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Whole genome sequence analysis and *in-vitro* probiotic characterization of *Bacillus velezensis* FCW2 MCC4686 from spontaneously fermented coconut water

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ABSTRACT

In this study, the probiotic potential of *B. velezensis* FCW2, isolated from naturally fermented coconut water, was investigated by *in vitro* and genomic characterization. Our findings highlight key features of the bacterium which includes, antibacterial activity, high adhesive potential, aggregation capacity, production of nutrient secondary metabolites. *In vivo* safety assessment revealed no adverse effects on zebrafish. WGS data of *B. velezensis* FCW2 revealed a complete circular genome of 4,147,426 nucleotides and a GC content of 45.87%. We have identified 4059 coding sequence (CDS) genes that encode proteins involved in stress resistance, adhesion and micronutrient production. The genes responsible for producing secondary metabolites, exopolysaccharides, and other beneficial nutrients were identified. The KEGG and COG databases revealed that genes mainly involved amino acid metabolism, carbohydrate utilization, vitamin and cofactor metabolism, and biological adhesion. These findings suggest that *B. velezensis* FCW2 could be a putative probiotic in the development of fermented foods.

1. Introduction

Globally, probiotics have gained attention for their health benefits and potential to ease or prevent diarrhea and other gastrointestinal disorders [1]. Microorganisms that provide health benefits to the host when administered in adequate quantities are known as probiotics [2]. Several microorganisms, such as yeast, fungi and bacteria, are known to function as probiotics. *Lactobacillus* species and *Bifidobacterium spp.* are the most popular probiotic strains. Many *Bacillus* species including *Bacillus coagulans, Bacillus subtilis, Bacillus licheniformis, Bacillus cereus, Bacillus cereus* var. toyoi, *Bacillus clausii, Bacillus pumilus*, and *Bacillus amyloliquefaciens* have been found to be beneficial, so these strains were used as commercial probiotics for humans and in farm animals [3]. *Bacillus* species occurs naturally in soil, air, fermented foods, human and animal gut [4]. Major probiotic characteristics of *Bacillus* species including their ability to form endospores. Endospores provide the bacteria indefinite shelflife and allow them to survive under massive stressful environment in gastrointestinal tract (GIT) [5,6]. A broad spectrum of secondary metabolites, such as surfactin and bacteriocins, are produced by *Bacillus* species [7]. With the advantage of storage stability and antimicrobial effects *Bacillus* species have been used in the fermentation of foods. The *Bacillus* species are gaining attention for their high stability, higher stress tolerance, antimicrobial, antioxidant, immunomodulatory and food fermentation properties [8].

Naturally fermented foods have many unique and undiscovered microorganisms. These microorganisms play a crucial role in the fermentation process and can offer many health benefits. Several studies reported that *Lactic acid bacteria* (LAB) and *Bacillus* are the dominant

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microorganisms isolated from Asian fermented food [9]. *B. velezensis are frequently found in various fermented foods, which produces various exo-enzymes, including proteases, amylases, glucanase, and glutamine transferase and enhance the sensory properties of fermented foods through macromolecule degradation. Soybean shuidouchi* [10] *and soybean meal* [11] *are fermented by B. velezensis.* In the study by Na et al., *Bacillus velezensis* DMB06 was found to be a safe and potentially useful strain as a starter culture in food fermentations [12]. *B. velezensis* promotes plant growth, combats plant pathogens, and detoxifies mycotoxins. In addition, this species used as a probiotic for fish and poultry. *B. velezensis* was recommended for Qualified Presumption of Safety (QPS) status in 2017 by European Food Safety Authority (EFSA), it has been qualified based on the absence of clinically relevant antimicrobial resistance genes, the inability to produce aminoglycoside and the absence of toxigenic potential [13].

Fermented coconut water is a functional beverage produced by fermentation of fresh coconut water using beneficial microorganisms, such as LAB or yeast. It is a rich source of nutrients, antioxidants, and electrolytes and has been consumed for its refreshing taste and potential health benefits in various regions, particularly Southeast Asia. It is also low in calories and sugar, making it a healthy alternative to traditional soft drinks and sugary beverages. Fermented coconut water has gained popularity due to its potential probiotic properties, as the fermentation process can lead to the production of beneficial metabolites and the proliferation of health-promoting bacteria. The probiotics in fermented coconut water can boost the immune system and promote digestive health, reduce inflammation, and better overall immune function. Several studies have explored the potential use of fermented coconut water as a probiotic beverage [14–18]. It was reported that fermented coconut water inhibited the formation of struvite crystals, had anti-uropathogenic effects, and antioxidant properties [19]. Prado et al., developed a fermented coconut water beverage using probiotic bacteria isolated from naturally fermented coconut water and proving that probiotic bacteria exist in naturally fermented coconut water [20]. Previously, we reported probiotic properties of *Staphylococcus gallinarum* with unique characteristics from naturally fermented coconut water [21].

In this study, *B. velezensis* FCW2 isolated from naturally fermented coconut water was investigated for its probiotic properties and safety assessment, including its resistance to acid and bile salts, enzymatic activity, adhesion ability, auto aggregation, antibiotic susceptibility, hemolysis. Whole genome sequencing analysis of strain FCW2 was also performed to investigate its genetic determinants in relation to its *in-vitro* probiotic properties.

2. Materials and methods

2.1. Isolation and identification of potential Bacillus isolate

Bacillus probiotic candidate isolated from naturally fermented coconut water, with high activity against bacterial pathogens, was selected for further identification and probiotic characterization. Initial identification of selected bacteria was made by morphological and biochemical characterization [22]. HiIMViC Biochemical Test Kit (Himedia, Mumbai, India) was used to measure carbohydrate utilization. Molecular identification of isolate FCW2 was done by 16srRNA sequencing. Genomic DNA was extracted from the selected strain FCW2 using NucleoSpin® Tissue Kit (Macherey-Nagel) as per the manufacturer's instructions. The quality of the DNA was checked using agarose gel electrophoresis. Amplification of 16 s rRNA was performed using universal primers: 16 s-RS-F (5′ CAGGCCTAACACATGCAAGTC-3′) and 16 s-RS-R (5′-GGGCGGWGTGTACAAGGC-3′) in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). Sequencing was performed with the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA).The sequence was compared with the NCBI database through BlastN (basic local alignment search tool – http://www.ncbi.nlm.nih.gov/BLAST) as well as Ezbiocloud blast (https://www.ezbiocloud.net/identify) and the sequence has been submitted in the GenBank data library (Accession number- MW453068). The phylogenetic analysis was performed with MEGA X software by using the Maximum Likelihood algorithm [23].

2.2. Whole genome sequencing and annotation

For the precise identification of the bacteria and to characterize the probiotic traits at genomic level, whole genome sequencing of the bacterial DNA was performed. Using QIAamp DNA Mini Kit (QiagenInc, USA), DNA was extracted from purified FCW2 culture broth according to manufacturer's instructions. The genome was sequenced on the Illumina NextSeq 2500 platform using 2 × 250 paired-end libraries. The raw reads quality control software,FastQC version 0.11.9 (Andrews 2010) and Trimmomatic (ver 0.35) was used to trim low-quality reads with a Phred cutoff of Q20 [24]. The primary *de novo* assembly of the sequences was performed using SPAdes genome assembler (ver 3.10) [25]. Assembled genome was then annotated using NCBI Prokaryotic Genome Annotation Pipeline. The OrthoANI application of EzBioCloud was used to compute the overall genome relatedness index, which measures the Orthologous Average Nucleotide Identity (OrthoANI) [26].

The genes associated with the probiotic characteristics were manually extracted from the annotated genome and confirmed by using BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against NCBI's non-redundant database. Essential enzyme functional prediction was obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) using blastKOALA server (http://www.kegg.jp/blastkoala/).The Protein coding genes were obtained by blasting genes against Clusters of Orthologous Groups (COGs) of proteins on WebMGA (http://weizhong-lab.ucsd.edu/webMGA/server/cog/).The OrthoVenn2 software was used to compare and analyze the whole-genome orthologous clusters of FCW2 and the closest genome (https://orthovenn2.bioinfotoolkits.net/) [27].Using Antismash 6.0, genes involved in secondary metabolites biosynthesis were detected and the antibiotic resistance genes were detected by Resistance Gene Identifier (RGI) (version 5.1.1) [28]. Genomic islands were detected by the program IslandViewer (https://www.pathogenomics.sfu.ca/islandviewer/browse/) [29].

2.3. Evaluation of probiotic characterization In-vitro

2.3.1. Tolerance to different stress conditions

The survivability of the bacterial isolate in high concentration of bile was assayed based on Somashekaraiah et al. with slight modifications [30]. The overnight culture of bacteria was centrifuged at 5000 rpm for 10 min, pellets were collected, washed thrice with Phosphate Buffered Saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, and 0.245 g KH2PO4 in 1000 ml distilled water, pH 7.4), and resuspended in sterile PBS. The bacterial suspension was inoculated in LB broth containing 0.3% bile salt and incubated for 4 h. The acid tolerance was estimated based on previous published method [31] with slight modifications by suspending the FCW2 pellets in 5 ml PBS solution after adjusting the pH to 2 with 1 M HCl and incubated for 4 h. To assess lysozyme resistance, bacterial suspension was resuspended in 10 mL LB broth with lysozyme (100 mg/L) and incubated for 120 min at 37 °C [32]. Atspecific intervals, the samples were examined for cell viability by plate count method and absorbance rate at 600 nm by spectrophotometer. Percentage survivability was calculated using the formula below:

$$\text{Survival rate } (\%) = \text{ODtest/OD control} \times 100$$

For the osmotic stress tolerance assessment, the strain was inoculated in LB containing 0, 3, and 6% NaCl and incubated at 37 °C for 24 h [33]. The ability of the isolate to grow at different temperatures was evaluated by inoculating overnight grown strain in LB broth and incubated at 0–4, 10–15, 25–28 and 37–40 °C for 24 h [34].The absorbance was measured at 600 nm, and the survival rate was calculated.

