. breve* strain BS2-PS2, *(C) Lc. paracasei* subsp. *paracasei* strain BS2-PB5, and *(D) Lb. plantarum* strain BR2-12.

C



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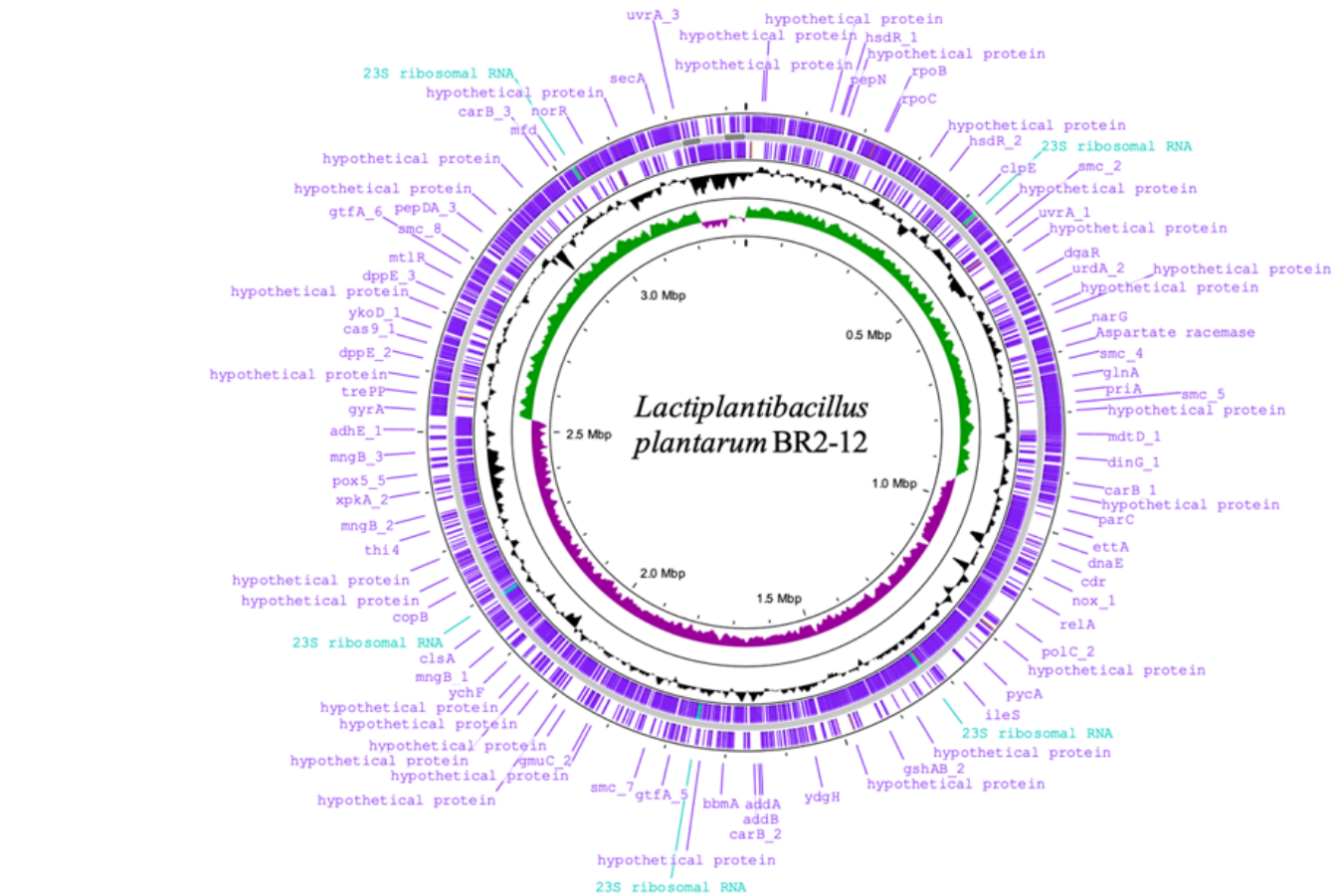

Figure 2. Contd.

