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Whole Genome Sequence and *In Silico* Analysis of Probiotic Potential of *Limosilactobacillus fermentum* PC-10 and PC-76 Isolated from Poultry Gut

Adnan Mehmood[1], Muhammad Nawaz[1]*, Masood Rabbani[1] and Muhammad Hassan Mushtaq[2]

[1]*Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, 54000, Pakistan*
[2]*Department of Epidemiology and Public Health, University of Veterinary and Animal Sciences, Lahore, 54000, Pakistan*

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Key words
***Limosilactobacillus fermentum*, Whole genome sequencing, Probiotic, Anti-Salmonella Gallinarum, Comparative genomic, Phylogenetic analysis**

ABSTRACT

The *Limosilactobacillus fermentum* (PC-10 and PC-76) originally isolated from poultry gut have displayed antibacterial activity against *Salmonella* Gallinarum and potential *in vitro* probiotic properties. The aim of this study was to elucidate the genetic traits associated with probiotic characteristics and to assess the safety of these strains. Using illumina sequencing technology the entire genome of isolates (PC-10 and PC-76) was sequenced and annotated for the characterization of probiotic proteins at genomic level. The genomic features of isolates were analyzed by Circular Genome (CG) View server. The results showed that complete circular chromosomes of strains are approximately 2MB including GC content, coding sequences, tRNA, ncRNA and mRNA. The comparison of genome by Orthovenn2 tool using orthologous cluster genes and evolutionary tree constructed by TYGS server showed their close association with *L. fermentum*. Several genes encoding for probiotic properties such as resistance to acidic pH and bile salts, oxidative stress, adhesion to gut and stress (heat and cold) conditions were identified by KEGG. *In silico* analysis evidenced the presence of probiotic markers that perform different functions in hosts such as acid tolerance and resistance to bile salts, aggregation and improved survival under stress conditions. Moreover, these isolates possess the CRISPR region in their genome and are considered safe, as they have no acquired antibiotic resistance genes or prophage loci. These findings suggest that *L. fermentum* (PC-10 and PC-76) presents promising probiotic traits, further investigation is required to evaluate their biological and health promoting effects in chicken.

INTRODUCTION

Probiotics are non-harmful microorganisms that provide health advantages to the host when administered in appropriate amounts (Hill *et al*., 2014; Rashid *et al*., 2023). Microbial genera that belong to *Lactobacillus*, *Bifidobacterium* and *Saccharomyces* are mainly used as probiotics. Among these the most familiar and well known are the *Lactobacillus*, that belong to phylum firmicutes and have recently been classified into 25 genera (Gul and Alsayeqh, 2022; Zheng *et al*., 2020). Lactobacilli are normal inhabitants of the human and animal gastrointestinal tract and prefer to live in an acidic environment. Various studies have reported that consumption of probiotic strains of *Lactobacillus* improves gut health and protects from intestinal infection (Yue *et al*., 2020).

The *Lactobacillus* are aerotolerant or anaerobic, beneficial microbes. They are considered as safe and are used in the fermentation of milk, vegetables and fruits while some species are also the members of healthy gut microbiota (Duar *et al*., 2017; Gasbarrini *et al*., 2016; Saito *et al*., 2019). Lactic acid bacteria produce various antimicrobial peptides and metabolites such as hydrogen peroxide, bacteriocins and organic acids (acetic and lactic acid) that inhibit the

* Corresponding author: muhammad.nawaz@uvas.edu.pk
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growth of pathogens (Patrignani *et al*., 2019). *L. fermentum*, a member of the *Lactobacillus* genus, is a Gram positive, hetero-fermentative bacterium that naturally inhabits the human and animal gastrointestinal tract (GIT). Some strains of this species have anti-inflammatory probiotic properties. It is also used for the control and treatment of various GIT infections, prevention of alcoholic disease and attenuation of colorectal cancer (Barone *et al*., 2016; Kim *et al*., 2022). Other studies have reported the anti-obesity effect (Kim *et al*., 2020), cholesterol reduction (Pan *et al*., 2011) and immune-modulatory capabilities of *L. fermentum* (Zhao *et al*., 2019).

The European Food Safety Authority (EFSA) has released guidelines to ensure the safety and efficacy of probiotics. According to these guidelines, probiotic strains must be identified using whole genome sequencing and their antibiotic resistance pattern must be determined by genotypic and phenotypic methods. Additionally, the ability of strain to withstand host associated stress resistance and binding capacity to the gut epithelium must be evaluated to ensure that it can colonize the host and exert its beneficial properties even under unfavorable conditions (de Melo Pereira *et al*., 2018).