2.3.2. Adhesion assays

Several factors contribute to the bacterial attachment to epithelial cells, including hydrophobicity, auto aggregation, and biofilm formation. FCW2 strain was tested for auto aggregation and hydrophobicity as described by Li et al. and Diale et al., respectively [35,36]. The biofilm forming ability of FCW2 isolate was determined following Zayed et al. with slight modifications by inoculating LB broth with FCW2 isolate and incubating for 48 h at 37 °C in a shaking incubator at 120 rpm [37]. Subsequently, the culture was decanted, washed in PBS buffer and stained with 0.1% crystal violet for 30 min, then again washed thrice in distilled water. The violet tint on the test tube walls indicates the formation of a biofilm.

2.3.3. Safety assessment

The antimicrobial activity of the cell free supernatant of *B. velezensis* FCW2 was determined against various pathogens (*Klebsiella pneumoniae, Streptococcus spp.*, *Escherichia coli*, *Pseudomonas aeurogenosa, Staphylococcus aureus*, and *Enterococcus faecalis* - All belonging to K.A.P.V. Govt. Medical College, Tiruchirappalli) by agar well diffusion method [31]. The wells were cut on the Muller Hinton agar (MHA) plate after swabbing pathogens and 100 ml of FCW2 culture supernatant was loaded. After incubation for 24 h at 37 °C, the zone of inhibition was measured. The antibiotic susceptibility test by Kirby Bauer's disc diffusion method was performed for screening of nonpathogenic functional strains as per the CLSI (Clinical and Laboratory Standards Institute, USA) guidelines. After incubation, the results were compared with the interpretative standards (sensitive (IZD $\geq$ 20 mm), intermediate (15 mm $\leq$ IZD $\leq$ 19 mm), and resistant (IZD $\leq$ 14 mm)) as described in Performance Standards for Antimicrobial Disc Susceptibility Tests [38]. The hemolytic activity of the isolate was performed by streaking FCW2 on blood agar plate and incubated at 37 °C for 48 h for screening of nonpathogenic functional strains [39]. The isolate FCW2 was streaked onto a deoxyribonuclease (DNase) agar medium to test the pathogenicity of bacteria by producing DNase enzyme as described by Kumari et al. [40].

2.4. In-vivo toxicological assay in zebrafish

Zebrafish model (*Danio rerio*) were used to examine the toxicology and biosafety of strain FCW2 [41]. All animals were handled following the guidelines of the Institution Animal Ethics Committee at Bharathidasan University (BDU/IAEC/P11/2021) (Tamil Nadu, India). The experiments were conducted in triplicate in aquaria at a temperature of 25–30 °C with a lighting schedule of Light Dark cycle (LD) 12:12. After an initial 10-days acclimation period, 30 fish (mean body weight: 0.188 g) were randomly assigned to the aquaria. A probiotic strain was inoculated into MRS broth for 24 h and harvested the cells by centrifuging at 6000 rpm for 20 min at 4 °C. Different concentrations (10^5, 10^6, and 10^7cells/mL) of the probiotic strains dissolved in sterile PBS were added to fish tanks labeled as experimental animals with appropriate concentrations. Control tanks were filled with sterile saline solution. Twice a day, fish were fed (35% protein) with 5% of their body weight. During the experimental period the symptoms, abnormalities, and mortality rates were recorded.

3. Results and discussion

3.1. Identification of potential Bacillus isolate

Strain identification is a crucial step for evaluating probiotic candidates. The primary identification of isolate FCW2 was performed by morphological and biochemical characteristics. The morphological view of strain FCW2 is given in Fig. 1. Based on the Gram-staining and scanning electron microscopy (SEM) the strain FCW2 is identified as Gram-positive bacilli. The biochemical profiles based on enzymatic activity and carbohydrate fermentation are given in Supplementary Table S1.

Molecular identification of strain FCW2 was done based on 16 s rRNA sequence comparison with NCBI BlastN and Ezbiocloud blast and the strain FCW2 shares 99.92% similarity with *B. velezensis* CR-502 followed by 99.84% similarity with *B. amyloliquefaciens* DSM 7. The maximum likelihood tree, constructed with bootstrap values out of 1000, based on 16 s rRNA sequence, shows that the strains FCW2, *B. velezensis* CR-502 and *B. amyloliquefaciens* DSM 7 are in the same branch (Fig. 2a).

3.2. Whole genome sequence analysis and comparison

The complete genome of *B. velezensis FCW2* consisted of 4,147,426 nucleotides with a GC content of 45.87%. The circular genome map and its general genomic characteristics are shown in Fig. 3a and Supplementary Table S2. There were no plasmids in the FCW2 genome. A total of 4133 genes and 4059 protein-coding genes (CDS) were predicted. These genes are grouped as subsystem proteins based on their predicted involvement in a specific biological process or structural complex using the PATRIC annotation tool. Fig. 3b provides an overview of the subsystems of the genome. There are 74 RNA genes in the genome, which include 57 tRNAs, 4 rRNAs, and 1 tmRNA. The phylogenetic tree based on WGS also suggests that the strain belongs to *B. velezensis* and showed a sequence similarity to the strain type of the species, *B. velezensis* FZB42 (Fig. 2b). To make in-depth analysis, genome-to-genome distance comparison has been done by GGDC web server (https://ggdc.dsmz.de/). The overall similarity between FCW2, DMS7 and FZB42 species, as estimated by the GGDC, is shown in Table 1. The pair-wise comparison of the FCW2 genome with DMS7 was found to be 87.85%, 36.87%, and 88.76% and with FZB42 96.78%, 93.95%, and 99.52% for the formulae HSP length/total length, identities/HSP length, and identities/total length ratios, respectively, in a probability DDG $\geq$70% index analysis. This indicates that FCW2 was more closely related to FZB42 than DMS7. The results from annotated protein sequences analysis using Orthovenn tool revealed that the FCW2, FZB4 and DMS7 consist of 3670

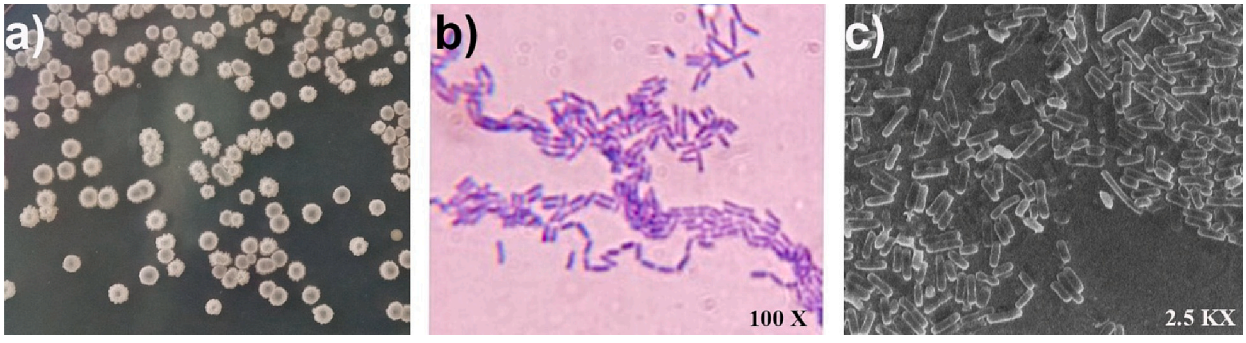

Fig. 1. Morphological view of FCW2 strain. (a) Colony morphology in LB agar media, (b) Gram-stained bacteria under 100× light microscope and (c) Bacterial morphology under scanning electron microscope.

