


Original Research

Whole Genome Analysis of Tibetan Kefir-Derived *Lactiplantibacillus Plantarum* 12-3 Elucidates Its Genomic Architecture, Antimicrobial and Drug Resistance, Potential Probiotic Functionality and Safety

Tariq Aziz¹ , Muhammad Naveed², Muhammad Aqib Shabbir^{2,3}, Abid Sarwar¹, Ayaz Ali Khan⁴, Ammarah Hasnain³, Taqweem Ul Haq⁴, Zhennai Yang^{1,*}, Abdellah Zinedine⁵, João Miguel Rocha^{6,*}, Metab Alharbi⁷

¹Key Laboratory of Geriatric Nutrition and Health of Ministry of Education, Beijing Advanced Innovation Center for Food Nutrition and Human Health, Beijing Engineering and Technology Research Center of Food Additives, Beijing Technology and Business University, 100048 Beijing, China

²Department of Biotechnology, Faculty of Science & Technology, University of Central Punjab, 54590 Lahore, Pakistan

³Department of Biotechnology, Faculty of Biological Sciences, Lahore University of Biological & Applied Sciences, 53400 Lahore, Pakistan

⁴Department of Biotechnology, University of Malakand Chakdara, 18800 Khyber Pakhtunkhwa, Pakistan

⁵BIOMARE Laboratory, Faculty of Sciences, Chouaib Doukkali University, 24000 EL Jadida, Morocco

⁶Universidade Católica Portuguesa, CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, 4169-005 Porto, Portugal

⁷Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, 11451 Riyadh, Saudi Arabia

*Correspondence: yangzhennai@163.com (Zhennai Yang); joao.rocha73@gmail.com (João Miguel Rocha)

Academic Editors: Teresa Semedo-Lemsaddek and Jen-Tsung Chen

Submitted: 24 October 2023 Revised: 15 December 2023 Accepted: 5 January 2024 Published: 11 April 2024

Abstract

Background: *Lactiplantibacillus plantarum* 12-3 holds great promise as a probiotic bacterial strain, yet its full potential remains untapped. This study aimed to better understand this potential therapeutic strain by exploring its genomic landscape, genetic diversity, CRISPR-Cas mechanism, genotype, and mechanistic perspectives for probiotic functionality and safety applications. **Methods:** *L. plantarum* 12-3 was isolated from Tibetan kefir grains and, subsequently, Illumina and Single Molecule Real-Time (SMRT) technologies were used to extract and sequence genomic DNA from this organism. After performing pan-genomic and phylogenetic analysis, Average Nucleotide Identity (ANI) was used to confirm the taxonomic identity of the strain. Antibiotic resistance gene analysis was conducted using the Comprehensive Antibiotic Resistance Database (CARD). Antimicrobial susceptibility testing, and virulence gene identification were also included in our genomic analysis to evaluate food safety. Prophage, genomic islands, insertion sequences, and CRISPR-Cas sequence analyses were also carried out to gain insight into genetic components and defensive mechanisms within the bacterial genome. **Results:** The 3.4 Mb genome of *L. plantarum* 12-3, was assembled with 99.1% completeness and low contamination. A total of 3234 genes with normal length and intergenic spacing were found using gene prediction tools. Pan-genomic studies demonstrated gene diversity and provided functional annotation, whereas phylogenetic analysis verified taxonomic identity. Our food safety study revealed a profile of antibiotic resistance that is favorable for use as a probiotic. Analysis of insertional sequences, genomic islands, and prophage within the genome provided information regarding genetic components and their possible effects on evolution. **Conclusions:** Pivotal genetic elements uncovered in this study play a crucial role in bacterial defense mechanisms and offer intriguing prospects for future genome engineering efforts. Moreover, our findings suggest further *in vitro* and *in vivo* studies are warranted to validate the functional attributes and probiotic potential of *L. plantarum* 12-3. Expanding the scope of the research to encompass a broader range of *L. plantarum* 12-3 strains and comparative analyses with other probiotic species would enhance our understanding of this organism's genetic diversity and functional properties.

Keywords: probiotics; CRISPR; *L. plantarum*; genotyping; functional studies

1. Introduction

Identification of microorganisms with potential probiotic application has become a focal point in the field of microbiology in recent years. Such efforts are motivated, in part, by the discovery that many microorganisms play a critical role in promoting health and avoiding disease. *L. plantarum* 12-3, a strain of lactic acid bacteria, has met with notable interest among potential microorganisms due to its reported probiotic properties. A thorough explanation of

the rationale for closely examining *L. plantarum* 12-3 as a probiotic would substantially benefit the strain's potential therapeutic contribution [1].

Understanding probiotic microbe activity and safety applications is inextricably tied to their genetic diversity. In keeping with this principal, the genetic composition of *L. plantarum* 12-3 has potential to understanding its probiotic efficacy and safety. Indeed, Mao *et al.* [1] emphasized the need to investigate the genetic landscape of *L. plantarum*



Table 1. The *L. plantarum* 12-3 strains utilized in the phylogenetic ANI studies and clustering analysis.

STRAINS	SIZE	CONTINGS	GC%
<i>L. plantarum</i> 12-3	3,403,608	8	44.4
<i>L. plantarum</i> CMPG5300	3,507,828	7	44.15
<i>L. plantarum</i> 2025	3,334,257	164	44.44
<i>L. plantarum</i> Nizo2802	3,372,514	89	44.45
<i>L. plantarum</i> 16	3,361,015	11	44.34
<i>L. plantarum</i> E2C2	3,603,563	121	43.99
<i>L. plantarum</i> IPLA88	3,254,055	208	44.43
<i>L. plantarum</i> L31-1	3,216,471	45	44.50
<i>L. plantarum</i> 90sk	3,371,458	47	44.26
<i>L. plantarum</i> subsp. <i>plantarum</i> CGMCC	3,273,801	4	44.42
<i>L. plantarum</i> WJL	3,503,067	13	44.21
<i>L. plantarum</i> SRCM101060	3,263,056	101	44.33
<i>L. plantarum</i> subsp. <i>plantarum</i> P -8 <i>plantarum</i>	3,246,630	8	44.55
<i>L. plantarum</i> JSA22	3,455,529	3	44.29
<i>L. plantarum</i> ZJ316	3,299,755	4	44.49
<i>L. plantarum</i> WCFS1	3,348,624	4	44.42
<i>L. plantarum</i> 80	3,224,773	67	44.36
<i>L. plantarum</i> WJL	3,477,495	102	44.24
<i>L. plantarum</i> DSM 13273	3,439,800	90	44.22
<i>L. plantarum</i> UCMA 3037	3,108,278	68	44.52
<i>L. plantarum</i> Nizo2891	3,469,171	78	44.13
<i>L. plantarum</i> B21	3,284,260	1	44.47
<i>L. plantarum</i> 8RA -3	3,330,093	18	44.37
<i>L. plantarum</i> ST -III	3,307,936	2	44.48
<i>L. plantarum</i> PS128	3,325,806	11	44.41
<i>L. plantarum</i> WLPL04	3,185,263	51	44.52
<i>L. plantarum</i> JDM1	3,197,759	1	44.66
<i>L. plantarum</i> DSM 20174	3,250,154	2	44.50
<i>L. plantarum</i> 2165	3,179,972	192	44.53

ANI, average nucleotide identity; GC, Guanine and Cytosine.

