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- 1 Genomic and Metabolic Properties of Staphylococcus gallinarum FCW1 MCC4687 Isolated
- 2 from Naturally Fermented Coconut Water towards GRAS Assessment

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- 35 Abstract
- Staphylococcus gallinarum FCW1 was isolated from naturally fermented coconut-water and 36 identified by biochemical and molecular methods. Probiotic characterization and safety 37 38 assessment were conducted through a series of in vitro tests. A high survival rate was observed when the strain was tested for resistance to bile, lysozyme, simulated gastric and intestinal fluid, 39 40 phenol, and different temperature and salt concentrations. The strain showed antagonism against some pathogens, was susceptible to all antibiotics tested except penicillin, and showed no 41 42 hemolytic and DNase activity. Hydrophobicity, autoaggregation, biofilm formation, and antioxidation tests indicated that the strain possessed a high adhesive and antioxidant ability. 43 44 Enzymatic activity was used to evaluate the metabolic capacities of the strain. In-vivo 45 experiment on zebrafish was performed to check its safety status. The whole-genome sequencing 46 indicated that the genome contained 2,880,305 bp with a GC content of 33.23%. The genome annotation confirmed the presence of probiotic-associated genes and genes for oxalate 47 degradation, sulfate reduction, acetate metabolism, and ammonium transport in the FCW1 strain, 48 adding to the theory that this strain may be helpful in treating kidney stones. This study revealed 49 50 that the strain FCW1 might be an excellent potential probiotic in developing fermented coconut
- 52 **Keywords**: *Staphylococcus gallinarum*, Whole genome sequencing, Zebrafish, Safety assessment, Probiotic characteristics, Kidney stone.

beverages and treating and preventing kidney stone disease.

#### 1. Introduction

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Growing consumer interest in probiotics and fermented foods has broadened their therapeutic and research opportunities. Over recent years, more focus has been shifted to searching for novel potential probiotics taxa from various sources and their therapeutic applications. Probiotics are beneficial microorganisms that provide health benefits to the host when consumed adequately (FAO/WHO, 2002; Cammarota et al., 2014). They are the core part of fermented foods that preserve and improve foods and maintain a healthy gut microbiome without adverse side effects

in humans and animals. Some traditional probiotics have marginal ameliorative effects on various diseases and are not disease-specific. Some exhibit safety issues also. An urgent need exists at this point for screening and characterizing of novel high-quality probiotics against specific diseases (Chang et al., 2019). Naturally fermented foods are a good source of probiotics, which break down complex chemical matrices into simpler components that are more nutrient dense and improve their bioactivity, bioavailability and safety, as well as textural and sensorial characteristics (Tamang et al., 2016). Lactic acid bacteria (LAB) and Gram-positive catalase-positive cocci (GCC<sup>+</sup>) are the predominant bacteria isolated from various naturally fermented foods. The most common and technologically relevant GCC<sup>+</sup> are non-pathogenic Coagulase-negative staphylococci (CNS), which are responsible for the flavour and aroma formation and colour stabilization of the fermented foods by their proteolytic and lipolytic activities and preventing rancidity by decomposition of peroxides (Talon et al., 2007). Besides improving the quality and sensory properties of the final product, GCC+CNS also provides nitrate-reductase, catalase and antioxidant activities (Talon et al., 1999; 2007). Owing to this, food-derived GCC+CNS have been widely used as a starter culture for the fermentation of sausages, meat, cheese, and soybean, suppressing the growth of poisoning and spoilage microorganisms. Numerous studies have reported the isolation and safety assessment of CNS from various fermented foods like meat (Landeta et al., 2013), milk (Irlinger, 2008), seafood (Jeong et al., 2014) and soybean (Jeong et al., 2016). However, the probiotic characterization and strain-specific safety evaluation of GCC<sup>+</sup>CNS are limited. Spontaneously or naturally fermented coconut water (CW), the reservoir and vehicle of beneficial bacteria, was investigated in the present study for the isolation and probiotic characterization of GCC+CNS. Generally, CW is believed to be sterile when it is in the nut cavity. Nevertheless, a recent study by Sriram et al. (2020) proved the presence of endophytic bacteria in coconut endosperm. They isolated and identified Staphylococcus cohnii from coconut endosperm, which could be responsible for synthesizing secondary metabolites. Furthermore, sugars and minerals in CW provide a suitable environment for the survival and growth of autochthonous microbiota, which influences safety, sensory and nutritional properties. Prado et al. (2015) successfully isolated seven autochthonous LAB from naturally fermented CW with

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- 92 probiotic properties and developed a potential fermented CW beverage. This evidenced the
- 93 presence of probiotic bacteria in naturally fermented CW.
- 94 Probiotic strain identification and characterization are crucial for their practical use. According
- of to "Guidelines for the Evaluation of Probiotics in Foods" the currently available *in vitro* tests are
- 96 inadequate to characterize a probiotic microorganism and its functionality (FAO/WHO, 2002).
- 97 Bacterial whole-genome sequence analysis is an advanced method for accurate phylogenetic and
- 98 taxonomic profiling, determining health-promoting activities, and safety evaluation of the
- 99 probiotic candidates. The main objective of the current study was isolation, identification, and in
- 100 vitro and in vivo probiotic characterization along with the whole genome sequencing of
- autochthonous bacteria from naturally fermented CW. This study provides the data necessary to
- understand whether the isolated bacterial strain has probiotic traits and is safe for use.

#### 103 2. Materials and Methods

## 104 2.1. Sample collection and processing

- Fresh green tender coconuts were purchased from the local market in Tiruchirappalli, Tamil
- Nadu, India. CW was collected in a sterile condition in a laminar airflow chamber and filtered
- through Whatman grade 42 filter paper (2.5 µm) (Whatman, GE Healthcare, UK). An aliquot of
- 108 CW was allowed for spontaneous or natural fermentation in aerobic conditions as described by
- 109 Prado et al. (2015) with slight modifications (16-24 h at room temperature). After the
- fermentation procedure, the sample was subjected to microbiological analyses.

#### 111 2.2. Isolation of GCC<sup>+</sup>CNS strains

- Serial dilutions (10<sup>-1</sup> to 10<sup>-9</sup>) of 1 ml naturally fermented CW sample were prepared in distilled
- water (DW) and then 100µl sample from different dilutions was spread plated over plates of
- nutrient agar (NA) enriched with CW (CW and DW in 1:1 ratio) in triplicate and incubated at
- 115 37°C for 24-48 h. Gram-staining, catalase and coagulase tests were performed with
- morphologically discrete colonies. Only Gram-positive, catalase-positive and coagulase-negative
- colonies were propagated twice and restreaked on NA to obtain pure cultures, and the cultures
- were stored at -20°C as 15% glycerol stock (15% glycerol supplemented nutrient broth) for
- further analysis. All the media and chemicals for the above experiments were purchased from
- 120 Himedia, Mumbai, India.

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## 2.3. Biochemical and Morphological Characterization

- Preliminary identification of selected bacteria was done by morphological and biochemical
- characterization. Morphological characterization of the isolate FCW1 was performed by colony
- morphology, Gram-staining, and motility. Biochemical characterization was carried out using the
- 125 IMVIC test, citrate utilization assay, catalase and oxidase test, urease test, nitrate reduction
- assay, triple sugar iron (TSI) test and hydrogen sulfide (H<sub>2</sub>S) production (Cappuccino and
- Sherman, 2005). The Carbohydrate utilization test was performed using the HiIMViC
- Biochemical Test Kit (Himedia, Mumbai, India), following the manufacturer's guidelines.

#### 129 2.4. Molecular identification of FCW1 strain

- Molecular identification of isolate FCW1 was made by 16S rRNA gene sequencing. The
- genomic DNA of strain FCW1 was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) as
- per the manufacturer's instructions. The quality of the DNA isolated was checked using agarose
- gel electrophoresis. The 16S rRNA gene amplification was carried out in a PCR thermal cycler
- 134 (GeneAmp PCR System 9700, Applied Biosystems) using universal primers: 16S-RS-F (5'-
- 135 CAGGCCTAACACATGCAAGTC-3') and 16S-RS-R (5'-GGGCGGWGTGTACAAGGC-3')
- (Selvin et al., 2019). The sequencing of 16S rRNA was done using the BigDye Terminator v3.1
- 137 Cycle sequencing Kit (Applied Biosystems, USA) in a PCR thermal cycler (GeneAmp PCR
- 138 System 9700, Applied Biosystems). The 16S rRNA sequence was compared with the NCBI
- 139 database through BlastN (basic local alignment search tool -
- 140 http://www.ncbi.nlm.nih.gov/BLAST) as well as Ezbiocloud blast were performed
- at https://www.ezbiocloud.net/identify web server and also the sequence has been submitted in
- the GenBank data library with an accession number: MW453067. Phylogenetic analysis was
- done with MEGA X software using the Maximum Likelihood algorithm. The evolutionary
- distances were calculated using the Tamura-Nei model and are in the units of the number of base
- substitutions per site. The rate variation among sites was uniform. Initial tree(s) for the heuristic
- search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix
- of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology
- with superior log likelihood value. The analysis involved 22 nucleotide sequences, 1000
- bootstrap replications and Codon positions 1st + 2nd + 3rd + Noncoding.

## 150 2.5. *In vitro* Evaluation of Probiotic Properties

#### 2.5.1. Safety Assessment

#### 152 2.5.1.1. Antagonistic activity

The screening of potential antagonistic activity was determined by the agar gel diffusion method 153 according to Oureshi et al. (2020). Cell-free supernatant was collected from the bacterial culture 154 grown overnight at 37°C in 50 ml Luria-Bertani broth (LB; Himedia, Mumbai, India) by 155 centrifugation at 5000 rpm for 10 min at 4°C and filtered through syringe filters (0.2 µm 156 Acrodisc® Syringe Filters, Pall). Six pathogenic bacteria Klebsiella pneumoniae, Streptococcus 157 spp., Escherichia coli, (these clinical isolates belonging to K.A.P.V. Govt Medical Collage, 158 Tiruchirappalli), Bacillus cereus (NCIM 2156), Staphylococcus aureus (NCIM 159 and Enterococcus faecalis (MTCC 439) were cultured in LB broth, incubated at 37°C for 18 h 160 and the cultures were swabbed over the Muller Hinton agar (MHA; Himedia, Mumbai, India) 161 plates. On the swabbed MHA plate, 4 mm diameter wells were cut, and 100 µl of bacterial 162 culture supernatant was added. Tetracycline (10 mcg) disc was used as a control since it is a 163 164 broad-spectrum antibiotic which is used extensively in the prophylaxis and therapy of human and animal infections. The inhibitory zone around the wells was measured after 24 h of incubation at 165 166 37°C. The assays were carried out in duplicate.