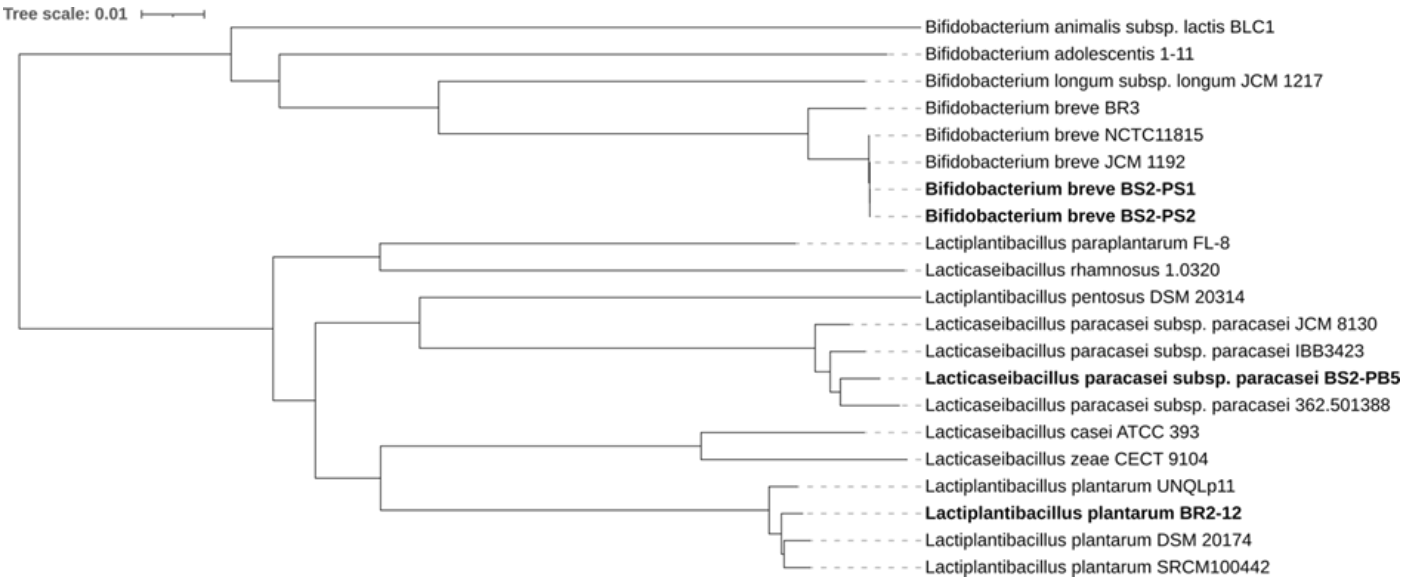

Figure 3. Phylogenetic tree of *B. breve* strain BS2-PS1 and BS2-PS2, *Lc. paracasei* subsp. *paracasei* strain BS2-PB5, and *Lb. plantarum* strain BR2-12. The comparisons of *B. breve* BS2-PS1, *B. breve* BS2-PS2, *Lc. paracasei* subsp. *paracasei* BS2-PB5 and *Lb. plantarum* BR2-12 with other strains of *Bifidobacterium* spp., *Lacticaseibacillus* spp., and *Lactiplantibacillus* spp. were carried out in the TYGS webserver (https://tygs.dsmz.de/) and were visualized by iTOL (https://itol.embl.de/). The tree was generated using TYGS, in which the MASH algorithm was used to compare all chosen genomes in the TYGS database. All pairwise comparisons were conducted using GBDP, and accurate intergenomic distances were deduced using the algorithm trimming and distance formula d5. The intergenomic distances were used to construct a balanced minimum evolution tree with branch support using FASTME 2.1.6.1, which included SPR postprocessing. Each branch support was inferred from 100 pseudo-bootstrap replicates. The tree was then visualised using iTOL.

with 98 % identity, whereas the ANI values of BR2-12 indicated that it belongs to *Lb. plantarum* with 99 % identity. This result is aligned with molecular identification using whole-genome sequencing, indicating that while isolates BS2-PS1 and BS2-PS2 were *B. breve*, BS2-PB5 and BR2-12 were *Lc. paracasei* subsp. *paracasei* and *Lb. plantarum*, respectively.

