Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2024.306

RESEARCH ARTICLE

In-vitro, i*n-vivo* and whole genome-based probe into probiotic potential of *Weissella viridescens* PC-45 against *Salmonella* Gallinarum

Adnan Mehmood[1], M. Nawaz[*1], Masood Rabbani[1] and M. 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
*Corresponding author: muhammad.nawaz@uvas.edu.pk

ARTICLE HISTORY (24-680)

Received: October 26, 2024
Revised: November 21, 2024
Accepted: November 22, 2024
Published online: January 10, 2025

Key words:
Illumina sequencing
Fowl typhoid
Probiotic
S. Gallinarum
Weissella viridescens

ABSTRACT

This study was aimed to isolate and screen *Weissella* spp. with anti-*Salmonella* Gallinarum (*S.* Gallinarum) activity from the poultry gut and to analyze their probiotic properties from phenotypic and genomic characterization aspects *in-vitro* and *in-vivo* in broiler. Six isolates were confirmed as *Wiessella* by 16S rDNA sequencing. These isolates showed variable *in-vitro* activity (10.00±0.57~17.00±1.00 mm) against *S.* Gallinarum and were further evaluated for probiotic traits. *In-vitro*, results revealed that *Weissella viridescens* (*W. viridescens*) PC-45 can tolerate acidic conditions (pH 3 and 4), resist bile salts (>0.5 OD at 600nm), auto-aggregate (58.98%±0.85), co-aggregate with *S.* Gallinarum (47.23±0.94%) and have no acquired antibiotic resistance. The strain also inhibited (>4 log10) *S.* Gallinarum in co-culture assays. Genomic analysis of *W. viridescens* PC-45 revealed a genome size of 1593258 bp with a 41% GC contents. Genes related to probiotic properties, including resistance to acidic pH, bile salts, oxidative stress, synthesis of exopolysaccharide, host-microbe interaction, and resistance to stress conditions, were identified by the Kyoto Encyclopedia of Genes and Genomes (KEGG). Moreover, it also contained various CRISPR-associated genes and no genes for acquired antibiotic resistance. *In-vivo* results revealed that *W. viridescens* PC-45 reduced (>2 log10) *S.* Gallinarum counts in broilers. It is concluded that this strain may be used to further develop anti-*S.* Gallinarum probiotic products to control fowl typhoid.

To Cite This Article: Mehmood A, Nawaz M, Rabbani M and Mushtaq MH, 2024. *In-vitro*, i*n-vivo* and whole genome-based probe into probiotic potential of *Weissella viridescens* PC-45 against *Salmonella* Gallinarum. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2024.306

INTRODUCTION

Salmonella enterica subsp. enterica serovar Gallinarum (*S.* Gallinarum) and *S.* Pullorum are host-adopted pathogens of chicken that cause fowl typhoid and pullorum disease in birds of all ages (Khan *et al.*, 2014; Sarba *et al.*, 2020). Various methods such as vaccines, biosecurity plans and antibiotic therapy are currently used in the poultry sector to prevent and control fowl typhoid. Management practices like using pathogen-free chicks, cleaning and disinfection, and strong biosecurity measures can help to reduce *Salmonella* in poultry. The use of antibiotics in poultry has led to the emergence of multidrug resistance in *S.* Gallinarum. However, multidrug-resistant pathogens are transferred from poultry to humans via contaminated food (Yin *et al.*, 2015; Shami *et al.*, 2024). Antibiotics are also used in poultry feed to improve the growth performance of animals by modulating the gut microbiota and boosting their health. However, due to public health concerns related to antibiotics in feed and their residue leading to the emergence of antibiotic-resistant bacteria, there is a current need to find alternatives to antibiotics (Khan *et al.*, 2022; Ali *et al.*, 2024; Wu *et al.*, 2024). Probiotics are now considered potential candidates for use in poultry (Noohi *et al.*, 2021).

Probiotics have been defined as viable microorganisms that, when administered in appropriate amounts, confer a health benefit on the host (Hill *et al.*, 2014). The *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Pediococcus* and *Weissella* are the most common genera used in the production of poultry (Vieco-Saiz *et al.*, 2019). They promote the activity of digestive enzymes and improve gut health by increasing the digestibility of nutrients. Moreover, probiotics protect the host from pathogenic organisms by regulating the immune response

and preventing their colonization in the gastrointestinal tract through a competitive exclusion strategy (Ahmad *et al.*, 2022; Mehmood *et al.*, 2023).

Weissella strains have received more attention in the last few years because of their technological and probiotic properties (Pabari *et al.*, 2020; Teixeira *et al.*, 2021). These bacteria naturally occur in fermented foods and are characterized as Gram-positive, short rods with pointed ends or coccoid, non-spore-forming and catalase-negative. *Weissella* belongs to lactic acid bacteria as they produce lactic acid using carbohydrates (Lee *et al.*, 2010; Rizzello *et al.*, 2019). Antimicrobial activity and probiotic characteristics of *Weissella* strains that provide health benefits to hosts have been described in various studies. *Weissella spp*. produce antimicrobial substances such as organic acids, hydrogen peroxide, carbon dioxide and bacteriocins (Liao and Nyachoti, 2017). Considering the importance of *Weissella*, this study was designed to screen *Weissella* as having antibacterial activity and further analyze *in-vitro*, *in-silico* and *in-vivo* probiotic properties.

MATERIALS AND METHODS

Isolation and identification of *Weissella:* A total of 60 intestinal parts (caeca and ileum, 30 from each) of healthy commercial and backyard (Golden Misri) poultry from a flock having an outbreak of fowl typhoid were collected from different areas of Punjab Province. The samples were cultured on selective media de Man Rogosa and Sharpe (MRS) agar (Noohi *et al.*, 2021). Gram staining and catalase test were used for preliminary identification. The isolates were further confirmed to specie level by 16S rDNA sequencing (Nawaz *et al.*, 2011).

***In vitro* determination of probiotic properties:** The antibacterial effect of the isolates against *S.* Gallinarum (accession number CP116616) was analyzed using a well diffusion technique (Mohankumar and Murugalatha, 2011). The low pH tolerance of *Weissella* strains was determined according to a previously described protocol (Cele *et al.*, 2022). To assess tolerance to bile salts, isolates (3×10^8 CFU/mL) were cultured in MRS broth supplemented with and without bile salts at concentrations of 0.3%, 1.0%, and 1.8% for 24 hours. Optical density (OD) values were recorded at 600nm on a spectrophotometer at 0 minutes and after 24 hours to analyze the isolate's ability to resist bile salts. The auto-aggregation and co-aggregation of *Weissella* with *S.* Gallinarum were analyzed following a described procedure (Bao *et al.*, 2010). The susceptibility of *Weissella* strains to different antibiotics was analyzed by broth micro-dilution (Saleem *et al.*, 2018; Issa, 2024) and the isolates were categorized as susceptible, or resistant following the standard criteria of the European Food Safety Authority (EFSA, 2012).