## 167 2.5.1.2. Antibiotic susceptibility test

- Antibiotic susceptibility profiles were determined by the Kirby-Bauer disc diffusion method according to CLSI guidelines (CLSI, 2022). The analysis was performed with eight antibiotics (Penicillin, Gentamycin, Tetracycline, Erythromycin, Ciprofloxacin, Chloramphenicol, Norfloxacin, Trimethoprim; Himedia, Mumbai, India), which were placed over the FCW1 inoculated MHA plates. The results were compared with the interpretative zone diameters described in Performance Standards for Antimicrobial Disc Susceptibility Tests.
- 174 2.5.1.3. Antioxidant assay by DPPH method
- 2,2-diphenylpicrylhydrazyl (DPPH; Himedia, Mumbai, India) assay was used to determine the antioxidant activity of bacterial cell-free supernatant (CFS) as described by Bhukya and Bhukya
- 177 (2021). The reference standard was ascorbic acid (1 mg/ml). The following formula was used to
- calculate antioxidant activity (%):

Antioxidant activity (%) = 
$$(A0-As/A0) \times 100$$
 (1)

A0 and As, respectively, represent the absorbance of controls and samples.

#### 181 2.5.1.4. Haemolytic and DNase activity

The Hemolytic assay was done by measuring the zone of hemolysis around FCW1 colonies on a blood-agar plate (Himedia, Mumbai, India) after 48 h incubation at 37°C as described by Zommiti *et al.* (2017). DNase agar plates (Himedia, Mumbai, India) were streaked with isolate FCW1 and the development of a pink halo or a clear zone around the bacterial colonies after 48 h incubation at 37°C indicates positive DNase activity (Shuhadha et al., 2017).

## 2.5.2. Tolerance to different stress conditions

Tolerance to low pH and bile salt was assessed as described by Somashekaraiah et al. (2019) and Nath et al. (2021), respectively, with slight modifications. Briefly, overnight culture FCW1 (10 ml) was centrifuged at 5000 rpm for 10 min before being twice rinsed with sterile phosphate-buffered saline (PBS). The pellets were resuspended in PBS to reach the initial volume. The ability of the strain to grow at low pH was evaluated by adding 100 µl bacterial suspension into 5 ml PBS solution after adjusting the pH to 2 with 1N hydrochloric acid (HCL) and pH 7 considered as control. For bile salt tolerance, bacterial suspension (100 µl) was added to 5 ml of LB broth with (0.3%) and without (control) bile salt (LobaChemie, India) and incubated at 37°C for 4 h. At every 1 h interval, the optical density (OD) was measured at 620 nm using a spectrophotometer (Lambda 35, Perkin Elmer) and the total viable cell count was determined by plating 100 µl of samples onto fresh LB agar plates. Percentage survivability was calculated by the formula given below:

Survivability 
$$\%$$
 = Survival rate ( $\%$ ) = OD test/OD control×100 (2)

Following Liu et al. (2021), the tolerance to gastrointestinal conditions was determined. Synthetic gastric juice was prepared using 3.0 g of pepsin dissolved in sterile PBS, pH 2.5 adjusted with hydrochloric acid. Trypsin (1.0 g/L) and bile salt (1.8%) were dissolved in sterile PBS and adjusted to pH 8.0 with 0.1 mol/L NaOH to prepare artificial intestinal fluid. Before use, the artificial gastric and intestinal fluids were filtered through a 0.22 µm filter membrane (Millipore, Massachusetts, USA). The overnight culture (10 ml) of bacteria was centrifuged at 5000 rpm for 10 min; pellets were collected, washed thrice with PBS, and resuspended in sterile PBS. To 4.5 ml of artificial gastric and intestinal fluid, 0.5 ml of bacterial suspension was added

- and then incubated at 37°C for 5 h. The PBS solution at pH 7 was used as a control. Percentage
- survivability was calculated after measuring the OD values at 620 nm at every 1 h interval.
- 211 The ability of FCW1 to grow at different temperatures was tested according to Macías-
- Rodríguez (2008) with minor modifications. The overnight grown strain (100 µl) was inoculated
- 213 in 5 ml LB broth and incubated at 4, 10, 25 and 37°C for 24 h and the growth rate was measured
- by reading absorbance at 620 nm spectroscopically.
- To assess lysozyme resistance, overnight FCW1 culture (5 ml) was centrifuged at 5000 rpm for
- 216 10 min and resuspended in 10 ml LB broth with lysozyme (100 mg/l) and incubated for 3 h at
- 217 37°C. At different time intervals (0, 1, 2 and 3 h), the percentage of inhibition was calculated by
- reading absorbance at 620 nm (Turchi et al., 2013).
- Tolerance to phenol and NaCl was determined by growing the bacterial suspension (100 µl) in 5
- 220 ml LB broth containing various concentrations of phenol (0.1%, 0.4%, and 0.6%), and NaCl
- 221 (3%, and 6%) for 24 h at 37 °C (Shehata et al., 2016, Qureshi et al., 2020). LB broth without
- phenol or NaCl was used as a control. The survival rate was determined by reading OD values
- spectroscopically at intervals of 0, 1, 2, 3, 4 and 24 h and the inhibition percentage of was
- calculated by using the following formula:

Inhibition % = 
$$[(OD control - OD test)/OD control] \times 100$$
 (3)

- Experiments were conducted in triplicate and results were expressed as mean + standard
- deviation. Statistical analysis was performed using a Paired two-sample t-test to compare the
- changes in stress tolerance of the isolate with the corresponding control. P value < 0.05 was
- 229 considered statistically significant.
- 230 2.5.3. Adhesion assays
- 231 *2.5.3.1. Auto Aggregation*
- The auto aggregation ability of the FCW1 strain was tested as per Li et al. (2020). The overnight
- bacterial culture was centrifuged at 5,000 rpm for 10 min at 4°C and washed twice with PBS
- buffer. The pellets were resuspended in 5 ml PBS buffer to an  $OD_{620nm}$  of  $0.33 \pm 0.015$ , vortexed
- 235 for 10 S, and incubated for 24 h at 37°C. The upper suspension was checked for absorbance at

- 236 600 nm at 0, 1, 2, 3, 4, 8 and 24 h. The auto aggregation percentage was measured using the
- 237 formula:

Auto aggregation 
$$\% = (1 - At/A0) \times 100$$
 (4)

- Where, At represents the absorbance for a particular incubation time and A0 is the absorbance at
- 240 Oh incubation.
- 241 2.5.3.2. Cell Surface Hydrophobicity
- The hydrophobicity of FCW1 was assayed using the microbial cell adhesion to solvents (MATS)
- method (Dlamini et al., 2018). The overnight bacterial cells were harvested by centrifugation at
- 5,000 rpm at 4°C for 10 min, washed twice with PBS, and resuspended in 3 ml PBS buffer,
- followed by absorbance (A0) measurement at 600 nm. The cell suspension was added with 1 ml
- of solvents (hexane, chloroform and ethyl acetate), vortexed for 1 min and incubated at 37°C for
- 1 h for the separation of aqueous and organic phases. The absorbance (A1) of the aqueous phase
- 248 (1 ml) was measured, and the percentage of hydrophobicity was calculated using the following
- 249 formula:
- % cell surface hydrophobicity =  $(1 A1/A0) \times 100$  (5)
- Isolate with 50% and above MATS was considered strong hydrophobic (Garc'ıa-Hern'andez et
- 252 al., 2016).
- 253 2.5.3.3. Biofilm formation assay
- A good biofilm-forming bacterium can adhere and colonize the intestinal epithelial cells. The
- biofilm formation ability of the isolate FCW1 was determined as in Zayed et al. (2021) with
- 256 modifications. Briefly, the LB broth in test tubes was inoculated with FCW1 isolate and
- incubated for 48 h at 37°C with shaking at 120 rpm. After incubation, the culture was decanted
- and the tubes were washed with PBS buffer, which was then air-dried at room temperature. The
- 259 tubes were then stained with 3ml of 0.1% crystal violet, kept for 30 min, and washed with
- distilled water thrice. The appearance of the violet colour on the test tube walls indicates biofilm
- formation. The stain was released with ethanol and read OD at 595nm. Cut-off values were
- 262 calculated as the mean OD of the negative controls (ODc). The biofilm production was
- determined based on the following classification:  $OD \le ODc = \text{non-biofilm producer}$ ; ODc < OD
- $\leq 2 \times ODc = \text{weak biofilm producer}, 2 \times ODc \leq OD \leq 4 \times ODc = \text{moderate biofilm producer}, and$
- 265 OD  $> 4 \times ODc = strong biofilm producer$ .
- 266 2.5.4. Enzymatic activity

The isolate FCW1 was inoculated on an appropriate agar medium to detect the amylase, 267 protease, lipase (Cappuccino and Sherman, 1983) and cellulase activity (Kasana et al., 2008). To 268 detect amylase activity, a starch agar plate was inoculated with FCW1 and incubated for 24 h at 269 37°C. After adding Gram's iodine solution to the culture, the plate was observed for a clear zone 270 around the colonies. The cellulolytic activity was detected by streaking the isolate on carboxy 271 methyl cellulose agar, and the zone of clearance was recorded after 24 h incubation. Tributyrin 272 agar was used for the detection of lipolytic activity. A cloudy zone around the colony shows 273 lipolytic activity. 274

## 275 2.6. *In vivo* Bio-safety assay

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Probiotic candidates were tested for pathogenicity and bio safety using the Immersion assay, 276 which involves challenging zebra fish and looking at their health conditions. The animals were 277 handled according to Bharathidasan University's (Tamil Nadu, India) Institution Animal Ethics 278 Committee guidelines (BDU/IAEC/P11/2021). Experiments were carried out in triplicates in 279 aquaria set at 25-30°C and a lighting schedule of LD 12:12. Ten Danio rario (mean body weight: 280 0.182g) were randomly distributed among the aquariums after an initial 10-day acclimation 281 282 period. The strain FCW1 was inoculated into MRS broth and incubated for 24 h. Cells were extracted by centrifugation at 5000 rpm for 10 min at 4°C in a cooling centrifuge. Sterile PBS 283 was used twice to wash and resuspend the cells. The concentrations of 10<sup>5</sup>, 10<sup>6</sup>, and 10<sup>7</sup>CFU/ml 284 were added to the fish tanks. Control aquariums were added with sterile PBS solution. Fish were 285 286 fed 5% of their body weight twice daily (35% protein). Symptoms, abnormalities, and mortality rates were measured at the end of the experiment. 287

## 2.7. Whole Genome Sequencing of S. gallinarum FCW1

Purified S. gallinarumFCW1 culture broth was used for further DNA extraction usingQIAamp 289 290 DNA Mini Kit (QiagenInc, USA) according to manufacturer's instructions. Illumina sequencing 291 libraries were prepared and genome was sequenced on the Illumina NextSeq 2500 platform using 2 × 250 paired-end libraries. The quality control of raw reads was carried out in FastQC version 292 0.11.9 (Andrews, 2010) and low quality reads were trimmed using Trimmomatic (ver 0.35), with 293 a phred cutoff of Q20 (Bolger et al., 2014). After quality filtering, the primary de novo assembly 294 295 of the reads was performed using SPAdes genome assembler (ver 3.10) (Nurk et al., 2013). Genome annotation was performed using the National Center for Biotechnology Information 296 297 (NCBI) Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) (Tatusova et al., 2013).