12-3, emphasizing the need for a more detailed understanding of its genomic composition, and Parlindungan *et al.* [2] commented that understanding the genetic variability of *L. plantarum* strain 12-3 is of importance in the investigation of its probiotic efficacy and safety implications. Here we sought to uncover subtle mechanisms that govern this bacterium's probiotic potency and safety by better understanding its genetic diversity. Furthermore, the use of advanced genotyping technologies provides a more detailed understanding of the strain's genetic variations, and may provide insights into its hereditary traits. As the role of probiotics in health promotion becomes more apparent, this work serves as a fundamental investigation, offering critical knowledge for exploiting the probiotic potential of *L. plantarum* 12-3 as this may result in an understanding of its genetic variability and possible implications in the area of probiotic use and risk evaluation [3].

The *L. plantarum* 12-3 species is recognized for its adaptable characteristics and extensive prevalence in diverse ecological habitats. *L. plantarum* 12-3 strains are frequently detected in fermented food items including dairy

products, pickles, and sauerkraut where they play a significant role in preserving the food and enhancing its flavor profile [4]. Furthermore, *L. plantarum* 12-3 has been acknowledged for its prospective health advantages and probiotic functionality. Probiotics are living microorganisms that, upon being administered in sufficient quantities, provide advantageous health effects to the host. The probiotic potential of *L. plantarum* 12-3 has garnered significant interest, owing to its ability to withstand the harsh conditions of the gastrointestinal tract and elicit favorable impact on gut health.

The use of modern genotyping techniques can provide valuable insight into genomic variations and hereditary traits, thereby shedding light on its potential adaptability and advantageous features. Applications of these technologies will provide a thorough understanding of *L. plantarum* 12-3 can be attained, by better understanding the genetic basis of its probiotic properties and ensuring its appropriateness for human consumption [5]. The principal aim of this study was to specifically investigate the genetic diversity, CRISPR-Cas system, and genotype of *L. plantarum*

12-3, and place emphasis on the potential probiotic properties and safety implications associated with this particular microorganism.

Investigation of *L. plantarum* 12-3 has yielded useful insight into its genetic diversity, probiotic potential, and genomic features. The dynamic character of this strain's genome has been revealed through predictive analysis of transposases, mobile elements, and genomic islands, and provides a solid platform for future research [6]. Needed information should include improved genomic analysis, resolve prior analytical flaws, and conduct in-depth functional studies. Furthermore, the prospective applications of *L. plantarum* 12-3 in probiotics, biotechnology, and medicines require further investigation as such additional understanding will surely advance our understanding of probiotic bacteria and their potential uses in health and biotechnology [7].

2. Materials and Methods

2.1 Strain Collection and Culture

L. plantarum 12-3 was isolated from Tibetan Kefir grains in 2015 and maintained as frozen (−20 °C) stocks in MRS broth (Beijing Aoboxing Co., Ltd., Beijing, China) supplemented with 20% (v/v) glycerol. These strains were identified primarily through Gram staining, catalase assays, cell morphology, and 16S rDNA sequencing analysis as previously described [8–13].

2.2 DNA Extraction and Whole Genome Sequencing

Genomic DNA was extracted using a Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) and then quantified using a TBS-380 fluorometer (Turner Bio Systems Inc., Sunnyvale, CA, USA). An OD_{260/280} ratio between 1.8 and 2.0 was obtained and a DNA quantity greater than 20 micrograms was utilized. Using the NEXTflex Rapid DNA-Seq Kit (Revvity, Waltham, MA, USA), sheared fragments were utilized to create Illumina sequencing libraries. Briefly, DNA ends were subjected to initial end repair and phosphorylation, and termini were subsequently A-tailed and ligated with sequencing adapters. Next, polymerase chain reaction (PCR)-based amplification of the adapter-ligated products was conducted for enrichment purposes. The constructed libraries were then sequenced utilizing a paired-end approach on an Illumina HiSeq X Ten platform, with 150 bp read length at each end. The genome of *L. plantarum* 12-3 was sequenced utilizing Single Molecule Real-Time (SMRT) technology and Illumina sequencing platforms. The accession number given to the completed and assembled *L. plantarum* 12-3 genome is GCF_004028335.1. The complexity of the genome was measured by examining the information provided by Illumina [14].

2.3 Phylogenetic and Pan-Genomic Analysis

To verify the taxonomic identity of the *L. plantarum* 12-3 strain, an analysis of the Average Nucleotide Identity (ANI) was conducted on various strains of *L. plantarum* 12-3 (Table 1). After plasmid removal, ANI analysis was undertaken using the publicly-available JSpecies Web Server (<http://jspecies.ribohost.com/jspeciesws/>) [15]. HemI version 2.0 (<http://hemi.biocuckoo.org/>) was utilized to create gene clustering and heatmap [16]. In addition, fully assembled genomes of *L. plantarum* 12-3 isolates available on PanX (<https://pangenome.org/>; accessed on 28 May 2023) were used for pan-genomic analysis. All genomes were re-annotated with the Prokka tool to eliminate biases in comparisons caused by the various annotation methods used. The output general feature format (GFF) was used to perform a pan-genome analysis using a BLASTp (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) threshold of 95% to identify core, accessory, and unique genes. As a result, four distinct gene classes were identified: core (99% strain 100%), soft-core (95% strain 99%), shell (15% strain 95%), and cloud (0% strain 15%) [17].

2.4 Food Safety Assessment

The Comprehensive Antibiotic Resistance Database (CARD) (<https://card.mcmaster.ca/>) was used as this database has a large number of annotated antibiotic resistance genes. Antibiotic resistance genes were identified by comparing the predicted genes with the CARD database using specific algorithms and tools. Additional analysis and filtering were performed on the discovered matches based on parameters such as sequence similarity, functional annotation, and resistance mechanisms. Based on their resistance patterns, the discovered antibiotic resistance genes were categorized and characterized [18]. Antimicrobial susceptibility tests and comparison with previously identified resistance genes were also included as validation phases.

Collectively, this approach enabled the prediction, and characterization, of antibiotic resistance genes and shed light on resistance profiles of bacterial isolates and added to our knowledge of antibiotic resistance. Human host pathogenicity predictions and the identification of acquired virulence genes were conducted using VirulenceFinder 2.0 (<https://cge.food.dtu.dk/services/VirulenceFinder/>) to provide a safety assessment of *L. plantarum* 12-3 [19,20].

2.5 Identification of Prophage, Genomic Islands, and Other Insertion Sequences

Phage Search Tool Enhanced Release (PHASTER) (<http://phaster.ca>) was utilized for the identification of genomic regions containing prophage. This tool provided the length, localization, guanine and cytosine (GC) content, and gene annotation of the prophage [21]. A comparison was made between the predicted intact prophage sequences and the Virus-Host DB database (<https://www.genome.jp/vi>

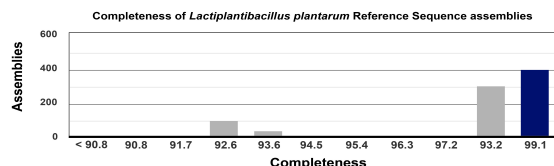


Fig. 1. The genome completeness overview of the genomic assemblies from *L. plantarum* 12-3. RefSeq, Reference Sequence Database.

[rushostdb](#)) to validate detected prophages. Using the VIP-tree application, a proteomics tree of the viral genome sequence was also generated. This tree was constructed using tBLASTx-calculated genome-wide sequence similarities. This analysis helped clarify the evolutionary relationships between viral genomes.

The presence of genomic islands (GI) was analyzed by searching multiple databases with Island Viewer 4 (<https://www.pathogenomics.sfu.ca/islandviewer>). This made it possible to identify and characterize genomic islands within the bacterial genome. Using these methods and databases, a thorough analysis of prophage regions and genomic islands was conducted. These efforts yielded valuable insights into genetic elements present in the *L. plantarum* 12-3 genome and their potential impact on bacterial evolution and virulence. ISFinder and ISSaga were subsequently utilized for the annotation of transposase and mobile elements [22].

