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Whole genome sequence of *Lactiplantibacillus plantarum* strain HA9 isolated from conventional Iraqi cheese: First report

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Abstract

Background: *Lactiplantibacillus plantarum* is a renowned probiotic known for its ability to enhance health when ingested in sufficient quantities. While *L. plantarum* typically exhibits unique characteristics, variations in strains and environments can occur. Therefore, it is crucial to conduct genetic identification of the strain before incorporating it into products or administering it for health benefits.

Methods: After being isolated from classic artisanal milk cheeses, the bacterial DNA extraction kit from GENE AID, Korea, was used, and strain HA9's genomic DNA was isolated. Then, the highly conserved section of ribosomal RNA was amplified with universal primers. The results were confirmed by a blast analysis of the NCBI database. The genome of HA9 was sequenced using the Illumina and PacBio Sequel II technologies (HN00194138; Psomagen/USA). The Prokaryotic Dynamic Programming Gene-Finding Algorithm software was applied to forecast coding genes. The Pathosystems Resource Integration Center constructed the *L. plantarum* genome.

Result: According to the findings, the genome of the *L. plantarum* HA9 strain has a length of 3,321,986 bp and a GC percentage of 44.40. This assembled genome contained a total of 1,420 coding proteins and 82 contigs, as discovered by the Comprehensive Genome Analysis. The isolate had been submitted to the NCBI and received the HA9 genome sequence (accession ID: [JAUTDL000000000.1](https://www.ncbi.nlm.nih.gov/nuclseq/JAUTDL000000000.1)).

Conclusion Microbes' whole-genome sequencing plays a crucial role in clarifying their probiotic properties and enables accurate identification down to the strain level. The authors argue that further research should be done on probiotics' whole genomes, with a special emphasis on second-generation strains.



Introduction

Lactiplantibacillus plantarum, previously known as *Lactobacillus plantarum*, is a type of lactic acid bacterium commonly encountered in various fermented foods, such as artisanal milk cheeses. *L. plantarum* is one of the predominant microbial species engaged in fermentation, which is a procedure used historically to create artisanal milk cheeses [1].

Depending on the type of milk used, the region, and the particular cheese-making methods utilized by the artisanal cheese manufacturers, isolates of *L. plantarum* from traditional artisanal milk cheeses might differ. Different *L. plantarum* strains or isolates may have unique traits, such as the capacity to create enzymes. *L. plantarum* strains isolated from various cheeses had various proteolytic and lipolytic capabilities as well as various levels of resistance to stress [2]. Some strains of *L. plantarum* are regarded as a probiotic, which means that when taken in sufficient levels, it gives health advantages. It is thought to strengthen immune system function, increase gut microbial balance, and support digestive health [3]. Low pH values, high salt concentrations, and a range of temperatures are only a few environmental variables to which *L. plantarum* is very tolerant. Due to its versatility, it may live and flourish in many gut and food conditions. For instance, according to [4], *L. plantarum* can withstand pH levels as low as 2.51, bile concentrations as high as 0.3%, pancreatin concentrations as high as 0.5%, NaCl concentrations as high as 5%, and temperatures as low as 4°C to 45°C [5]. Due to these qualities, *L. plantarum* is able to colonize a variety of fermented foods as well as resist the challenging circumstances of the gastrointestinal system. In response to several environmental cues including pH, bile, and oxygen, *L. plantarum* may also alter the expression of its genes. Due to its adaptability, *L. plantarum* is able to retain its functioning and viability in a variety of conditions [6]. The *L. plantarum* creates bacteriocins, which are antimicrobial peptides that can stop the growth of other hazardous bacteria. The capacity of these bacteriocins to outcompete harmful bacteria and aid in food preservation makes it especially beneficial [7]. *L. plantarum* has a flexible metabolic route that enables it to ferment several types of carbohydrates and generate a range of byproducts, such as carbon dioxide, ethanol, and other organic acids [8]. The *L. plantarum* is capable of forming biofilms. According to [9], the creation of biofilms can be advantageous in some situations, such as boosting adherence to probiotic colonization in the gut or enhancing during fermentation processes. *L. plantarum* has the capacity to produce a number of vitamins, including several B-group vitamins, which may improve the nutritional content of food overall and be

advantageous to the host [10]. Despite the fact that *L. plantarum* often demonstrates these traits, there may be variances between different strains and depending on the particular circumstances it faces. As a result, the *L. plantarum* genome may exhibit structural variants (SVs), particularly in genes involved in metabolism, stress tolerance, biofilm formation, and virulence [11]. The phenotypic traits of *L. plantarum* strains, such as their glucose metabolism, ethanol tolerance, anti-fungal activity, and interactions with other microorganisms, can also be affected by these genetic differences [12]. For instance, the *L. plantarum* B2 strain has a mutation in the FMN-aptamer of the riboswitch, which regulates the expression of the rib operon. The strain benefits from the increased production and accumulation of riboflavin and FMN as sources of vitamin B2, but this mutation also decreases the riboswitch's regulatory activity and makes the strain more susceptible to roseoflavin, a riboflavin analog that prevents bacterial growth [13]. Thus, it is essential to establish the genetic identity of the strain both before and throughout manufacturing.