- 298 Overall genome relatedness index, measured as the Orthologous Average Nucleotide Identity
- 299 (OrthoANI), was calculated using the OrthoANI application of EzBioCloud (Lee et al., 2016).
- 300 The genes of probiotic characteristics were retrieved manually from the annotated genome and
- 301 confirmed using BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against a non-redundant
- database of National Center for Biotechnology Information (NCBI). The Clusters of Orthologous
- 303 Groups of proteins (COG) functional categories of protein coding genes were done using
- database WebMGA (http://weizhong-lab.ucsd.edu/webMGA/server/cog/). Genes involved in
- secondary metabolites biosynthesis were detected by Antismash 6.0 (Blin et al., 2021).
- 306 Antibiotic resistance gene was detected by PATRIC's AMR classifier.

## 307 Strain Deposition and Complete Genome Sequence Data Accession Number

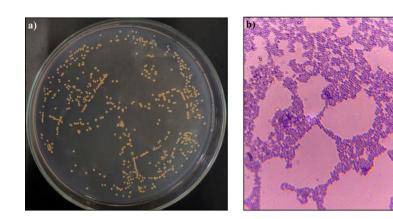
- The whole genome sequence data of *S. gallinarum* FCW1 has been deposited at GenBank under
- the accession number CP086207. The strain has been deposited at National Centre for Microbial
- 310 Resource, India (Accession no. MCC 4687).

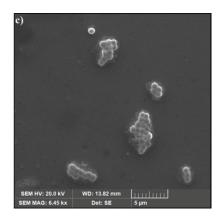
## 311 **3.** Results

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## 3.1. Isolation and screening of GCC+CNS strains

- 313 A total of seven morphologically discrete colonies were isolated from the naturally fermented
- coconut water and tested for GCC+CNS. Of these, only one isolate (FCW1) with Gram-positive,
- 315 cocci-shaped (Fig. 1), catalase-positive and coagulase-negative properties was selected for
- further analysis. Scanning electron microscopic (SEM) analysis of *S. gallinarum* FCW1 confirms
- 317 its spherical shape. The biochemical characterization revealed that isolate FCW1 does not utilize
- 318 citrate as a carbon source, but could reduce the nitrate in nitrite. The Carbohydrate fermentation
- pattern showed that the isolate FCW1 was able to ferment glucose, sucrose, mannitol, adonitol,
- arabinose, and rhamnose, whereas lactose and sorbitol were not utilized (Table 1). According to
- 321 the phenotypic and biochemical characterization, the isolate FCW1 was confirmed as
- 322 GCC<sup>+</sup>CNS.





**Fig. 1**. Morphological view of GCC<sup>+</sup>CNS strain FCW1. (a) Colony morphology of FCW1 on nutrient agar after 24 h incubation at 37°C, (b) Cell morphology of FCW1 based on Gram staining under the light microscope (100X magnification) and (c) SEM imaging of FCW1 with HV 20 kv and 6.45 kx magnification.

Table 1. Phenotypic and Biochemical Characterization of Strain FCW1

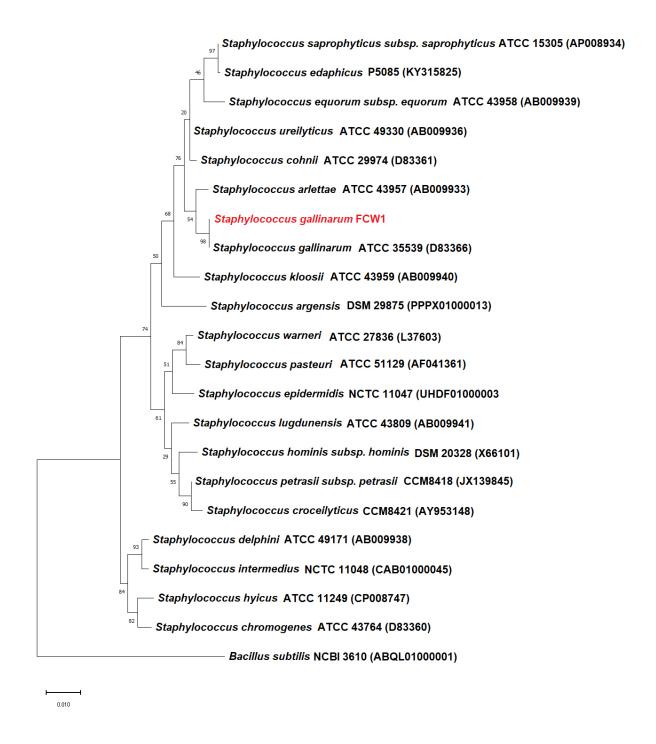
Tests	FCW1		
Gram Staining	$G^+$		
Shape	Cocci		
Motility	Non-motile		
Indole	Negative		
MR	Positive		
VP	Negative		
Citrate	Negative		
TSI	K/K		
H <sub>2</sub> S production	Negative		
Nitrate Reduction test	Positive		
Urease	Positive		
Catalase	Positive		
Oxidase	Positive		
Coagulase	Negative		
Carbohydrate Fermentation			
Glucose	Positive		
Adonitol	Positive		

Arabinose	Positive
Lactose	Negative
Sorbitol	Negative
Mannitol	Positive
Rhamnose	Positive
Sucrose	Positive

G<sup>+</sup> indicates Gram-positive, MR indicates methyl red, VP indicates Voges Proskauer

## 3.2. Molecular Identification of FCW1 strain

Based on the 16S rRNA sequencing comparison with NCBI BlastN as well as Ezbiocloud blast, the FCW1 was identified as *S. gallinarum*. Using MEGA X software, a maximum likelihood phylogenetic tree based on 16S rRNA gene sequences, including 1000 bootstrap replications, was built to reveal the exact phylogenetic position of *S. gallinarum* FCW1 within 20 *Staphylococcus* species(Fig. 2). Moreover, we have used *Bacillus subtilis* as a outgroup . *S. gallinarum* FCW1 is closely related to *S. gallinarum* D35539/ATCC 35539, isolated from chicken skin (Devriese et al., 1983).



**Fig. 2.** Phylogenetic tree of *S. gallinarum* FCW1 based on Ezbiocloud database using MEGA X software. This maximum likelihood tree illustrates the phylogenetic relationships of *S. gallinarum* FCW1 and closely-related strains of the genus *Staphylococcus*. 1000 bootstrap replications were used to generate the tree and *Bacillus subtilis* strain NCBI 3610 was used as an out-group. The percentage of tree in which the associated taxa clustered together is shown next

to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Rate variations were uniform per sites. This analysis involved 22 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding.

## In vitro Evaluation of Probiotic Properties

## 3.2.1. Safety Assessment

### 3.2.1.1. **Antagonistic activity**

- Antimicrobial activities of isolates are a suitable way of screening potential probiotic bacteria.
- 351 The isolate FCW1 showed moderate inhibition of the growth of E. coli, B. cereus, and weak
- inhibition towards *K. pneumoniae* (Table 2).

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**Table 2.** Antagonistic activity of *S. gallinarum* FCW1 against common human pathogens

Pathogens	Zone of Inhibition (mm)	_
Staphylococcus aureus	* -	_
Streptococcus spp.	*	
Escherichia coli	$15 \pm 0.71$	
Enterococcus faecalis	*	
Klebsiella pneumoniae	$6\pm0$	
Bacillus cereus	$13\pm0.71$	

The zone diameter values (mm) are the average of two experiments and  $\pm$  indicates the standard deviation from the means; \*No zone of inhibition

## 3.2.1.2. Antibiotic Susceptibility Profiles

Table 3 shows the antibiotic susceptibility profile of FCW1 using the disc diffusion method. Based on the findings, isolate FCW1 was susceptible to all the selected antibiotics except erythromycin (intermediated susceptible) and penicillin (resistant).

Table 3. Antibiotic Susceptibility Profile of Strain FCW1

Class	Antibiotics	Disc Content	Inhibitio n Zone (mm)*	Susceptibili ty Profile	CLSI Susceptibility breakpoints
Penicillin	Penicillin (P)	10 units	$20\pm0$	Resistant	≥29
Aminoglycosides	Gentamycin (GEN)	10 mcg	$24 \pm 1$	Susceptible	≥15
Tetracycline	Tetracycline (TE)	30 mcg	$26 \pm 1$	Susceptible	≥19
Diaminopyrimidi	Trimethoprim (TR)	5 mcg	$29 \pm 1$	Susceptible	≥16
nes Macrolides	Erythromycin (E)	15 mcg	$20\pm1$	Intermediate	≥23
Fluoroquinolones	Ciprofloxacin (CIP)	5 mcg	$31 \pm 1$	Susceptible	≥21
Chloramphenicol	Chloramphenicol (C)	30 mcg	$26 \pm 1$	Susceptible	≥18
Quinolone	Norfloxacin (NX)	10 mcg	$19 \pm 1$	Susceptible	≥17

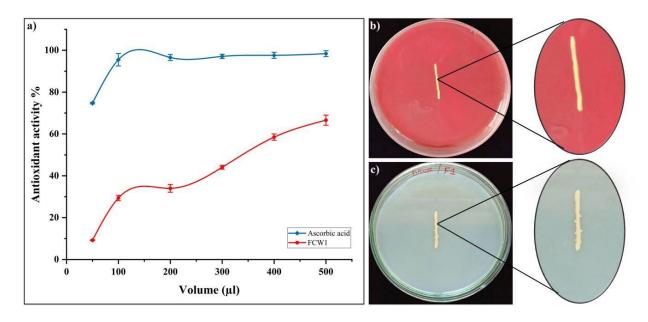
<sup>\*</sup>The zone diameter values (mm) are the average of two experiments and ± indicates the standard deviation from the means

## 3.2.1.3. Antioxidant Assay by DPPH Method

An *in vitro* antioxidant activity of cell-free supernatants FCW1 is shown in Fig. 3a. The DPPH radical scavenging activity increased from  $9.22 \pm 0.22\%$  to  $66.57 \pm 2.43\%$  with increasing volume of CFS from 50 to 500  $\mu$ L. The radical scavenging activity percentage of ascorbic acid was  $74.73 \pm 0.39\%$  at a concentration of  $50 \mu$ g/L and  $98.4 \pm 1.4\%$  at  $500 \mu$ g/L. Various secondary metabolites, peptides, phenolic compounds, etc., may contribute to the radical scavenging activity.