2.6 CRISPR-Cas Sequence Analysis

The identification and verification of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated genes (Cas) was accomplished with the aid of specialized bioinformatic tools. The CRISPRCas-Finder v.1.1.2 (<https://crisprcas.i2bc.paris-saclay.fr/CrisprCasFinder/Index>) tool was used to search for CRISPR and Cas genes within the genome's coding sequences and enabled the detection of arrays and associated Cas genes in genomic sequences of interest. For validating the identified arrays, the CRISPRdb database (<https://crisprdb.org>) was queried. The predicted results were cross-referenced with the CRISPRdb database to assure their accuracy and alignment with previously identified CRISPR sequences. This methodology enabled the identification and confirmation of arrays and associated Cas genes within the studied genomes, and provided valuable information concerning the presence and organization of essential genetic elements involved in bacterial defense and potential approaches to future genome engineering applications [23].

3. Results

3.1 Genomic Landscape of *L. Plantarum* 12-3

Mobile elements and repetitive sequences present in the *L. plantarum* 12-3 genome make it challenging to assemble the entire genome using only short-read sequences obtained using the Illumina NextGen sequencing platform. The G+C content of the 3,403,608 bp *L. plantarum* 12-3 whole genome was 44.4% within 8 assembled contigs. The genome's quality analysis was estimated at 99.1% genome completeness with only 1.39% contamination. Fig. 1 indicates the *L. plantarum* 12-3 reference sequence (RefSeq) assembly completeness. The circular map of the whole genome is given below in Fig. 2.

MetaGeneMark (http://exon.gatech.edu/meta_gmh_mmp.cgi) was used to predict the location of genes within the *L. plantarum* 12-3 genome. A total of 3234 genes were predicted, with typical lengths ranging from 1020 to 2500 base pairs. An average intergenic spacing of 1000 bp indicates that the genome underwent gene dispersion.

Closer examination of the *L. plantarum* 12-3 genome revealed a wide variety of tRNA, tmRNA, and rRNA sequences. There are several different varieties of tRNA, including tRNA-Val, tRNA-Met, and tRNA-Ala genes. Examining the predicted tRNA secondary structures revealed sequence differences in the anticodon loops and stems. In addition to transfer RNA, transfer-messenger RNA (tmRNA) genes were also expected to be present in the genome. The breakdown of abnormal proteins can occur during translation and tmRNA is essential in this process, and the mechanisms directing protein quality control can be better understood by examining tmRNA. The genome of *L. plantarum* 12-3 has 43 specialty genes which are specialized for various bioactivities (Supplementary Table 1).

3.2 Phylogenetic and Comparative Analysis

ANI analysis was used to confirm the taxonomy and phylogenetic association of *L. plantarum* 12-3, utilizing the platform's comprehensive genome assembly tool. In our ANI analysis, various strains of *Lactobacillus plantarum* were incorporated (see Supplementary Table 1). The results of the ANI analysis indicated that LPG1 exhibited a high degree of similarity with other *L. plantarum* 12-3 strains, with an ANI value exceeding the established threshold for species identity (>95%). Fig. 3 depicts the clustering and heatmaps of various strains of *L. plantarum* 12-3.

Pan-genome analysis revealed a considerable magnitude and heterogeneity in *L. plantarum* 12-3. We observed that genomes can be categorized into distinct types, including a collection of genes that are universally present across all living organisms and another collection of genes that are exclusively shared among a particular subset of organisms. Discrepancies observed in the analysis of prokaryotic organism genomes imply variations in their genetic makeup and evolutionary trajectories. Pan-genome functional anno-

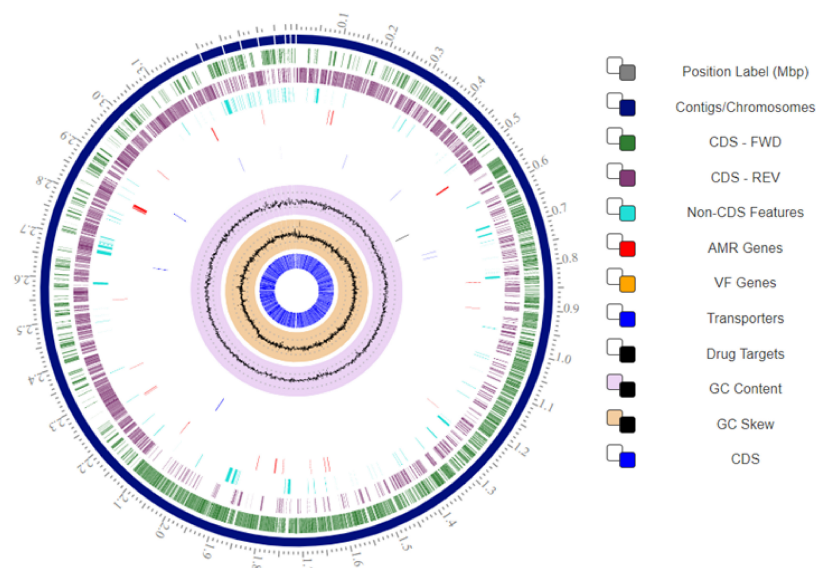


Fig. 2. The genetic characteristics and circular map of the *L. plantarum* 12-3 genome. CDS, Coding Sequence; AMR, Antimicrobial Resistance; VF, Virulence Factor; FWD, Forward; REV, Reverse; GC, Guanine-Cytosine; Mbp, Million Base Pairs.