Whole genome sequencing combined with bioinformatics has gained more attention in recent years, as it provides the in-depth genome characteristics, diversity and evolution of probiotic strains with precise taxonomy identification (Li *et al*., 2022). It also enables us to understand the metabolic potential and probiotic properties of each strain, such as adaptation to the gut, safety, composition of the cell membrane, binding to epithelial cells, virulence factor identification, and presence of bacteriocins (Maiden *et al*., 2013). Moreover, the identification of functional genes that act as molecular markers can be used in the selection of effective probiotic strains (Kandasamy *et al*., 2022). *L. fermentum* (PC-10 and PC-76) were originally isolated from backyard poultry in our previous study and exhibited potential *in vitro* probiotic properties under simulated gut conditions such as tolerance to low pH and bile salts, auto aggregation as well as co-aggregation, and also possessed an inhibitory effect against *Salmonella* Gallinarum (Mehmood *et al*., 2023). In order to gain a better understanding of the probiotic potential on genetic basis, the entire genome of both isolates was sequenced in this study. We also identified the metabolic properties and compared the whole genome sequence of these isolates with other *L. fermentum* strains to identify their possible mechanism of action.

MATERIALS AND METHODS

Culture conditions of Limosilactobacillus fermentum

The *L. fermentum* (PC-10 and PC-76) isolated from caeca of backyard poultry were cultured on de Man Rogosa Sharpe Agar at 37°C under anaerobic conditions. Isolates were identified by biochemical and molecular techniques including *Lactobacillus* genus specific PCR and identified to species level by 16SrDNA sequencing. Following this, the isolates were subjected to tests for *in vitro* probiotic properties such as low pH tolerance, bile salt activity, auto-aggregation, co-aggregation activity with *Salmonella* Gallinarum and antimicrobial activity against *Salmonella* Gallinarum (Mehmood *et al*., 2023). Purified isolates were stored in MRS broth supplemented with 30% glycerol. Isolates were routinely cultured in MRS broth for further analysis.

Whole genome sequencing and assembly

Genomic DNAs of the isolates (PC-10 and PC-76) were extracted by following the protocol of a commercially available kit (Thermo Fisher Scientific, USA). The quality of extracted DNAs was analyzed through agarose gel electrophoresis (1%) while concentration and purity of DNAs were assessed by a nano-drop apparatus (Mutiskan Sky Microplate, Spectrophotometer, USA). The whole genome sequencing of the isolate using illumina technology was outsourced (Macrogen, South Korea). The read quality was checked using FastQC version 0.11.9, and paired end reads were trimmed using FastP version 0.39 and were *de novo* assembled into contigs using Unicycler version 3.0 (Tatusova *et al*., 2016). The quality of contigs and scaffolds was evaluated by QUAST 5.02 and the assembled contigs were annotated by using Prokka software (Seemann, 2014) in addition to the prokaryotic genome annotation pipeline by NCBI for rapid annotation of prokaryotic genome. The tRNA genes were identified by tRNAscan-SE (Holt *et al*., 2002) and the rRNA genes were identified by Barnap. Variants were called using the snippy tool.

Taxonomy analysis

The *L. fermentum* (PC-10 and PC-76), coding the cluster of orthologous genes (COG) were analyzed using the Orthovenn 2 tool and the COG similarity was compared with the reference genome *L. fermentum* ASM2281924V1 (accession number CP094655) retrieved from the NCBI database (Wang *et al*., 2015). The average nucleotide identity (ANI) was calculated by BLASTN tool and identified the species with more than >95% sequence similarity based on the established criteria. Further, a phylogenetic tree was constructed based on whole genome sequence data using the TYGS server (Meier-Kolthoff and Göker, 2019).

Analysis of metabolic pathways

The graphical maps of whole genome sequences were constructed using a circular genome (CG) viewer server (Boucard *et al*., 2022). The comparison and identity

of proteins were analyzed by SEED server version 2.0 (Overbeek *et al*., 2014). The PHASTER (phage search tool enhanced release) web server was used for rapid annotation and identification of prophage loci within the bacterial genome (Arndt *et al*., 2016). To identify the CRISPR repeats and spacer region, the CRISPR finder tool was used (Grissa *et al*., 2007).

Analysis of probiotic determinants

Genes related to probiotic properties were retrieved from the whole genome sequence using different publicly available tools. Antimicrobial gene clusters encoding bacteriocin proteins were analyzed using BAGEL4 (van Heel *et al*., 2018). Using the Resistance Gene Identifier (RGI) tool, fasta files of whole genome sequences were comprehensively scanned in the CARD database to find the acquired antibiotic resistance gene and mutations (Jia *et al*., 2016). Genes that play a role in host microbe interaction and stress conditions were analyzed by the basic local alignment search tool (BLAST) (Tegopoulos *et al*., 2021). Functional annotation of genes was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al*., 2022).

RESULTS

Genomic features

The genome characteristics and probiotic properties of *L. fermentum* (PC-10 and PC-76) were investigated using the cutting edge whole genome sequencing and comprehensive bioinformatics tools. The genome sequences of the isolates (PC-10 and PC-76) were submitted in the NCBI database under the Bioproject numbers PRJNA904097 and PRJNA921838, respectively. Fasta files of the assembled genome and annotated genome files were also deposited in Genbank and obtained the accession numbers CP116618 and CP116617 respectively. General characteristics of the whole genome of each isolate are presented in Table I.

Table I. Whole genome features of *Limosilactobacillus fermentum* isolates.