## 3.2.1.4. Haemolytic and DNase assay

After 48 h incubation, strain FCW1 showed  $\alpha$  haemolysis or no haemolysis on blood agar, indicating its weak or null ability to lyse blood cells (Fig. 3b). The strain demonstrated no zone of inhibition on DNase agar also indicates its safety for probiotic usage (Fig. 3c).



**Fig. 3**. Antioxidant activity and Safety assessment of Strain FCW1. a) DPPH radical scavenging activity of strain FCW1. Ascorbic acid used as positive control. The data were expressed as mean  $\pm$  SD (n=3) b) Hemolytic activity of FCW1 shows  $\alpha$  hemolysis on blood agar after 48 h incubation (zoomed imaged on right), c) DNase activity of FCW1 shows no pink or clear zone of inhibition around the colonies on DNase agar after 48 h incubation indicates no DNase enzyme activity (zoomed imaged on right).

## 3.2.2. Tolerance to different stress conditions

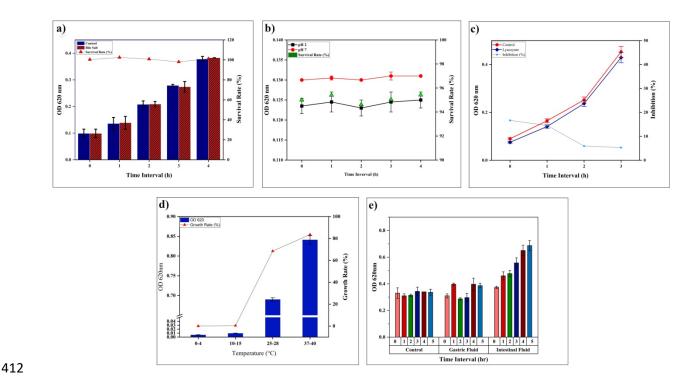
The strain FCW1 was resistant to 0.3% of bile salt (P > 0.05) and was viable even after 4 h incubation (Fig. 4a). In the presence of bile salt, FCW1 showed 101.15% survivability when compared to the control.

The isolate FCW1 tolerated to pH 2, which was be comparable to pH 7 (Fig. 4b) and showed stable growth (P > 0.05) after 4 h incubation without any significant loss of viability. The survival rate was calculated as 99.65%.

The lysozyme had less inhibitory effect on strain FCW1 (Fig. 4c) and significantly grew well in the presence of lysozyme (P < 0.01), with viable cell count 8.32 log CFU/ml after 3 h incubation, while the control having 9.04 log CFU/ml viable cells.

The growth performance of isolate FCW1 at different temperatures was shown in Fig. 4d. At 0-4 and 10-15°C the strain showed no or poor growth. However, the cells were viable after 24 h

- incubation. The best growth was seen at 37-40°C when compared to 25-28°C (P > 0.05). Based
- on the results, the optimum temperature for FCW1 strain is 37°C.
- 396 The preliminary analysis of the isolate's tolerance to artificial gastric fluid and intestinal fluid
- 397 was shown in Fig. 4e. The isolate showed excellent growth in the gastric and intestinal
- 398 environments. The bacterial inoculation to gastric juice results in the rapid bacterial division
- during the 1<sup>st</sup> hour, and then growth rates drop in the 2<sup>nd</sup> hour, which then gradually increases (P
- 400 > 0.05). The survival rate after 5 h exposure to gastric and intestinal fluid (P < 0.005) was
- 401 estimated as >100% compared to the control.
- The ability of the FCW1 isolate to endure phenolic conditions was assayed in the presence of
- 403 0.1, 0.4 and 0.6% phenol. The bacterial growth is highly affected by 0.4% and 0.6% of phenol at
- 3 h of exposure with respect to corresponding control (P < 0.05). At these higher concentrations,
- 405 the inhibition rates at 3 h and 24 h are similar. However, the bacteria could grow well at 0.1%
- 406 phenol (P > 0.05). The percentage of inhibition is given in Table 4.
- NaCl is an inhibitory substance which prevents the growth of probiotic bacteria. The NaCl
- 408 tolerance was performed at 3% and 6% NaCl concentrations. The isolate FCW1 was able to
- grow at 3 and 6% NaCl concentrations (P < 0.05) and the inhibition percentage was given in
- 410 Table 4.



**Fig. 4**. Strain FCW1 tolerance to different stress conditions. (a) The growth of FCW1 was remained unaffected in the presence of 0.3 % bile salt (P > 0.05) when compared to control, (b) FCW1 showed stable growth at pH 2 and pH 7 (P > 0.05), (c) Significant growth was observed in the presence of lysozyme (100 mg/l) with respect to corresponding control (P < 0.01), (d) growth at different temperatures shows 37°C is the optimum temperature for FCW1, (e) FCW1 showed insignificant growth (P > 0.05) in the presence of gastric and highly significant (P < 0.005) growth in the presence of intestinal fluid tolerance.

**Table 4**. Growth performance of FCW1 strain in the presence of different concentrations of NaCl and phenol.

Incubation		*Growth Inh			
time (h)	3% NaCl <sup>a</sup>	6% NaCl <sup>a</sup>	0.1% Phenol <sup>b</sup>	0.4% Phenol <sup>a</sup>	0.6% Phenol <sup>a</sup>
0	0	2.78	0	2.78	5.56
1	14.54	21.81	1.78	20	32.72

2	13.15	27.5	1.72	46.25	56.25
3	25.53	45.74	1.56	77.65	89.36
4	26.81	47.49	27.56	74.30	82.12
24	21.99	26.97	28.23	77.59	89.21

<sup>\*</sup>The values (Inhibition %) are the mean of two experiments, 'a' indicates Significant (P < 0.05)

## 424 3.2.3. Adhesion assays

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## 3.2.3.1. Auto Aggregation ability

As shown in Fig. 5a, the auto aggregation percentage of isolate FCW1 increased as time

progressed. Isolate FCW1 had a very low initial auto aggregation percentage of 8.77±7.38%,

which increased gradually over time and reached 95.04±1.6% after 24 h incubation.

## 429 3.2.3.2. Cell Surface Hydrophobicity

430 The ability of probiotics to adhere to intestinal mucosal cells is associated with their

hydrophobicity. In this study, the isolate FCW1 have moderate to strong adhesion capacity to all

the tested solvents. The isolate showed high adherence to chloroform (87.31%), 57.62%

hydrophobicity to hexane, and moderate adherence to ethyl acetate (32.16%) (Fig. 5b).

## 3.2.3.3. Biofilm formation assay

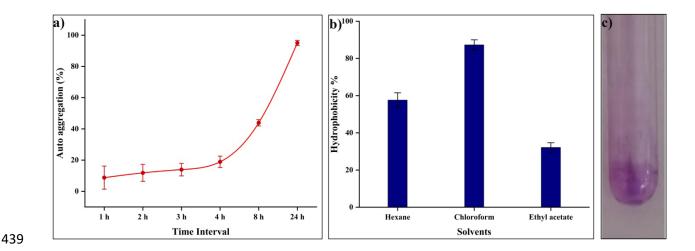
The biofilm formation property promotes adherence and colonization of bacteria on the host

intestinal epithelium and thus prevents the colonization and reproduction of pathogens (Salas-

Jara et al., 2016). The isolate FCW1 was found positive for biofilm formation (Fig. 5c) and based

on the OD value, it was found that the isolate exhibited modest biofilm formation.

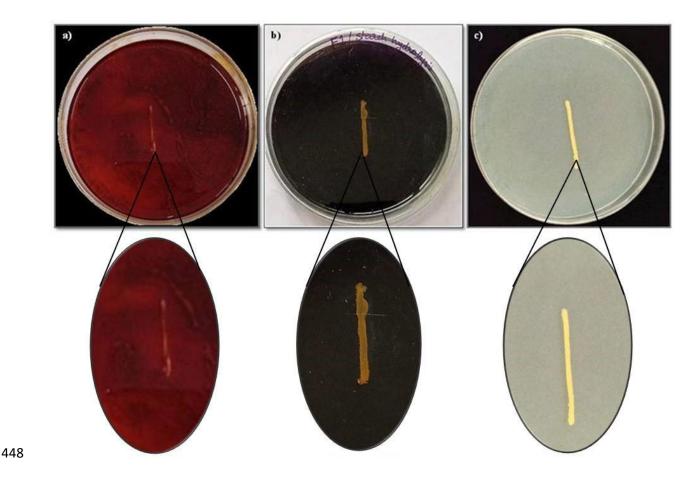
and 'b' indicates Not significant (P > 0.05) with corresponding controls



**Fig. 5**. Adhesion assays of strain FCW1. a) Auto aggregation. The auto aggregation % of FCW1 increased over time b) Hydrophobicity ability. FCW1 showed high hydrophobicity to chloroform and hexane whereas moderate to ethyl acetate. Error bar represents the mean value of three independent experiments  $\pm$  SD, c) Stained tubes showing biofilm formation ability of FCW1.

## **Enzymatic activity**

The enzyme-producing property of isolate FCW1 was tested by inoculating on specific media. The results showed that the isolate failed to produce the enzymes amylase, cellulase and lipase (Fig. 6).



**Fig. 6**. Metabolic capacities through enzymatic activity of Isolate FCW1. No clear zone around the colonies indicates lack of a) cellulase, b) amylase, c) lipase activity. The zoomed image shows absence of enzymatic activity.

## 3.3. *In vivo* bio safety assay

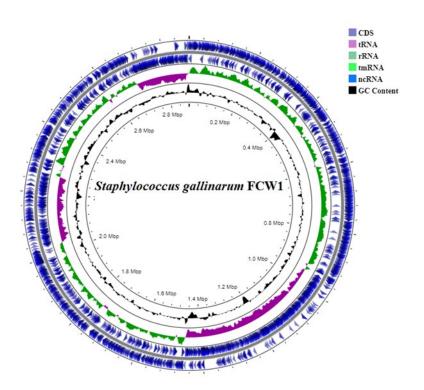
The *in vivo* bio safety assay revealed no behavioural changes, disease symptoms such as external lesions, edema, haemorrhage, loss of scales or mucus and mortalities in either experimental or control groups after 10 days.