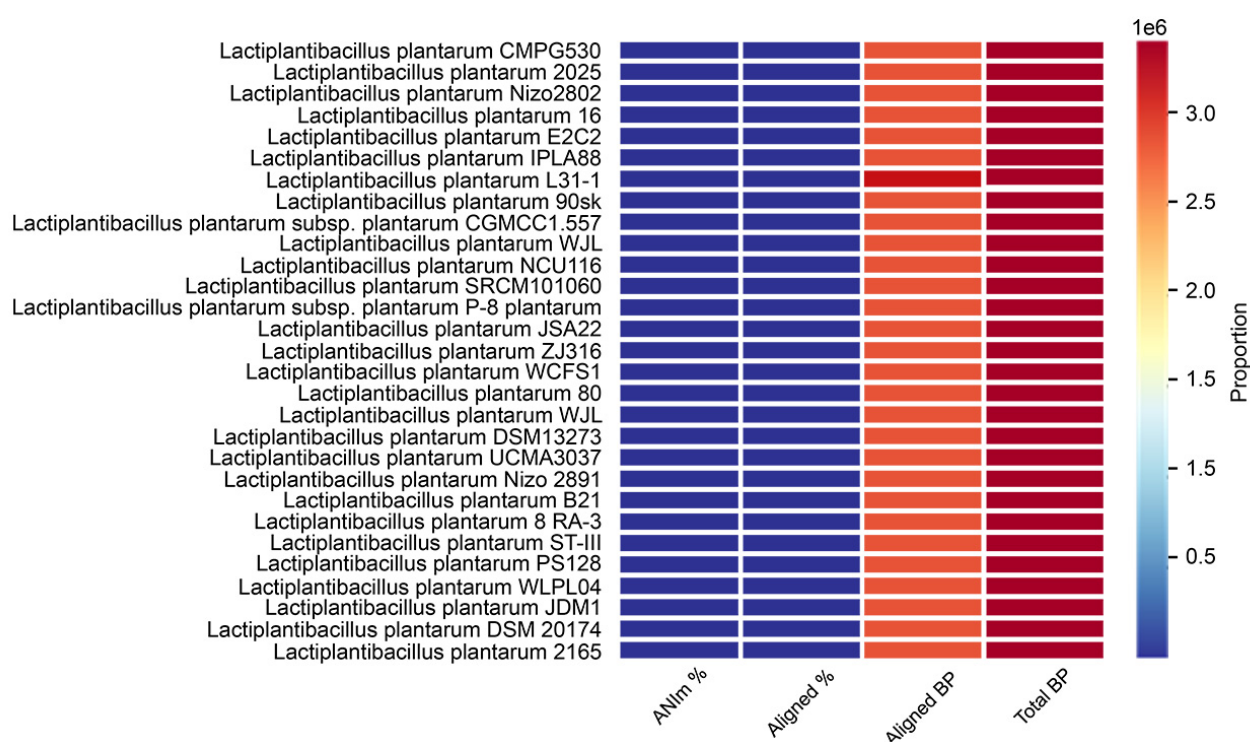


Fig. 3. Clustering and heatmaps of various strains of *L. plantarum* 12-3 indicating the ANIm percentage, aligned percentage, aligned basepairs, and total basepairs. BP, basepairs; ANIm, Average Nucleotide Identity based on MUMmer.

tation has exposed numerous pathways that are involved in diverse biological processes, including metabolism, transport, and regulation. Moreover, such variability has been observed in the distribution of genes associated with virulence and antibiotic resistance across species [24].

The utilization of the pan-genome to construct a phylogenetic strain tree can provide significant insights into the evolutionary relationships among the organisms. The results obtained from the analysis of the strain tree can uncover discernible clades, which can be interpreted as distinct manifestations of genomic diversity and evolutionary

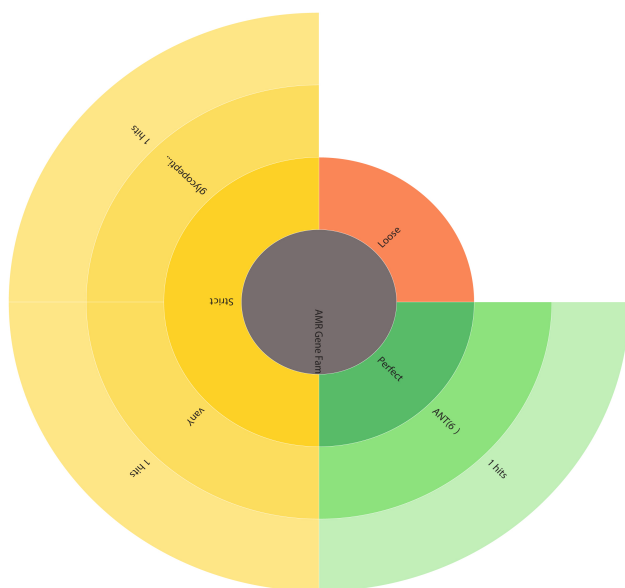


Fig. 5. Antibiotic resistance wheel depicting the antimicrobial resistance (AMR) gene families in *L. plantarum* 12-3. ARO, antibiotic resistance ontology.

dict the length, localization, GC content, and gene annotation of prophage genomes, among other characteristics. To validate the predicted intact prophages, the identified regions were further compared to the Virus-Host database. This comparison confirmed the prophages uncovered and shed further light on their potential host range. A total 5 prophage regions were identified as outlined in Table 2. Furthermore, a circular genome map depicting the genomic regions containing prophage is provided in Fig. 6.

Using the VIPtree tool (<https://www.genome.jp/viptree/>), a proteomic tree of the viral genome sequence was also generated. This tree, which is based on genome-wide sequence similarities, visualized the evolutionary relationships between viral genomes. By using the PHASTER tool to perform database comparisons in conjunction with VIPtree, a comprehensive analysis of prophage regions was conducted. This analysis provided an enhanced understanding of the genetic elements present in the investigated genomes and their potential impact on bacterial evolution and host-pathogen interactions. The circular proteomic tree of the viral genome sequences is provided in Fig. 7.

Using the IslandViewer 4 tool, the presence of genomic islands (GI) within the *L. plantarum* 12-3 genome was investigated. To identify and characterize GI within the genome, multiple databases were queried. This analysis enabled the comprehensive identification of GIs and provided insight into their potential structural and functional functions. The results of this study advance our understanding of the genomic landscape of *L. plantarum* 12-3 and cast light on the potential role of GI in bacterial evolution, adaptation, and virulence [27]. A total of 18 GI were predicted by IslandViewer 4, and the various region sizes and GI pre-

diction methods are provided in Table 3. Furthermore, a circular map, which depicts the GI within the *L. plantarum* 12-3 genome is given in Fig. 8.

Transposases, the enzymatic catalyst of genetic transposition, and mobile elements are critical in orchestrating dynamic gene positioning within the genome. IS-Finder and ISSaga are well-established bioinformatic resources designed to identify and annotate insertion sequences (IS) and other mobile genetic elements within a queried genome. Transposase genes and other mobile elements present within the *L. plantarum* 12-3 genome were accurately identified and annotated using these tools [28]. This annotation procedure yielded useful information regarding the diversity and distribution of transposase genes in the *L. plantarum* 12-3 genome. ISFinder and ISSaga facilitated a comprehensive analysis of mobile genetic elements and their potential influence on the evolution and genetic plasticity of *L. plantarum* 12-3. The predicted mobile elements are provided in Table 4.

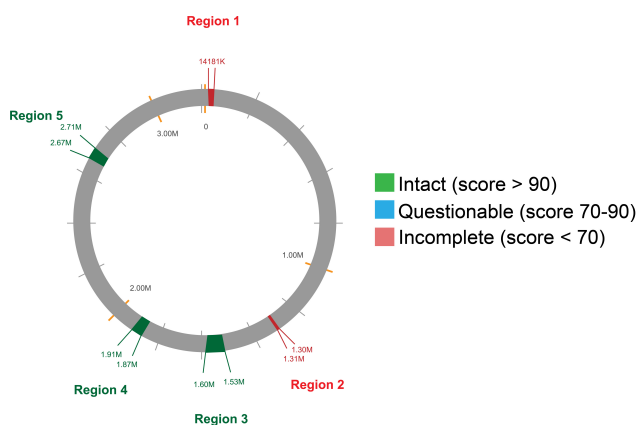


Fig. 6. Circular genome map highlighting the predicted prophage regions within the *L. plantarum* 12-3 genome.

3.5 CRISPR-Cas Sequence Analysis

Using the specialized bioinformatics tool CRISPRCasFinder v.1.1.2 (<https://crisprcas.i2bc.paris-saclay.fr/CrisprCasFinder/Index>), the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated genes (Cas) were identified and validated within the *L. plantarum* 12-3 genome. Furthermore, the presence and arrangement of CRISPR systems within the examined genome were meticulously identified and characterized [29]. Our analysis revealed 1 spacer sequence with a Conservation DR of 97.30% and a Conservation Spacer of 100%. This analysis provided insight into the molecular machinery that underlies this bacterial defense mechanism in *L. plantarum* 12-3. Fig. 9 outlines the spacer sequence uncovered by this analysis.

Table 2. Predicted prophage region, length, localization, and GC Content using PHASTER.