Features	PC-10	PC-76
Genome size (bp)	1,955,042	1,954,763
GC content (%)	52.2	51.6
Total genes	1,975	1,973
CDS (Protein)	1,761	1,748
Pseudogene	138	149
tRNA	38	58
nc RNA genes	3	3
rRNA	5	5

The genomic features of *L. fermentum* (PC-10 and PC-76) encoding the coding sequences (ORF), GC Skew+, GC Skew-, resistance gene of antibiotics and CRISPR region are illustrated with different color ring in Figure 1.

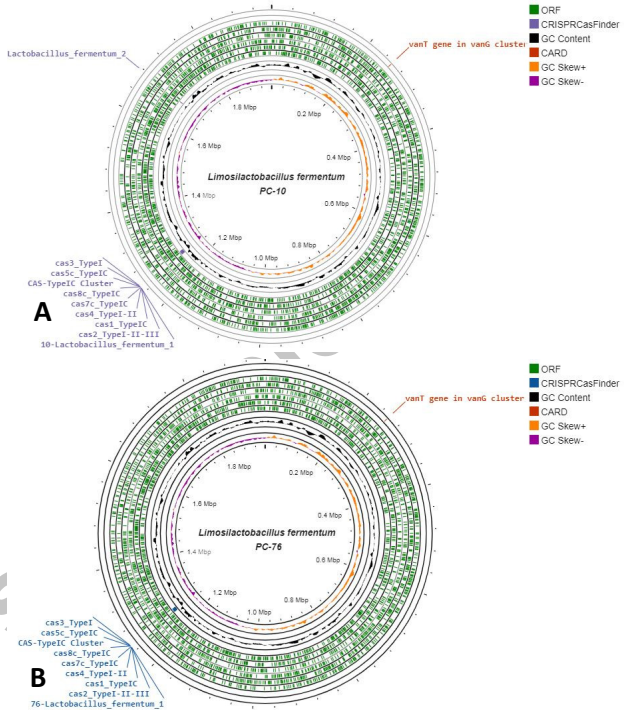

Fig. 1. Circular view of whole genome sequence of *Limosilactobacillus fermentum* PC-10 (A) and PC-76 (B). It describes the following information, rings from outer to inner side of open reading frame (green), GC content (black), GC Skew$^-$ (purple), GC Skew$^+$ (orange), antibiotic resistance gene (red) and gene of CRISPR (blue).

Identification of metabolic and structural genes

Metabolic features of the isolates were analyzed by rapid annotation using subsystem technology (RAST). The genome of *L. fermentum* (PC-10) comprises 312 subsystems that are involved in various biological processes. The largest subsystem was encoded by 188 genes for protein metabolism, followed by 173 genes for amino acids and derivative and carbohydrate metabolism that encoded 152 genes. Other subsystems that are involved in response to various stress conditions (52), dormancy and sporulation (5), phosphorus metabolism (32), cofactors, vitamins, prosthetic groups and pigments (140). Moreover, it also encoded the gene for biosynthesis of structural elements such as capsule and cell wall (81), nucleosides and nucleotides (107) and membrane transport proteins (17). Subsystems of proteins that are involved in various biological processes and structural elements are

represented with different colors in the pie chart as shown in Figure 2.

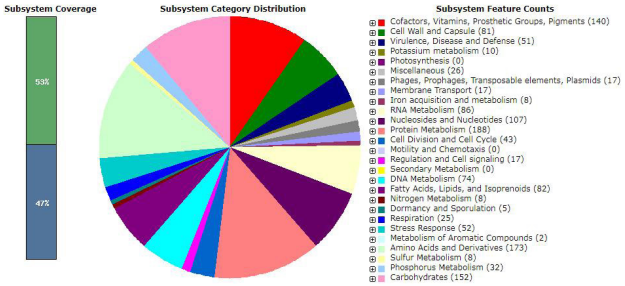

Fig. 2. Subsystems identified in *Limosilactobacillus fermentum* PC-10 after annotation of whole genome sequence by RAST.

Genome annotation of *L. fermentum* PC-76 by RAST showed the presence of 1931 coding sequences (CDS) which are distributed in 309 subsystems. These CDS are found to be associated with different subsystems. As shown in Figure 3, the higher number of genes are related to the metabolism of proteins (187), carbohydrates (156), and are also involved in the synthesis of vitamins, prosthetic groups and pigment production (139). Many of the proteins that respond to stress conditions (52), cell division and cell cycle (42), and phosphorus metabolism (36) were analyzed. Subsystems of phage, prophage, transposable elements, plasmids encoded 25 genes, while other 14 genes are related to respiration subsystems as shown in Figure 3.