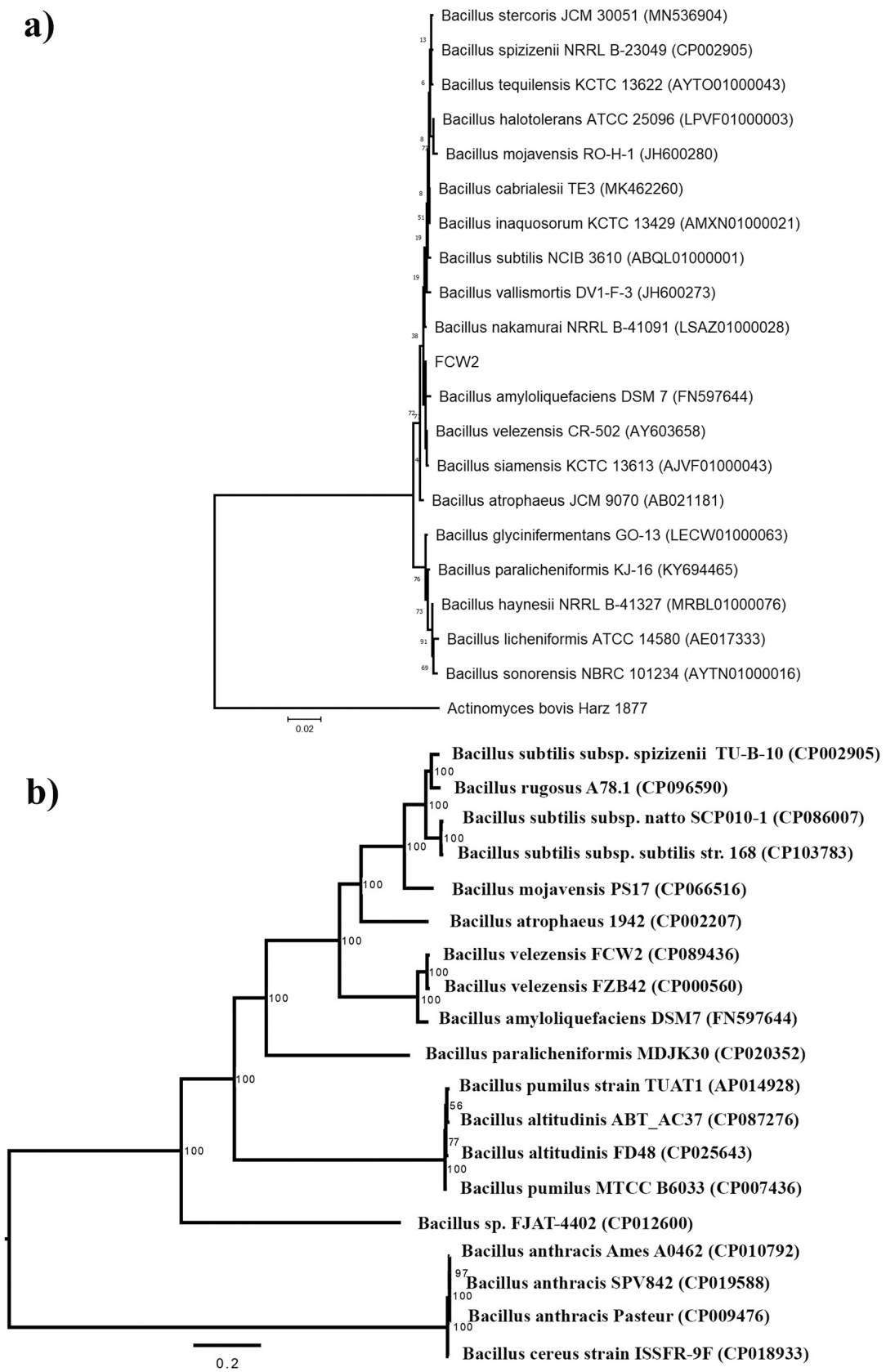

Fig. 2. The phylogenetic tree based on 16 s rRNA and WGS. a) The phylogenetic relationships between strain FCW2 and 23 closely related strains of the genus *Bacillus*. The out-group species used was *Actinomyces bovis* Harz 1877 (X81061). b) Phylogenetic tree based on WGS containing 20 strains constructed using PATRIC annotation tool. The multiple alignment was performed by mafft and the phylogenetic tree was constructed with RAxML fast Bootstrapping branch supporting method.

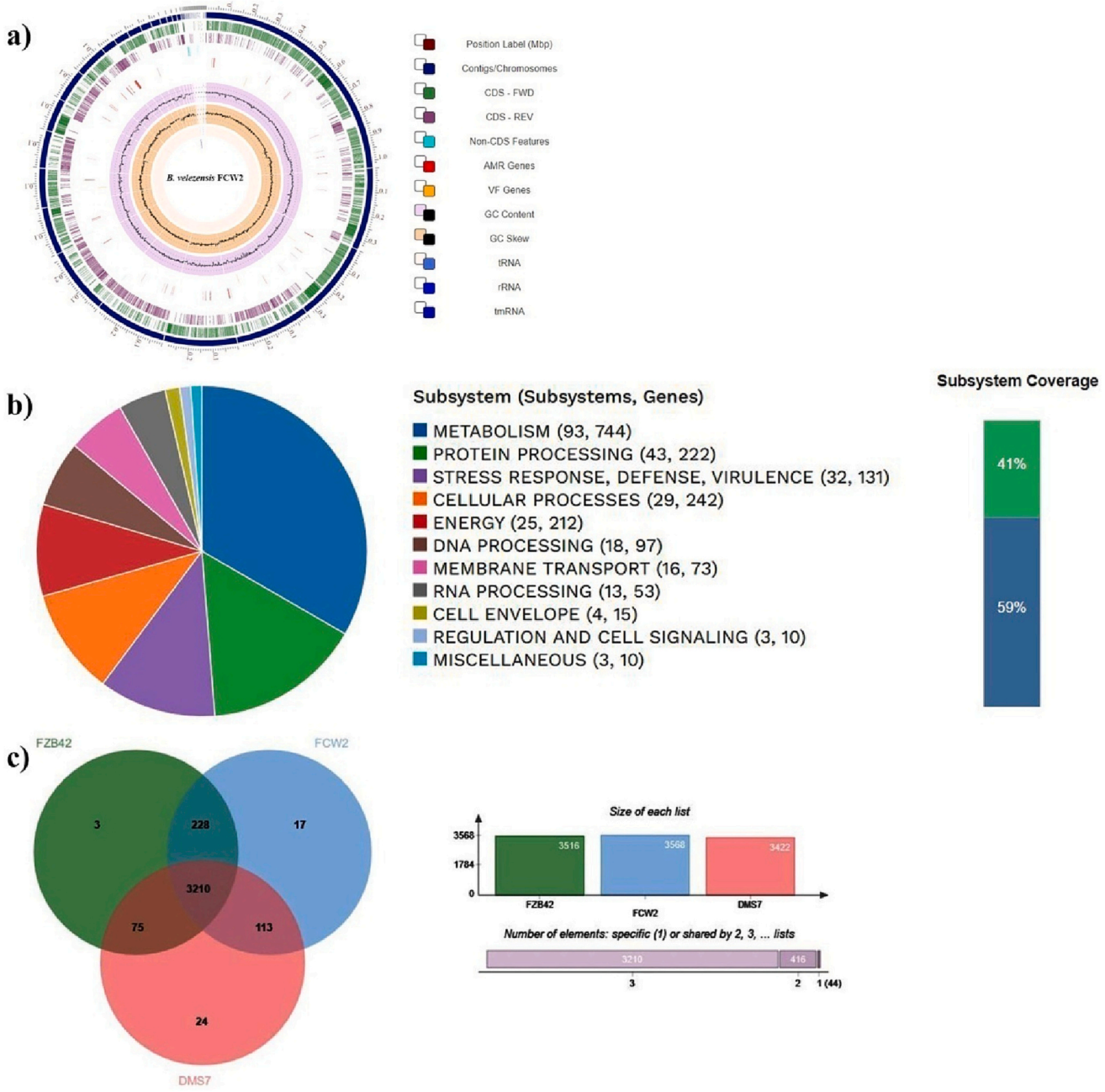

Fig. 3. a)Circular graphical representation of *B. velezensis* FCW2 genome. From outer to inner rings, the contigs, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to known virulence factors, GC content and GC skew. b) An overview of Patric annotated subsystem gene distribution. The bar diagram represents the subsystem coverage, and the pie chart represents the distribution of subsystem features. c) Venn diagram showing the distribution of shared and unique gene clusters of FCW2 strain with the genome of *B. velezensis* FZB42 and *B. amyloliquefaciens* DMS7.

clusters, 472 orthologous clusters (at least contains two species) and 3198 single-copy gene clusters (Fig. 3c). A total of 3210 ortholog clusters shared by all three species, 416 clusters were shared by at least two genomes.

3.3. Functional annotation of FCW2 Genome

The functional annotation of CDS was performed by KEGG and COG database analysis. A total of 2309 genes were classified into 11 KEGG functional categories, with the highest abundance of genes assigned to genetic information processing, signaling and cellular processing, carbohydrate metabolism, environmental information processing, amino acid, vitamin and cofactor metabolism (Fig. 4a).Based on the COG annotation results, we found that all CDS are classified into 20 functional categories. A majority of CDS in the genome were classified for general function prediction only (R, 13.52%), amino acid transport and metabolism (E, 9.65%), transcription (K, 8.79%), and carbohydrate transport and metabolism (G, 7.54%) as shown in Fig. 4b. This strain can degrade a wide range of carbohydrates and proteins.

3.4. Evaluation of probiotic characterization

Probiotics are considered to have good adhesion capacity to mucosal membranes in the intestine, letting them to colonize, compete with

Table 1
Pairwise comparison of strains FCW2, DMS7 and FZB42.

Query	Reference	HSP length/total length				Identities/HSP length				Identities/total length ratios				G + C difference
		Model C.I.	DDH	Distance	Prob. DDH ≥70%	Model C.I.	DDH	Distance	Prob. DDH ≥70%	Model C.I.	DDH	Distance	Prob. DDH ≥70%	
CP089436	CP000560	[85.3 - 91.4%]	88.7	0.0881	96.78	[82.7–87.8%]	85.4	0.0171	93.95	[88.2–93%]	90.9	0.1037	99.52	0.6
CP089436	FN597644	[73.3 - 80.8%]	77.3	0.1462	87.85	[52.8–58.3%]	55.5	0.0598	36.87	[71.6–78.3%]	75.1	0.1972	88.76	0.21

pathogens, and modulate the immune system [42]. Probiotics must survive the harsh digestive environment, such as gastric pH, bile salt, lysozyme and osmotic stress. Bacteria respond to stress by expressing genes or activating proteins. The probiotic characteristics of the strain FCW2 were checked by combining WGS analysis with *in-vitro* tests.