The antimicrobial susceptibility profile of the strain and its ability to spread AMR genes or virulence factors to other bacteria are important criteria in establishing a probiotic's safety for use in people and/or animals. Any bacterium employed as a probiotic must be demonstrated to be devoid of AMR and virulence factors, both of which can be transmitted by ingestion, and all probiotics should have their antimicrobial susceptibility profiles assessed [14,15]. If a probiotic is shown to be the cause or is suspected of being the source of any invasive illness, knowledge of AMR patterns can help clinicians make treatment options. Additionally, understanding a microbe's AMR phenotype can aid in the development of innovative multi-component treatment regimens, which is crucial in light of the growing body of research supporting the simultaneous administration of probiotics and antibiotics [16-18].

The goals of this study were to determine the genome sequence of *L. plantarum* isolated from classic artisanal milk cheese and to confirm their taxonomic, strain-level identities and more details.

Methods

L. plantarum HA9 culture conditions

Lactic acid bacteria (LAB) were isolated from classic artisanal milk cheeses. Isolation was done using, with minor modifications, a previously published approach [19]. In short, we plated each sample on MRS agar after diluting and homogenizing 1 gram of each sample in MRS broth. Then incubated the plates anaerobically at 37 °C for 48 hours and selected colonies with different morphologies. The colonies were purified by sub-

culturing and confirmed them as *Lactobacillus* spp. based on their catalase-negative, gram-positive, rod-shaped appearance. The bacterial isolates stored in MRS broth with 20% glycerol.

Extraction of genomic DNA from *L. plantarum* HA9

Using the bacterial DNA extraction kit from GENE AID, Korea, genomic DNA was isolated from strain HA9 [20]. Thermo Fisher Scientific's NanoDrop 2000 spectrophotometer and Life Technologies' Qubit 3.0 were used for DNA quantification and concentration measurements, respectively, and 0.8% (w/v) agarose gel electrophoresis was used to assess the purity of the DNA. Then, the highly conserved section of ribosomal RNA was amplified with universal primers 27F and 1492R, the PCR product was sequenced with these primers [21], and the results were confirmed by a blast analysis of the NCBI database [22].

Whole-genome sequence analysis of *L. plantarum* HA9

The genome of *L. plantarum* HA9 was sequenced using the Illumina and PacBio Sequel II technologies (HN00194138; Psomagen/USA). After passing quality control (QC), the DNA from the samples was randomly fragmented and ligated with 5' and 3' adapters to produce a library. The generated NGS library will then be sequenced. Following the completion of the sequencing, the raw data was analyzed for parameters such as GC (percentage), total bases, and total reads. To reduce analytical biases, quick QC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and quality filtering were utilized. We used the phred quality score (Q20(%) and Q30(%) to assess data quality at the end of each cycle [23].

Genome annotation of *L. plantarum* HA9

Coding genes were predicted using Prodigal (Prokaryotic Dynamic Programming Gene-finding Algorithm) software [24]. Filtering for proteins with the highest sequence similarity using annotations with $e = 1e-5$, the Diamond software [25] was employed to contrast expected coding sequences in order to provide functional annotation information. The HMMER software [26] was utilized for comparing these sequences with protein group models in order to identify the families that scored the highest. The predicted code sequence regions were compared against the KEGG (Kyoto Encyclopedia of Genes and Genomes), COG (Cluster of Orthologous Groups of proteins), GO (Gene Ontology), CAZy (Carbohydrate-Active Enzymes Database), and NR (non-redundant) databases in order to complete the foundational functional prediction and annotation. Using the Circos (v0.69) program [27] many data sets were shown in one single genomic circular map.

Genome analysis

PATRICS, a sophisticated genome analysis service, constructed *L. plantarum* genome [28]. The *L. plantarum* reference genome mapped sequencing reads. Functional genes in subsystem categories were annotated in the genome, mapping results were compared, conserved and unique sequencing properties were identified, and high-quality maps were created to verify and display the described features. Bioinformatics techniques removed duplicates, found variants, and mapped filtered data reads to the reference genome [29-31].