N50 was related to the median and mean lengths of a set of sequences, representing the length of the shortest read in the group of longest sequences, which account for at least 50 % of the nucleotides in the set of sequences. L50 was related to N50, indicating the number of sequences that when they arranged from longest to shortest are needed to reach or exceed 50 % of the total assembly size. bp, base pairs; GC, guanine–cytosine; CDS, coding sequence; RNA, ribonucleic acid; CRISPR, clustered regularly interspaced short palindromic repeats.

Tolerance to bile salt exposure

Bile salts contain bile acids and salts synthesised in the liver and stored in the gallbladder. Bile salts are essential for the digestion and absorption of dietary fats in the small intestine. Probiotics should be able to withstand bile salts as they disrupt the structure of cell membranes and damage DNA because they act as a natural detergent (Urdaneta & Casadesús 2017; Hagi et al. 2020). The average concentration of bile salts is approximately 0.3 % in the human intestinal tract, whereas concentrations as high as 0.5 % can be found in the duodenum (Grimm et al. 2014). Therefore, resistance to bile salts is important for the survival of probiotics in the digestive system. The growth response of all isolates to various concentrations of bile salts is shown in Figure 4.

Upon comparison between exposure to 0.3 % and no bile salts, the isolates BS2-PS1, and BS2-PB5 showed a reduction of 41 % and 8 %, respectively. Upon comparison between exposure to 0.5 % and no bile salts, the isolates BS2-PS1 and BS2-PB5 showed a reduction of 70 % and 47 %, respectively.

Indeed, the CFU reduction of BS2-PS1 upon exposure to 0.5 % of bile salt was statistically significant when compared to a condition without bile salt ($p = 0.0219$). Intriguingly, no substantial reduction on BS2-PS2 and BR2-12 was observed upon bile salt exposure. These were plausibly due to the poor growth of both isolates; hence their survivability rates could not be assessed. Taken together, these findings demonstrated that the isolates BS2-PS1 and BS2-PB5 were able to survive exposure to 0.3 % and 0.5 % of bile salts for 3 h, in which BS2-PB5 demonstrated a stronger resilience.

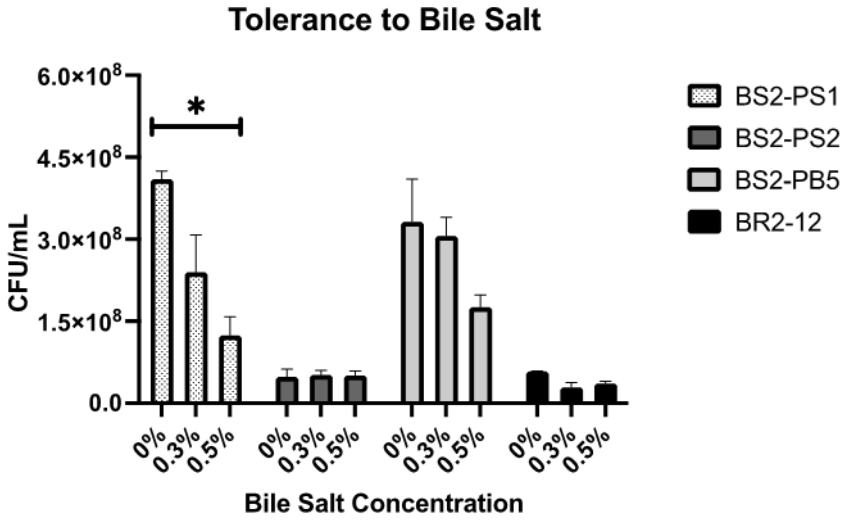

Figure 4. The bile salt tolerance of *B. breve* BS2-PS1 and BS2-PS2, *Lc. paracasei* subsp. *paracasei* BS2-PB5 and *Lb. plantarum* BR2-12 strains. Each isolate was cultured at 37 °C in TPY broth supplemented with either 0.3 % or 0.5 % (w v^{-1}) of bile salt. The control was the condition without any bile salt. Upon incubation for 3 h under obligate anaerobic condition, each culture was sampled and cultured on TPY agar for enumeration. The median of CFU mL^{-1} is displayed as a bar graph. The upper error bar indicates the maximum value. Results of each bacterial strain were analysed individually using the Kruskal–Wallis test with p-values <0.05 considered statistically significant. Results of *B. breve* strain BS2-PS1 were subsequently analysed using Dunn's multiple comparison test to determine which comparison between two groups was significantly different. An asterisk sign indicates p-values <0.05. The values were obtained from three experiments.