Co-culture assay: The inhibitory effect of *Weissella* on the growth of *S.* Gallinarum was analyzed in a broth culture as described in a previous study (Todoriki *et al.*, 2001). In summary, a freshly grown culture (1mL) of *S.* Gallinarum and *Weissella* isolate was mixed in 10mL of nutrient broth and then incubated at 37°C for 24 hours. The growth kinetics of *S.* Gallinarum and *Weissella* from the mixed culture were enumerated on their selective media MRS agar, and salmonella shigella agar (SSA) at different time intervals (0 hour, 6 hours, 12 hours, and 24 hours), respectively.

Whole genome sequencing and *in-silico* analysis of *Weissella viridescens* PC-45: The genomic DNA of *W. viridescens* (PC-45) was sequenced using Illumina technology (Macrogen, South Korea). The complete genome was annotated using the prokaryotic genome annotation pipeline of NCBI and Prokka software (Seemann, 2014). The species identification was achieved using the online tool BLAST ANI and following the identity criteria of more than 95-96% similarity (Xu *et al.*, 2019). The bacteriocin-encoding genes were identified by the BAGEL4 database (Van Heel, 2018). To search for antibiotic resistance genes and mutations, the entire genome was scanned in three freely available databases, which are given as follows: 1) ResFinder tool, 2) Resistance Gene Identifier (RGI) tool, 3) BlastKoala in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa *et al.*, 2016; Alcock *et al.*, 2020; Bortolaia *et al.*, 2020).

Functional annotation of genome: The circular genome map was constructed to analyze the genomic features of *Weissella viridescens* PC-45 using the Circular Genome Viewer (CG view) web. The metabolic features and structural genes were analyzed by Rapid Annotation using Subsystem Technology (RAST) web server (Overbeek *et al.*, 2014). The genes associated with probiotic characteristics were searched manually in Prokka and NCBI-derived annotation files. Using KEGG, the functions and pathways of predicted genes were identified (Kanehisa *et al.*, 2022).

***In vivo* evaluation of probiotic effect in broiler challenged with *S.* Gallinarum:** One day old chicks (n=60) were randomly divided into four groups (A-D), with 15 birds in each group. Group A served as control (no probiotics or challenge). Group B received *S.* Gallinarum (10^7 CFU/bird) on day 21. Group C was administered with *W. viridescens* (10^8 CFU/bird) from day 1 to day 35 and the challenge organism on day 21. Group D received a commercial probiotic (HAN-LACVET) at 0.6g/15 birds from day 1 to 35 and challenge of *S.* Gallinarum on day 21. After the challenge, *S.* Gallinarum was enumerated from fecal samples and mortality rate and symptoms of birds in each group were recorded daily.

Statistical analysis: The microbial enumeration and optical density data are expressed as mean±Standard Deviation (SD). Statistical significance ($P<0.05$) between isolates was analyzed using one-way ANOVA followed by post hoc Tukey's Multiple Comparison Test with GraphPad Prism software version 8.0.

RESULTS

Isolation and identification of *Weissella*: Fifteen isolates were presumptively identified as *Weissella* based on their

morphological characteristics (Gram-positive short rods with pointed ends) and catalase test (negative). Out of these fifteen, five isolates (PI-85, PI-86, PI-88, PI-91, and PI-95) were confirmed as *Weissella confusa* by 16S rRNA gene sequencing (GenBank accession numbers ON819871, ON819872, ON819873, ON908489 and ON908490, respectively) and one isolate (PC-45) was identified as *W. viridescens* (Accession number ON819866). A phylogenetic tree was constructed using the neighbor-joining method based on the 16S rRNA gene sequences, as illustrated in Fig.1.

Antibacterial activity of *Weissella*: The antibacterial activity of the CFSs of the isolates was analyzed using the well diffusion technique. The CFSs of six isolates at acidic pH displayed inhibition zones ranging from (10.00±0.57~17.00±1.00 mm against *S.* Gallinarum. Isolate PI-85 showed the highest zone of inhibition (17.00±1.00 mm), as given in Table 1. The CFSs adjusted to a pH of 6.5 showed no antibacterial effect against *S.* Gallinarum. Following the antibacterial activity, the isolates were further evaluated for *in vitro* probiotic properties, including survival at acidic pH, resistance to bile salts, auto-aggregation, and co-aggregation properties.

***In vitro* characterization of *Weissella*:** The survival ability of isolates under acidic conditions was assessed. Table 1 presents the percentage viability of isolates after 2 hours of pH 3 and 4 exposure. Isolates PC-45, PI-85, PI-88, PI-91, and PI-95 exhibited 50-80% survival at both pH 3 and 4, whereas isolate PI-86 showed significantly lower tolerance ($P<0.05$) with only 32% growth at pH 3 and 38% growth at pH 4. The isolates displayed varying degrees of resistance to bile salts, with 5 out of 6 isolates showing good growth (OD increase >0.5) in the presence of 0.3% bile salts and three isolates demonstrating good growth at 1.0%. The growth rate tended to decrease with higher concentrations of bile salts.

The isolates had a variable range of auto-aggregation (19.40±0.25~58.98±0.85%) and co-aggregation with *S.* Gallinarum (07.20±0.66~47.23±0.94%) as given in Table 1. The *W. viridescens* PC-45 has higher autoaggregation activity (58.98±0.85%) and co-aggregation activity with *S.* Gallinarum (47.23±0.94%) in comparison to other *Weissella confusa* strain and control *Lactobacillus rhamnosus* GG isolate (39.03±0.22,42.07±0.71% respectively). The results of antibiotic susceptibility profile indicates that the isolates have higher level of resistance to streptomycin (100%), vancomycin (100%), gentamicin (83%), oxytetracycline (66%), erythromycin (50%), ciprofloxacin (50%), doxycycline (50%), and lower level of resistance to penicillin (33%), chloramphenicol (33%), bacitracin (33%). The isolates having acquired antibiotic resistance to penicillin (PI-86, PI-88), erythromycin (PI-85, PI-88, and PI-95) and tetracycline (PI-85, PI-88, PI-91, PI-95) were discontinued due to the safety concern. The *W. viridescens* PC-45 is sensitive to most commonly used antibiotics and has no acquired antibiotic resistance; it was selected for *in-vitro* inhibition in broth culture. As shown in Fig. 2, the isolate PC-45 had a non-significant effect on the growth of *S.* Gallinarum after 6 hrs (8.30 log10CFU/mL). It caused a remarkable reduction after 12 hrs (4.11 log10CFU/mL) and 24 hrs (3.11 log10CFU/mL) as compared to the initial count (8.39 log10CFU/mL). The result revealed *W. viridescens* PC-45 inhibited the >4 log10 reduction of *S.* Gallinarum in the co-culture assay.

