



## RESEARCH ARTICLE

### *In-vitro, in-vivo* and Whole Genome-Based Probe into Probiotic Potential of *Weissella viridescens* PC-45 against *Salmonella Gallinarum*

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#### 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 *Weissella* 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 log<sub>10</sub>) *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 log<sub>10</sub>) *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.

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#### 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 ( $3 \times 10^8$  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

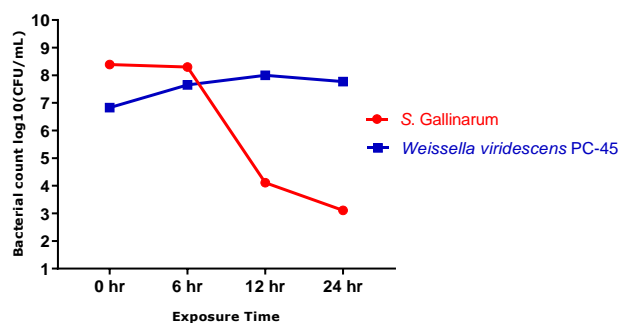
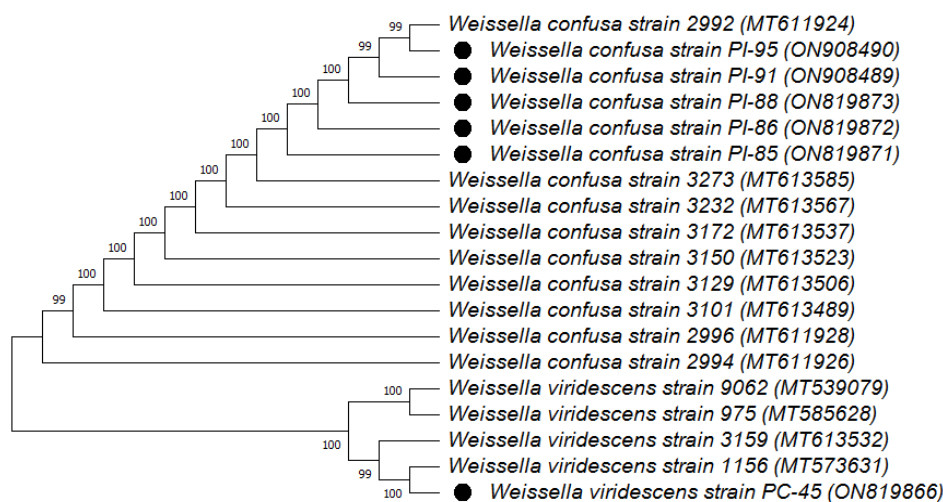
shown in Fig. 2, the isolate PC-45 had a non-significant effect on the growth of *S. Gallinarum* after 6 hrs (8.30 log<sub>10</sub>CFU/mL). It caused a remarkable reduction after 12 hrs (4.11 log<sub>10</sub>CFU/mL) and 24 hrs (3.11 log<sub>10</sub>CFU/mL) as compared to the initial count (8.39 log<sub>10</sub>CFU/mL). The result revealed *W. viridescens* PC-45 inhibited the  $>4$  log<sub>10</sub> 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<sup>+</sup>, GC Skew<sup>-</sup>, 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 log<sub>10</sub>CFU/g) to day 35 (6.88±0.22 mean log<sub>10</sub>CFU/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 log<sub>10</sub>CFU/g) compared to both the positive control group (6.88±0.22 mean log<sub>10</sub>CFU/g) and the commercial probiotic group (5.35±0.45 mean log<sub>10</sub>CFU/g).