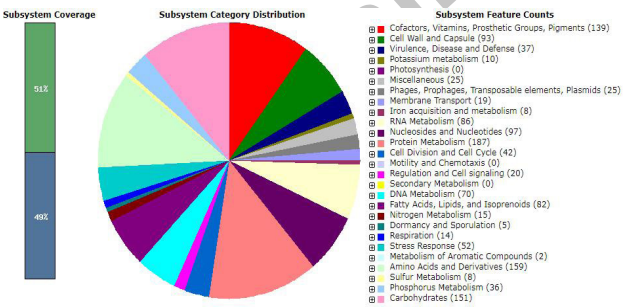

Fig. 3. Subsystems in *Limosilactobacillus fermentum* PC-76 analyzed by annotation of whole genome sequence by RAST.

Species confirmation

The evolutionary tree constructed on the basis of the whole genome sequence showed the close association of *L. fermentum* (PC-10 and PC-76) with *L. fermentum* ATCC 14931 and *Lactobacillus cellobiosus* DSM 20055. The current study isolates are represented with (+) blue color in phylogenetic tree as shown in Figure 4.

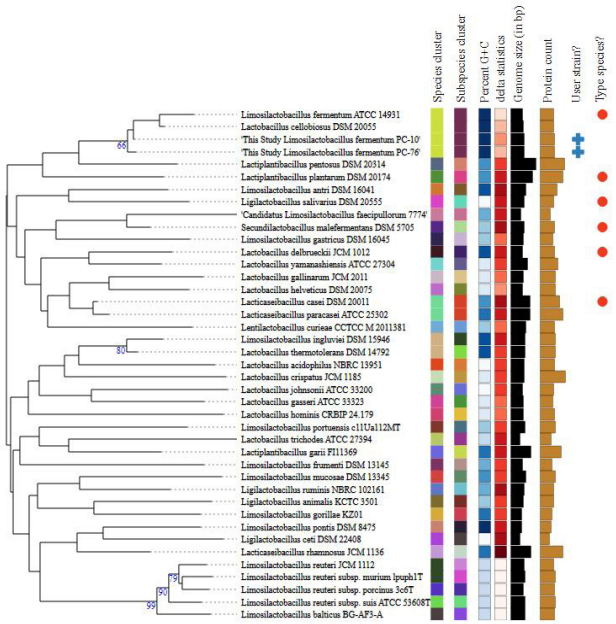

Fig. 4. Evolutionary tree of *Limosilactobacillus fermentum* (PC-10 and PC-76) and other bacterial genera available in the TYGS database server. The tree was developed by FastME 2.1.6.1 based on the whole genome sequence. Numbers above the branches indicate GBDP pseudo bootstrap value above 60% of 1000 replications. The tree rooted at the midpoint and branch length was scaled using GBDP distance formula d5.

Histograms with different colors showed the total number of gene clusters in each isolate. The isolate PC-10 contained 1703, PC-76 has 1639 and the reference genome has 1742 total orthologous gene clusters as shown in Figure 5. The isolates PC-10 and PC-76 along with the reference genome shared 1537 common orthologous cluster genes and 212 exclusive genes cluster as shown in venn diagram. Moreover, the pairwise comparison of genome was analyzed to identify the similarity index between isolates. The overlapping region in heat map describes the cluster of orthologous genes that are common between isolates. Moreover, it also showed the similarity matrix as shown in Figure 6.

Identification of prophage and CRISPR genes

The complete sequence of prophage loci was not found in either of the two isolates (PC-10 or PC-76). The CRISPR regions were detected by PGAP and Prokka annotation pipelines. Analysis by Prokka indicated the following CRISPR genes: The 5 crisper endonucleases (CAS 6, CAS10/Csm1 single strand specific

deoxyribonuclease, Subtype IIIA, CRISPR associated endonucleases CAS1, Cms protein (Cms^3, Cms^4, Cms^5), csm^2, Cms endoribonuclease Csm^3, CRISPR associated protein (CAS8c/Csd1, Cas5c), and CRISPR associated helicase Cas3 in isolates PC-10 and PC-76.

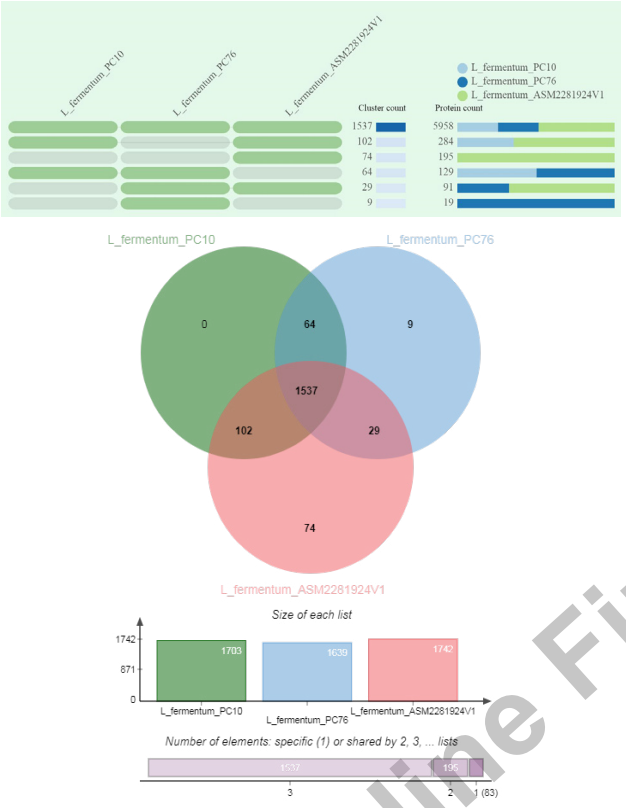

Fig. 5. Venn diagram represents the orthologous clusters of common genes distribution in *Limosilactobacillus fermentum* PC-10, PC-76, and the reference genome *Limosilactobacillus fermentum* ASM2281924V1. The total number of orthologous cluster genes in each isolate is also illustrated in the form of a histogram.