***In silico* analysis of probiotic properties of *W. viridescens* PC-45:** The complete genome of *W. viridescens* PC-45 contains a circular chromosome of 1,593,258bp with a guanine cytosine (GC) content of 41%. The annotated genome includes 1449 coding genes, 78 tRNA, 3 ncRNA, 9 rRNA and 12 pseudogenes. The whole genome sequence has been submitted in the NCBI repository under BioProject accession number PRJNA936630, and the genome assembly is available in the GenBank database under accession number CP118690. The average nucleotide identity value of *W. viridescens* PC-45 against the reference genome *W. viridescens* NZ_CP061835 (Retrieved from the NCBI database) was 98.4%. The pangenome of *W. viridescens* PC-45 comprises 1455 clusters of orthologous genes. Moreover, general characteristics of the genome, including open reading frame (ORF), GC content, GC Skew$^+$, GC Skew$^-$, CRISPR region and antibiotic resistance genes, are presented in a circular view in Fig. 3(a).

The genome annotation by RAST provides an overview of coded biological features, as shown in Fig. 3(b). A total of 270 functional subsystems were identified in *W. viridescens* PC-45, which include genes involved in the synthesis and metabolism of proteins, amino acids, lipids, fatty acids, isoprenoids, carbohydrates, DNA metabolism, cofactors, vitamins, prosthetic groups, and pigments production. The presence of membrane transport genes indicates the organism's ability to transport molecules across the cell membrane. Moreover, identifying stress-resistance genes indicates the probiotic properties of *W. viridescens* PC-45.

The genome analysis by BAGEL4 revealed that *W. viridescens* PC-45 has no bacteriocin activity. The functional annotation of the genome was analyzed by KEGG, which identified the following probiotic genes (resistance to acidic pH, bile salts, oxidative stress, toxic compounds and host-microbe interaction) that facilitate the survival of *W. viridescens* PC-45 in the host Gastrointestinal Tract (GIT), as given in Table 2.

Effect of *W. viridescens* on broiler gut challenged with *S.* Gallinarum: *Salmonella* counts were enumerated on alternate days following the infection with *S.* Gallinarum in all experimental groups. The results showed a significant increase in *Salmonella* count in the positive control group from day 23 (6.31±0.27 mean log10CFU/g) to day 35 (6.88±0.22 mean log10CFU/g), as shown in Table 3. A significant difference ($P<0.05$) in *Salmonella* counts was observed on day 35 in all experimental groups. The group administered (day 1 to 35) with *W. viridescens* PC-45 showed significantly less growth (4.35±0.10 mean log10CFU/g) compared to both the positive control group (6.88±0.22 mean log10CFU/g) and the commercial probiotic group (5.35±0.45 mean log10CFU/g).

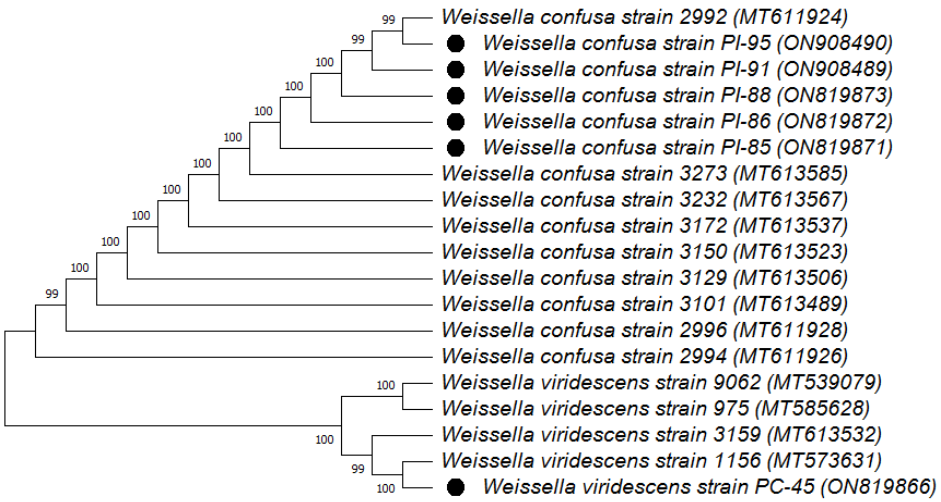


Fig. 1: A phylogenetic tree was built using the neighbor-joining method at the bootstrap value of 1000. It shows the genetic relatedness of *Weissella* strains isolated in this study with reference to previous studies based on the 16S rRNA gene sequencing. Strains represented with filled circles are isolated in this study, while the numbers in parentheses are accession numbers obtained from the NCBI database.