Region	Region length	Completeness	Score	Proteins	Region position	Most common phage	GC%
1	16.2 Kb	incomplete	30	26	14366-30637 info_outline	PHAGE_Lactob_LfeSau_NC_029068	40.68%
2	9.6 Kb	incomplete	60	14	1296322-1306018 info_outline	PHAGE_Staphy_phiPV83_NC_00248	44.51%
3	75.2 Kb	intact	150	71	1529017-1604260 info_outline	PHAGE_Lactob_phig1e_NC_004305	41.99%
4	40.3 Kb	intact	150	55	1869418-1909799 info_outline	PHAGE_Lactob_Sha1_NC_019489	41.46%
5	40.4 Kb	intact	150	55	2671326-2711736 info_outline	PHAGE_Lactob_Sha1_NC_019489	40.67%

Table 3. Predicted genomic islands, their sizes, and prediction methods. These genomic islands (GIs), or distinct regions of the genome, include a variety of genetic components and are crucial in understanding the strain's particular traits.

Start	End	Size	Gi Prediction Method
351,551	393,860	42,309	Predicted by at least one method
389,368	393,860	4492	Predicted by at least one method
695,664	753,081	57,417	Predicted by at least one method
731,210	736,351	5141	Predicted by at least one method
1,691,750	1,749,038	57,288	Predicted by at least one method
1,749,032	1,777,308	28,276	Predicted by at least one method
2,170,145	2,178,397	8252	Predicted by at least one method
2,638,102	2,648,174	10,072	Predicted by at least one method
2,920,631	2,929,067	8436	Predicted by at least one method
1,749,032	1,777,308	28,276	IslandPick
389,368	393,860	4492	SIGI-HMM
731,210	736,351	5141	SIGI-HMM
2,170,145	2,178,397	8252	SIGI-HMM
351,551	393,860	42,309	IslandPath-DIMOB
695,664	753,081	57,417	IslandPath-DIMOB
1,691,750	1,749,038	57,288	IslandPath-DIMOB
2,638,102	2,648,174	10,072	IslandPath-DIMOB
2,920,631	2,929,067	8436	IslandPath-DIMOB

4. Discussion

The study of the *L. plantarum* 12-3 genome demonstrates its potential as a probiotic strain for a variety of applications. Our genomic analysis revealed a wide variety of genes and functional elements, indicating the potential functional properties of the organism. The strain exhibited favorable probiotic properties, such as resistance to hostile environments, antimicrobial properties, epithelial cell adhesion, and immune modulation abilities [11]. The investigation of the CRISPR-Cas system disclosed the presence and organization of these essential genetic elements involved in bacterial defense mechanisms, indicating its potential for applications in genome engineering. These findings contribute to our understanding of the genetic diversity, genotype, and functional insight into *L. plantarum* 12-3, and amply support its potential as a safe and beneficial probiotic candidate for future use [30,31]. These findings underscore promising attributes to *L. plantarum* 12-3 for probiotic function, and suggest its potential use in a variety of safety applications and, thereby, pave the way for future probiotics research. *L. plantarum* 12-3's potential ranges from improving digestive health to helping produce novel functional foods and medicinal formulations.

The identification of *L. plantarum* 12-3 genetic diversity and favorable probiotic properties provides a foundation for further investigation into its potential therapeutic and preventative applications for human health. The strain's resistance to severe conditions and antimicrobial properties bode well for its application in the development of probiotic formulations for specific health conditions [32]. Our findings clearly contribute to the expanding body of knowledge regarding probiotics, as well as paving the way for future research and applications in the field of probiotic functionality and safety, ultimately benefiting the health and well-being of humans [33].

It is very important to discuss the limitations of this study. First, our analysis is limited to the genomic data, and additional experimental validation is required to corroborate *L. plantarum* 12-3 functional characteristics and probiotic potential. Second, although bioinformatics tools were used to identify and annotate genetic elements, the accuracy and completeness of the annotations is dependent on the accuracy of databases and algorithms used [34]. Third, our investigation is restricted to a single strain of *L. plantarum* 12-3; thus, generalizations to other strains or species should be made with caution. Future studies using larger sample sizes, *in vitro* and *in vivo* models, and comparative

Table 4. Predicted transposases and mobile elements within the genome of *L. plantarum* 12-3.

Sequences producing significant alignments	Is family	Group	Origin	Score (bits)	E. Value
ISP2	IS1182		<i>Lactobacillus plantarum</i>	3418	0.0
ISP1	ISL3		<i>Lactobacillus plantarum</i>	2833	0.0
ISLpl1	IS30		<i>Lactobacillus plantarum</i>	1972	0.0
ISPP1	IS30		<i>Pediococcus pentosaceus</i>	1853	0.0
ISLpl3	IS5	IS427	<i>Lactobacillus plantarum</i>	1651	0.0
ISDha13	IS200/IS605	IS200	<i>Desulfotobacterium hafniense</i>	89.7	3×10^{-14}
ISSsu4	IS200/IS605	IS200	<i>Streptococcus suis</i>	71.9	7×10^{-9}
ISStin10	IS200/IS605	IS200	<i>Streptococcus iniae</i>	67.9	1×10^{-7}
ISLpl2	IS3	IS150	<i>Lactobacillus plantarum</i>	61.9	6×10^{-6}
ISLd13	IS30		<i>Lactobacillus delbrueckii</i>	61.9	6×10^{-6}
ISPlu5	IS200/IS605	IS200	<i>Photorhabdus luminescens</i>	58.0	1×10^{-4}
ISEcl11	IS30		<i>Enterobacter cloacae</i>	56.0	4×10^{-4}
ISPlu7	IS5	IS427	<i>Photorhabdus luminescens</i>	52.0	0.006
ISEnfa200	IS200/IS605	IS200	<i>Enterococcus faecium</i>	52.0	0.006
ISWco1	IS3	IS150	<i>Weissella confusa</i>	50.1	0.024
ISPy52	IS3	IS51	<i>Paracoccus yeei</i>	48.1	0.095
ISSmi2	IS1182		<i>Streptococcus mitis</i>	46.1	0.37
ISBce18	IS3	IS150	<i>Bacillus cereus</i>	46.1	0.37
ISHar1	IS3	IS150	<i>Hermiimonas arsenicoxydans</i>	46.1	0.37
ISSth6	IS3	IS150	<i>Streptococcus thermophilus</i>	46.1	0.37
ISLpn1	IS4	IS10	<i>Legionella pneumophila</i>	46.1	0.37
IS1648	IS5	IS427	<i>Streptomyces coelicolor</i>	46.1	0.37
ISCLta1	IS1202	IS1202	<i>Clostridium tagluense</i>	44.1	1.5
ISLmo11	IS3	IS150	<i>Listeria monocytogenes</i>	44.1	1.5
ISAlw31	IS5	IS427	<i>Acinetobacter lwoffii</i>	44.1	1.5
ISSlu1	IS30		<i>Streptococcus lutetiensis</i>	44.1	1.5
ISWsu1	IS3	IS407	<i>Wolinella succinogenes</i>	44.1	1.5
IS1548	ISAs1		<i>Streptococcus agalactiae</i>	44.1	1.5
ISGalb2	IS1202	ISTde1	<i>Gallionellaceae bacterium</i>	42.1	5.8
ISSph16	IS630		<i>Sphingopyxis</i> sp.	42.1	5.8
ISPSko1	IS66		<i>Pseudomonas koreensis</i>	42.1	5.8
ISBli34	IS3	IS150	<i>Brevibacterium linens</i>	42.1	5.8
ISMyr1	IS1595	ISSod11	<i>Myroides</i> sp.	42.1	5.8
ISSbr1	ISNCY	ISDo1	<i>Candidatus Scalindua</i>	42.1	5.8
ISAsp6	IS200/IS605	IS605	<i>Anabaena</i> sp.	42.1	5.8
ISHbo9	IS200/IS605	IS1341	<i>Halogeometricum borinquense</i>	42.1	5.8
ISKpn26	IS5	IS5	<i>Klebsiella pneumoniae</i>	42.1	5.8
ISCst1	ISLre2		<i>Cylindrospermum stagnale</i>	42.1	5.8
ISRfsp3	IS5	ISL2	<i>Roseiflexus</i> sp.	42.1	5.8
ISStma15	IS3	IS51	<i>Stenotrophomonas maltophilia</i>	42.1	5.8
ISRel24	IS66		<i>Rhizobium etli</i>	42.1	5.8
ISYen4	IS1182		<i>Yersinia enterocolitica</i>	42.1	5.8
ISLsp4	ISAz13		<i>Leptospirillum</i> sp.	42.1	5.8
ISClbu1	IS1182		<i>Clostridium butyricum</i>	42.1	5.8
ISCARN112	IS1202	ISTde1	Metagenomic data	42.1	5.8
ISNha5	IS1595	ISNha5	<i>Nitrobacter hamburgensis</i>	42.1	5.8
ISPTo5	ISAs1		<i>Psychroflexus torquis</i>	42.1	5.8
ISBthe4	ISAs1		<i>Bacteroides thetaiotaomicron</i>	42.1	5.8
ISBaov2	ISAs1		<i>Bacteroides ovatus</i>	42.1	5.8
ISvEsV1_1	IS4	IS4	<i>Ectocarpus siliculosus</i>	42.1	5.8
ISFac2	IS5	IS903	<i>Ferroplasma acidarmanus</i>	42.1	5.8
ISC1173a	IS1		<i>Sulfolobus solfataricus</i>	42.1	5.8
IS1419	IS481		<i>Burkholderia glumae</i>	42.1	5.8
IS1070	IS30		<i>Leuconostoc lactis</i>	42.1	5.8