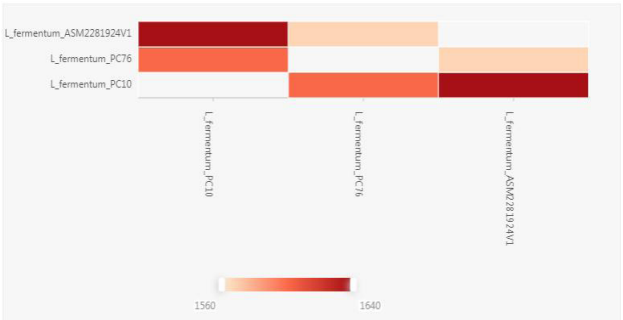

Fig. 6. Heat map shows the similarity matrix of an ortholog cluster via the pairwise comparison of isolates.

Analysis of probiotic genes

The probiotic properties of *L. fermentum* (PC-10 and PC-76) have already been evaluated on the basis of phenotypic characteristics in our previous study (Mehmood *et al*., 2023). These isolates exhibited the probiotic properties when tested *in vitro,* good growth and survival rate under simulated condition of gut such as tolerance to low pH, high bile salt concentration, resistance to stomach and intestinal fluid, ability of autoaggregation and co-aggregation with indicator organism (*Salmonella* Gallinarum).

In silico analysis by annotation of the genome evidenced the presence of acid tolerance determinants such as D-alanine-poly (phosphoribitol) ligase subunit dltA, D-alanyl-lipoteichoic acid biosynthesis protein DltB, D-alanine-poly (phosphoribitol) ligase subunit DltC and D-alanyl-lipoteichoic acid biosynthesis protein DltD. In addition to this, glucose 6, phosphate isomerase also known as acid shock protein and eight subunits of ATP synthase proteins that help in acid tolerance were also found in the genome of *L. fermentum* (PC-10 and PC-76). All of these genes indicate their ability to survive acidic conditions.

The whole genome sequence of the isolates also contained a gene for bile salt tolerance such as choloylglycine hydrolase family protein, GNAT family N-acetyl transferase and linear amide C-N hydrolase as given in Table II. The hydrolase enzyme catalyzes the hydrolysis of bile salts and along with GNAT family proteins provides bile salt resistance in bacteria.

Another pre-requisite selection criterion of probiotics is their ability to attach and colonize epithelial cells. We identified the 10 different genes, including elongation factor Tu, LPXTG cell wall anchor domain-containing protein, mucBP domain containing protein, L-lactate dehydrogenase, PTS system, ATP dependent Clp protease, GTP pyrophosphokinase, pyruvate kinase, phosphoglycerate mutase and CTP synthase in the annotated genome of *L. fermentum* (PC-10 and PC-76) that function as adhesion proteins. In addition, it also contains a gene for autoaggregation (LysM peptidoglycan binding domain containing protein) and other structural proteins such as exopolysaccharide biosynthesis protein and tyrosine protein kinases that maintain the structure and integrity of membrane proteins under adverse environmental conditions.

The *L. fermentum* (PC-10 and PC-76) also possessed the 11 genes related to heat shock proteins, including molecular chaperons (DnaJ, DnaK, GrpE, GroEL, GroES, Hsp33, Hsp20), protease encoding genes (clpX, clpP), ATP dependent Zinc metalloprotease and elongation factor G that play a key role in the stabilization of membrane

Table II. Probiotic proteins identified in whole genome sequence of *Limosilactobacillus fermentum*.