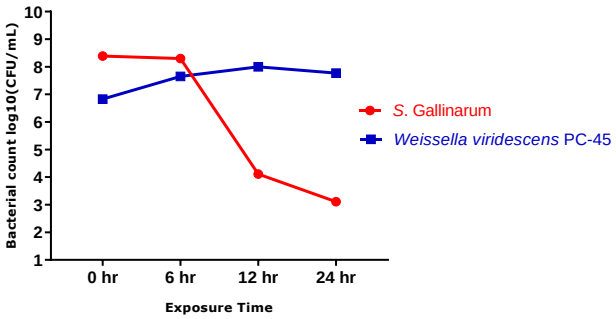


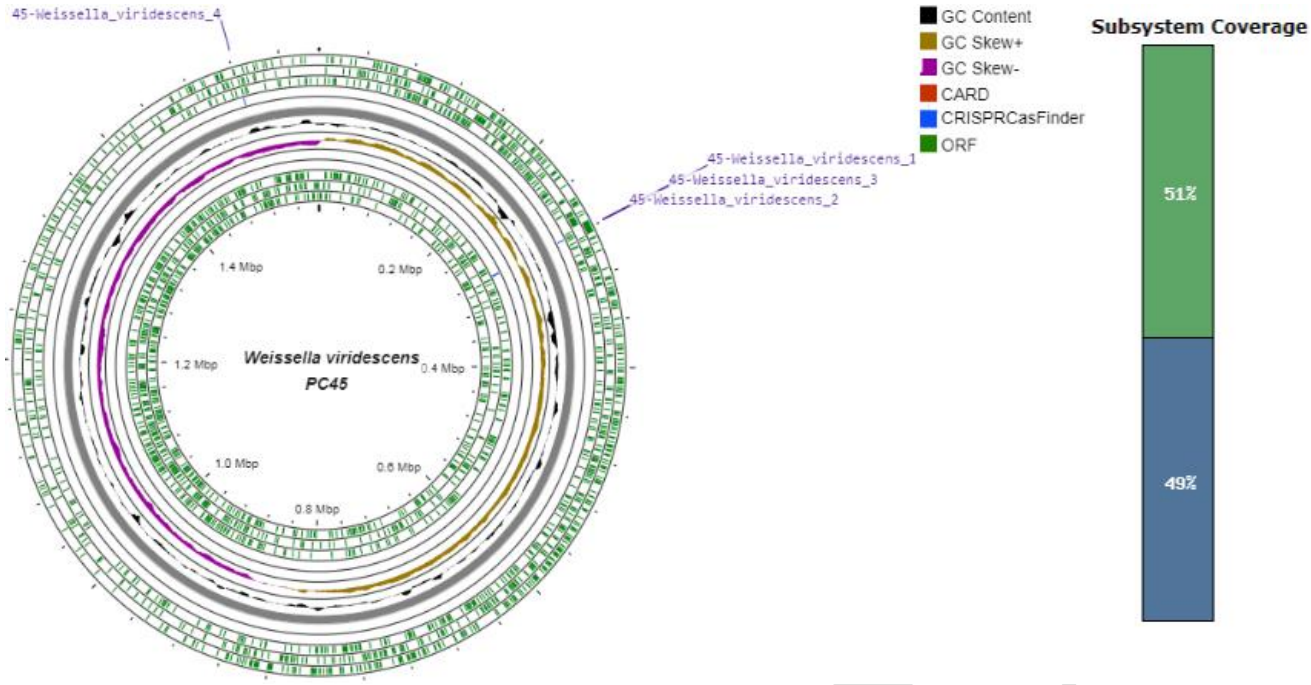
Fig. 2: Growth kinetics of *S.* Gallinarum and *W. viridescens* PC-45 at different time intervals in co-culture assay.

DISCUSSION

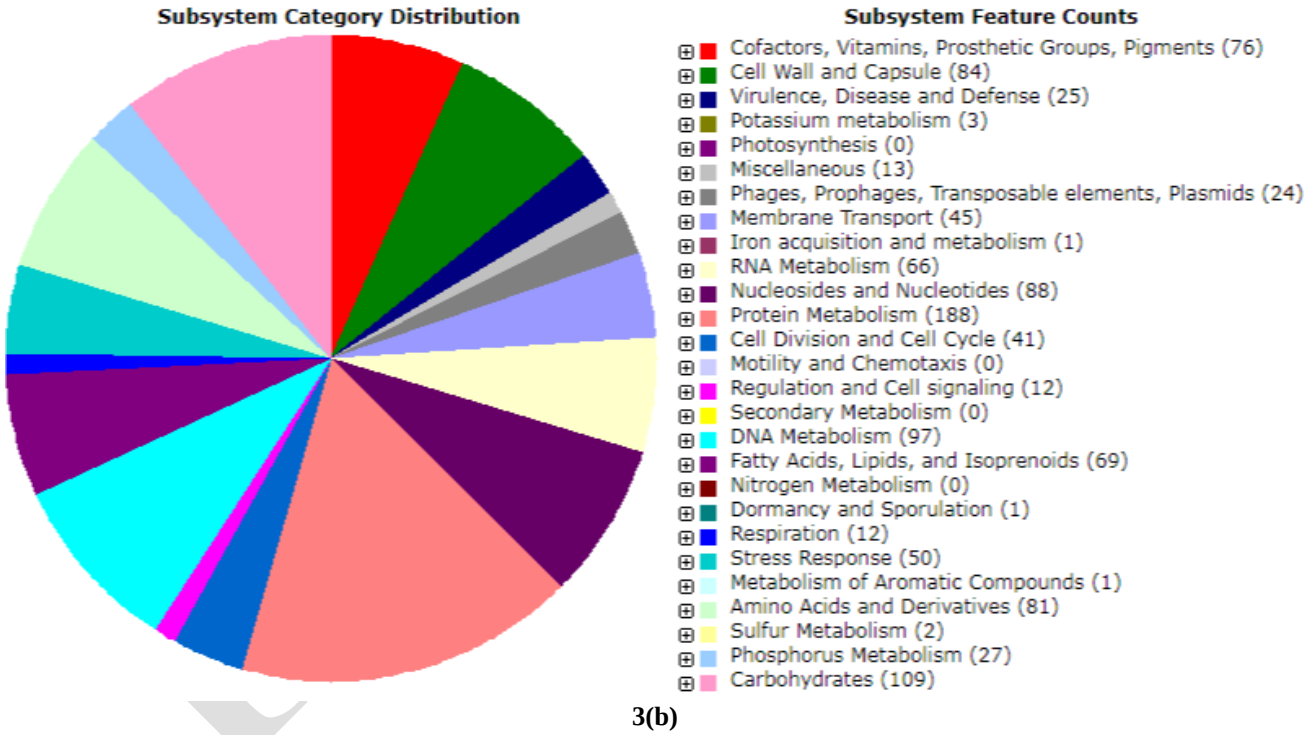
In the current study, 15 presumptive *Weissella* isolates were selected from healthy poultry caeca and ileum from flocks suffering from fowl typhoid, assuming that these healthy birds have diverse microbiota that can be explored as probiotics. Six isolates were confirmed as *Weissella confusa* or *W. viridescens* by 16S rRNA gene sequencing. These six isolated had varying antibacterial activity against *S.* Gallinarum. The antibacterial effect of *W. viridescens* against *Salmonella* has been described in previous studies (Ye *et al*., 2018; Pelyuntha *et al*., 2019). Similarly, Espinoza-Monje *et al*. (2021) found the inhibitory effect of *W. viridescens* UCO-SMC3 on *Cutibacterium acne* and *Staphylococcus aureus*. Their study did not find the antibacterial peptides, while inhibition was primarily mediated due to the production of lactic acid and hydrogen peroxide. These findings are consistent with our results, where CFSs at acidic pH effectively inhibited the growth of *S.* Gallinarum. The viability of bacterial strains in gastrointestinal conditions is an important prerequisite for the selection of probiotics (Zheng *et al.*, 2021). Tolerance to low pH (3) and bile salt concentration of 0.10 to 0.30% are considered standard criteria for evaluating probiotic strains (Shokryazdan *et al.*, 2014). The *Weissella* strains isolated in this study exhibited good growth (up to 80%) after exposure to acidic pH for 2 hours. Similar acid tolerance and variations have also been observed among lactobacilli in previous studies (Zheng *et al.*, 2021; Mehmood *et al*., 2023). The resistance to bile salt concentration decreased with increased bile salt concentrations in all isolates. Growth of isolates in bile salts (an increase of 0.5 $O.D_{600}$) was considered good, as described previously (Mehmood *et al.*, 2023). At 0.3% bile salt concentration, five isolates showed more than 0.5 OD, and three isolates demonstrated resistance up to 1.0%. The resistance to bile salts and acidic pH were strain-specific and in accordance with previous studies (Mustafa *et al.*, 2022).

