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

Adnan Mehmood1, Muhammad Nawaz1*, Masood Rabbani1 and Muhammad Hassan Mushtaq2

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

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).

* Corresponding author: muhammad.nawaz@uvas.edu.pk
0030-9923/2024/0001-0001 $ 9.00/0

Copyright 2024 by the authors. Licensee Zoological Society of Pakistan.
This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).
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 16S rDNA 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 (MultiSkans, 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 tRNA 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 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
Online First Article

Online First Article

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 \textit{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 \textit{Limosilactobacillus fermentum} isolates.

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

The genomic features of \textit{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 \textit{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).](image)

Identification of metabolic and structural genes

Metabolic features of the isolates were analyzed by rapid annotation using subsystem technology (RAST). The genome of \textit{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.

![Pie chart showing subsystem coverage and distribution](image)

**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.

![Subsystems in Limosilactobacillus fermentum PC-76 analyzed by annotation of whole genome sequence by RAST](image)

**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.

![Evolutionary tree of Limosilactobacillus fermentum (PC-10 and PC-76) and other bacterial genera available in the TYGS database server](image)

**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 crispr endonucleases (CAS 6, CAS10/Csm1 single strand specific

---

A. Mehmood et al.

---
deoxyribonuclease, Subtype IIIA, CRISPR-associated endonucleases CAS1, Cms protein (Cms3, Cms4, Cms5), csm2 Cms endoribonuclease Csm1, CRISPR-associated protein (CAS8c/Csd1, Cas5c), and CRISPR-associated helicase Cas3 in isolates PC-10 and PC-76.

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

<table>
<thead>
<tr>
<th>PC-10</th>
<th>PC-76</th>
<th>Proteins*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Locus_tag</strong></td>
<td><strong>locus_tag</strong></td>
<td><strong>Acid tolerance</strong></td>
</tr>
<tr>
<td>OS090_00355 PE049_00360</td>
<td>D alanine poly(phosphoribitol) ligase subunit DltA</td>
<td></td>
</tr>
<tr>
<td>OS090_00350 PE049_00355</td>
<td>D alanyl lipoteichoic acid biosynthesis protein DltB</td>
<td></td>
</tr>
<tr>
<td>OS090_00345 PE049_00350</td>
<td>D alanine poly(phosphoribitol) ligase subunit DltC</td>
<td></td>
</tr>
<tr>
<td>OS090_00340 PE049_00345</td>
<td>D alanyl lipoteichoic acid biosynthesis protein DltD</td>
<td></td>
</tr>
<tr>
<td>OS090_02450 PE049_02460</td>
<td>ATP synthase F0 sector subunit A</td>
<td></td>
</tr>
<tr>
<td>OS090_02460 PE049_02470</td>
<td>ATP synthase F0 sector subunit B</td>
<td></td>
</tr>
<tr>
<td>OS090_02455 PE049_02465</td>
<td>ATP synthase F0 sector subunit C</td>
<td></td>
</tr>
<tr>
<td>OS090_02470 PE049_02480</td>
<td>ATP synthase alpha chain</td>
<td></td>
</tr>
<tr>
<td>OS090_02480 PE049_02490</td>
<td>ATP synthase beta chain</td>
<td></td>
</tr>
<tr>
<td>OS090_02465 PE049_02495</td>
<td>ATP synthase delta chain</td>
<td></td>
</tr>
<tr>
<td>OS090_02475 PE049_02485</td>
<td>ATP synthase gamma chain</td>
<td></td>
</tr>
<tr>
<td>OS090_02485 PE049_02495</td>
<td>ATP synthase epsilon chain</td>
<td></td>
</tr>
<tr>
<td>OS090_09190 PE049_09175</td>
<td>Glucose-6-phosphate isomerase</td>
<td></td>
</tr>
<tr>
<td>OS090_02295 PE049_02300</td>
<td>Glucose-6-phosphate isomerase</td>
<td></td>
</tr>
<tr>
<td>OS090_02040 PE049_02045</td>
<td>Proteolytic (pta-AckA Pathway) for long term acid and bile stress resistance</td>
<td></td>
</tr>
<tr>
<td>OS090_07075 PE049_04365</td>
<td>MFS transporter (mdrt)</td>
<td></td>
</tr>
</tbody>
</table>

**Bile salt resistance**

| OS090_00150 PE049_00150 | Choloylglycine hydrolase family protein |
| OS090_01485 PE049_01495 | GNAT family N-acetyltransferase |
| OS090_04860 PE049_04855 | Linear amide C-N hydrolase |

**Aggregation**

| OS090_09725 PE049_09715 | LysM peptidoglycan-binding domain-containing protein |

**Exopolysaccharide**

| OS090_00520 PE049_00525 | Exopolysaccharide biosynthesis protein |
| OS090_00515 PE049_00520 | CpsD/CapB family tyrosine-protein kinase |

**Adhesion**

| OS090_03455 PE049_03465 | Elongation factor Tu |
| OS090_05225 PE049_05210 | LPXTG cell wall anchor domain-containing protein |
| OS090_07310 PE049_07295 | MucBP domain-containing protein |
| OS090_07415 PE049_07400 | L-lactate dehydrogenase |
| OS090_03610 PE049_03325 | L-lactate dehydrogenase |
| OS090_04125 PE049_03620 | L-lactate dehydrogenase |
| OS090_02240 PE049_02245 | PTS system, cellobiose-specific IIC component/PTS system oligo-beta-mannoside-specific EIIC component |
| OS090_03465 PE049_03475 | ATP-dependent Clp protease ATP-binding subunit ClpX |
| OS090_03160 PE049_03170 | GTP pyrophosphokinase |
| OS090_04465 PE049_04455 | Pyruvate kinase |
| OS090_00795 PE049_00800 PE049_01470 | Phosphoglycerate mutase/2,3-bisphosphoglycerate-dependent phosphoglycerate mutase |
| OS090_01135 PE049_01140 | CTP synthase |

Table continued on next page.................
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 CRISPR 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 CRISPR 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 cholyglycine 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-*Salmonella Gallinarum* probiotics for prevention and control of fowl typhoid in poultry after evaluating their health benefiting properties in poultry.

**ACKNOWLEDGMENT**

We would like to acknowledge “ABO scientific” for their valuable assistance in transporting our samples for sequencing to Macrogen, Seoul, South Korea.

**DECLARATIONS**

**Funding**

This research was partially supported by a grant from the Higher Education Commission, Islamabad (Pakistan), under the National Research Program for Universities (Project No. 7069/Punjab/NRPU/RandD/HEC/2017).

**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.

REFERENCES


Ebrahimi, M.T., 2020. Evaluation of lactic acid bacteria isolated from poultry feces as potential probiotic and its in vitro competitive activity against Salmonella typhimurium. Vet. Res. Forum, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran, pp. 67.