The ability to withstand bile salt stems from the presence of relevant gene(s), such as *bsh*, *cbh*, *dps*, *glf*, *cfa*, or *nagB* (Wall et al. 2014; Albarracin et al. 2022). Those genes confer bile salt resistance by breaking it down and increasing the tolerance to bile salt. One of the well-known enzymes that reduce bile salt toxicity is Bile Salt Hydrolase (BSH), encoded by the *bsh* gene. BSH enzyme catalyzes the hydrolysis of conjugated bile salts into deconjugated bile acids by breaking the bond between bile acid and amino acid. Therefore, BSH can decrease the overall concentration of bile salts, reducing their toxicity to bacterial cells (Bourgin et al. 2021). The breakdown of bile salts can reduce its toxicity through various mechanisms. It reduces membrane damage by limiting the penetration of bile salts into bacterial cells. It also minimises the DNA damage by lowering the concentration of bile salts, while the hydrolysis products, such as amino acids, provide nutrients that promote bacterial growth and survival (Jarocki et al. 2014; Song et al. 2019). As shown in Table 3, while BS2-PS1 and BS2-PS2 possess the same genes (i.e., *bsh*, *cbh*, *dps*, *glf* and *cfa*), BR2-12 has four of the five genes (i.e., *bsh*, *cbh*, *dps*, and *cfa*). It was of interest that among these three isolates, BS2-PS1 exhibited the highest resistant phenotype *in vitro*, suggesting that the expression of bile acid-resistant genes in BS2-PS1 was better than in BS2-PS2 and BR2-12. However, as BS2-PS2 and BR2-12 did not grow well in this assay, further studies are

Table 3. Bile resistance genes identified in the isolates.

Isolate	Gene	Mechanism	Identity (%)	Gene ID
BS2-PS1	*bsh*	Bile salt hydrolase	99.6	89503319
	cbh	Breakdown bile salt	84	13069843
	dps	DNA protection during starvation protein	99	69566565
	glf	UDP-galactopyranose mutase	98.6	8950422
	cfa	Cyclopropane-fatty-acyl-phospholipid synthase	72.3	6653298
BS2-PS2	*bsh*	Bile salt hydrolase	99.6	89503319
	cbh	Breakdown bile salt	84	13069843
	dps	DNA protection during starvation protein	99	69566565
	glf	UDP-galactopyranose mutase	98.6	8950422
	cfa	Cyclopropane-fatty-acyl-phospholipid synthase	72.3	6653298
BS2-PB5	*nagB*	Glucosamine-6-phosphate deaminase	86.4	45549971
BR2-12	*bsh*	Bile salt hydrolase	100	77216564
	cbh	Breakdown bile salt	99.5	14532344
	dps	DNA protection during starvation protein	99.5	77216492
	cfa	Cyclopropane-fatty-acyl-phospholipid synthase	99.6	77218098

required to investigate this speculation. Interestingly, BS2-PB5 only had one relevant gene (*nagB*), but it displayed a similar level of resistance phenotype to BS2-PS1. Therefore, the actual mechanism of *nagB* to confer resistance to bile salt in future studies should also be investigated.

Tolerance to acidity

Another criterion to consider is the resistance of probiotics to acidity in the gastrointestinal tract. The pH ranges from pH 2 (in stomach) and pH 4–5 (in small intestine) to pH 7–7.4 (in large intestine) (Henze et al. 2021). Hu and Li (2018) suggested that low pH reduces the cell growth by potentially activating the G1 cell cycle arrest. Mendonça et al. (2022) also indicated that low pH can disturb the proton motive force and other cellular processes, which leads to cell damage and death. Therefore, tolerance to acidity is a fundamental capability that probiotics possess to provide benefits to their host. The growth response of all isolates to various pH is illustrated in Figure 5.