3.5. Stress tolerance

Figure 5a-e represents the growth of FCW2 at different stress conditions. During the stomach transit, probiotic bacteria should resist high acidic (pH 1.5 to 2) environment in stomach. *In-vitro* results showed that the strain FCW2 was found to be tolerant to acid stress. Compared to the initial time (0 h), the strain FCW2 showed stable growth after 4 h incubation at pH 2 and pH 7, without any significant loss of viability.

The high concentration of bile secreted by cholesterol catabolism in the small intestine destroys bacteria cell membranes, which reduces the survival of bacteria. After 4 h incubation in the presence of 0.3% bile salt, the strain showed 98.59% survivability. Lysozyme, an enzyme that is present in gastrointestinal secretions and saliva, is another obstacle for probiotics. The survival rate of FCW2 after exposure to lysozyme for 120 min was estimated as 90.32%. This suggests that FCW2 showed high resistance to lysozyme.

Analysis of the whole genome sequence reveals that FCW2 contains several bile, acid, and other stress-resistant genes, including *nhaX, nhaK, nhaC, yveA, bsaA, addAB, recDGQ, pcrAB, cshA*, and *dnaC*. The Na^+/H^+ antiporter (*nhaC*), $Na^+(K^+, Li^+, Rb^+)/H^+$ antiporters (*nhaX* and *nhaK*) and *mrpABCDEF* genes plays a major role in Na^+ resistance, pH homeostasis, and osmoregulation, thusaids the bacteria to survive in acidic conditions [43]. The aspartate proton symporter (*yveA*) which promotes proton efflux to protect bacteria from acid stress [44] and glutathione peroxidase (*bsaA*) helps to protect the cells from oxidative stress by reducing hydrogen peroxide to water [45]. The ATP-synthase gene complexes play an important role in bile resistance and in maintaining pH levels in cells [46]. The gene *luxS* that plays a crucial role in quorum sensing could get expressed under oxidative stress and acidic conditions thereby enhancing the quorum sensing ability of the bacteria to tolerate stressful conditions. The *bsh* genes (*bshA, bshB, bshC*) encode the enzyme bile salt hydrolase that catalyzes the deconjugation of bile salts like glycine and taurine. Many resistance mechanisms are common for bile and acid stress. Adaptive response to acid and bile stress also includes the upregulation of genes that encode proteins that protect and repair DNA. Genes encoding DNA repair proteins such as *uvrABC* system proteins, ATP-dependent helicase/nuclease subunit, ATP-dependent DNA helicase (*recDGQ* and *pcrA*), DEAD-box ATP-dependent RNA helicase (*cshAB*) could be upregulated under acid stress thereby enable functional DNA repair mechanisms [47–49]. Genes encoding ATP-dependent *clp* protease expression could be triggered by acid and bile stress and refold or degrade the denatured proteins [50].

The presence of chaperones such as *dnaK*, *dnaJ*, *dnaC* and *grpE* enables FCW2 to withstand prolonged stress conditions. The expression of these molecular chaperone proteins is induced under acid stress, enabling the strain to tolerate heat, cold shock, and osmotic stress (different NaCl concentrations), and aid in the repair of damaged proteins [51–53]. The *cspBCD* genes are RNA chaperones that support cells in transcription and translation under cold conditions [54]. These findings corroborate with the *in-vitro* results. The strain FCW2 exhibited stable growth at 0–4 °C and 10–15 °C, and excellent growth was observed at 25–30 °C and 37–40 °C. The optimum temperature for FCW2 growth is 37–40 °C. In response to osmotic stress, FCW2 was allowed to grow at different concentrations (0, 3, and 6%) of NaCl, and it grew well at 3% NaCl with a growth rate of 78.18%. However, when the concentration of NaCl was increased to 6%, the growth rate decreased to 30.91%. This means higher concentrations of NaCl inhibit FCW2 growth.

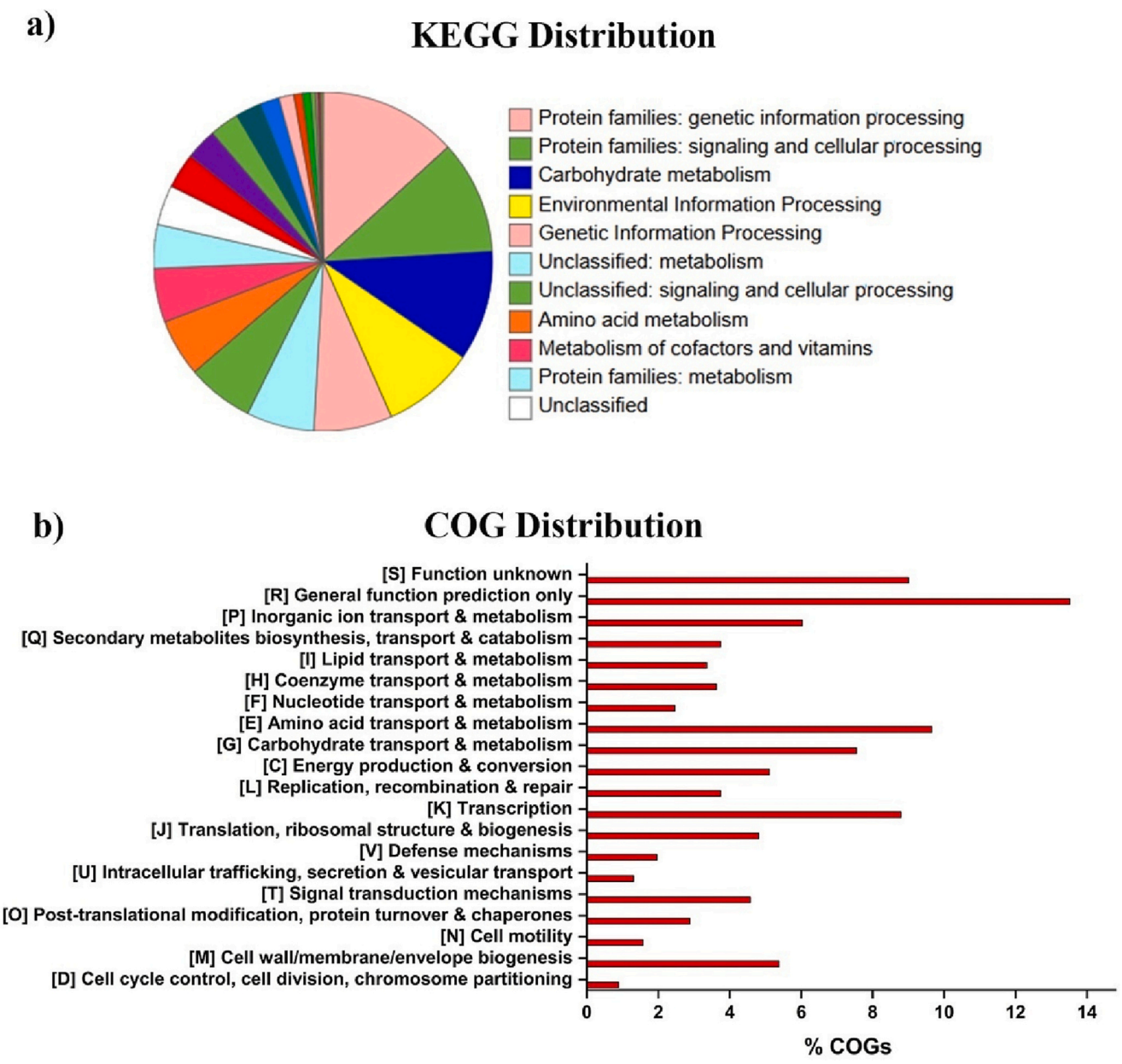

Fig. 4. Functional classification of FCW2 genome. a)KEGG functional distribution of gene families in strain FCW2 genome. b) COG annotation of FCW2 genome. COG functional categories are listed in Y axis and percentage of genes for corresponding COG categories are listed in X axis.

3.6. Adhesion assays

The ability of FCW2 to adhere to epithelial surfaces is evident by its strong hydrophobicity to ethyl acetate (53.99%) and moderate hydrophobicity to chloroform (44.27%) and hexane (24.61%) (Fig. 6a). The auto aggregation rate of FCW2 was 74.5 ± 5.6% after 24 h incubation, and biofilm formation ability was high, indicative of potential probiotic activity (Fig. 6b&c). Many cell surface proteins that contribute to adhesion, colonization, and biofilm formation exist in genome sequence of FCW2, including *srtA* and *fbp* genes, as well as genes encoding pyruvate dehydrogenase. Sortase (*srtA*) cleaves the cell wall sorting molecule (LPXTG motif) between threonine and glycine and attaches covalently to the peptidoglycan [55]. The *fbp* gene encodes fibronectin binding protein, a key adhesins, that attach to fibronectin found on host cell surface and thus mediate bacterial adherence and colonization [56]. The genes that encode ABC transporters and proteins involved in the PTS system have also been identified in the genome, whose expression were induced by mucin, thereby allowing bacteria to colonize the GIT. The *adh* gene cluster is involved in the adhesion and secretion of mucin [57].

3.7. Safety assessments

An *in-vitro* anti-microbial assay has shown that FCW2 can inhibit most of the pathogens tested (Supplementary Table S2). This is due to the organic acids or secondary metabolites produced by the isolate, which compete for adhesion sites and nutrients with pathogens, further suppressing pathogen growth. [58]. The FCW2 genome analysis by antiSMASH software showed that the polyketide gene clusters were present in FCW2, which is responsible for the biosynthesis of macrolactin H, difficidin, and bacillaene. Non-ribosomal peptides (NRP) gene clusters for surfactin, bacillibactin, fengycin and plipastatin, and other genes responsible for the biosynthesis of bacilysin were also found in the genome [59].