PC-10	PC-76	Proteins*
Locus_tag	**locus_tag**	**Acid tolerance**
OS909_00355	PE049_00360	D alanine poly(phosphoribitol) ligase subunit DltA
OS909_00350	PE049_00355	D alanyl lipoteichoic acid biosynthesis protein DltB
OS909_00345	PE049_00350	D alanine poly(phosphoribitol) ligase subunit DltC
OS909_00340	PE049_00345	D alanyl lipoteichoic acid biosynthesis protein DltD
OS909_02450	PE049_02460	ATP synthase F0 sector subunit A
OS909_02460	PE049_02470	ATP synthase F0 sector subunit B
OS909_02455	PE049_02465	ATP synthase F0 sector subunit C
OS909_02470	PE049_02480	ATP synthase alpha chain
OS909_02480	PE049_02490	ATP synthase beta chain
OS909_02465	PE049_02495	ATP synthase delta chain
OS909_02475	PE049_02485	ATP synthase gamma chain
OS909_02485	PE049_02495	ATP synthase epsilon chain
OS909_09190	PE049_09175	Glucose-6-phosphate isomerase
OS909_02295	PE049_02300	Glucose-6-phosphate isomerase
OS909_02040	PE049_02045	Proteolytic (pta-AckA Pathway) for long term acid and bile stress resistance
OS909_07075	PE049_04365	MFS transporter (mdrt)
Bile salt resistance		
OS909_00150	PE049_00150	Choloylglycine hydrolase family protein
OS909_01485	PE049_01495	GNAT family N-acetyltransferase
OS909_04860	PE049_04855	Linear amide C-N hydrolase
Aggregation		
OS909_09725	PE049_09715	LysM peptidoglycan-binding domain-containing protein
Exopolysaccharide		
OS909_00520	PE049_00525	Exopolysaccharide biosynthesis protein
OS909_00515	PE049_00520	CpsD/CapB family tyrosine-protein kinase
Adhesion		
OS909_03455	PE049_03465	Elongation factor Tu
OS909_05225	PE049_05210	LPXTG cell wall anchor domain-containing protein
OS909_07310 OS909_07415	PE049_07295 PE049_07400	MucBP domain-containing protein
OS909_03610	PE049_03325	L-lactate dehydrogenase
OS909_04125	PE049_03620	L-lactate dehydrogenase
OS909_02240	PE049_02245	PTS system, cellobiose-specific IIC component/PTS system oligo-beta-mannoside-specific EIIC component
OS909_03465	PE049_03475	ATP-dependent Clp protease ATP-binding subunit ClpX
OS909_03160	PE049_03170	GTP pyrophosphokinase
OS909_04465	PE049_04455	Pyruvate kinase
OS909_00795	PE049_09255 PE049_00800 PE049_01470	Phosphoglycerate mutase/2,3-bisphosphoglycerate-dependent phosphoglycerate mutase
OS909_01135	PE049_01140	CTP synthase

Table continued on next page.....

PC-10	PC-76	Proteins*
Resistance to antibiotics/Toxic Compounds		
OS909_03220	PE049_06650	PBP1A family Penicillin-binding protein
OS909_06905	PE049_06895	
OS909_08150	PE049_08140	Elongation factor G
OS909_09300	PE049_09285	Heavy metal translocating P-type ATPase
OS909_06720	PE049_06710	MATE family efflux transporter
OS909_09295	PE049_09280	Cation transporter
Oxidative stress		
OS909_09230	PE049_02860	Thioredoxin genes (trx A)
OS909_01940	PE049_01950	Thioredoxin reductase (trxB)
OS909_01615	PE049_01625	Glutathione reductase (gshR1)
OS909_04065	PE049_04060	Methionine sulfoxoid reductase (msrA)
OS909_04665	PE049_04655	Methionine sulfoxoid reductase (mrsB)
Protein integrity under heat stress conditions		
OS909_03465	PE049_03475	ATP dependent Clp protease ATP binding subunit ClpX
OS909_02040	PE049_02045	ATP dependent Clp endopeptidase proteolytic subunit ClpP
OS909_01840	PE049_01850	Co-Chaperone GroES
OS909_01845	PE049_01855	Chaperonin GroEL,
OS909_03950	PE049_03945	Nucleotide exchange factor GrpE
OS909_01245	PE049_01250	Hsp33 family molecular chaperone
OS909_07115	PE049_07100	Hsp 20/alpha crystalline family protein
OS909_03955	PE049_03950	Molecular Chaperone DnaK
OS909_03960	PE049_03955	Molecular Chaperone DnaJ
OS909_01240	PE049_01245	ATP dependent zinc metalloprotease FtsH
OS909_08150	PE049_08140	Elongation factor G
OS909_08345	PE049_08335	Cold Shock Proteins

*Gene function annotation was done using KEGG. Permission to use KEGG was obtained from Kanehisa Laboratories

structure and intracellular protein aggregation at higher temperatures. In addition to this, both isolates PC-10 and PC-76 also encode the oxidative stress genes (trxA, trxB, msrA, msrB, and glutathione sulfoxide reductase) and resistance to toxic compounds, including penicillin binding protein, elongation factor G, heavy metals translocating P-type ATPase that response to stress conditions and improved the survival of bacteria in the GIT.

The annotated genomes of *L. fermentum* (PC-10 and PC-76) were also found to contain cold shock proteins that help their survival at low temperatures as mentioned in Table II.

Antibiotic resistance

The data obtained from the Centre for Genomic Epidemiology and CARD server revealed *L. fermentum* (PC-10 and PC-76) strains not have any acquired antibiotic resistance, while resistance to glycopeptide (Vant) has been detected in both isolates using the RGI server.