All the isolates displayed variable autoaggregation (23.29±0.99~58.98±0.85%) and co-aggregation activity (07.20±0.66~47.23±0.94%). These results agree with previous studies and indicate the adhesion potential of these strains (Mustafa *et al.*, 2022; Mehmood *et al.*, 2023). Assessment of antibiotic resistance in probiotics is important concerning their safety (Nawaz et *al.*, 2011). All the isolates showed resistance to vancomycin and streptomycin. The resistance to kanamycin, streptomycin, vancomycin and nalidixic acid is generally intrinsic in *Weissella* species (Le and Yang, 2018; Fhoula *et al.*, 2022). The antibiotic susceptibility profile of isolates exhibited higher levels of resistance to gentamicin (83%), oxytetracycline (66%), erythromycin (50%), ciprofloxacin (50%), doxycycline (50%), and lower levels of resistance to chloramphenicol (33%), penicillin (33%), and bacitracin (33%). A similar antibiotic resistance pattern has been reported previously by Rajoka *et al*. (2018). The *W. viridescens* PC-45 inhibited (>4 log**10**) the *S.* Gallinarum in *in-vitro* assay, which may be attributed to the production of lactic acid since the pH of media was reduced to 3.7 in co-culture assay. Inhibition of the *Salmonella* by probiotic lactic acid bacteria is reported in many previous studies (Mustafa *et al.*, 2022; Mehmood *et al.*, 2023).

The probiotic genes and safety of *W. viridescens* PC-45 was further assessed through whole genome sequencing. The complete genome of *W. viridescens* PC-45 contains a circular chromosome of 1593258bp with a GC content of 41% and 1449 coding genes. Interestingly, bacteriocin production genes were not detected from *W. viridescens* PC-45. These findings are similar to another study that reported the lack of bacteriocin genes in *W. paramesenteroids* (Apostolakos *et al.*, 2022).



3(a)



3(b)

Fig. 3: Genome characteristics and metabolic features of *W.* viridescens: Fig. 3(a) Circular view of *Weissella viridescens* PC-45 genome. The circular ring from outer to inner side described the following information: ring of ORF (green), GC content (black), GC Skew^{+} (brown), GC Skew^{-} (purple), CARD resistance gene (red), CRISPR gene (blue) and inner circle with numerical digits showed the genome size 3(b) The genome of *Weissella viridescens* PC-45 annotated by RAST shows the different subsystems that encoded the different biological features.

Table 1: Antibacterial activity and *in vitro* probiotic properties of *Weissella* isolates.

Antibacterial activity against *S.* Gallinarum		Percentage Survival at low pH		Bile Salt tolerance after 24 hours			After 2 hours		
Isolate	Zone of Inhibition (mm)	pH=4	pH=3	MRS Broth	0.3%	1.0%	1.8%	% Auto-aggregation	% Co-aggregation	Antibiotic Resistance
PC-45	13±0.57^{bc}	68.11	71.89	1.08±0.02	0.80±0.03^{a}	0.63±0.04^{a}	0.40±0.02^{a}	58.98±0.85^{a}	47.23±0.94^{a}	S, CN, CIP, VA
PI-85	17±1.00^{a}	71.03	74.22	1.19±0.04	0.76±0.04^{a}	0.52±0.05^{b}	0.27±0.02^{c}	27.02±0.55^{d}	20.23±1.21^{c}	ERY, S, CIP, VA, OT, BAC, DA
PI-86	10±0.57^{d}	38.31	32.35	0.98±0.02	0.71±0.03^{a}	0.62±0.02^{a}	0.38±0.04^{ab}	37.10±0.33^{c}	28.15±0.26^{b}	S, CN, P, VA, DA
PI-88	14±0.57^{b}	58.82	60.01	1.11±0.03	0.60.±0.02^{b}	0.44±0.04^{bc}	0.29±0.02^{bc}	41.70±0.89^{b}	07.20±0.66^{e}	ERY, S, CN, P, VA, OT, BAC
PI-91	10±1.00^{d}	61.13	65.10	1.32±0.05	0.22±0.04^{c}	0.16±0.01^{d}	0.12±0.04^{d}	23.29±0.99^{e}	19.05±0.45^{c}	S, CN, VA, OT, CHL, DA
PI-95	11±0.57^{cd}	80.37	78.83	1.26±0.03	0.58±0.04^{b}	0.40±0.02^{c}	0.23±0.05^{c}	19.40±0.25^{f}	12.53±0.67^{d}	ERY, S, CN, VA, CIP, OT, CHL

Mean values that do not share a letter in the same column are significantly different ($p<0.05$) from each other.

Table 2: Probiotic genes encoded by *Weissella viridescens* PC-45 involved in stress response and host-microbe interaction.