## 3.4. Whole Genome Sequencing

#### 3.4.1. Genomic features of FCW1 strain

Based on the *in vitro* probiotic characteristics, the whole genome sequencing of strain FCW1 was performed to evaluate the probiotic properties at the genomic level. The complete genome of *S. gallinarum* FCW1 consisted of 2,880,305 nucleotides with a GC content of 33.23%. The circular genome map and its general genomic features are shown in Fig. 7 and Table 5. Plasmids

were not present in the FCW1 genome. The assembled reads consist of a single contig. The genome comprises a total of 2770 genes and 2708 protein-coding genes (CDS). Among the predicted CDS (an average length of 897.45bp), 1792 (66.17%) genes were functional genes, and 916 (33.83%) genes were hypothetical genes. The genome contains 62 RNA genes, including 57 tRNAs, 4 rRNAs and 1 tmRNA. It also contains one CRISPR repeat and 11 pseudogenes. The identified 57 tRNAs representing 19 amino acids: Arg (4), Asn (3), Ser (5), Glu (2), Gly (7), His (2), Phe (2), Asp (3), Met (4), Leu (5), Csy (1), Gln (2), Trp (1), Tyr (1), Thr (3), Ala (2), Pro (2), Lys (3), and Val (2).



**Fig. 7**. Whole genome sequencing of *S. gallinarum* FCW1. The circular graphical representation refers to the genome annotations of FCW1 strain. This includes, from outer to inner rings, the contigs, CDS on the forward strand, CDS on the reverse strand, RNA genes, and GC content. The circular genome was generated using CG server.

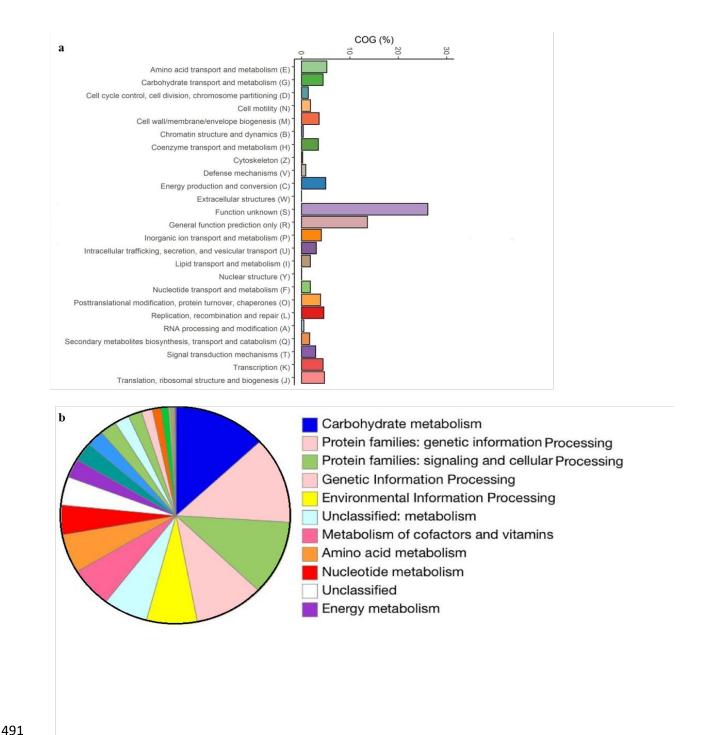
 Table 5. Genomic Statistics of FCW1 genome

Species Attribute	
Genome size (bp)	2,880,305

G + C %	33.23%
Contigs	1
Scaffolds	1
Total genes	2770
Protein coding genes	2708
RNA genes	62
Pseudo genes	11
CRISPR repeats	1

#### 3.4.2. Functional classification

The protein-coding genes involved in the major metabolic pathways were assigned to COG categories (Wu et al., 2011) (Fig. 8a). In the FCW1 genome, major COGs were assigned into the following categories i) amino acid transport and metabolism (5.23%), ii) energy production and conversion (5%), iii) translation, ribosomal structure and biogenesis (4.75%), iv) replication, recombination and repair (4.61%), v) transcription (4.47 %), vi) carbohydrate transport and metabolism (4.45%), vii) Inorganic ion transport and metabolism (4.11%), viii) posttranslational modification, protein turnover, chaperones (3,93%), ix) cell wall/membrane/envelope biogenesis (3.64%), x) coenzyme transport and metabolism (3.47%). The summarized COG classification is included in Supplementary Table 1. Based on the KEGG annotation results, a total of 1684 protein families were mapped in the KEGG database. This result reveals a higher number of protein families classified into carbohydrate metabolism, genetic information processing, signaling and cellular processing, environmental information processing, metabolism of cofactors and vitamins and amino acid metabolism (Fig. 8b).



**Fig. 8**. a) Number of genes associated with general COG functional categories b) Analysis of KEGG distribution in *S. gallinarum* FCW1 using Blast KOALA algorithm.

## 3.4.3. Genome analysis for probiotic traits

- We performed comprehensive genomic data analysis to evaluate stains FCW1's probiotic
- 496 potency. The relevant genes, their function and locus tag in the genome are listed in
- 497 Supplementary Table 2.

## 3.4.3.1. Genes encoding surface proteins

- Genome analysis of FCW1 reveals the presence of different surface protein-encoding genes. For
- example, gene encoding sortase-dependent surface proteins (srtA) has been identified at Locus
- tag SG 01663. The adhesion gene (fbp), which encodes fructose-1,6-bisphosphatase class 3, has
- also been found at locus tag SG 01689. The pdhABCD genes (SG 00617 to SG 00620)
- 503 encoding pyruvate dehydrogenase components are also responsible for fibronectin binding
- 504 (Vastano et al., 2014). In addition, the analysis of the FCW1 genome reveals the presence of
- 505 gndA gene encoding 6-phosphogluconate dehydrogenase, decarboxylating proteins (Locus tag
- SG 01015) and lactate dehydrogenase (ldh1 and ldhD; Locus tag: SG 01280 and SG 01600) are
- responsible for the bacterial attachment to epithelial mucin (Qureshi et al., 2020).

## 508 3.4.3.2. Mucin secreting genes

- The adh genes clusters at locus tag: SG\_02307, SG\_02050, and SG\_00095, encode alcohol
- dehydrogenase, which is involved in the adhesion and mucin secretion. The genes responsible
- for the phosphotransferase (PTS) system and ABC transporters (SG 02068; SG 02485) will get
- induced in the presence of mucin and help the bacteria to colonize the human gastrointestinal
- 513 tract.

#### 514 3.4.3.3. Stress related genes

- 515 The probiotic bacteria have to face a harsh environment in the stomach and intestine. The
- presence of Na(+)/H(+) antiporter subunit ABCDEF, which is encoded by *nhaC* gene
- 517 (SG 01628) and *mrpABCDEF* genes (SG 00161 to SG 00166 and SG 00482 to SG 00488)
- play a major role in Na<sup>+</sup>-resistance, pH homeostasis, and osmoregulation. The genes *nhaP2* and
- 519 nhaK (SG 01688 and SG 00169) encoding Na<sup>+</sup>, K<sup>+</sup>, Li<sup>+</sup>, Rb<sup>+</sup>/H<sup>+</sup> antiporters are also present,
- which help to survive in acidic conditions (Fujisawa et al., 2005). Heat shock proteins such as
- 521 dnaK, groS, groL, and grpE are present at locus tag SG 01085, SG 02502, SG 02503 and
- SG 01086. Genes encoding ATP-dependent *clp* protease (*clpBCPX*; Locus tag: SG 00513;
- 523 SG\_00008; SG\_00378; SG\_01193) are also expressed by acid and bile stress and refold or

degrade the denatured proteins (Ferreira et al., 2013). The main function of vveA gene encoding 524 asparate proton symporter (SG 01520) and bsaA gene encoding glutathione peroxidase 525 (SG 00822) is to protect the bacterium from the acid stress response. Genes encoding DNA 526 repair proteins such as uvrABC system proteins (Locus tag: SG 00297; SG 00296; SG 00665), 527 ATP-dependent helicase/nuclease subunit (addAB genes at Locus tag: SG 00504, SG 00505), 528 ATP-dependent DNA helicase (recDGO and pcrA at Locus tag: SG 00739, SG 01120, 529 SG 00257, SG 02538), DEAD-box ATP-dependent RNA helicase (cshABat SG 00994; 530 SG 01063), replicative DNA helicase (dnaC at Locus tag: SG 02179) are present which 531 upregulated under acid stress and involved in DNA repair mechanisms (Jin et al., 2012; Petit et 532 al., 1998; Lehnik-Habrink et al., 2013). The gene luxS encoding S-ribosylhomocysteine lyase 533 located at Locus tag: SG 02650 contributes acid stress resistance through quorum sensing. The 534 535 presence of fab genes (fab BDFHIGZ) responsible for fatty acids and cell envelop biosynthesis will have a specific role in acid stress tolerance. The bsh genes (bshABC) at locus tag SG 00973, 536 SG 00107 and SG 00686 encode the enzyme bile salt hydrolase, which deconjugates bile salts 537 like glycine and taurine. Another gene involved in the deconjugation of bile salt is betA gene 538 539 encoding oxygen-dependent choline dehydrogenase is present at SG 01510.

## 3.4.3.4. Genes involved in Exopolysaccharide synthesis

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The glm genes involved in the biosynthesis of UDP-GlcNAc, the building blocks of 541 peptidoglycan, glucosamine-6-phosphate synthase (glmS), phosphoglucosamine mutase (glmM), 542 and N-acetylglucosamine-1-phosphate uridyltransferase (glmU) are present at Locus tag: 543 SG 02672, SG 02677 SG 02714. and The dapAgenes encoding 4-hydroxy-544 tetrahydrodipicolinate synthase present at Locus tag SG 00908, SG 00073, and SG 02241 are 545 also involved in peptidoglycan biosynthesis. Acetyl-CoA carboxylase genes (SG 01216, 546 SG 01032, SG 01108, SG 01107, SG 01217) encode biotin carboxyl carrier proteins which 547 participate in lipid metabolism and fatty acids biosynthesis. The eps genes encode putative 548 549 regulatory proteins such as putative glycosyl transferase epsD (SG 00046), putative sugar transferase epsL (SG 00047), putative acetyl transferase epsM(SG 00048), and putative 550 pyridoxal phosphate-dependent amino transferase epsN (SG 00049) are involved in the 551 biosynthesis of exopolysaccharide and lipoteichoic acids. The gene ftsW is the SEDS-family 552 553 protein putative peptidoglycan glycosyltransferase located at SG 00638 and SG 02369 are also

554 involved in cell wall or peptidoglycan biosynthesis. The dlt genes (dltACD) encode D-alanine-D-

alanyl carrier protein ligase at SG 00468, SG 00470, and SG 00471, involved in the

556 lipoteichoic acid biosynthetic process.