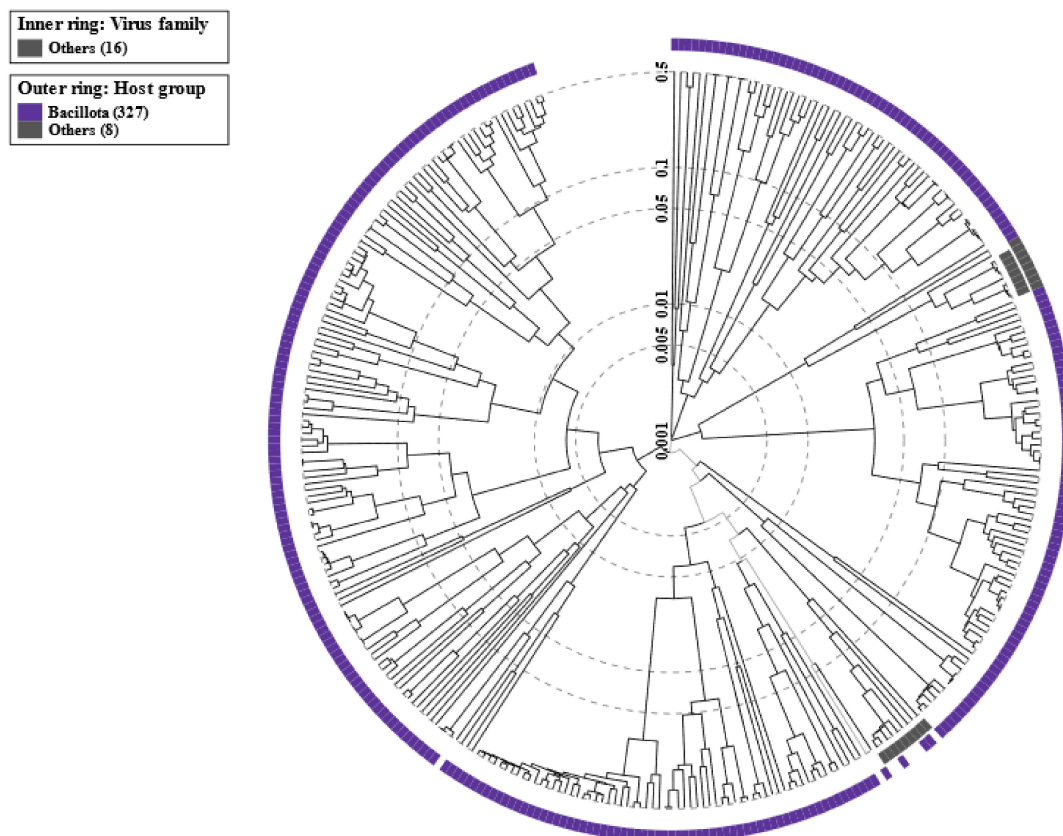


Fig. 7. Circular proteomic tree of viral genomes. The outermost ring outlines the host group of bacteriophages, while the innermost ring outlines the various phage families. The outermost ring provides a panoramic perspective of the bacteriophage host groups, highlighting the different habitats these viruses inhabit. Meanwhile, the innermost ring methodically categorizes the phages according to their viral family, providing a thorough understanding of the taxonomic relationships among these microbial predators.

analyses with other closely related strains may provide a more comprehensive understanding of the probiotic functionality, safety, and potential applications of *L. plantarum* 12-3 [35].

Based on the findings and limitations of this study concerning the genome of *L. plantarum* 12-3, several recommendations for future research can be made. As we have demonstrated in our previous studies, *L. plantarum* 12-3 can convert linoleic acid to various fatty acid metabolites and is also capable of producing exopolysaccharides [9]. Additional experimental evidence is required to validate the functional characteristics and probiotic potential of this *L. plantarum* 12-3 strain, including *in vitro* and *in vivo* evaluation with relevant model systems. In addition, broadening the scope of the study to include a variety of *L. plantarum* 12-3 strains and a comparative analysis with other probiotic species would improve our understanding of genetic diversity and functional properties [36–41]. A closer investigation of the CRISPR-Cas system in *L. plantarum* 12-3, and its application in genome engineering, may result in the development of novel probiotic strains with improved functionalities. Additionally, comprehensive safety evalu-

ations, including evaluation of potential allergenicity and long-term effects, are necessary to ensure the safe use of *L. plantarum* 12-3 and other probiotic strains. These recommendations are intended to advance our understanding of probiotics and maximize their potential health benefits for humans.

5. Conclusions

This investigation has shed light on the genetic variability, CRISPR-Cas mechanism, genotype, and functional implications of the genome of *L. plantarum* 12-3, thereby highlighting its potential for utilization as a probiotic agent. Our comprehensive genomic analysis unveiled a diverse array of genes and functional elements within the genome, suggesting its potential functional capabilities. This *L. plantarum* 12-3 strain exhibited several advantageous probiotic characteristics, including resilience to adverse surroundings, antimicrobial capability, adhesion to epithelial cells, and the ability to modulate the immune system. Additional research focused on understanding the functional characteristics and safety considerations of *L. plantarum* 12-3 through *in vitro* and *in vivo* investigations for its pro-