Locus Tag	Proteins
	Acid Tolerance
PWA48_04595	D-alanine-poly(phosphoribitol) ligase subunit Dlt A
PWA48_04590	D-alanyl-lipoteichoic acid biosynthesis protein Dlt B
PWA48_04585	D-alanine-poly(phosphoribitol) ligase subunit Dlt C
PWA48_04580	D-alanyl-lipoteichoic acid biosynthesis protein Dlt D
PWA48_00670	F0F1-ATP synthase subunit A
PWA48_00680	F0F1-ATP synthase subunit B
PWA48_00675	F0F1-ATP synthase subunit C"
PWA48_00690	F0F1-ATP synthase subunit alpha
PWA48_00700	F0F1-ATP synthase subunit beta
PWA48_00695	F0F1-ATP synthase subunit gamma
PWA48_00705	F0F1-ATP synthase subunit epsilon
PWA48_06525	Glucose-6-phosphate isomerase
PWA48_01680	MFS transporter
	Bile Salt Tolerance
PWA48_01895	GNAT family N-acetyltransferase
	Aggregation
PWA48_05150	LysM peptidoglycan-binding domain-containing protein
	Exopolysaccharide
PWA48_06920	CpsD/ CapB family tyrosine-protein kinase
	Adhesion
PWA48_03440	Elongation factor Tu
PWA48_05690	LPXTG cell wall anchor domain-containing protein
PWA48_06010	LPXTG cell wall anchor domain-containing protein
PWA48_07295	LPXTG cell wall anchor domain-containing protein
PWA48_07395	LPXTG cell wall anchor domain-containing protein
PWA48_00605	MucBP domain-containing protein
PWA48_01400	L-lactate dehydrogenase
PWA48_04025	L-lactate dehydrogenase
PWA48_05130	L-lactate dehydrogenase
PWA48_06175	PTS system mannose/fructose/sorbose family transporter subunit IID
PWA48_00745	ATP-dependent Clp protease ATP binding subunit
PWA48_03455	ATP-dependent Clp protease ATP binding subunit ClpX
PWA48_03540	GTP pyrophosphokinase family protein
PWA48_02725	Pyruvate kinase
PWA48_06930	Phosphoglycerate mutase
PWA48_07115	CTP synthase
	Resistance to Toxic Compounds and antibiotics
PWA48_05555	PBP1A family penicillin-binding protein
PWA48_06480	Elongation factor G
PWA48_00185	Heavy metal translocating P-type ATPase
PWA48_05265	Cation:proton antiporter
	Oxidative Stress
PWA48_03480	Thioredoxin trxA
PWA48_06420	Thioredoxin-disulfide reductase trxB
PWA48_06555	Glutathione peroxidase
PWA48_03720	Peptide-methionine-S-oxide reductase Msr A
PWA48_06005	Peptide-methionine-S-oxide reductase Msr B
	Protein integrity under stress conditions
PWA48_03455	ATP-dependent Clp protease ATP-binding subunit ClpX
PWA48_02265	Co-chaperone GroES
PWA48_02270	Chaperonin GroEL
PWA48_05410	Nucleotide exchange factor GrpE
PWA48_07565	Hsp33 family molecular chaperone HslO
PWA48_02090	Hsp20/alpha-crystallin family protein
PWA48_05405	Molecular chaperone DnaK
PWA48_05400	DnaJ C-terminal domain-containing protein
PWA48_07570	ATP-dependent zinc metalloprotease FtsH
PWA48_06480	Elongation factor G

In silico analysis and genome annotation of *W. viridescens* PC-45 revealed the presence of acid tolerance determinants, which indicate the strain's ability to survive acidic conditions. The whole genome sequence of the isolates also contained genes related to bile salt tolerance, such as GNAT family N-acetyl transferase and linear amide C-N hydrolase. These genes have been previously reported from *Weissella* and *Lactobacillus* (Dos-Santos *et al.,* 2021; Tegopoulos *et al.,* 2021; Fanelli *et al.,* 2023).

Genes associated with adhesion and structural integrity contributes to probiotic potential in *W. viridescens* PC-45. The mucBP domain-containing protein, in particular, is known for its role in binding peptidoglycan, thereby enhancing the bacteria's ability to adhere to the gut lining (Montoro *et al.,* 2016; Abriouel *et al.,* 2017). Additionally, the gene for autoaggregation (LysM peptidoglycan-binding domain-containing protein) and other structural proteins such as exopolysaccharide biosynthesis protein and tyrosine protein kinases maintain the membrane protein structure and integrity under adverse environmental conditions as described in previous study (Patrone *et al.*, 2021).

Table 3: Effect of *W. viridescens* PC-45 on *Salmonella* count in broiler gut.

Groups	*Salmonella* count on SS agar mean log10 (CFU/g)						
	Day 23	Day 25	Day 27	Day 29	Day 31	Day 33	Day 35
Negative control	-	-	-	-	-	-	-
Positive control	6.31± 0.27^{a}	6.39± 0.20^{a}	6.45.± 0.70^{a}	6.47± 0.30^{a}	6.59± 0.10^{a}	6.71± 0.10^{a}	6.88± 0.22^{a}
PC-45	6.02± 0.18^{a}	5.28± 0.10^{c}	5.19± 0.15^{b}	5.18± 0.19^{b}	5.13± 0.02^{c}	4.77± 0.24^{c}	4.35± 0.10^{c}
HAN-LACVET	5.95± 0.60^{a}	5.83± 0.11^{b}	5.69± 0.08^{ab}	5.56± 0.19^{b}	5.40± 0.15^{b}	5.43± 0.20^{b}	5.35± 0.45^{b}

Means that do not share a letter in the same column are significantly different ($p<0.05$).

W. viridescens PC-45 also possesses genes related to heat shock proteins, which play a key role in stabilizing membrane structure and intracellular protein aggregation at higher temperatures, as described in previous research work (Boucard *et al*., 2022; Kandasamy *et al*., 2022). The strain's ability to withstand the oxidative challenges is reinforced by genes involved in oxidative stress response. Additionally, the genes responsible for resistance to toxic compounds and heavy metals enhance the survival of bacteria during stress conditions in the gastrointestinal tract (Dos-Santos *et al*., 2021; Kandasamy *et al*., 2022).

Assessing probiotic strains for transferable antibiotic resistance genes is vital for public health concerns. The genomic analysis of *W. viridescens* PC-45 through the CARD database revealed its susceptibility to a broad range of antibiotics. However, the RGI server identified the presence of the glycopeptide resistance gene (Vant), which is known to be intrinsic to *Lactobacillus* and *Weissella*, as documented in previous studies (Zhang *et al*., 2018; Campedelli *et al*., 2019). Therefore, this strain is deemed safe because it lacks transmissible antibiotic-resistance genes.