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## 3.4.3.5. Genes involved in the production of nutrients and other beneficial processes

558 The presence of genes involved in the production of vitamins, Biotin, and other cofactors synthesis evince the ability of probiotics to produce bioactive compounds. The vitamin B12 559 import ATP-binding protein encoding gene btuDF are present at locus tag: SG\_00258, 560 SG 00928, SG 01061, SG 02367, SG 02511, SG 02513 and SG 00148, which aids in the In 561 situ production of important nutrients. The genes (bioBDY) responsible for Biotin synthase 562 563 (SG 01536, SG 02354) and Biotin transporter (SG 01901) play a major role in biotin and cofactor biosynthesis. The gene responsible for molybdenum cofactor biosynthesis such as moeA 564 565 encoding molybdopterin molybdenum transferase (SG 01915), moaA encoding GTP 3',8-cyclase (SG 01920), moaB encoding molybdenum cofactor biosynthesis protein B (SG 01913), moaC 566 567 encoding cyclicpyranopterin monophosphate synthase (SG 01914), moaD molybdopterin synthase sulfur carrier subunit (SG 01918), moaE encoding molybdopterin 568 569 synthase catalytic subunit (SG 01917) and mobA gene for putative molybdenum cofactor guanylyl transferase (SG 01919). The catabolite control protein A gene (ccpA) is present at 570 571 locus tag SG 00866, SG 01254, which plays a major role in cholesterol reduction. The gene

### 573 3.4.3.6. Disease-specific genes

Our bacterial genome contained a unique frc gene encoding Formyl-CoA: oxalate CoA-574 transferase protein at Locus tag SG 02283. This gene has been associated with oxalate 575 metabolism. Additionally, an ammonium transporter gene (nrgA) is present at SG 02455, which 576 577 will facilitate passive ammonium uptake in low pH environments. This genome also contains 578 genes involved in sulfate reduction, such as sulfate adenylyl transferase encoding gene (sat) at locus tag SG 02342, and genes for phosphoadenosine phosphosulfate reductase (cysC, cysH) at 579 580 locus tags SG 02343 and SG 02335. Phosphotransacetylase (pta 1, pta 2) and acetate kinases (ack) involved in acetate metabolism are also found at locus tags SG 00128, SG 01023, and 581 582 SG 01229, respectively.

ccpN is a transcriptional repressor (SG 01069) that controls the carbon catabolite repression.

#### 3.4.4. Secondary metabolite identification

According to the antiSMASH tool, seven metabolites' regions were detected by FCW1 secondary metabolites (Table 6). These include a biosynthetic siderophore cluster with high overall similarity to staphyloferrin A and two T<sub>3</sub>PKS (Type III polyketide synthases) and NRPS (Non-ribosomal peptide synthetases) clusters resembling capsular polysaccharide and rhizocticin A, respectively.

**Table 6**. Genes responsible for Secondary metabolites by AntiSMASH tool

			Metabolit		Similarit	Function
Region	Type	Location	es	Gene cluster	y %	
Region 1	<u>Terpene</u>	902,072- 921,889	-	-	-	-
Region 2	<u>Terpene</u>	1,461,570- 1,481,615	-	-	-	-
Region 3	T <sub>3</sub> PKS	1,624,274- 1,665,443	Capsular polysacchari de	Exopolysacc haride	3%	Antimicrob ial activity
Region 4	Siderophore	2,012,549- 2,027,535	Staphyloferrin A	Other:Non- NRP siderophore	100%	Antimicrob ial activity
Region 5	<u>Terpene</u>	2,201,938- 2,222,819	-	-	-	-
Region 6	<u>NRPS</u>	2,451,92- 2,508,725	Rhizocticin A	Other	6%	Antifungal activity
Region 7	Cyclic- lactone- autoinducer	2,600,285- 2,620,994	-	-	-	-

"-" indicates not available.

## 3.4.5. Antibiotic-resistant genes

The PATRIC's AMR classifier module revealed that *S. gallinarum* FCW1 contained 46 resistant genes categorized into different strategies such as Antibiotic target in susceptible species, Antibiotic target modifying enzymes, Antibiotic resistance gene cluster, cassette, or operon, Efflux pump conferring antibiotic resistance, Protein altering cell wall charge conferring antibiotic resistance, Regulator modulating expression of antibiotic resistance genes (Table 7). The genome contained *blaZ* gene responsible for penicillin which supports our *in vitro* antibiotic

susceptibility results. The list of genes responsible for antibiotic resistance based on PATRIC annotation is provided in the supplementary Table 3.

 Table 7. Antibiotic-Resistant genes present in FCW1 genome based on PATRIC genome

 annotation

AMR Mechanisms	Genes
Antibiotic target in susceptible species	alr, ddl, EF-G, EF-Tu, folA, dfr, folP, gyrA, gyrB, inhA, fabI, Iso-tRNA, kasA, murA, rho, rpoB, rpoC, S10p, S12p
Antibiotic target modifying enzymes	blaZ
Antibiotic resistance gene cluster, cassette,or operon	tcaB, tcaB2, tcaR
Efflux pump conferring antibiotic resistance	norA, ykkCD
Gene conferring resistance via absence	gidB
Protein altering cell wall charge conferring antibiotic resistance	gdpD, mprF, pgsA
Regulator modulating expression of antibiotic resistance genes	bceR, bceS, liaF, liaR, liaS

#### 4. Discussion

Fermented food products possess many health benefits, including antioxidant, anti-microbial, anti-inflammatory, anti-diabetic, and anti-cancer properties. They are the large reservoir of beneficial microorganisms or starter cultures which enhance the sensory properties and safety of fermented foods by accelerating the acidification process of their matrix and producing secondary metabolites. Food-derived CNS species are often used as starter cultures since they are non-pathogenic and are native to fermented foods. CNS *S. gallinarum* is associated with Japanese fermented foods, like miso (Onda et al., 2003), and African alkaline fermented foods, like maari, dawadawa and soydawadawa (Parkouda et al., 2009). Considering the role of *S. gallinarum* in food fermentation, this study aimed to evaluate its safety and probiotic capabilities. In recent studies, CNS isolated from various fermented foods exhibited probiotic

characteristics (Khusro et al., 2017; Sung et al., 2010; Mangrolia et al., 2020). However, only limited information available on the safety of *S. gallinarum* and its probiotic properties.

In the current study, of the 7 bacterial isolates recovered from naturally fermented coconut water, only one was confirmed as GCC+CNS bacteria and was processed further. The isolate was susceptible to most of the antibiotics tested except penicillin, a crucial characteristic for probiotic selection. In the antagonistic study, the isolate showed activity against E. coli, K. pneumoniae, and B. cereus. This is due to the organic acids or secondary metabolites produced by the isolate, which suppress the pathogen growth (Kosin and Rakshit, 2006). Researchers have previously reported CNS's antagonistic role against Mycobacterium tuberculosis (Khusro et al., 2017), S. aureus (Sung et al., 2010), E. coli (Mangrolia et al., 2020), Salmonella spp. (Sathyabama et al., 2012). In addition, antioxidant properties were used to confirm the probiotic's potential and safety. The antioxidants protect cells from oxidative damage. FCW1 showed antioxidant activity in a concentration-dependent manner, which is in par with previous study by Khusra et al. (2017). Although hemolysins and DNase are major virulence factors, many other factors may contribute to the virulence of a strain (Yasmin et al., 2020; Somashekaraiah et al. 2019). The isolate FCW1 was found to be non-hemolytic and DNase negative, which indicates its nonpathogenic nature. Our findings were consistent with those of Somashekaraiah et al. (2019), who found that LAB isolated from naturally fermented coconut palm nectar lacked hemolytic and DNase activity. Um et al. (1996) also reported similar observations with Staphylococcus spp. isolated from fermented fish.

Probiotics ought to survive the harsh digestive environment for at least 3-5 h before reaching the colon, where they colonize and confer benefits. The high gastric pH, bile salt, and lysozyme pose extreme hindrances for probiotic microorganisms. In the present study, FCW1 showed a higher survival rate (99.65%) in gastric acidity (pH 2) even after 3 h of incubation. According to Borah et al. (2016), *Staphylococcus* spp. can survive up to pH 2, which supports our findings. The next obstacle for probiotics is bile salt tolerance, which is secreted by cholesterol catabolism. Probiotics must resist the high concentration of bile (0.3%) in the small intestine for at least 4 h. Despite being resistant to 0.3% bile salt, FCW1 grew well in the presence of bile salt. In a study, the researchers found that *Staphylococcus* spp. isolated from Slovak meat products, could tolerate 1% bile salt. (Simonova et al., 2006). In another study, Khusro et al. (2017) also

confirmed that six CNS from koozh could survive 0.5% bile salt. These reports support our findings that bile salt boosted FCW1's survivability (101.15%). Another critical property of probiotics is their ability to tolerate gastric and intestinal conditions, such as the presence of lysozyme, acid pH, pepsin, trypsin, and bile salt. We found that isolate FCW1 was remarkably resistant to pepsin and trypsin in gastric and intestinal fluids. As well, FCW1 showed a high level of resistance to lysozyme, indicating that it can tolerate lysozyme in saliva. Previously, Borah et al. (2016) and Kushra et al. (2017) reported similar results for Staphylococcus spp. in the presence of lysozyme. Phenol is a toxic metabolic byproduct of the deamination of aromatic amino acids by gut bacteria during digestion. At a low phenol concentration (0.1%), the isolate FCW1 remained viable even after 24 h of incubation, and the growth inhibition rate was 28.23%, while 0.4% and 0.6% of phenol caused a higher inhibition rate, 77.58% and 89.21%, respectively. A similar result was reported by Parlindungan et al. (2021), the plantarum, Pediococcusacidilactici and Lactobacillus strains *Lactobacillus* coryniformis exhibited a greater growth rate at 0.2% phenol and decreased at 0.5% phenol. They also found that some strains, such as Lactobacillus curvatus and Lactobacillus sakei exhibited a low growth rate even in the presence of 0.2% phenol. We found that FCW1 displayed a high tolerance level to 3% and 6% NaCl concentrations, with a growth inhibition rate of 21.99% and 26.97%. In addition, the strain showed considerable growth at 4°C, 15°C and 25°C, with 37°C being the optimum temperature. These findings coincide with a previous report by Qureshi et al. (2020). Probiotics should possess hydrophobicity and auto aggregation properties, which aid in adhesion and biofilm formation, thereby hindering enteropathogens (Ku et al., 2016). FCW1 exhibits high hydrophobicity to chloroform (87.31%) and hexane (57.62%) and moderate hydrophobicity to ethyl acetate (32.16%), indicating its ability to adhere to epithelial surfaces. The auto aggregation rate of FCW1 was  $95.04 \pm 1.6\%$  after 24 h incubation, and biofilm formation ability was moderate, indicative of potential probiotic activity.

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An *in-vivo* testing of strain FCW1 on zebrafish was conducted for safety evaluation and no mortality or disease symptoms were observed during the 10 days study period, confirming the non-pathogenic nature of the strain.