Results

The quality of the HA9 DNA

To guarantee the concentration and quality of DNA, the bioinformatics tools provided by PATRIC were employed. The genome isolates of strain HA9 displayed a coarse consistency score of 97.8, was complemented by a fine consistency score of 96.1, and achieved a checkM completeness rating of 100. As a result, this genome exemplified exceptional overall quality.

Genomic characteristics of HA9

The cutting-edge PacBio Sequel II sequencing technology was employed to unveil the entire genome sequence of *L. plantarum* strain HA9. This breakthrough resulted in NCBI receiving the HA9 genome sequence (accession ID: [JAUTDL000000000.1](https://www.ncbi.nlm.nih.gov/nuclseq/JAUTDL000000000.1)). Impressively, the HA9 genome spans 3,321,986 base pairs, consisting of a singular circular chromosome with a GC content of 44.40%, and notably, no plasmids were detected. Table 1 encapsulates the anticipated 3,138 genes, along with 2 rRNAs (5S, 16S, and 23S) and 59 tRNAs present in this comprehensive genome. Meanwhile, the strain HA9 whole-genome circle map is shown in Figure 1, meticulously delineating key genomic features such as homologous genes, GC content, genomic islands, gene distribution across forward and antisense strands, and COG functional classification of genes, and more intricate details. Noteworthy is the genome's high quality, substantiated by annotation statistics and comparison with PATRIC genomes of the same species. Following meticulous read filtering, the phred quality scores of bases exceeded Q20 and Q30 at an impressive rate of 97.8% and 94.0%, respectively. Furthermore, Comprehensive Genome Analysis unveiled 81 contigs spanning 3,314,413 base pairs, housing 1,420 coding proteins in this meticulously assembled genome, as detailed in Table 2. The genome's GC content averages 44.3%, with Figure 1 providing a schematic representation of GC content and GC skew analysis.

Each protein subsystem within the genome contributes to specific biological activities or structural complexes, with a striking 278 genome-specific subsystems found across annotation. Figure 2 offers a comprehensive overview of the genome's subsystem organization, highlighting its intricate biological functionality and structural complexity.

Genome Annotation Pipeline (PGAP)	Results
Whole length	3,321,986 bp
GC content %	44.40
Contig count	81
Subsystem count	278
Genes count	2,530
CDSs count	5,074
Genes (coding) proteins	2,454
CDSs (with protein)	3,299
(RNA) Genes	61
rRNA	2
tRNAs	59
Contig L50	4
Contig N50	345,302
Plasmids	0

Table 1: Summary of the genome assembly and details of the annotated characteristics.

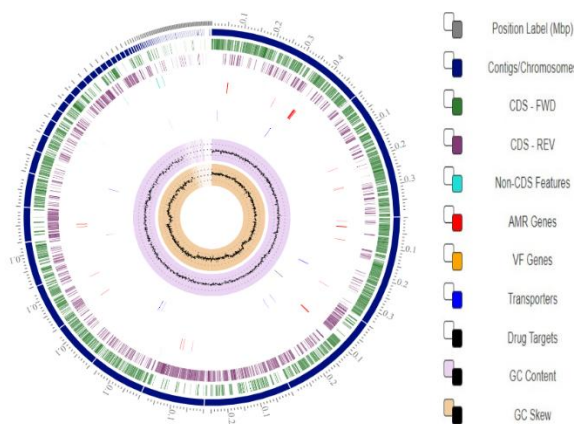


Figure 1: The circular genome of HA9. Chromosome location is reflected by the innermost ring, while the genomic backbone (contigs) is indicated by the blue frame.

Features of proteins	Count
Hypothetical proteins	1,487
Proteins with functional assignments	1,812
Proteins with EC number assignments	695
Proteins with GO assignments	585
Proteins with pathway assignments	490
Proteins with Subsystem assignments	48691740
Proteins with PATRIC genus-specific family (PLfam) assignments	0
Proteins with PATRIC cross-genus family (PGfam) assignments	4,159

Table 2: Summary proteins features details.

Phylogenetic Analysis

For phylogenetic research, PATRIC offers representative and reference genomes. Based on 16S rRNA sequences, Figure 3 shows a neighbor-joining phylogenetic tree; Figure 4 shows the nearest reference and typical genotypes found during the research.