The isolated strain *W viridescens* PC-45 with potential phenotypic and genotypic properties was further evaluated to analyze its effect in broilers infected with *S.* Gallinarum. It was found that *W. viridescens* PC-45 can reduce (>2 log10) *S.* Gallinarum in broiler. We have reported similar results using lactobacilli as well (Mehmood *et al*., 2023). The results of another study reported lactobacilli with the ability to reduce (>4 log10) *Salmonella* in mice with a possible mechanism of lactic acid production or competitive exclusion (Mustafa *et al.,* 2022).

Conclusions: In conclusion, *W. viridescens* PC-45 isolated from chicken has potential *in-vitro* phenotypic characteristics such as antimicrobial activity, ability to withstand low pH, bile salts, autoaggregation, co-aggregation activity and no transmissible antibiotic resistance. It also reduced more than 4 log reduction growth of *S.* Gallinarum in co-culture assay. Moreover,

the size of the assembled genome of *W. viridescens* PC-45 is 1593258 bp, including the GC content of 41%, coding sequence 1449. *In-silico* analysis showed various metabolic and structural genes and CRISPR regions encoded in the genome of *Weissella viridescens* PC-45. The various putative genes involved in stress conditions (tolerance to low pH, bile salts, oxidative stress, and toxic compounds) and adhesion to the gut indicated the probiotic properties. An *in-vivo* study revealed that *W. viridescens* PC-45 reduced the growth of *S.* Gallinarum (>2 log 10) in chickens. The *in-vitro*, *in-silico* and *in-vivo* findings suggest that *W. viridescens* PC-45 has potential probiotic properties and can be used for further development of anti-*S.* Gallinarum probiotics to control fowl typhoid in poultry.

Credit authorship contribution statement: AM: Methodology, Formal analysis, Writing–original draft, Writing–review & editing. M N: Conceptualization, Formal analysis, Writing–original draft, Writing–review & editing. M R: Methodology, Writing–review & editing. M H M: Methodology–review & editing.

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/R&D/HEC/2017).

REFERENCES

Abriouel H, Pérez Montoro B, Casimiro-Soriguer CS, *et al.*, 2017. Insight into potential probiotic markers predicted in *Lactobacillus pentosus* MP-10 genome sequence. Front Microbiol 8:891.

Ahmad R, Yu YH, Hsiao FSH, *et al.,* 2022. Probiotics as a friendly antibiotic alternative: assessment of their effects on poultry's health and productive performance. Fermentation 8:672.

Alcock BP, Raphenya AR, Lau TT, *et al.,* 2020. CARD 2020:antibiotic resistome surveillance with the comprehensive antibiotic resistance database. Nucleic Acids Res 48:D517-D525.

Ali H, Tohamy HG, Al-Hattali R, *et al.*, 2024. Encephalitic listeriosis in small ruminants in Oman: pathophysiology, antimicrobial sensitivity and molecular characterization. Pak Vet J 44(1):87-92.

Apostolakos I, Paramithiotis S and Mataragas M, 2022. Functional and safety characterization of *Weissella paramesenteroides* strains isolated from dairy products through whole-genome sequencing and comparative genomics. Dairy 3(4):799-813.

Bao Y, Zhang Y, Zhang Y, *et al.,* 2010. Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy products. Food control 21:695-701.

Bortolaia V, Kaas RS, Ruppe E, *et al.,* 2020. ResFinder 4.0 for predictions of phenotypes from genotypes. J Antimicrob Chemother 75:3491-3500.

Boucard AS, Florent I, Polack B, *et al.,* 2022. Genome sequence and assessment of safety and potential probiotic traits of *Lactobacillus iohnsonii* CNCM I-4884. Microorganisms 10:273.

Campedelli I, Mathur H, Salvetti E, *et al.*, 2019. Genus-wide assessment of antibiotic resistance in *Lactobacillus* spp. App Env Microbiol 85(1): e01738-18.

Cele N, Nyide B and Khoza T, 2022. *In Vitro* Characterisation of Potential Probiotic Bacteria Isolated from a Naturally Fermented Carrot and Ginger Brine. Fermentation 8:534.

Dos-Santos CI, Campos CD, Nunes-Neto WR, *et al.*, 2021. Genomic analysis of *Limosilactobacillus fermentum* ATCC 23271, a potential probiotic strain with anti-*Candida* activity. J Fungi 7(10):794.

EFSA, 2012. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). Guidance on assessing bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J 10:2740.

Espinoza-Monje M, Campos I, Alvarez Villamil E, *et al.*, 2021. Characterization of *Weissella viridescens* UCO-SMC3 as a potential probiotic for the skin: its beneficial role in the pathogenesis of *acne vulgaris*. Microorganisms 9 (7):1486.

Fanelli F, Montemurro M, Verni M, *et al.*, 2023. Probiotic potential and safety assessment of type strains of *Weissella* and *Periweissella* species. Microbiol Spectr 11(2):e03047-22.

Fhoula I, Boumaiza M, Tayh G, *et al.*, 2022. Antimicrobial activity and safety features assessment of *Weissella spp*. from environmental sources. Food Sci Nutr 10(9):2896-2910.

Hill C, Guarner F, Reid G, *et al.,* 2014. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 11:506-514

Issa NA, 2024. Evaluation the antimicrobial activity of essential oils against veterinary pathogens, multidrug-resistant bacteria and dermatophytes. Pak Vet J 44(2):260-265.

Kandasamy S, Yoo J, Yun J, *et al.*, 2022. Probiogenomic in-silico analysis and safety assessment of *Lactiplantibacillus plantarum* DIF10 strain isolated from Korean raw milk. Int J Mol Sci 23(22):14494.

Kanehisa M, Sato Y and Morishima K, 2016. BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. J Mol Biol 428:726-731.

Kanehisa M, Sato Y and Kawashima M, 2022. KEGG mapping tools for uncovering hidden features in biological data. Prot Sci 31:47-53.

Khan A, Mahmood M, Hussain I, *et al.,* 2014. Bacteriological and epidemiological investigations of Pullorum disease in selected poultry farms of Faisalabad, Pakistan. Glob Vet 12:455-460.

Khan RU, Fatima A, Naz S, *et al.,* 2022. Perspective, opportunities and challenges in using fennel (foeniculum vulgare) in poultry health and production as an eco-friendly alternative to antibiotics: a review. Antibiotics 11(2):278.

Le B, and Yang SH, 2018. Isolation of *Weissella* strains as potent probiotics to improve antioxidant activity of salted squid by fermentation. J Appl Biol Chem 61:93-100.