DISCUSSION

Genome sequencing and analysis of potential probiotic strains are now incredibly useful for gaining appropriate information regarding safety and evaluating functional features. Precise identification of the taxonomy of a probiotic strain is the first step in its evaluation (Tarrah *et al*., 2020a). The 16S rDNA sequence is insufficient for species attribution due to the complex taxonomy of the *Lactobacillus* group (Hill *et al*., 2018). However, whole genome sequencing can be used not only to determine the accurate taxonomic placement of strains, it also provides insight into identifying genetic determinants of probiotic properties that provide health benefit to the host (Tarrah *et al*., 2020b). Therefore, the main focus of this study was to assess the safety and probiotic features of *L. fermentum*

(PC-10 and PC-76) through whole genome sequencing. The genome size of isolates PC-10 and PC-76 were found to be 1,955,042 bp and 1,954,763 bp, respectively each containing a total number of 1973 and 1975 genes. The GC content was found to be 52.2% in PC-10 and 51.6% in PC-76, which are consistent with previous study conducted on *L. fermentum* (Brandt *et al*., 2020).

The phylogenetic tree of *L. fermentum* (PC-10 and PC-76) along with other strain of *L. fermentum* (ATCC 14931) and *Lactobacillus cellobiosus* (DSM, 20055) are represented in the clade of same species as shown in Figure 5. Both *Lactobacillus cellobiosus* and *L. fermentum* are heterofermentative and share similar phenotypic characteristics. They belong to the subgenus Betabacterium. The scientists Orla-Genus were in favor of combining both organisms under one name while *L. fermentum* was used earlier as a synonym (Dellaglio *et al*., 2004). Various previous studies have found a close linkage between *L. fermentum* and *Lactobacillus cellobiosus*. However, *Lactobacillus cellobiosus* has recently been classified as a biovar of *L. fermentum*.

The production of antimicrobial substances such as bacteriocins, organic acids, and hydrogen peroxide inhibits the growth of pathogenic/undesirable bacteria. This activity is necessary to maintain the balance of healthy microorganisms in the gut (Cárdenas *et al*., 2015; Salehizadeh *et al*., 2020). In our study, genes coding for bacteriocin activity were not detected in both isolates. Identification of L-lactate dehydrogenase in *L. fermentum* (PC-10 and PC-76) indicates their ability to produce lactic acid that may display the inhibitory effect against *Salmonella* Gallinarum. Similar to our findings, another study reported activity of organic acids (lactic and acetic acids) against Gram positive bacteria, and did not find any proteins that are involved in the biosynthesis of bacteriocins and hydrogen peroxide in genome of the *L. fermentum* (Cárdenas *et al*., 2015).

Considering the safety of isolates, probiotics should not have any acquired antibiotic resistance. Based on phenotypic analysis, *L. fermentum* (PC-10 and PC-76) showed resistance to vancomycin and aminoglycosides in our previous study. However, the data obtained from *in silico* analysis of the genome using the online server centre of genomic epidemiology showed that isolates have not acquired antibiotic resistance. Both isolates were found to have resistance to glycopeptide antibiotics and the *Vant* resistance gene using the RGI server which is in accordance with the phenotypic characteristics of isolates. The resistance to vancomycin is intrinsically present in *Enterococcus*, *Lactobacillus* and *Bifidobacterium* and is not transferable to other bacteria (Zhang *et al*., 2018).

The presence of the CRISPR-Cas region in bacteria prevents bacteriophage infection and conjugation (He *et al*., 2018). It also prevents the natural transfer of foreign genomic fragments and provides genotypic stability to the strain (Tarrah *et al*., 2020b). The whole genome sequence of *L. fermentum* PC-10 and PC-76 comprised 1 CRSIPR array, CRISPR associated endonuclease (Cas2, CAS1c), CRISPR associated protein (Cas4, Cas7/Csd2, CAS8/Csd1, CAS5c), and CRISPR associated helicase/endonuclease (Cas3) analyzed by the CRISPR finder tool. Similar to our findings, another study evidenced the presence of a CRISPR array, a CRIPSR spacer, and CRISPR repeats in *L. fermentum* AGA52 (Yetiman *et al*., 2023). The nucleotides of the CRISPR spacer region recognize the foreign genetic fragments and form CRISPR-CAS complexes that help in the degradation of foreign genetic material (Toropov *et al*., 2020). The presence of the CRISPR region in the current study isolates revealed the absence of acquired antibiotic resistance genes.

A prerequisite criterion for probiotics is their ability to survive the unfavorable conditions of the chicken gut. We identified various proteins that are associated with tolerance to acidic pH and bile salt resistance. The annotated genome of *L. fermentum* (PC-10 and PC-76) possesses the eight different ATP synthase proteins that are involved in acid tolerance. These are essential for the pumping of protons outside of the bacterial cytoplasm and maintaining the pH homeostasis in the cytosol, which in turn protects the bacterial from cell death due to acidic conditions (Duary *et al*., 2010). Glucose 6 phosphate isomerase also known as acid shock protein, was also analyzed in the PC-10 and PC-76 genomes. All of these genes that are involved in acid tolerance have previously been recognized in *L. fermentum* ATCC no. 23271 (Jayashree *et al*., 2014). In addition to this, we also found four genes (dltA to dltD), and mft transporter, which encode different proteins and are involved in the acid tolerance mechanism. Proteolytic (pta-AckA Pathway) helps in the long-term acid and bile stress resistance.