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

## DISCUSSION

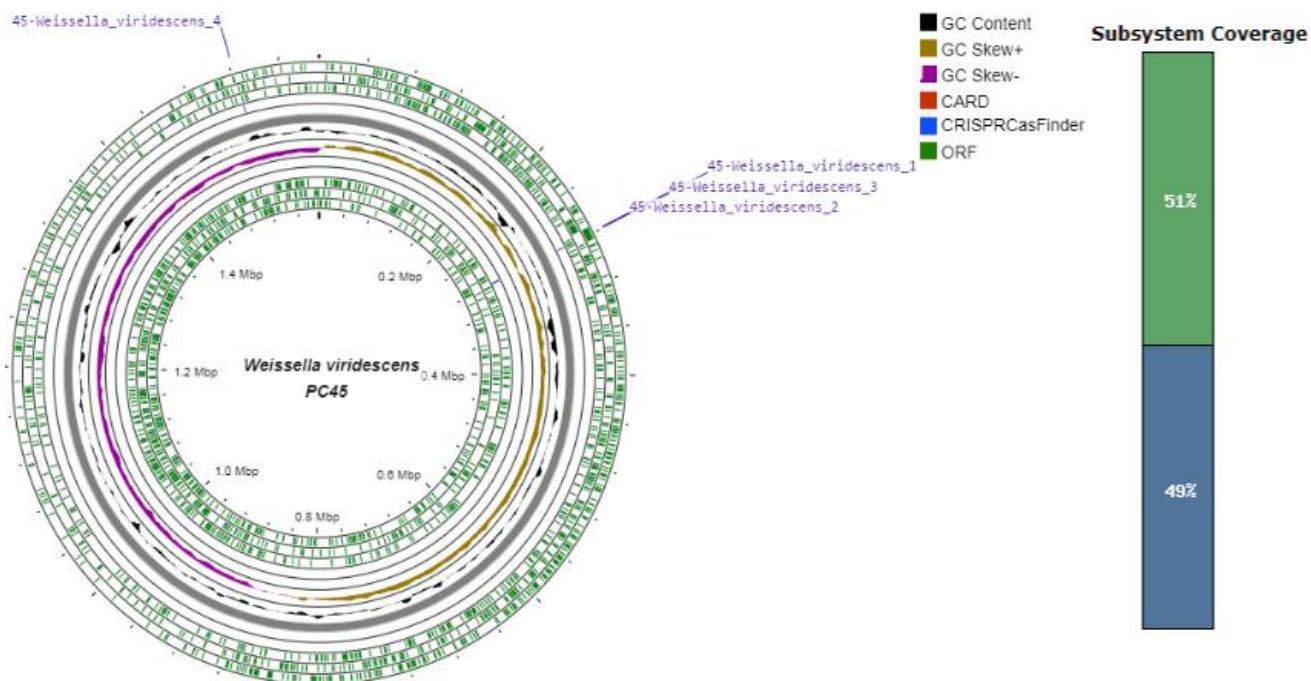
In the current study, 15 presumptive *Weissella* isolates were selected from healthy poultry caeca and ileum from flocks suffering from fowl typhoid, assuming that these healthy birds have diverse microbiota that can be explored as probiotics. Six isolates were confirmed as *Weissella confusa* or *W. 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

**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.

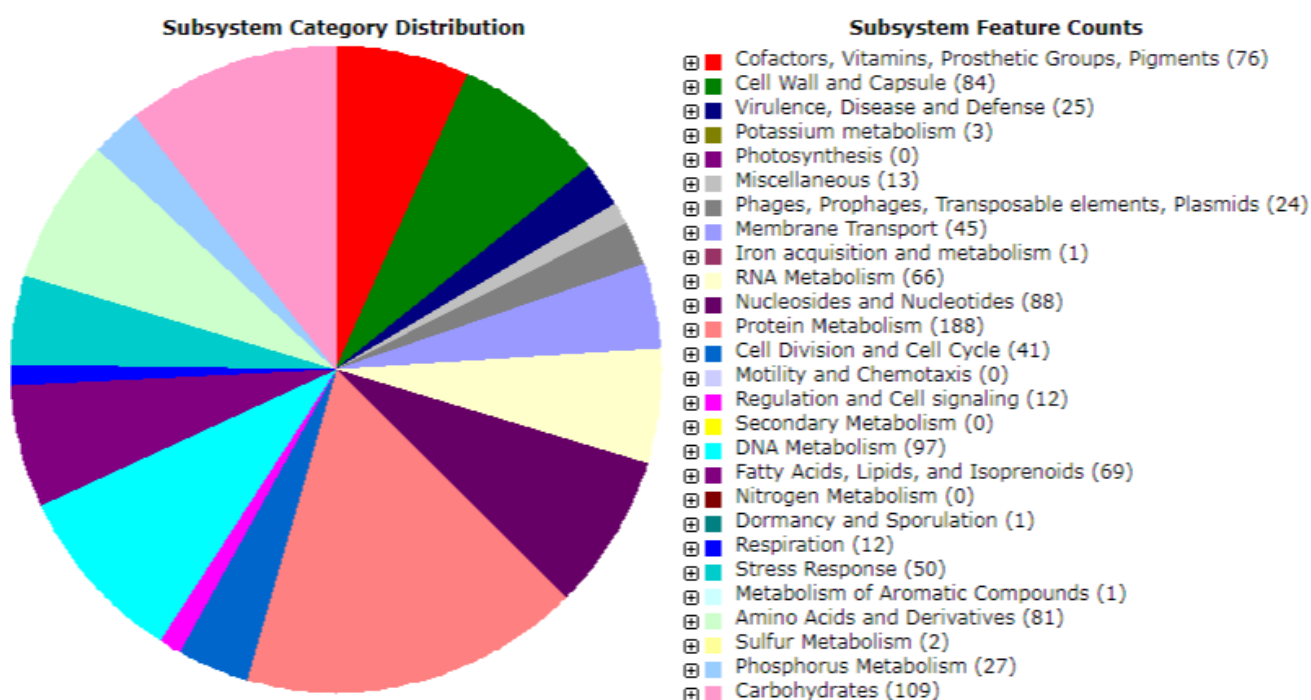
concentration decreased with increased bile salt concentrations in all isolates. Growth of isolates in bile salts (an increase of 0.5 O.D<sub>600</sub>) was considered good, as described previously (Mehmood *et al.*, 2023). At 0.3% bile salt concentration, five isolates showed more than 0.5 OD, and three 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<sub>10</sub>) 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<sup>+</sup> (brown), GC Skew<sup>-</sup> (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