Probiotic candidates must not carry transmissible virulence or

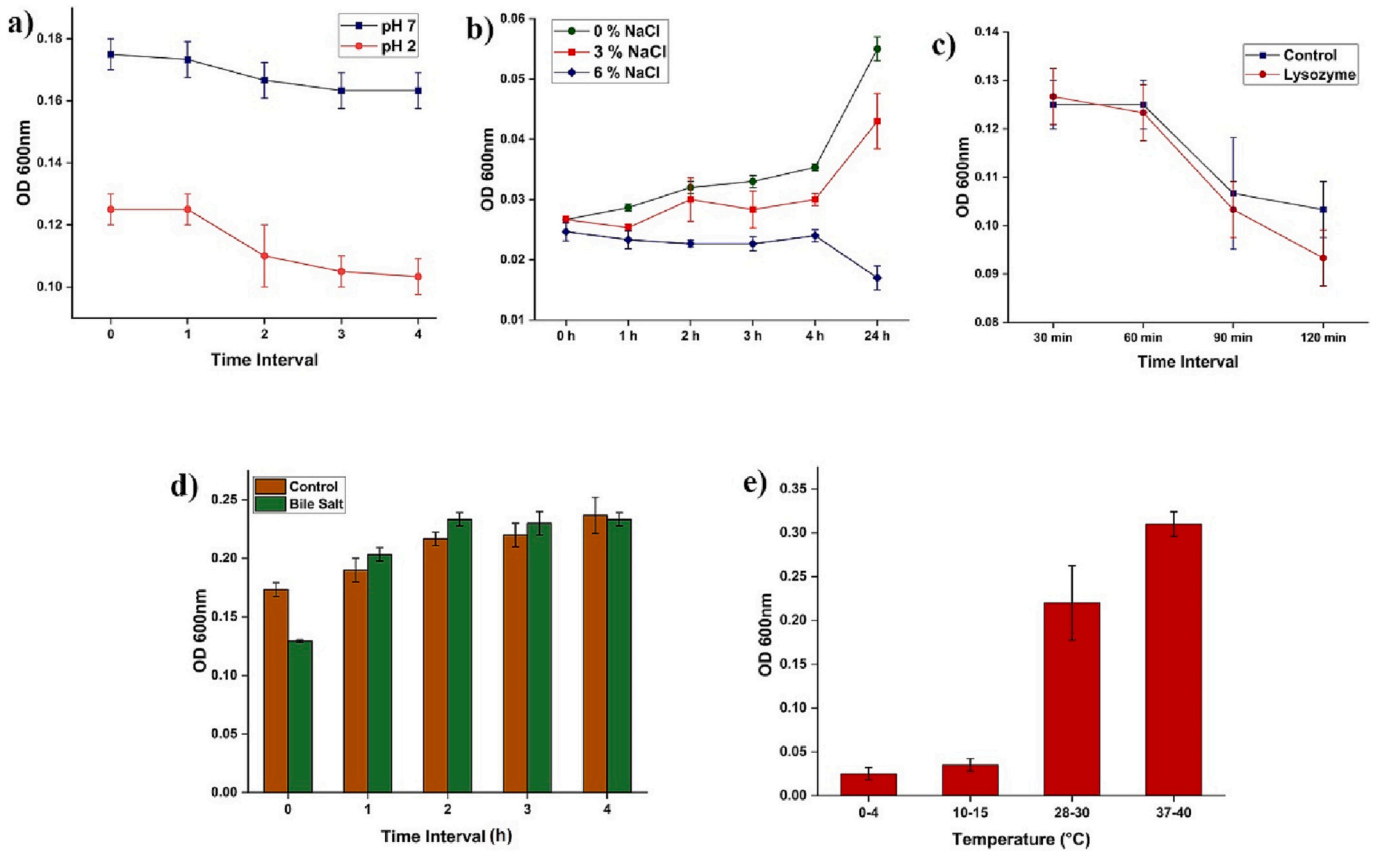

Fig. 5. Strain FCW2 tolerance to different stress conditions. a)acid tolerance (pH 2 and pH 7), b) Osmotic stress tolerance (different concentrations of NaCl), c) growth in the presence of lysozyme, e) growth at different temperatures, d) Bile salt tolerance. (A standard deviation of three independent experiments were shown by the error bar).

antibiotic resistance genes to be safe and effective. Antibiotic resistant genes associated with cephalosporin (*BcI*) and tetracycline (*tet*) antibiotics were identified in the genome by CARD analysis (See Table 2). Genes resistant to disinfecting agents and antiseptics were also present in the genome. Also, genomic islands that contain AMR and virulent genes in clusters were not identified in the FCW2 genome. However, an *in-vitro* antibiotic sensitivity test revealed that the strain was susceptible to cephalosporine and tetracycline, which could indicate that the resistant gene could be non-functional or could be resistant to a different antibiotic. The strain found to be susceptible for tetracyclin, gentamycin, ciprofloxacin, penicillin, cephalexin, erythromycin, cefotaximeandan intermediate susceptible to doxycycline hydrochloride and streptomycin (Supplementary Table S3).

A toxicity and virulence assay performed by VFanalyser showed that the genome lacks genes for the cytokine K, hemolysin (*hblD/A*) and non-hemolytic enterotoxins (*nheB*), emetogenic toxin (*ces*) and enterotoxin FM (*entFM*) but contains a hemolysin III gene (*hlyIII*). Genes encoding hemolysin alone won't define bacterial pathogenicity and were not located on transmissible plasmids. An *in-vitro* study was conducted to observe hemolytic and DNase enzyme production, which destroy RBCs and hydrolyze DNA, respectively. The isolate FCW was found to be α-hemolytic and DNase negative, which indicates its non-pathogenic nature (Fig. 6d&e). A safety evaluation of strain FCW2 was conducted *in-vivo* on zebrafish models. There were no mortality or disease symptoms observed during the 10-days study period, indicating that the strain is non-pathogenic.

Other important probiotic properties of *B. velezensis* were its ability to produce exopolysaccharide (EPS) and beneficial metabolites. The presence of genes responsible for vitamins, biotin, and other cofactors synthesis indicates the ability of probiotics to produce bioactive compounds. The genome contains *rib* genes (*ribD, ribBA, ribE, ribH, ribT*) involved in riboflavin synthesis. The genes (*ribZ_1, rib_2, rib_3* and *fmnp*) encodes riboflavin transporter that facilitate the uptake of riboflavin from outside the cell membrane. The *ribF* gene encodes bifunctional riboflavin kinase/FMN adenyly transferase catalyzes the conversion of riboflavin to FMN and FDA [6]. The genes responsible for biotin synthase (*bioD*, *bioB*, and *bioI*) and biotin transporter (*bioY_1, bioY_2*) play a major role in biotin biosynthesis. The genes (*btuD* and *btuF*) responsible for vitamin B6 production and formolybdenum cofactor biosynthesis such as molybdopterin molybdenum transferase (*moeA*), GTP 3′,8-cyclase (*moaA*), molybdenum cofactor biosynthesis protein B (*moaB*), cyclic pyranopterin monophosphate synthase (*moaC*), molybdopterin synthase sulfur carrier subunit (*moaD*), molybdopterin synthase catalytic subunit (*moaE*) and putative molybdenum cofactor guanylyl transferase (*mobA*) were present in the bacterium.

EPS gene clusters (*epsDEFGHIJKLMNO*) and a transcriptional regulator gene (*slrA*) were present in the genome [60,61]. The *eps* genes are involved in the biosynthesis of EPS by transferring nucleotide sugars and modifying polysaccharide repeating units. *slrA* directly regulated EPS biosynthesis. The genes encoding 4-hydroxy-tetrahydrodipicolinate synthase (*dapA*) involved in peptidoglycan biosynthesis, D-alanine-D-alanyl carrier protein ligase (*dltACD*) involved in lipoteichoic acid biosynthesis, *N*-acetylglucosamine-1-phosphate uridyltransferase (*GlmU*), glucosamine-6-phosphate synthase (*GlmS*) and phosphoglucosamine mutase (*GlmM*) involved in the biosynthesis of UDP-GlcNAc, the building blocks of peptidoglycan. EPS are important metabolites that provide strong immunomodulatory, antioxidant, and antimicrobial effects in the host's gut [62]. It can help the bacteria to adhere and colonize gut mucosa, strengthen the gut barrier, and help to prevent the overgrowth of harmful bacteria. They can also scavenge harmful toxins