Bile tolerance improves the colonization ability of bacteria in the gut. The presence of bile salts resistance genes, linear amide C-N hydrolase, belong to the choloylglycine hydrolase family of protein and the GNAT family N-acetyltransferase in *L. fermentum* (PC-10 and PC-76) indicate their ability to survive in bile salts. These enzymes detoxify the bile through the hydrolysis of the amide bond between glycine and the steroid in bile acids. Tolerance to bile salts is strain specific and many probiotic strains belonging to *Bifidobacterium* and *Lactobacillus* that cause enzymatic hydrolysis of bile salts have been described (Horáčková *et al*., 2018).

The ability of probiotic strains to bind with the gut epithelial surface is one of the key features of probiotics

(Nath *et al*., 2021). *L. fermentum* (PC-10 and PC-76) encode a plethora of cell surface proteins, such as LPXTG a cell wall anchor domain containing and mucus binding protein with adhesion-like activity. In the genome analysis of PC-10 and PC-76, elongation factor, CpsD/capB and exopolysaccharide act as adhesion proteins. Elongation factor (Tu), acts as aminoacyl transfer RNA and binds to ribosomes, indicating the adhesive ability of isolates to mucin. Exopolysaccharide biosynthesis proteins that form a polysaccharide capsule have been recognized in both isolates of PC-10 and PC-76. It protects the probiotic strains from adverse environmental conditions.

L. fermentum (PC-10 and PC-76) also harbors various genes that play a key role in response to stress conditions. There are a large number of chaperones such as GroES, GroEL, GrpE, DnaK, DnaJ, and other proteins such as Hsp 33, Hsp 20, an ATP dependent zinc metalloprotease, that facilitate the survival of bacteria during heat stress conditions. Genes encoded for antioxidant activity such as thioredoxin genes (*trxA* and *trxB*), glutathione reductase and methionine sulfoxide reductase (*msrA* and *msrB*) protect the bacteria in response to oxidative stress conditions. In addition, identification of cold shock proteins indicates their survival at low temperatures. Various previous studies have reported numerous proteins that respond to stress conditions in different potential probiotic strains, including *L. fermentum* ATCC 23271 and *Lactobacillus Johnsonii* (Boucard *et al*., 2022; Dos Santos *et al*., 2021). To the best of our knowledge, this is the first study in Pakistan that has explained the *in silico* probiotic properties of *L. fermentum* and studied their antibacterial activity against *Salmonella* Gallinarum. Overall, the results of this study described that *L. fermentum* (PC-10 and PC-76) contains several probiotic genes and has a defense system against oxidative stress, toxic compounds and unsuitable temperature conditions.

CONCLUSION

L. fermentum (PC-10 and PC-76) has harbored in its genome various putative genes that enable the probiotic strains to survive in the chicken gastrointestinal tract. Following the *in vitro* characterization of these strains in the laboratory, *in silico* analysis also described the various probiotic genes that are associated with the viability of probiotics at low pH, bile salt, oxidative stress, adaption, toxic compounds and in response to heat and cold conditions. In current study, no genes for bacteriocin activity were detected; while genes encoded for organic acid synthesis indicate their inhibitory effect against *Salmonella* Gallinarum. Moreover, these isolates also encoded the genes for CRISPR, which indicate their genotypic stability and the presence of exopolysaccharides revealed their ability to survive under adverse environmental conditions. Related to safety concerns, these isolates are considered safe as they have no transferrable antibiotic resistance genes, virulence genes or prophage loci in their genome. These potential probiotic strains can be used for further development of anti-*Salmonell*a Gallinarum probiotics for prevention and control of fowl typhoid in poultry after evaluating their health benefiting properties in poultry.

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DECLARATIONS

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IRB approval

The study was conducted according to the guidelines approved by the Animal ‚Ethical Review Committee' UVAS, Lahore, Pakistan (no. DR/1103, Dated: 11 October 2017).

Ethics approval and consent to participate

The study was conducted according to the guidelines approved by the Animal “Ethical Review Committee” UVAS, Lahore, Pakistan and the study protocols were approved by “Ethical Review Committee” of University of Veterinary and Animal Sciences (vide letter no. DR/1103, Dated: 11 October 2017). All methods are reported in accordance with ARRIVE guidelines for the reporting of animal experiments. Informed consent was obtained from the owners or an authorized agents (poultry farms) for the collection of intestinal samples from poultry birds for this study.

Availability of data and material

The whole genome sequencing datasets generated and analyzed for each *Limosilactobacillus fermentum* isolate included in the current study were deposited at the National Center for Biotechnology Information (NCBI) with the following Gen Bank accession numbers CP116617- CP116618. Data will be made available upon manuscript publication.

Statement of conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The funding agency in our research project only provided the financial support and has no competing interest in the design of the study and collection, analysis, interpretation of data, and the writing of the manuscript.

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