Genomics provides in-depth knowledge of bacteria's physiology, metabolism, functions, and ability to adapt to varying environmental conditions. Genome size and GC content may reflect

the bacteria's lifestyle and preferred environment (Merino et al., 2019). The isolate FCW1 has a genome size with 2,880,305 bp and a GC content of 33.23%. However, the absence of plasmids in FCW1 indicates it has a stable genome (Wang et al., 2021). FCW1 contains many cell surface proteins, including srtA and fbp genes, as well as genes encoding pyruvate dehydrogenase and lactate dehydrogenase, which contribute to adhesion, colonization, and biofilm formation. The sortase (srtA) cleaves the cell wall sorting signal molecule (LPXTG motif) between threonine and glycine and then covalently adhered to the cell wall peptidoglycan (Muñoz-Provencio et al., 2012). The fbp gene encoding cell surface protein binds to fibronectin, a glycoprotein of the extracellular matrix of epithelial cells (Azcarate-Peril et al., 2008). The biofilm protects bacteria from the host immune system and antagonistic factors of enteropathogens (Toropov et al., 2020). Bacteria should be tolerated in the harsh environment of the stomach. In order to resist such stress conditions, the bacteria arise suitable responses in the expression of genes and protein activity according to the environmental changes. Several bile, acid, and other stress-resistant genes are identified in the FCW1 genome, including nhap2, nhaK, clp, yveA, addAB, recDGQ, and pcrA, cshAB, and dnaC. In addition to the presence of bile salt hydrolase genes (bshA, bshB, bshC), FCW1 is theoretically resistant to bile salts. The findings are consistent with our in vitro results, which confirm that the bacteria are resistant to high salt, acid, and bile salt conditions.

Furthermore, the presence of chaperones and proteases, such as *dnaK*, *groS*, *groL*, *grpE*, and *clp* proteins, enables FCW1 to withstand acid stress for an extended time. These molecular chaperone proteins get induced under acid stress, tolerate heat and osmotic shock, and repair damaged proteins (Prasad et al., 2003; Hamon et al., 2014). Furthermore, Arena et al. (2019) and Skinner et al. (2001) also reported upregulation of these chaperones and proteases in response to heat and cold shock. The gene *luxS* encoding quorum sensing will also get expressed under oxidative stress and acidic conditions and enhanced quorum sensing to tolerate the stress conditions. Also play an important role in the induction of anti-inflammatory cytokines adhesion, bacterial growth and biofilm formation (Koponen et al., 2012). The presence of *glm* and *dapA* genes for exopolysaccharide synthesis protects bacteria from environmental damage by producing a polysaccharide capsule. In addition, these exopolysaccharides stimulate the host's immune system, are involved in cell adhesion and biofilm formation, act as antioxidants and anti-inflammatory agents, promote auto aggregation and enhance the sensory properties of fermented foods (Saadat et al., 2019; Stergiou et al., 2021). The PTS system is a distinctive

method used by bacteria to acquire sugar, which uses phosphoenol pyruvate as an energy source and phosphoryl donor. The PTS system for maltose/fructose/glucose was found in the FCW1 strain, which is involved in sugar uptake. Besides regulating carbohydrate metabolism, PTS also governs colonization, biofilm formation, stress response, chemotaxis, and virulence (Wanna et al., 2021). The strains also contain genes that regulate the synthesis of vitamin B12, biotin, and other beneficial nutrients.

Antibiotic-resistant genes associated with fluoroquinolone resistance (*gyrA*, *gyrB*) and trimethoprim resistance (*dfrc*) were identified in the PATRIC annotation. Due to the absence of plasmids in the FCW1 genome, plasmid-mediated horizontal gene transfer may not be possible. Also an *in vitro* antibiotic susceptibility test revealed that the strain was susceptible to ciprofloxacin (fluoroquinolone) and trimethoprim. A preliminary antibiotic sensitivity assay showed that the strain is resistant to penicillin and the *blaz* gene responsible for penicillin resistance is also detected in the genome. In addition to genome screening, *in vitro* antibiotic tests, hemolytic assays, and DNase tests, the strain was confirmed to be safe.

An *in vitro* anti-microbial assay has shown that FCW1 can inhibit some pathogens. The FCW1 genome analysis by antiSMASH software showed that it produces Staphyloferrin A, an iron-chelating siderophore that suppresses the growth of pathogenic bacteria through iron chelation, as iron is essential in virulence and bacterial interactions. Siderophore-producing non-pathogenic *Staphylococcus* strains are generally considered as safe (GRAS), are promising candidates for probiotics that fight pathogens with low iron uptake capabilities, and are often used as starter cultures for fermented meat (Raaska and MattilaSandholm, 1995). Moreover, the KEGG and COG annotation results showed that our strain possesses many beneficial characteristics.

The *frc* gene-bearing bacterial pool is also identified as Oxalate Metabolizing Bacterial Species (OMBS), which prevents oxalate toxicity in the gastrointestinal tract (GIT) by converting it to formic acid and CO<sub>2</sub>. It is also possible for bacteria like acetogenic, methanogenic, and sulfate-reducing bacteria (AMS) to use formic acid and CO<sub>2</sub> as carbon and energy sources. As FCW1 contains sulfate-reducing genes *sat*, *cysC*, and *cysH*, as well as acetate-metabolizing genes such as *ppa* and *ack*, the bacterium can reduce oxalate toxicity by utilizing formic acid. FCW1 genome also contains ammonium transporter genes (*nrgA*), which enhance passive ammonium

uptake as an energy source. It has been hypothesized that bacteria may oxidize ammonium in 735 struvite stones, releasing hydrogen ions that enhance the dissolution process. Based on the 736 737 presence of these genes in FCW1, this strain might be beneficial in treating and preventing kidney stone disease through its role as OMBS, AMS and Ammonia oxidizing bacteria (AOB). 738

#### 5. Conclusion 739

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This study has highlighted the probiotic strain of S. gallinarum FCW1, which is convincing by the presence of crucial probiotic genes as annotated on the draft genome sequence. The strain FCW1 meets the probiotic selection criteria, including antibiotic susceptibility, survivability to 743 GI conditions, and non-hemolytic properties. In addition, we found the frc genes responsible for oxalate degradation, sulfate-reducing genes, acetate-metabolizing genes and ammonium transporter genes in S. gallinarum FCW1, which adds to the idea that this strain can contribute to treating kidney stones. Taking into account in vitro probiotic capabilities, in vivo bio safety and probiotic-associated genes, the strain FCW1 offers a variety of potential applications in the medical and nutritional industries. In this context, in future studies, the FCW1 strain of S. gallinarum will be studied for its potential as a starter culture for developing coconut water beverages since it was isolated from fermented coconut water. Further investigation of strain FCW1's role in preventing and treating kidney stones will be conducted.

#### **Ethics Statement**

- Animal experiment was conducted in accordance with the Institutional Animal Ethics Committee 753
- 754 (IAEC) guidelines of Bharathidasan University. The study was approved by IAEC,
- 755 Bharathidasan University (Ref No: BDU/IAEC/P11/2021, dated 04.09.2021).

## **Authors Contributions**

- 757 Conceptualization - DRCT, RAJ; Formal analysis - DRCT; Investigation and Methodology -
- DRCT; Resources RAJ, MVS; Software; MVS, DRCT; Supervision; RAJ; Roles/Writing -758
- 759 original draft - DRCT, KPR; Writing - review & editing- SK, KPR, RRS, RAJ.

## **Declaration of Competing Interest**

- 761 The authors declare that they have no competing interests or personal relationships that could
- have appeared to influence the work reported in this paper.

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## 1018 Supplementary Materials

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Supplementary Table 1: Number of genes associated with general COG functional categories in
 FCW1

Class	No_families	Coverage	Abundance	Description
J	245	0.542857	0.068502	Translation, ribosomal structure and biogenesis
A	25	0	0	RNA processing and modification

K	231	0.341991	0.084429	Transcription
L	238	0.352941	0.046236	Replication, recombination and repair
В	19	0.052631	0.000444	Chromatin structure and dynamics
D	72	0.194444	0.00815	Cell cycle control, cell division, chromosome partitioning
Y	2	0	0	Nuclear structure
V	46	0.347826	0.012928	Defense mechanisms
T	152	0.276315	0.031664	Signal transduction mechanisms
M	188	0.393617	0.053083	Cell wall/membrane/envelope biogenesis
N	96	0.052083	0.001684	Cell motility
Z	12	0	0	Cytoskeleton
W	1	0	0	Extracellular structures
U	158	0.132911	0.010017	Intracellular trafficking, secretion, and vesicular transport
Ο	203	0.270935	0.030244	Posttranslational modification, protein turnover, chaperones
C	258	0.317829	0.065648	Energy production and conversion
G	230	0.465217	0.105124	Carbohydrate transport and metabolism
E	270	0.585185	0.114633	Amino acid transport and metabolism
F	95	0.663157	0.034228	Nucleotide transport and metabolism
Н	179	0.502793	0.052684	Coenzyme transport and metabolism
I	94	0.478723	0.035889	Lipid transport and metabolism
P	212	0.415094	0.067791	Inorganic ion transport and metabolism
Q	88	0.272727	0.02196	Secondary metabolites biosynthesis, transport and catabolism
R	702	0.282051	0.146984	General function prediction only
S	1347	0.146993	0.103742	Function unknown