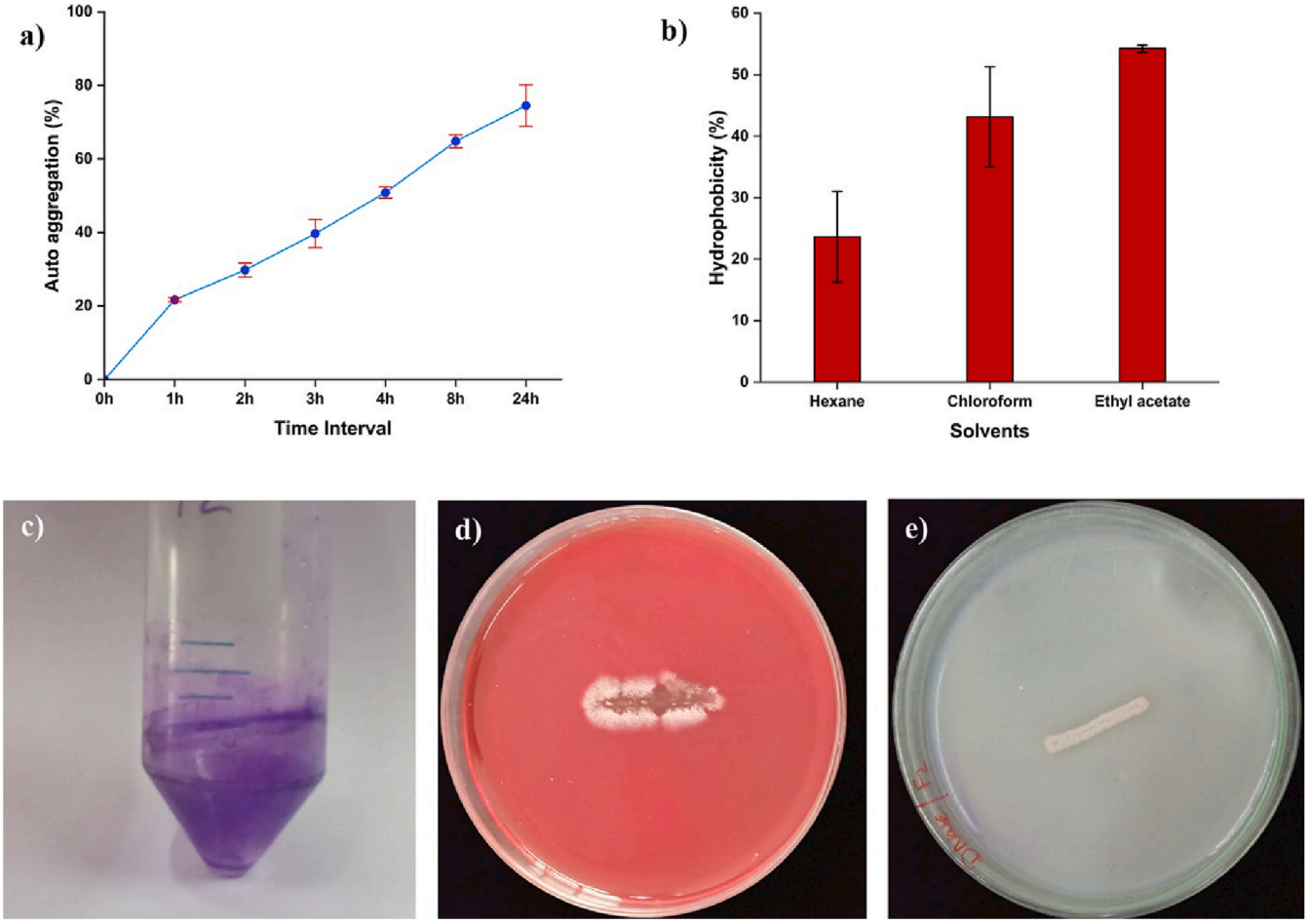

Fig. 6. Adhesion and Safety assessment. a) Autoaggregation assay, b) Hydrophobicity assay, c) Biofilm formation, d) Hemolytic property and e) DNase activity of FCW2 strain. (A standard deviation of three independent experiments is shown by the error bar).

and reduce inflammation. Overall, EPS and other metabolites produced by the probiotic bacteria play an important role in shaping the gut microbiota and maintaining health. In the food fermentation process EPS and other beneficial metabolites inhibit the growth of competing bacteria, leading to a more uniform fermentation, increasing the bioavailability of nutrients, and providing a higher quality product with longer shelf life.

4. Conclusion

The *in-vitro* and genomic studies of FCW2 have provided valuable insights into the efficacy of FCW2 as a probiotic. In addition, the strain was found to be stress tolerant, sensitive to antibiotics. The genes encoded by our bacterial strain, ensures that safety measures are in place in order to enable FCW2 to resist colonization by potentially harmful organisms and to remain safe for human consumption. The strain was also found to have genes that encodes secondary metabolites, EPS, and other beneficial nutrients, and enzymes that aid them to ferment complex carbohydrates. All these features would contribute to the overall health benefits that the strain could provide and make them qualify to be a probiotic strain. This strain could be used to fortify food products, thereby enhancing the health benefits of the consumer. It has been reported that some *B. velezensis* strains can contribute to food spoilage, but our findings showed that *B. velezensis* FCW2 can produce antimicrobial compounds and exhibit broad-spectrum antimicrobial activity. Furthermore, in our pilot study on testing the potential probiotic nature of bacterial strains isolated from fermented coconut water in combating urolithiasis, we have observed antilithiatic properties with *B. velezensis* FCW2, as reduced crystal growth was observed in the growth inhibition study of struvite crystals (Data not shown). From our observation and genetic characterization, this strain, could be a potential probiotic candidate for use in food preservation and fermentation, though further functional studies are required to validate the probiotic nature of our isolated *Bacillus* strain.

Strain deposition and complete genome sequence data accession number

The draft genome sequence of *B. velezensi* FCW2 has been deposited at GenBank under the accession number CP089436. The strain has been deposited at National Centre for Microbial Resource, India (Accession number- MCC 4686).

CRediT authorship contribution statement

C.T. Dhanya Raj: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Data curation. **Mangesh V. Suryavanshi:** Methodology, Writing – review & editing, Software, Data curation. **Surabhi Kandaswamy:** Conceptualization, Methodology, Supervision, Writing – review & editing, Funding acquisition. **KesavaPriyan Ramasamy:** Writing – review & editing, Data curation. **Rathinam Arthur James:** Conceptualization, Resources, Supervision, Writing – review & editing.

Table 2
AMR and Secondary metabolites gene clusters determined in *B. velezensis* FCW2.

Antibiotic Resistant Genes				
Gene	AMR Gene Family	Antibiotic Class	Similarity %	Mode of action
BcI	Class A Betalactamase	Cephalosporin/ Penem	62.42	Antibiotic inactivation
Tet	Major facilitator superfamily (MFS) efflux pump	Tetracyclin	75.05	Antibiotic efflux
qacJ/ G	Small multidrug resistance (SMR) efflux pump	Disinfectants/ antiseptics	44.9/ 42.45	Antibiotic efflux

Genes responsible for Secondary metabolites				
Synthetase	Gene Cluster	Metabolites	Similarity %	Mode of action
TransAT-PKS	Polyketide	Macrolactin H	100	Antibacterial
TransAT-PKS, NRPS	Polyketide + NRP	Bacillaene	100	Antibacterial
Other	Other	Bacilysin	100	Antibacterial; Antiyeast
RiPP-like, NRPS	NRP	Bacillibactin	100	Antimicrobial
TransAT-PKS	Polyketide+ NRP	Difficidin	86	Antibacterial
NRPS phosphonate	NRP: Lipopeptide	Surfactin	86	Antiviral; antifungal
NRPS, betalactone, transAT-PKS	NRP	Fengycin	86	Antifungal
NRPS,transAT-PKS-like	NRP	Plipastatin	46	Antifungal
PKS_like	Sacchaide	Butirosin A/ butirosin B	7	Antibacterial

Declaration of Competing Interest

The authors declare no financial and other competing interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2023.110637.

References

[1] Y. Cui, S. Wang, S. Ding, J. Shen, K. Zhu, Toxins and mobile antimicrobial resistance genes in Bacillus probiotics constitute a potential risk for one health, J. Hazard. Mater. 382 (2020), 121266, https://doi.org/10.1016/j.jhazmat.2019.121266.

[2] C. Hill, F. Guarner, G. Reid, G.R. Gibson, D.J. Merenstein, Morelli L. Pot, R. B. Canani, H.J. Flint, S. Salminen, P.C. Calder, M.E. Sanders, The international scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic, Nat. Rev. Gastroenterol. Hepatol. 11 (8) (2014) 506–514, https://doi.org/10.1038/nrgastro.2014.66.

[3] G. Khullar, R. Det-udom, P. Prombutar, C. Prakitchaiwattana, Probiogenomic analysis and safety assessment of Bacillus isolates using omics approach in combination with in-vitro, LWT 159 (2022), 113216, https://doi.org/10.1016/j.lwt.2022.113216.

[4] I. Sorokulova, Modern status and perspectives of Bacillus Bacteria as probiotics, J. Probiot. Health 01 (2013), https://doi.org/10.4172/2329-8901.1000e106.

[5] T.T. Hoa, L.H. Duc, R. Isticato, L. Baccigalupi, E. Ricca, P.H. Van, S.M. Cutting, Fate and dissemination of Bacillus subtilis spores in a murine model, Appl. Environ. Microbiol. 67 (2001) 3819–3823, https://doi.org/10.1128/AEM.67.9.3819-3823.2001.

[6] N.T. Quach, T.H.N. Vu, N.A. Nguyen, V.T. Nguyen, T.L. Bui, S.C. Ky, T.L. Le, H. Hoang, C.C. Ngo, T.T.M. Le, T.N. Nguyen, H.H. Chu, Q.T. Phi, Phenotypic features and analysis of genes supporting probiotic action unravel underlying perspectives of Bacillus velezensis VTX9 as a potential feed additive for swine, Ann. Microbiol. 71 (2021) 36, https://doi.org/10.1186/s13213-021-01646-4.

[7] A. Emam, C. Dunlap, Genomic and phenotypic characterization of Bacillus velezensis AMB-y1; a potential probiotic to control pathogens in aquaculture, Antonie Van Leeuwenhoek 113 (2020), https://doi.org/10.1007/s10482-020-01476-5.

[8] F.M.F. Elshaghabee, N. Rokana, R.D. Gulhane, C. Sharma, H. Panwar, Bacillus as potential probiotics: status, concerns, and future perspectives, Front. Microbiol. 8 (2017) 1490, https://doi.org/10.3389/fmicb.2017.01490.