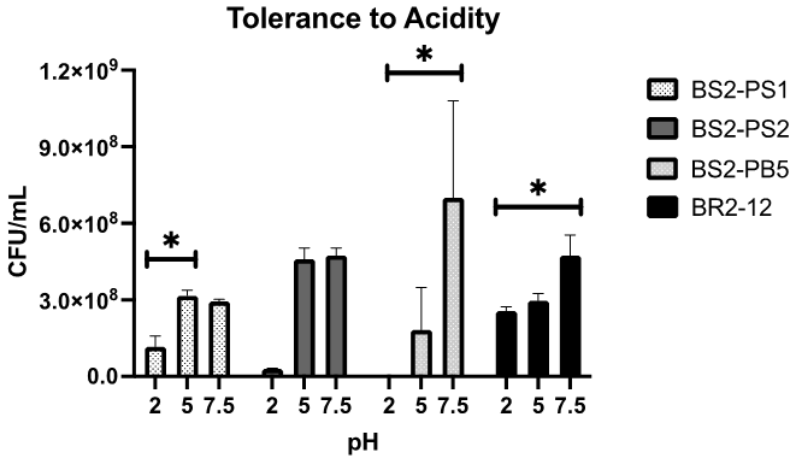

Figure 5. The acid tolerance of *B. breve* BS2-PS1 and BS2-PS2, *Lc. paracasei* subsp. *paracasei* BS2-PB5, and *Lb. plantarum* BR2-12 strains. Each isolate was cultured at 37 °C in TPY broth with their pH adjusted to 2 and 5. The control was a condition with pH 7.5. Upon incubation for 3 h under obligate anaerobic condition, each culture was sampled and cultured on TPY agar for enumeration. The median of CFU mL^{-1} is displayed as a bar graph. The upper error bar indicates the maximum value. Results of each bacterial strain were analysed individually using the Kruskal–Wallis test, with a p-value <0.05 considered statistically significant. Results of *B. breve* BS2-PS1,

Lc. paracasei subsp. *paracasei* BS2-PB5, and *Lb. plantarum* BR2-12 strains were subsequently analysed using Dunn's multiple comparison test to determine which comparison between two groups differed significantly. An asterisk sign indicated a p-value <0.05. The values were obtained from three experiments.

Compared to results for pH 7.5, *B. breve* BS2-PS1 and BS2-PS2 strains were interestingly not affected by pH 5. This finding is in contrast with the results of *Lc. paracasei* subsp. *paracasei* BS2-PB5 and *Lb. plantarum* BR2-12 strains in pH 5, in which both isolates underwent substantial reduction in their cellular counts (74 % and 38 % reduction, respectively). However, upon exposure to pH 2 for 3 h, all isolates contracted substantial reduction in their cellular counts. Specifically, the isolate BS2-PB5 and BR2-12 contracted significant reduction when compared to their cellular counts in pH 7.5 ($p = 0.0190$ and $p = 0.0219$, respectively). These findings indicated that all tested isolates were substantially reduced by a very low pH during 3 h of exposure. In compliance with the results of Mendonça et al. (2022), bacterial growth significantly decreased at a pH <4, and no growth or metabolic activity was observed at a pH <3. Nevertheless, it had been reported that the duration of normal gastric emptying was significantly faster than 3 hours because the meal retention within the stomach could vary between 30–90 % at the first hour of intake, and the meal retention was subsequently ≤10 % at the 4th hour of intake (Abell et al. 2008), suggesting that the transit time of ingested probiotics should not last longer than 3 h among normal individuals. Hence, the effects of stomach acid on cellular viability should arguably be less substantial than our findings due to a shorter duration.

Table 4 shows that BS2-PS1, BS2-PS2, and BR2-12 isolates were able to withstand low pH (2 and 5) due to the presence of several genes (e.g., *argC*, *argH*, *dapA*, *pyk*, or *pyrG*) (Yin et al. 2012; Tang & Matsuda 2024). In addition, the presence of eight subunit genes that encode the F_0F_1-ATPase enzyme, such as *atpC*, *atpB*, *atpE*, *atpF*, *atpH*, *atpA*, *atpG*, and *atpD* (Wu et al. 2022; Kingkaew et al. 2023), would confer resistance to acid as well. Although the presence of all eight subunit genes is not necessary for encoding the F_0F_1-ATPase enzyme, several subunits (e.g., α, β, and c subunits) are crucial for building this enzyme (Xu et al. 2015). The BS2-PS1, BS2-PS2, and BR2-12 isolates were found to possess all eight subunit genes of this enzyme. The F_0F_1-ATPase enzyme mediates an important role in maintaining the pH balance within the cell when exposed to acidic conditions. F_0F_1-ATPase functions by transferring proton (H^+) from the cytoplasm to the extracellular environment with the aid of energy generated by ATP synthesis reactions. By pumping protons out of the cell, F_0F_1-ATPase helps maintain intracellular pH balance under acidic conditions (Aoi & Marunaka 2014; Tran et al. 2021). Interestingly, although BS2-PB5 exhibited moderate levels of resistance to pH 5 (but not to pH 2), its whole-genome sequencing revealed the absence of any gene mediating acid resistance. Nonetheless, it had been reported that *Lc. paracasei* could survive in pH as low as 5 (Wu et al. 2021). Thus, there might be an unidentified mechanism of acid resistance in *Lc. paracasei*.