Supplementary Table 2: Genes responsible for probiotic properties

S No	Category	Gene	Product	Locus Tag	Function
1	Cell Surface Proteins				Cleaves the signal molecule between threonine and glycine and then attaches the covalent residue to
		srtA	Sortase A	SG 01663	peptidoglycan
		gnd	6- phosphogluconate dehydrogenase, decarboxylating	SG 01015	Promote bacterial adhesion to mucin and epithelial cells
		ldh1	L-lactate dehydrogenase 1	SG 01280	Promote bacterial adhesion to mucin and epithelial cells
		ldhD	D-lactate dehydrogenase		Cleaves the signal molecule between threonine and glycine and then attaches the covalent residue to
				SG_01600	peptidoglycan
		fbp	Fructose-1,6-bisphosphatase class 3	SG 01689	Fibronectin binding protein
			4- diphosphocytidyl- 2-C-methyl-D- erythritol kinase	SG_02718	Large surface protein, Putative mucus- binding
		lspE			
2	Active removal of	copA	Copper-exporting P-type ATPase	SG_01602	
	stressors	copZ	Copper chaperone CopZ	SG_01601	
		bshA; bshB2 ; bshC	putative N-acetyl- alpha-D- glucosaminyl L- malate deacetylase	SG_00107; SG_00686	Deconjugation of bile salts
3	Stress related genes	dnaK	Chaperone protein	SC 01095	Tolerate heat and osmotic shock, Repair of damaged proteins
	genes	groS	10 kDa chaperonin	SG_01085 SG_02502	of damaged proteins  Repair of damaged proteins
		groL	60 kDa chaperonin	SG_02503	Repair of damaged

					proteins
		grpE	Protein		Repair of damaged
		8.1-		SG 01086	proteins
		clpB;	ATP-dependent	SG 00513;	Refold or degrade the
		clpC;	Clp protease ATP-	SG 00008;	denatured proteins
		clpP;	binding subunit	SG 00378;	proteins
		clpY	omanig sacami	SG 01193	
		Cipii		01173	
		gltT_1		SG_01792;	Help the bacterium to
		,2; gltP	Proton/sodium-	SG_02213 SG_01418	survive in acidic
		giii	glutamate symport	50_01410	environment of
			protein		gastrointestinal tract
			Sodium/proton-	SG_02247;	"
		acp_1	dependent alanine	SG_00540	
		<i>; acp_</i>	carrier protein		
			Asparate proton	SG_01520	Protect bacteria from
		yveA	symporter		acid stress
			Glutathione	SG_00822	Protect bacterium from
		bsaA	peroxidase		Acid Stress response
1	Call			CC 00460	d Alemaistics of TA
4	Cell	11, 4.	D -1 D	SG_00468;	d-Alanylation of LTA
	Envelope	dltA;	D-alanineD-	SG_00470;	
	and	dltC; dltD	alanyl carrier	SG_00471	
	Lipoteichoic acids	anD	protein ligase Bifunctional		Dantida alyaan
	acius	glmU	protein	SG 02714	Peptidoglycan biosynthesis
		gimo	protein	30_02/14	blosynthesis
					Cell wall biogenesis/
		epsM	Putative	SG 00048	degradation, Teichoic
		1	acetyltransferase	_	acid biosynthesis
					Cell wall biogenesis/
			Monofunctional	SG_00536	degradation, Teichoic
		mgtE	glycosyltransferase		acid biosynthesis
					Cell wall biogenesis/
			Peptidoglycan	SG_02600	degradation, Teichoic
		rodA	glycosyltransferase		acid biosynthesis
			4,4'-		Cell wall biogenesis/
			diaponeurosporeno		degradation, Teichoic
			ate	SG_02154	acid biosynthesis
		crtQ	glycosyltransferase		
			putative		Cell wall biogenesis/
			peptidoglycan	SG_02369	degradation, Teichoic
		ftsW	glycosyltransferase		acid biosynthesis
	<b>D</b>	1	II ADC	GG 00007	DMA :
5	Protection	uvrA;	UvrABC system	SG_00297;	DNA repair

	and	uvrB;	protein	SG 00296;	
	repairDNA	uvrC		SG_00665	
	and proteins	dps	Protein		DNA protection
				SG 02656	during starvation
		msrB	Peptide methionine		DNA repair
			sulfoxide reductase	SG_00945	
			ATP-dependent		DNA repair
		addA	helicase/nuclease	SG_00505;	
		;addB	subunit	SG_00504	
		~	ATP-dependent		DNA repair
		recG	DNA helicase	SG_00739	
		cshA_			DNA repair
		1;	DEAD 1 AED		
		cshA_	DEAD-box ATP-	GG 00004	
		2;	dependent RNA	SG_00994;	
		cshB	helicase	SG_01063	DNA ronoir
			ATP-dependent RecD-like DNA		DNA repair
		recD2	helicase	SG 01120	
		ruvA;	Hericase	30_01120	DNA repair
		ruvB ,	Holliday junction		DIVATepan
		1;	ATP-dependent		
		ruvB	DNA helicase	SG 01143;	
		$\frac{1}{2}$	RuvB	SG 01142	
			Replicative DNA	<u> </u>	DNA repair
		dnaC	helicase	SG 02179	1
			ATP-dependent	_	DNA repair
			DNA helicase		1
		pcrA	PcrA	SG 02538	
		1	ATP-dependent	_	DNA repair
			DNA helicase		1
		recQ	RecQ	SG_00257	
6	Quorum				Induction of anti-
	sensing and				inflammatory
	Antipathoge				cytokines, Adhesion
	nic effects				and competitive
					exclusion of
					pathogens; Direct role
			C		in the production of
			S-	SG 02650	AI-2; indirect in the
		luxS	ribosylhomocystei ne lyase	30_02030	production of AI-3- like agonist molecules
		iuns	ne iyase		TIKE agoinst molecules
7	Immunomo				Potential
(	dulation	clpB	Chaperone protein	SG 00513	immunogenic proteins
		2.72	protein		

			D-3-		Inhibition of elastases
			phosphoglycerate	SG 01243	
		serA	dehydrogenase	_	
			SerinetRNA		Inhibition of elastases
		serS	ligase	SG 02186	
				_	
8	Degradation				Adhesion and
	of Mucin		Alcohol		stimulation of mucin
		adh	dehydrogenase	SG 02050	secretion
		•			
9	Protein		S-		Putative vitamin B12 -
	metabolism		adenosylmethionin	SG_01300	independent
		metK	e synthase	_	methionine synthase
		•			
10	Production	btuD	Vitamin B12		
	of nutrients	1 to 6	import ATP-	SG 00928;	In situ production of
	and other	&btuF	binding protein	SG_01061	important nutrients
	beneficial		Putative ABC		
	process		transporter		High production of
		ytrE,	permease and ABC		acetate and protection
		yheS,	transporter ATP-	SG_02068;	from enteropathogenic
		ylmA	binding protein	SG_02485	infection
			Biotin carboxyl		
		accB_	carrier protein of		
		1,	acetyl-CoA	SG_01032;	Fatty acid biosynthesis
		accC	carboxylase	SG_01107	and lipid metabolism
		bioB,			Biotin and cofactor
		bioD	Biotin synthase	SG_01536	biosynthesis
					interacts with the
					energy-coupling factor
					(ECF) ABC-
		bioY	Biotin transporter	SG_01901	transporter complex
				SG_01920;	
				SG_01913;	
			Molybdenumcofact	SG_01914;	
		moaA	orbiosynthesisprote	SG_01918;	Biosynthesis of
		to E	in B	SG_01917	molybdopterin
			putative		
			molybdenum		Mo-
		_	cofactor		molybdopterin co-
		mobA	guanylyltransferase	SG_01919	factor biosynthesis
		ccpA_	Catabolite control		Influencing blood
		1	protein A	SG_00866	cholesterol
					Processing of health-
			Metallothiol		promoting
		fosB	transferase	SG_01676	fructoligosaccharides

12	Carbohydra			SG_01860;	Sugar uptake
	te	malP;		SG_02673;	
	metabolism	mtlA;	PTS system for	SG_00237;	
		fruA;	(maltose/fructose/	SG_01529	
		ptsG	mannitol/glucose)		
			UDP-N-		Glucosyltransferase
			acetylglucosamine-		
			-peptide N-		
			acetylglucosaminyl	SG_01821;	
		gtfA	transferase	SG_02748	
		treP;	PTS system	SG_02462;	Trehalose
		treA;	trehalose-specific	SG_02463;	
		treR	EIIBC component	SG_02464	

## 

## **Supplementary Table 3**: AMR genes of *S. gallinarum*FCW1

Source	Gene	Product	Identity	E-value
PATRIC	S12p	SSU ribosomal protein S12p (S23e)	-	-
	rho	Transcription termination factor <i>Rho</i>	-	-
	bceS	Two-component sensor histidine kinase <i>BceS</i>	-	-
	kasA	3-oxoacyl-[acyl-carrier-protein] synthase, <i>KASII</i> (EC 2.3.1.179)	-	-
	liaR	Cell envelope stress response system LiaFSR, response regulator LiaR(VraR)	-	-
	inhA, fabI	Enoyl-[acyl-carrier-protein] reductase [NADH] (EC 1.3.1.9)	-	-
	tcaB2	Teicoplanin resistance transporter, <i>TcaB</i> family => TcaB2	-	-
	gdpD	Glycerophosphoryl diester phosphodiesterase (EC 3.1.4.46)	-	-
	tcaR	Teicoplanin-resistance associated HTH-type transcriptional regulator <i>TeaR</i>	-	-
	S10p	SSU ribosomal protein S10p (S20e)	_	-
	alr	Alanine racemase (EC 5.1.1.1)	_	-
	tcaB	Teicoplanin resistance transporter, <i>TcaB</i> family => <i>TcaB</i>	-	-
	bceR	Two-component response regulator <i>BceR</i>	-	-
	norA	MFS-type transporter quinolone resistance protein <i>NorA</i>	-	-
	gyrB	DNA gyrase subunit B (EC 5.99.1.3)	-	-
	liaF	Membrane protein <i>LiaF(VraT)</i> , specific inhibitor of <i>LiaRS(VraRS)</i> signaling	-	-

		pathway		
	EF-Tu	Translation elongation factor Tu	-	-
	mprF	L-O-lysylphosphatidylglycerol synthase	-	-
	1	(EC 2.3.2.3)		
	bla	Class A beta-lactamase (EC 3.5.2.6)	-	-
	ddl	D-alanineD-alanine ligase (EC	-	-
		6.3.2.4)		
	liaS	Cell envelope stress response system	-	-
		<i>LiaFSR</i> , sensor histidine kinase		
		LiaS(VraS)		
	murA	UDP-N-acetylglucosamine 1-	-	-
		carboxyvinyltransferase (EC 2.5.1.7)		
	ykkCD	Broad-specificity multidrug efflux	-	-
		pump YkkC		
	folP	Dihydropteroate synthase (EC 2.5.1.15)	_	-
	Iso-tRNA	Isoleucyl-tRNA synthetase (EC 6.1.1.5)	_	-
	gidB	16S rRNA (guanine(527)-N(7))-	_	-
		methyltransferase (EC 2.1.1.170)		
	EF-G	Translation elongation factor G	-	-
NDARO	dfrc	Dihydrofolate reductase (EC 1.5.1.3)	82	
CARD	sav1866	Efflux ABC transporter, permease/ATP-	84	1e-283
		binding protein <i>YgaD</i>		
	pgsA	CDP-diacylglycerolglycerol-3-	87	6e-89
		phosphate 3-phosphatidyltransferase		
		(EC 2.7.8.5)		
	rpoB	DNA-directed RNA polymerase beta	94	0.0
		subunit (EC 2.7.7.6)		
	arlR	Putative response regulator ArlR	81	2e-100
	pare	DNA topoisomerase IV subunit B (EC	90	0.0
		5.99.1.3)		
	tuf	Translation elongation factor Tu	87	1e-180
	mgrA	Transcriptional regulator MgrA	85	4e-63
		(Regulator of autolytic activity)		
	rpoC	DNA-directed RNA polymerase beta'	94	0.0
		subunit (EC 2.7.7.6)		
	gyrA	DNA gyrase subunit A (EC 5.99.1.3)	89	0.0

"-" indicates not available