[9] J. Meerak, H. Lida, Y. Watanabe, M. Miyashita, H. Sato, Y. Nakagawa, Y. Tahara, Phylogeny of gamma-polyglutamic acid-producing Bacillus strains isolated from fermented soybean foods manufactured in Asian countries, J. Gen. Appl. Microbiol. 53 (2007), https://doi.org/10.2323/jgam.53.315.

[10] H. Liu, S. Luo, J. Liu, Q. Yan, S. Yang, Z. Jiang, Novel green soybean shuidouchi fermented by Bacillus velezensis with multibioactivities, Food Sci. Nutr. 9 (2021) 6538–6547, https://doi.org/10.1002/fsn3.2579.

[11] Z. Liu, X. Guan, X. Zhong, X. Zhou, F. Yang, Bacillus velezensis DP-2 isolated from Douchi and its application in soybean meal fermentation, J. Sci. Food Agric. 101 (2020), https://doi.org/10.1002/jsfa.10801.

[12] H.E. Na, S. Heo, Y.S. Kim, T. Kim, G. Lee, J.H. Lee, D.W. Jeong, The safety and technological properties of Bacillus velezensis DMB06 used as a starter candidate were evaluated by genome analysis, LWT 161 (2022), 113398, https://doi.org/10.1016/j.lwt.2022.113398.

[13] EFSA Panel on Biological Hazards (BIOHAZ), K. Koutsoumanis, A. Allende, A. Alvarez-Ordóñez, D. Bolton, S. Bover-Cid, M. Chemaly, R. Davies, A. De Cesare, F. Hilbert, R. Lindqvist, M. Nauta, L. Peixe, G. Ru, M. Simmons, P. Skandamis, E. Suffredini, P.S. Cocconcelli, P.S. FernándezEscámez, M.P. Maradona, A. Querol, J.E. Suarez, I. Sundh, J. Vlak, F. Barizzone, S. Correia, L. Herman, Update of the List of QPS-Recommended Biological Agents Intentionally Added to Food or Feed as Notified to EFSA 11: Suitability of Taxonomic Units Notified to EFSA until September 2019. EFS2 18, 2020, https://doi.org/10.2903/j.efsa.2020.5965.

[14] P.R. Lee, C.X. Boo, S.Q. Liu, Fermentation of coconut water by probiotic strains *Lactobacillus acidophilus* L10 and *Lactobacillus casei* L26, Ann. Microbiol. 63 (2013) 1441–1450, https://doi.org/10.1007/s13213-013-0607-z.

[15] S.S. Giri, V. Sukumaran, S.S. Sen, S.C. Park, Front Microbiol, Sec. Food. Microbiol. 9 (2018), https://doi.org/10.3389/fmicb.2018.01976.

[16] D. Kantachote, A. Ratanaburee, W. Hayisama-ae, A. Sukhoom, T. Nunkaew, The use of potential probiotic Lactobacillus plantarum DW12 for producing a novel functional beverage from mature coconut water, J. Funct. Foods 32 (2017) 401–408, https://doi.org/10.1016/j.jff.2017.03.018.

[17] M.I. Watawana, N. Jayawardena, C.B. Gunawardhana, V.Y. Waisundara, Enhancement of the antioxidant and starch hydrolase inhibitory activities of king coconut water (Cocos nucifera var. aurantiaca) by fermentation with kombucha 'tea fungus', Int. J. Food Sci. Technol. 51 (2016) 490–498, https://doi.org/10.1111/ijfs.13006.

[18] A.S. Gangwar, A. Bhardwaj, V. Sharma, Fermentation of tender coconut water by probiotic bacteria Bacillus coagulans, Int. J. Food Stud. 7 (2018), https://doi.org/10.7455/ijfs/7.1.2018.a9.

[19] C.T.D. Raj, V. Palaninathan, R.A. James, Anti-uropathogenic, antioxidant and struvite crystallization inhibitory potential of fresh and fermented coconut water, Biocatal. Agric. Biotechnol. 47 (2022), 102555, https://doi.org/10.1016/j.bcab.2022.102555.

[20] F.C. Prado, J.D. Lindner, J. Inaba, V. Thomaz-Soccol, S.K. Brar, C.R. Soccol, Development and evaluation of a fermented coconut water beverage with potential health benefits, J. Funct. Foods 12 (2015) 489–497, https://doi.org/10.1016/j.jff.2014.12.020.

[21] C.T.D. Raj, S. Kandaswamy, M.V. Suryavanshi, K.P. Ramasamy, R. Rajasabapathy, R.A. James, Genomic and metabolic properties of *Staphylococcus gallinarum* FCW1 MCC4687 isolated from naturally fermented coconut water towards GRAS assessment, Gene 867 (2023), 147356, https://doi.org/10.1016/j.gene.2023.147356.

[22] J.G. Cappuccino, N. Sherman, Microbiology: A Laboratory Manual, Pearson Education, Singapore, 2004, p. 491.

[23] S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura, MEGA X: molecular evolutionary genetics analysis across computing platforms, Mol. Biol. Evol. 35 (2018) 1547–1549.

[24] A.M. Bolger, M. Lohse, B. Usadel, Trimmomatic: A flexible trimmer for Illumina sequence data, Bioinform. 30 (2014) 2114–2120, https://doi.org/10.1093/bioinformatics/btu170.

[25] S. Nurk, A. Bankevich, D. Antipov, A.A. Gurevich, A. Korobeynikov, A. Lapidus, A. D. Prjibelski, A. Pyshkin, A. Sirotkin, Y. Sirotkin, R. Stepanauskas, S.R. Clingenpeel, T. Woyke, J.S. McLean, R. Lasken, G. Tesler, M.A. Alekseyev, P.A. Pevzner, Assembling single-cell genomes and mini-metagenomes from chimeric MDA products, J. Comput. Biol. 20 (2013) 714–737, https://doi.org/10.1089/cmb.2013.0084.

[26] I. Lee, Y. Ouk Kim, S.-C. Park, J. Chun, OrthoANI: an improved algorithm and software for calculating average nucleotide identity, Int. J. Syst. Evol. Microbiol. 66 (2016) 1100–1103, https://doi.org/10.1099/ijsem.0.000760.

[27] L. Xu, Z. Dong, L. Fang, Y. Luo, Z. Wei, H. Guo, G. Zhang, Y.Q. Gu, D. Coleman-Derr, Q. Xia, Y. Wang, OrthoVenn2: a web server for whole-genome comparison and annotation of orthologous clusters across multiple species, Nucleic Acids Res. 47 (W1) (2019) W52–W58, https://doi.org/10.1093/nar/gkz333.

[28] B. Jia, A.R. Raphenya, B. Alcock, N. Waglechner, P. Guo, K.K. Tsang, B.A. Lago, B. M. Dave, S. Pereira, A.N. Sharma, S. Doshi, M. Courtot, R. Lo, L.E. Williams, J. G. Frye, T. Elsayegh, D. Sardar, E.L. Westman, A.C. Pawlowski, T.A. Johnson, F.S. L. Brinkman, G.D. Wright, A.G. McArthur, CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database, Nucleic Acids Res. 45 (2017) D566–D573, https://doi.org/10.1093/nar/gkw1004.

[29] C. Bertelli, M.R. Laird, K.P. Williams, Group SFURC, B.Y. Lau, G. Hoad, G. L. Winsor, F.S. Brinkman, IslandViewer 4: expanded prediction of genomic islands for larger-scale datasets, Nucleic Acids Res. 45 (2017) 30–35, https://doi.org/10.1093/nar/gkx343.

[30] R. Somashekaraiah, B. Shruthi, B.V. Deepthi, M.Y. Sreenivasa, Probiotic properties of lactic acid Bacteria isolated FromNeera: A naturally fermenting coconut palm nectar, Front. Microbiol. 10 (2019), https://doi.org/10.3389/fmicb.2019.01382.

[31] N. Qureshi, Q. Gu, P. Li, Whole genome sequence analysis and in vitro probiotic characteristics of a Lactobacillus strain *Lactobacillus paracasei* ZFM54, J. Appl. Microbiol. 129 (2020) 422–433, https://doi.org/10.1111/jam.14627.

[32] B. Turchi, S. Mancini, F. Fratini, F. Pedonese, R. Nuvoloni, F. Bertelloni, V. Ebani, D. Cerri, Preliminary evaluation of probiotic potential of Lactobacillus plantarum strains isolated from Italian food products, World J. Microbiol. Biotechnol. 29 (2013), https://doi.org/10.1007/s11274-013-1356-7.

[33] I. Yasmin, M. Saeed, W.A. Khan, A. Khaliq, M.F.J. Chughtai, R. Iqbal, S. Tehseen, S. Naz, A. Liaqat, T. Mehmood, S. Ahsan, S. Tanweer, In vitro probiotic potential and safety evaluation (hemolytic, cytotoxic activity) of Bifidobacterium strains isolated from raw camel Milk, Microorganisms 8 (2020) 354, https://doi.org/10.3390/microorganisms8030354.

[34] M.E. Macías-Rodríguez, M. Zagorec, F. Ascencio, M. Rojas, Potential probioticLactobacillus strains for piglets from an arid coast, Ann. Microbiol. 58 (2008) 641–648, https://doi.org/10.1007/BF03175569.

[35] M. Li, Y. Wang, H. Cui, Y. Li, Y. Sun, H.-J. Qiu, Characterization of lactic acid Bacteria isolated from the gastrointestinal tract of a wild boar as potential probiotics, Front. Vet. Sci. 7 (2020) 49, https://doi.org/10.3389/fvets.2020.00049.