Lee KW, Park JY, Chun JY, *et al.,* 2010. Importance of *Weissella* species during kimchi fermentation and future works. Microb Biotechnol Lett 38:341-348.

Liao SF and Nyachoti M, 2017. Using probiotics to improve swine gut health and nutrient utilization. Anim Nutr 3:331-343.

Mehmood A, Nawaz M, Rabbani M, *et al.,* 2023. *In Vitro* Characterization of probiotic potential of *Limosilactobacillus* fermentum against *Salmonella* Gallinarum causing fowl typhoid. Animals 13:1284.

Mehmood A, Nawaz M, Rabbani M, *et al.*, 2023. Probiotic Effect of *Limosilactobacillus fermentum* on growth performance and competitive exclusion of *Salmonella* Gallinarum in Poultry. Pak Vet J 43(4):659-664

Mohankumar A and Murugalatha N, 2011. Characterization and antibacterial activity of bacteriocin producing *Lactobacillus* isolated from raw cattle milk sample. Int J Biol 3:128.

Montoro BP, Benomar N, Lavilla Lerma L, *et al.,* 2016. Fermented Aloreña table olives as a source of potential probiotic *Lactobacillus pentosus* strains. Front Microbiol 7:1583.

Mustafa A, Nawaz M, Rabbani M, *et al.,* 2022. Characterization and evaluation of anti-*Salmonella* enteritidis activity of indigenous probiotic lactobacilli in mice. Open Life Sci 17:978-990.

Nawaz M, Wang J, Zhou A, *et al.,* 2011. Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. Curr Microbiol 62:1081-1089.

Noohi N, Papizadeh M, Rohani M, *et al.,* 2021. Screening for probiotic characters in lactobacilli isolated from chickens revealed the intra-species diversity of *Lactobacillus brevis*. Anim Nutr 7:119-126.

Overbeek R, Olson R, Pusch GD, *et al.,* 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res 42:D206-D214.

Pabari K, Pithva S, Kothari C, *et al.,* 2020. Evaluation of probiotic properties and prebiotic utilization potential of *Weissella paramesenteroides* isolated from fruits. Probiotics Antimicrob Proteins 12:1126-1138.

Patrone V, Al-Surrayai T, Romaniello F, *et al.*, 2021. Integrated phenotypic-genotypic analysis of candidate probiotic *Weissella cibaria* strains isolated from dairy cows in Kuwait. Probiotics Antimicrob. Proteins 13:809-823.

Pelyuntha W, Chaiyasut C, Kantachote D, *et al.*, 2019. Cell-free supernatants from cultures of lactic acid bacteria isolated from fermented grape as biocontrol against *Salmonella Typhi* and *Salmonella Typhimurium* virulence via autoinducer-2 and biofilm interference. Peer J 7: e7555.

Rajoka MSR, Hayat H, Sarwar S, *et al.,* 2018. Isolation and evaluation of probiotic potential of lactic acid bacteria isolated from poultry intestine. Microbiology 87:116-126.

Rizzello CG, Coda R, Wang Y, *et al.,* 2019. Characterization of indigenous *Pediococcus pentosaceus*, *Leuconostoc kimchii*, *Weissella cibaria* and *Weissella confusa* for faba bean bioprocessing. Int J Food Microbiol 302:24-34.

Saleem N, Nawaz M, Ghafoor A, *et al.,* 2018. Phenotypic and molecular analysis of antibiotic resistance in lactobacilli of poultry origin from Lahore, Pakistan. Pak Vet J 38(4):409-413.

Sarba EJ, Kudama K, Dandecha M, *et al.,* 2020. Prevalence, organ distribution and antimicrobial susceptibility profile of *Salmonella* isolated from chickens purchased from markets in selected districts of West Shoa, Ethiopia. Ethiop Vet J 24:73-89.

Seemann T, 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068-2069.

Shami A. Abdallah M. Alruways MW. *et al.*. 2024. Comparative Prevalence of Virulence Genes and Antibiotic Resistance in *Campylobacter jejuni* Isolated from Broilers, Laying Hens and Farmers. Pak Vet J 44(1):200-204.

Shokryazdan P, Kalavathy R, Sieo C, *et al.,* 2014. Isolation and characterization of *Lactobacillus* strains as potential probiotics for chickens. Pertanika J Trop Agric Sci 37(1):141-157.

Tegopoulos K. Stergiou OS. Kiousi DE. *et al.*. 2021. Genomic and phylogenetic analysis of *Lactiplantibacillus plantarum* L125, and evaluation of its anti-proliferative and cytotoxic activity in cancer cells. Biomedicines 9(11):1718.

Teixeira CG, Fusieger A, Milião GL, *et al.,* 2021. *Weissella*: An emerging bacterium with promising health benefits. Probiotics Antimicrob Proteins 13:915-925.

Todoriki K, Mukai T, Sato S, *et al.,* 2001. Inhibition of adhesion of food-borne pathogens to Caco-2 cells by *Lactobacillus* strains. J Appl Microbiol 91:154-159.

Van Heel AJ, de-Jong A, Song C, *et al.,* 2018. BAGEL4: a user-friendly web server to thoroughly mine RiPPs and bacteriocins. Nucleic Acids Res 46(W1): W278-W281.

Vieco-Saiz N, Belguesmia Y, Raspoet R, *et al.,* 2019. Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. Front Microbiol 10:57.

Wu H, Tao M, Liu J, *et al.*, 2024. *In vitro* Evaluation of antibiotic--matrine combinations against clinically relevant bacteria. Pak Vet J 44(1):162-168

Xu L, Dong Z, Fang L, *et al.,* 2019. OrthoVenn2: a web server for whole-genome comparison and annotation of orthologous clusters across multiple species. Nucleic Acids Res 47: W52-W58.

Ye K, Liu J, Liu M, *et al.,* 2018. Effects of two *Weissella viridescens* strains on *Listeria monocytogenes* growth at different initial inoculum proportions. CyTA-J Food 16(1):299-305.

Zhang S, Oh JH, Alexander LM, *et al.,* 2018. d-Alanyl-d-alanine ligase as a broad-host-range counterselection marker in vancomycin-resistant lactic acid bacteria. J Bact 200(13):10-1128.

Zheng J, Du M, Jiang W, *et al.,* 2021. *In vitro* probiotic characteristics and whole genome sequence analysis of *Lactobacillus* strains isolated from cattle-yak milk. Biology 11:44.