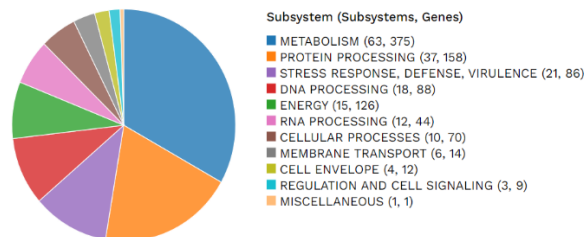


Figure 2: Distribution of *L. plantarum* subsystems. The genome is annotated by RAST. Subsystem feature counts and coverage were displayed using the pie chart and SEED viewer. Proteins included in the subsystems are indicated by the green bar of the subsystem coverage, whilst proteins excluded are indicated by the blue bar.

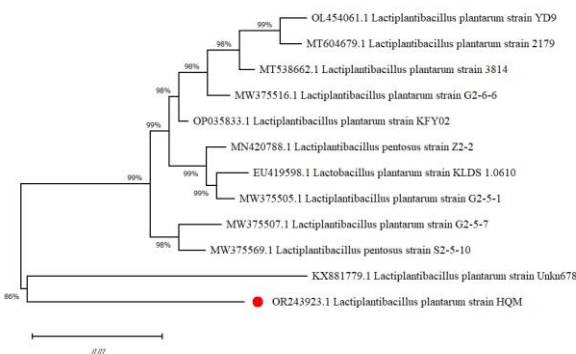


Figure 3: The Maximum Likelihood method and Tamura-Nei model were used for phylogenetic relationship representation of the *L. plantarum* with other *L. plantarum* using 16S rRNA sequences.

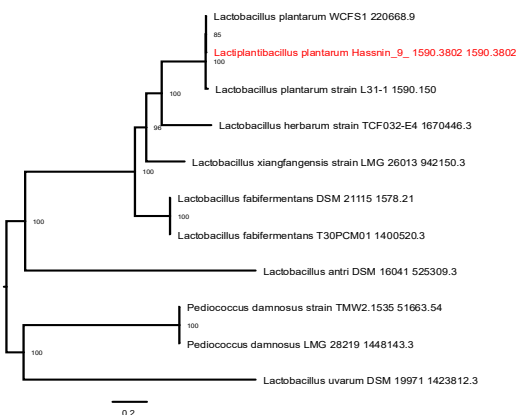


Figure 4: The phylogenetic tree for HA9 (indicated by the Red) and related species strains based on whole gene sequences. The closest reference and representative genomes to were identified by Mash/MinHash.

Discussion

16S rRNA analysis was used to identify the HA9 strain and classify it as *L. plantarum*. This taxonomy corresponds to the species commonly present in sauerkraut [32].

The size of the HA9 genome and the proportion of its GC content were identical to those previously reported for *L. plantarum* strains. Within the lactobacilli group, the genome of the *L. plantarum* species is among the largest genomes overall, ranging in size from 2.91 to 3.7 Mb. The whole genome of *L. plantarum* has a median length of 3.27272 Mb, a median protein counts of 2997, and a GC content of 44.5%, according to the NCBI database. It is considered that the large genomes of *L. plantarum* are directly related to the environmental flexibility and extraordinary adaptability it possesses to a wide variety of settings [33].

The HA9 strain of *L. plantarum* occupies a distinct position on the evolutionary tree when compared to other strains of the same species. Its closest counterparts include strains such as *L. plantarum* WCFS1, which is among the most extensively researched lactobacilli. Notably, the genome of *L. plantarum* WCFS1 was unveiled many years ago. This particular strain of *L. plantarum* demonstrates remarkable resilience and a relatively high survival rate when passing through the digestive system, and it can be cultivated to high densities. Additionally, it is easily modifiable genetically [34]. Furthermore, the genome of *L. plantarum* WCFS1 has not been found to include any transferable antibiotic resistance genes [35]. To understand the distinctions between HA9 and WCFS1 better, further comprehensive comparative genomic investigations are required.

A viable technique to enrich fermented meals and boost the health-promoting impact of probiotics is to make use of starter cultures that produce vitamins or probiotics [36]. On the other hand, future investigations both in silico and in vitro should shed light on the manner in which several biochemical biosynthesis processes are linked to the genetic ability to biosynthesize vitamins. We also evaluated genetic data linked to safety evaluation, like genes that encode toxins and virulence factors, however we were unable to find any risk-associated sequences in the genome of *L. plantarum* HA9.

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Author Contributions

Al-kaabi H. Q. and Chelab R. L. designed the study and performed the experiments. In addition, both authors analyzed the data and wrote the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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