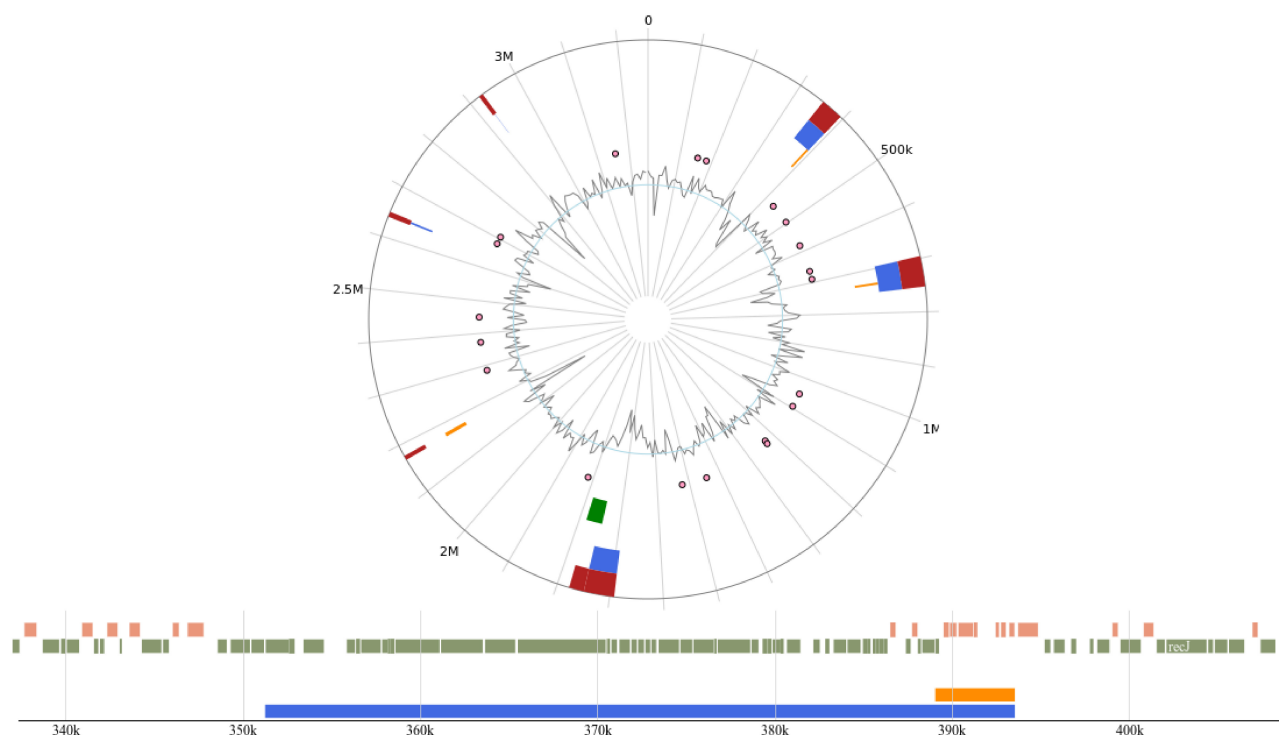


Fig. 8. Circular map of the genomic islands predicted using IslandViewer 4.



Fig. 9. The identified CRISPR-Cas sequences in the genome of *L. plantarum* 12-3.

biotic functionality and various bio-functional properties is warranted. Such studies may involve analysis of its interactions with the host microbiome, assessing its probiotic properties, and conducting a thorough safety assessment. Furthermore, a continuous exploration of the genome can provide insights into the adaptive evolution and genetic flexibility of *L. plantarum* 12-3 species, thereby enhancing our understanding of their potential applications as probiotic-based therapies and food safety measures.

Availability of Data and Materials

All the data generated in this study will be available on demand from the corresponding author.

Author Contributions

Conceptualization, TA, MN, MAS, AS, AAK, and ZY; methodology, TA, MN, MAS, AS, AH, and ZY; software, MA; validation, MA; formal analysis, TA; investigation, JMR and AZ; resources, ZY; MA and AH; data curation, TUH; writing—original draft preparation, AZ and MN; writing—review and editing, AH and MN; visualization, AH; supervision, ZY; project administration, AAS and

MA; funding acquisition, ZY. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

The authors extend their appreciation to the Deputyship for Research and Innovation, “Ministry of Education” in Saudi Arabia for funding this research (IFKSUOR3- 619-3).

Funding

This research work was financially supported by National Natural Science Foundation of China (Project No. 32272296) and the Deputyship for Research and Innovation, “Ministry of Education” in Saudi Arabia (IFKSUOR3-619-3).

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.fbl2904147>.