Isolate	Zone of Inhibition (mm)	Antibacterial activity Percentage		Bile Salt tolerance after 24 hours			After 2 hours		Antibiotic Resistance	
		pH=4	pH=3	MRS	0.3%	1.0%	1.8%	% Auto-aggregation		% Co-aggregation
PC-45	13±0.57 <sup>bc</sup>	68.11	71.89	1.08±0.02	0.80±0.03 <sup>a</sup>	0.63±0.04 <sup>a</sup>	0.40±0.02 <sup>a</sup>	58.98±0.85 <sup>a</sup>	47.23±0.94 <sup>a</sup>	S, CN, CIP, VA
PI-85	17±1.00 <sup>a</sup>	71.03	74.22	1.19±0.04	0.76±0.04 <sup>a</sup>	0.52±0.05 <sup>b</sup>	0.27±0.02 <sup>c</sup>	27.02±0.55 <sup>d</sup>	20.23±1.21 <sup>c</sup>	ERY, S, CIP, VA, OT, BAC, DA
PI-86	10±0.57 <sup>d</sup>	38.31	32.35	0.98±0.02	0.71±0.03 <sup>a</sup>	0.62±0.02 <sup>a</sup>	0.38±0.04 <sup>ab</sup>	37.10±0.33 <sup>c</sup>	28.15±0.26 <sup>b</sup>	S, CN, P, VA, DA
PI-88	14±0.57 <sup>b</sup>	58.82	60.01	1.11±0.03	0.60±0.02 <sup>b</sup>	0.44±0.04 <sup>bc</sup>	0.29±0.02 <sup>bc</sup>	41.70±0.89 <sup>b</sup>	07.20±0.66 <sup>e</sup>	ERY, S, CN, P, VA, OT, BAC
PI-91	10±1.00 <sup>d</sup>	61.13	65.10	1.32±0.05	0.22±0.04 <sup>c</sup>	0.16±0.01 <sup>d</sup>	0.12±0.04 <sup>d</sup>	23.29±0.99 <sup>e</sup>	19.05±0.45 <sup>c</sup>	S, CN, VA, OT, CHL, DA
PI-95	11±0.57 <sup>cd</sup>	80.37	78.83	1.26±0.03	0.58±0.04 <sup>b</sup>	0.40±0.02 <sup>c</sup>	0.23±0.05 <sup>c</sup>	19.40±0.25 <sup>f</sup>	12.53±0.67 <sup>d</sup>	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	<i>Salmonella</i> count on SS agar mean log <sub>10</sub> (CFU/g)						
	Day 23	Day 25	Day 27	Day 29	Day 31	Day 33	Day 35
Negative control	-	-	-	-	-	-	-
Positive control	6.31± 0.27 <sup>a</sup>	6.39± 0.20 <sup>a</sup>	6.45± 0.70 <sup>a</sup>	6.47± 0.30 <sup>a</sup>	6.59± 0.10 <sup>a</sup>	6.71± 0.10 <sup>a</sup>	6.88± 0.22 <sup>a</sup>
PC-45	6.02± 0.18 <sup>a</sup>	5.28± 0.10 <sup>c</sup>	5.19± 0.15 <sup>b</sup>	5.18± 0.19 <sup>b</sup>	5.13± 0.02 <sup>c</sup>	4.77± 0.24 <sup>c</sup>	4.35± 0.10 <sup>c</sup>
HAN-LACVET	5.95± 0.60 <sup>a</sup>	5.83± 0.11 <sup>b</sup>	5.69± 0.08 <sup>ab</sup>	5.56± 0.19 <sup>b</sup>	5.40± 0.15 <sup>b</sup>	5.43± 0.20 <sup>b</sup>	5.35± 0.45 <sup>b</sup>

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 log<sub>10</sub>) *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 log<sub>10</sub>) *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.

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