CONCLUSIONS

The whole-genome analyses of tested four isolates demonstrated that they were from three different species, that is, *B. breve* (BS2-PS1 and BS2-PS2), *Lc. paracasei* subsp. *paracasei* (BS2-PB5), and *Lb. plantarum* (BR2-12). Different degrees of sensitivity to bile salt (for isolates BS2-PS1 and BS2-PB5) and low pH exposures (for all isolates) were observed, of which the differences could be contributed by the presences of certain genes, such as *bsh*, *nagB*, *atpD*, and *atpC*. Thus, our findings demonstrate the benefits of combining *in vitro* and whole-genome data in assessing potentials of bacterial isolates to be further developed as probiotic candidates.

Table 4. Acid resistance genes identified in the isolates.

Isolate	Gene	Mechanism	Identity (%)	Gene ID
BS2-PS1	*atpC*	ATP synthase F1 sector subunit ε	98.6	14789701
	atpB	ATP synthase F0 sector subunit a	100	89502650
	atpE	ATP synthase F0 sector subunit c	96.4	89502651
	atpF	ATP synthase F0 sector subunit b	100	12168982
	atpH	ATP synthase F1 sector subunit δ	100	12168983
	atpA	ATP synthase F1 sector subunit α	99.7	89502654
	atpG	ATP synthase F1 sector subunit γ	100	12168985
	atpD	ATP synthase F1 sector subunit β	98.1	89502656
	argC	Tolerate acid environments	99.7	89503025
	argH	Tolerate acid environments	100	89503035
	dapA	Tolerate acid environments	99.8	89502831
BS2-PS2	*atpC*	ATP synthase F1 sector subunit ε	98.6	14789701
	atpB	ATP synthase F0 sector subunit a	100	89502650
	atpE	ATP synthase F0 sector subunit c	96.4	89502651
	atpF	ATP synthase F0 sector subunit b	100	12168982
	atpH	ATP synthase F1 sector subunit δ	100	12168983
	atpA	ATP synthase F1 sector subunit α	99.8	89502654
	atpG	ATP synthase F1 sector subunit γ	100	12168985
	atpD	ATP synthase F1 sector subunit β	98.2	89502656
	argC	Tolerate acid environments	99.7	89503025
	argH	Tolerate acid environments	100	89503035
	dapA	Tolerate acid environments	99.8	89502831
BR2-12	*atpC*	ATP synthase F1 sector subunit ε	100	77215740
	atpB	ATP synthase F0 sector subunit a	100	77215747
	atpE	ATP synthase F0 sector subunit c	100	77215746
	atpF	ATP synthase F0 sector subunit b	99.8	77215745
	atpH	ATP synthase F1 sector subunit δ	100	77215744
	atpA	ATP synthase F1 sector subunit α	100	77215743
	atpG	ATP synthase F1 sector subunit γ	100	14531356
	atpD	ATP synthase F1 sector subunit β	99.9	77215741
	pyk	Pyruvate kinases involved in acid resistance	99.9	77218256
	pyrG	CTP synthase	99.6	77217110

AUTHOR CONTRIBUTION

T.F.H., M.S., T.T.J., and J.J. designed the study. T.F.H., A.B.R., I.S.K., and R.T.L conducted the experiments and collected the data. M.S., T.T.J., and J.J. supervised the study. T.F.H., J.S.P., and J.J. analysed the data and drafted the manuscript. All authors approved the final version.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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