[36] M.O. Diale, E. Kayitesi, M.H. Serepa-Dlamini, Genome InSilico and in vitro analysis of the probiotic properties of a bacterial endophyte, Bacillus Paranthracis strain MHSD3, Front. Genet. 12 (2021), 672149, https://doi.org/10.3389/fgene.2021.672149.

[37] S.M. Zayed, M.M. Aboulwafa, A.M. Hashem, S.E. Saleh, Biofilm formation by Streptococcus mutans and its inhibition by green tea extracts, AMB Express 11 (2021) 73, https://doi.org/10.1186/s13568-021-01232-6.

[38] Clinical and Laboratory Standards Institute (CLSI), Performance Standards for Antimicrobial Susceptibility Testing, 30th ed., Clinical and Laboratory Standards Institute, Wayne, PA, USA, 2020. CLSI supplement M100 (ISBN 978-1-68440-066-9 [Print]; ISBN 978-1-68440-067-6 [Electronic]).

[39] H. Hanchi, W. Mottawea, K. Sebei, R. Hammami, The genus Enterococcus: between probiotic potential and safety concerns—an update, Front. Microbiol. 9 (2018) 1791, https://doi.org/10.3389/fmicb.2018.01791.

[40] V.B.C. Kumari, S.S. Huligere, R. Ramu, S. NaikBajpe, M.Y. Sreenivasa, E. Silina, V. Stupin, R.R. Achar, Evaluation of probiotic and antidiabetic attributes of Lactobacillus strains isolated from fermented beetroot, Front. Microbiol. 13 (2022), 911243, https://doi.org/10.3389/fmicb.2022.911243.

[41] R. Thirunavukkarasu, P. Pandi, D. Balaraman, F. Albalawi, N. Ahmad, M. Panagal, T. Nageswara Rao, K. Subramanian, E.G.J. George, M.A. Rajan, P.S. Renuga, W. Aruni, S.Y. AlOmar, Influence of extracellular protein isolated from fish gut associated bacteria as an enhancer of growth and innate immune system in Mugilcephalus, Sci. Rep. 12 (1) (2022) 3217, https://doi.org/10.1038/s41598-022-05779-7.

[42] S. Ku, M.S. Park, G.E. Ji, H.J. You, Review on Bifidobacterium bifidum BGN4: functionality and nutraceutical applications as a probiotic microorganism, Int. J. Mol. Sci. 17 (9) (2016) 1544, doi: 10.3390%2Fijms17091544.

[43] M. Fujisawa, A. Kusumoto, Y. Wada, T. Tsuchiya, M. Ito, NhaK, a novel monovalent cation/H+ antiporter of *Bacillus subtilis*, Arch. Microbiol. 183 (2005) 411–420, https://doi.org/10.1007/s00203-005-0011-6.

[44] N. Guan, L. Liu, Microbial response to acid stress: mechanisms and applications, ApplMicrobiolBiotechnol 104 (2020) 51–65, https://doi.org/10.1007/s00253-019-10226-1.

[45] E. Lubos, J. Loscalzo, D.E. Handy, Glutathione peroxidase-1 in health and disease: from molecular mechanisms to therapeutic opportunities, Antioxid. Redox Signal. 15 (2011) 1957–1997, https://doi.org/10.1089/ars.2010.3586.

[46] M.G. Alekseeva, T.A. Mironcheva, D.A. Mavletova, S.M. Elizarov, N. V. Zakharevich, V.N. Danilenko, FoF1-ATP synthase of Streptomyces fradiae ATCC 19609: structural, biochemical, and functional characterization, Biochemistry (Mosc) 80 (2015) 296–309, https://doi.org/10.1134/S0006297915030050.

[47] J. Jin, B. Zhang, H. Guo, J. Cui, L. Jiang, S. Song, M. Sun, F. Ren, Mechanism analysis of acid tolerance response of bifidobacteriumlongum subsp. longum BBMN 68 by gene expression profile using RNA-sequencing, PLoS One 7 (2012), e50777, https://doi.org/10.1371/journal.pone.0050777.

[48] M.A. Petit, E. Dervyn, M. Rose, K.D. Entian, S. McGovern, S.D. Ehrlich, C. Bruand, PcrA is an essential DNA helicase of *Bacillus subtilis* fulfilling functions both in repair and rolling-circle replication, MolMicrobiol 29 (1998) 261–273, https://doi.org/10.1046/j.1365-2958.1998.00927.x.

[49] M. Lehnik-Habrink, L. Rempeters, Á.T. Kovács, C. Wrede, C. Baierlein, H. Krebber, O.P. Kuipers, J. Stülke, DEAD-box RNA helicases in *Bacillus subtilis* have multiple functions and act independently from each other, J. Bacteriol. 195 (2013) 534–544, https://doi.org/10.1128/JB.01475-12.

[50] A.B. Ferreira, M.N.V. De Oliveira, F.S. Freitas, P. Alfenas-Zerbini, D.F. Da Silva, M. V. De Queiroz, A.C. Borges, C.A. De Moraes, Increased expression of clp genes in *Lactobacillus delbrueckii* UFV H2b20 exposed to acid stress and bile salts, Benefic. Microbes 4 (2013) 367–374, https://doi.org/10.3920/BM2013.0022.

[51] J. Prasad, P. McJarrow, P. Gopal, Heat and osmotic stress responses of probiotic *Lactobacillus rhamnosus* HN001 (DR20) in relation to viability after drying, Appl. Environ. Microbiol. 69 (2003) 917–925, https://doi.org/10.1128/AEM.69.2.917-925.2003.

[52] B. Zeng, C. Wang, P. Zhang, Z. Guo, L. Chen, K. Duan, Heat shock protein DnaJ in *Pseudomonas aeruginosa* affects biofilm formation via Pyocyanin production, Microorganisms 8 (2020) 395, https://doi.org/10.3390/microorganisms8030395.

[53] E. Hamon, P. Horvatovich, E. Marchioni, D. Aoudé-Werner, S. Ennahar, Investigation of potential markers of acid resistance in *Lactobacillus plantarum* by comparative proteomics, J. Appl. Microbiol. 116 (2014) 134–144, https://doi.org/10.1111/jam.12339.

[54] R. Guddimalli, A.K. Somanaboina, S.R. Palle, S. Edupuganti, D. Kummari, S. R. Palakolanu, J. Naravula, J. Gandra, I.A. Qureshi, N. Marka, R. Polavarapu, P. B. KaviKishor, Overexpression of RNA-binding bacterial chaperones in rice leads to stay-green phenotype, improved yield and tolerance to salt and drought stresses, Physiol. Plant. 173 (2021) 1351–1368, https://doi.org/10.1111/ppl.13369.

[55] D. Muñoz-Provencio, J. Rodríguez-Díaz, M.C. Collado, P. Langella, L.G. Bermúdez-Humarán, V. Monedero, Functional analysis of the *Lactobacillus casei* BL23 sortases, Appl. Environ. Microbiol. 78 (2012) 8684–8693, https://doi.org/10.1128/AEM.02287-12.

[56] J.L. Baugher, T.R. Klaenhammer, Invited review: application of omics tools to understanding probiotic functionality, J. Dairy Sci. 94 (2011) 4753–4765, https://doi.org/10.3168/jds.2011-4384.

[57] K. Papadimitriou, G. Zoumpopoulou, B. Folignac, V. Alexandraki, M. Kazou, B. Pot, E. Tsakalidou, Discovering probiotic microorganisms: in vitro, in vivo, genetic and omics approaches, Front. Microbiol. 6 (2015), https://doi.org/10.3389/fmicb.2015.00058.

[58] B. Kosin, S. Rakshit, Microbial and processing criteria for production of probiotics: a review, Food Technol. Biotechnol. 44 (2006).

[59] O.N. Reva, D.Z.H. Swanevelder, L.A. Mwita, A.D. Mwakilili, D. Muzondiwa, M. Joubert, W.Y. Chan, S. Lutz, C.H. Ahrens, L.V. Avdeeva, M.A. Kharkhota, D. Tibuhwa, S. Lyantagaye, J. Vater, R. Borriss, J. Meijer, Genetic, epigenetic and phenotypic diversity of four *Bacillus velezensis* strains used for plant protection or as probiotics, Front. Microbiol. 10 (2019) 2610, https://doi.org/10.3389/fmicb.2019.02610.

[60] R. Wu, Y. Qin, Q. Shen, P. Li, The complete genome sequence of *Bacillus velezensis* LPL061, an exopolysaccharide-producing bacterium, 3 Biotech 10 (2020) 243, https://doi.org/10.1007/s13205-020-02228-y.

[61] O.S. Stergiou, K. Tegopoulos, D.E. Kiousi, M. Tsifintaris, A.C. Papageorgiou, C. C. Tassou, N. Chorianopoulos, P. Kolovos, A. Galanis, Whole-genome sequencing, phylogenetic and genomic analysis of *Lactiplanti bacilluspentosus* L33, a potential probiotic strain isolated from fermented sausages, Front. Microbiol. 12 (2021), 746659, https://doi.org/10.3389/fmicb.2021.746659.

[62] K. Abdhul, M. Ganesh, S. Shanmughapriya, M. Kanagavel, K. Anbarasu, K. Natarajaseenivasan, Antioxidant activity of exopolysaccharide from probiotic strain Enterococcus faecium (BDU7) from Ngari, Int. J. Biol. Macromol. 70 (2014) 450–454, https://doi.org/10.1016/j.ijbiomac.2014.07.026.