References

- [1] Mao B, Yin R, Li X, Cui S, Zhang H, Zhao J, *et al.* Comparative Genomic Analysis of *Lactiplantibacillus plantarum* Isolated from Different Niches. *Genes*. 2021; 12: 241.
- [2] Parlindungan E, Dekiwadia C, Jones OA. Factors that influence growth and bacteriocin production in *Lactiplantibacillus plantarum* B21. *Process Biochemistry*. 2021; 107: 18–26.
- [3] Kurata A, Kiyohara S, Imai T, Yamasaki-Yashiki S, Zaima N, Moriyama T, *et al.* Characterization of extracellular vesicles from *Lactiplantibacillus plantarum*. *Scientific Reports*. 2022; 12: 13330.
- [4] Yang SY, Chae SA, Bang WY, Lee M, Ban OH, Kim SJ, *et al.* Anti-inflammatory potential of *Lactiplantibacillus plantarum* IDCC 3501 and its safety evaluation. *Brazilian Journal of Microbiology: [publication of the Brazilian Society for Microbiology]*. 2021; 52: 2299–2306.
- [5] Barbosa J, Albano H, Silva B, Almeida MH, Nogueira T, Teixeira P. Characterization of a *Lactiplantibacillus plantarum* R23 Isolated from Arugula by Whole-Genome Sequencing and Its Bacteriocin Production Ability. *International Journal of Environmental Research and Public Health*. 2021; 18: 5515.
- [6] Prete R, Garcia-Gonzalez N, Di Mattia CD, Corsetti A, Battista N. Food-borne *Lactiplantibacillus plantarum* protect normal intestinal cells against inflammation by modulating reactive oxygen species and IL-23/IL-17 axis. *Scientific Reports*. 2020; 10: 16340.
- [7] Villena J, Li C, Vizoso-Pinto MG, Sacur J, Ren L, Kitazawa H. *Lactiplantibacillus plantarum* as a Potential Adjuvant and Delivery System for the Development of SARS-CoV-2 Oral Vaccines. *Microorganisms*. 2021; 9: 683.
- [8] Aziz T, Sarwar A, Fahim M, Al-Dalali S, Din ZU, Din JU, *et al.* Conversion of linoleic acid to different fatty acid metabolites by *Lactobacillus plantarum* 13-3 and in silico characterization of the prominent reactions. *Journal of the Chilean Chemical Society*. 2020; 65: 4879–4884.
- [9] Aziz T, Sarwar A, Fahim M, Al Dalali S, Ud Din Z, Ud Din J, *et al.* In silico characterization of linoleic acid biotransformation to rumenic acid in food derived *Lactobacillus plantarum* YW11. *Acta Biochimica Polonica*. 2020; 67: 99–109.
- [10] Aziz T, Sarwar A, Fahim M, Ud Din J, Al Dalali S, Ud Din Z, *et al.* Dose-dependent production of linoleic acid analogues in food derived *Lactobacillus plantarum* K25 and in silico characterization of relevant reactions. *Acta Biochimica Polonica*. 2020; 67: 123–129.
- [11] Aziz T, Sarwar A, Naveed M, Shahzad M, Aqib Shabbir M, Dabool AS, *et al.* Bio-Molecular analysis of selected food derived *Lactiplantibacillus* strains for CLA production reveals possibly a complex mechanism. *Food Research International (Ottawa, Ont.)*. 2022; 154: 111031.
- [12] Aziz T, Sarwar A, Ud Din J, Al Dalali S, Khan AA, Din ZU, *et al.* Biotransformation of linoleic acid into different metabolites by food derived *Lactobacillus plantarum* 12-3 and in silico characterization of relevant reactions. *Food Research International (Ottawa, Ont.)*. 2021; 147: 110470.
- [13] Zhang J, Cai D, Yang M, Hao Y, Zhu Y, Chen Z, *et al.* Screening of folate-producing lactic acid bacteria and modulatory effects of folate-biofortified yogurt on gut dysbacteriosis of folate-deficient rats. *Food & Function*. 2020; 11: 6308–6318.
- [14] Churro C, Pereira P, Vasconcelos V, Valério E. Species-specific real-time PCR cell number quantification of the bloom-forming cyanobacterium *Planktothrix agardhii*. *Archives of Microbiology*. 2012; 194: 749–757.
- [15] Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J. JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. *Bioinformatics (Oxford, England)*. 2016; 32: 929–931.
- [16] Ning W, Wei Y, Gao L, Han C, Gou Y, Fu S, *et al.* HemI 2.0: an online service for heatmap illustration. *Nucleic Acids Research*. 2022; 50: W405–W411.
- [17] Kim Y, Gu C, Kim HU, Lee SY. Current status of pan-genome analysis for pathogenic bacteria. *Current Opinion in Biotechnology*. 2020; 63: 54–62.
- [18] Alcock BP, Raphenya AR, Lau TTY, Tsang KK, Bouchard M, Edalatmand A, *et al.* CARD 2020: antibiotic resistance surveillance with the comprehensive antibiotic resistance database. *Nucleic Acids Research*. 2020; 48: D517–D525.
- [19] Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, *et al.* BLAST+: architecture and applications. *BMC Bioinformatics*. 2009; 10: 421.
- [20] Joensen KG, Scheut F, Lund O, Hasman H, Kaas RS, Nielsen EM, *et al.* Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. *Journal of Clinical Microbiology*. 2014; 52: 1501–1510.
- [21] Arndt D, Grant JR, Marcu A, Sajed T, Pon A, Liang Y, *et al.* PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Research*. 2016; 44: W16–W21.
- [22] Bertelli C, Laird MR, Williams KP, Simon Fraser University Research Computing Group, Lau BY, Hoad G, *et al.* IslandViewer 4: expanded prediction of genomic islands for larger-scale datasets. *Nucleic Acids Research*. 2017; 45: W30–W35.
- [23] Couvin D, Bernheim A, Toffano-Nioche C, Touchon M, Michalik J, Néron B, *et al.* CRISPRCasFinder, an update of CRISPRFinder, includes a portable version, enhanced performance and integrates search for Cas proteins. *Nucleic Acids Research*. 2018; 46: W246–W251.
- [24] Huehn S, La Ragione RM, Anjum M, Saunders M, Woodward MJ, Bunge C, *et al.* Virulotyping and antimicrobial resistance typing of *Salmonella enterica* serovars relevant to human health in Europe. *Foodborne Pathogens and Disease*. 2010; 7: 523–535.
- [25] Yin Z, Yuan C, Du Y, Yang P, Qian C, Wei Y, *et al.* Comparative genomic analysis of the *Hafnia* genus reveals an explicit evolutionary relationship between the species *alvei* and *paralvei* and provides insights into pathogenicity. *BMC Genomics*. 2019; 20: 768.
- [26] McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ, *et al.* The comprehensive antibiotic resistance database. *Antimicrobial Agents and Chemotherapy*. 2013; 57: 3348–3357.
- [27] Ricaldi JN, Fouts DE, Selengut JD, Harkins DM, Patra KP, Moreno A, *et al.* Whole genome analysis of *Leptospira icterohaemolyticum* provides insight into leptospiral evolution and pathogenicity. *PLoS Neglected Tropical Diseases*. 2012; 6: e1853.
- [28] Durrant MG, Li MM, Siranosian BA, Montgomery SB, Bhatt AS. A Bioinformatic Analysis of Integrative Mobile Genetic Elements Highlights Their Role in Bacterial Adaptation. *Cell Host & Microbe*. 2020; 27: 140–153.e9.
- [29] Nazir R, Mandal S, Mitra S, Ghorai M, Das N, Jha NK, *et al.* Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated genome-editing toolkit to en-

hance salt stress tolerance in rice and wheat. *Physiologia Plantarum*. 2022; 174: e13642.

- [30] Aziz T, Naveed M, Makhdoom SI, Ali U, Mughal MS, Sarwar A, *et al*. Genome Investigation and Functional Annotation of *Lactiplantibacillus plantarum* YW11 Revealing Streptin and Ruminococcin-A as Potent Nutritive Bacteriocins against Gut Symbiotic Pathogens. *Molecules* (Basel, Switzerland). 2023; 28: 491.
- [31] Huang X, He Y, Zhong C, Zhao K, Shah NP, Tao X, *et al*. Screening of probiotic strains of *L. plantarum* from Hu sheep and its ability to inhibit *Listeria monocytogenes* in pasteurized milk. *LWT*. 2023; 182: 114883.
- [32] Yepez L, Tenea GN. Genetic diversity of lactic acid bacteria strains towards their potential probiotic application. *Rom Biotechnol Lett*. 2015; 20: 10191–10199.
- [33] Seddik HA, Bendali F, Gancel F, Fliss I, Spano G, Drider D. *Lactobacillus plantarum* and Its Probiotic and Food Potentialities. *Probiotics and Antimicrobial Proteins*. 2017; 9: 111–122.
- [34] Yetiman AE, Keskin A, Darendeli BN, Kotil SE, Ortakci F, Dogan M. Characterization of genomic, physiological, and probiotic features *Lactiplantibacillus plantarum* DY46 strain isolated from traditional lactic acid fermented shalgam beverage. *Food Bioscience*. 2022; 46: 101499.
- [35] Yang S, Liu L, Wang J, Guo S, Liu G, Chen X, *et al*. Antimicrobial activity against *Staphylococcus aureus* and genome features of *Lactiplantibacillus plantarum* LR-14 from Sichuan pickles. *Archives of Microbiology*. 2022; 204: 637.
- [36] Jiang Y, Zhang J, Zhao X, Zhao W, Yu Z, Chen C, *et al*. Complete genome sequencing of exopolysaccharide-producing *Lactobacillus plantarum* K25 provides genetic evidence for the probiotic functionality and cold endurance capacity of the strain. *Bioscience, Biotechnology, and Biochemistry*. 2018; 82: 1225–1233.
- [37] Jung BJ, Kim H, Chung DK. Differential immunostimulatory effects of lipoteichoic acids isolated from four strains of *Lactiplantibacillus plantarum*. *Applied Sciences*. 2022; 12: 954.
- [38] Min Z, Xiaona H, Aziz T, Jian Z, Zhennai Y. Exopolysaccharides from *Lactobacillus plantarum* YW11 improve immune response and ameliorate inflammatory bowel disease symptoms. *Acta Biochimica Polonica*. 2020; 67: 485–493.
- [39] Skotniczny M, Satora P. Molecular Detection and Identification of Plant-Associated *Lactiplantibacillus plantarum*. *International Journal of Molecular Sciences*. 2023; 24: 4853.
- [40] Yilmaz B, Bangar SP, Echeagaray N, Suri S, Tomasevic I, Manuel Lorenzo J, *et al*. The Impacts of *Lactiplantibacillus plantarum* on the Functional Properties of Fermented Foods: A Review of Current Knowledge. *Microorganisms*. 2022; 10: 826.
- [41] Zhang J, Zhao W, Guo X, Guo T, Zheng Y, Wang Y, *et al*. Survival and effect of exopolysaccharide-producing *Lactobacillus plantarum* YW11 on the physicochemical properties of ice cream. *Polish Journal of Food and Nutrition Sciences*. 2017; 67.