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The safety and potential probiotic properties analysis of *Streptococcus alactolyticus* strain FGM isolated from the chicken cecum

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Abstract

Purpose: *Streptococcus alactolyticus* strain FGM is used to ferment *Astragalus membranaceus* to develop a novel feed additive for animals in China. This study aimed at characterizing the safety and potential probiotic features of the strain FGM in vitro.

Methods: The genome of *S. alactolyticus* strain FGM was sequenced and used for genomic in silico studies. It was evaluated for morphology, antibiotic susceptibility, hemolytic activity, acid tolerance, bile salt tolerance, adherence ability to Caco-2, and inhibitory pathogens activity.

Result: The GC content of the strain FGM was 40.38% and composed of 29 contigs. The annotation of coding genes revealed important characteristics of the germs, especially 151 genes annotated to biological adhesion. The strain FGM forecasted 43 amino acid sequences to be VF, but did not have a hemolytic gene, and neither did it show hemolytic activity in phenotypic analysis. Although 30 amino acid sequences were predicted to aid in resisting some antibiotics, the strain FGM just showed the resistance to trimoxazole and oxytetracycline, and intermediate resistance to kanamycin. FGM cells were showed the tolerance to pH 2 broth within 4 h, and 0.15~0.30% bile salt medium with the latter being attributed to the presence of bile-salt hydrolase. The strain FGM was shown to have the ability to adhere to Caco-2 cells and the adherence rate of 1.0×10^9 CFU/mL bacterial suspensions was 37.51%. Compared with *Lactobacillus acidophilus*, *Lactobacillus reuteri*, and *Lactobacillus casei*, the strain FGM showed a high capability to inhibit the diffusion of *Escherichia coli O78* and reduce its adhesion on Caco-2 cells.

Conclusion: The results demonstrated the presence of probiotic potential and absence of adverse effects for the *Streptococcus alactolyticus* strain FGM in vitro, thus contributing to develop a safety and effective fermentation feed for animals.

Keywords: *Streptococcus alactolyticus*, Strain FGM, Genomes, Probiotic, Resistance, Inhibition, Adhesion, *Escherichia coli O78*

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Background

Lactic acid bacteria (LAB) are gram-positive, anaerobic, and catalase-negative rods or cocci bacteria which can ferment carbohydrate and produce plenty of lactic acids (Rinkinen et al. 2004). It was reported that many LAB increase the stability of intestinal environment and contribute to the health of the host by enhancing the gut microbiota balance through secretion of antibacterial substance (organic acids, bacteriocins) and competitive adhesion to the epithelium or by the stimulation of the GI immune system (Tallon et al. 2007). LAB of the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Bifidobacteria*, *Pediococcus*, *Saccharomyces*, and *Streptococcus*, are being widely used as veterinary drugs and feed additives (Vitetta et al. 2017; Aponte et al. 2020; Shin et al. 2019; Maria et al. 2014). These beneficial bacteria possess anti-inflammatory activity and antimicrobial properties, protect against oxidative stress and diabetes, as well as reducing the accumulation of reactive oxygen metabolites (Todorov 2009; Mazzoli et al. 2014; Lossa and Pollice 2019).

S. alactolyticus, a member of the genera Streptococcus, was originally described among isolates obtained from the intestinal tract of pigs and chickens and has been reported no hemolytic or α-hemolytic activity (Farrow et al. 1984). It has been proved as a dominant culturable LAB species of the commensal gut microbiota in animals such as chicken, pigs, dogs, ducks, pigeons, and fishes (Czerwinski et al. 2010; Baele et al. 2002). However, its characteristics related to survive and bioactivity in the animal guts were rarely reported.

In the broiler chicken gastrointestinal tract, the cecum is the main site of fermentation (Jozefiak et al. 2006). *S. alactolyticus* strain FGM was domesticated, screened, and isolated from bacterial load in the chicken cecum to ferment *Astragalus membranaceus* and produce fermented *Astragalus polysaccharides* (FAPS) in China (Hao et al. 2013). Previous studies showed that polysaccharide content of *A. membranaceus* fermented by S. *alactolyticus* strain FGM is four-fold higher than that before fermentation, and the biological effect of *A. membranaceus* after fermentation is the similar as that before fermentation, such as body weight gain and immunopotentiation of broiler chicken, obvious antagonism against hepatic fibrosis induced by CCl4 exposure on rats (Qin et al. 2012; Zhang et al. 2011). In view of the good fermentation performance and application prospect of *S. alactolyticus* strain FGM, its own characteristics and safety were further studied. The comparison against GenBank of the 16S rRNA gene showed that the strain belongs to *S. alactolyticus*, and a similarity index higher than 96% was presented between the strain FGM and *S. alactolyticus* EU728776.1 (Wang 2012). The strain was proved without toxic reaction and causing death on mice in acute oral toxicity test (Wang et al. 2020), and could improve the health of broilers infected by *E. coli O78* through protecting the intestinal villi structure, balancing the intestinal immunity, reducing the number of *E. coli*, improving the anti-inflammatory and anti-oxidation (Wu et al. 2020). From these pieces of evidence, *S. alactolyticus* appears to be a promising strain. So we characterized its safety and potential probiotic properties in vitro using genomic analyses and phenotypic tests in this study.

Materials and methods

Strains used and growth conditions

S. alactolyticus strain FGM (GenBank accession No. JX435470; China Patent No. 20120141827.5) was isolated from chicken cecum in Lanzhou City of China and preserved in the Lanzhou Institute of Husbandry and Pharmaceutical Sciences, Chinese Academy of Agricultural Sciences. The strain FGM, *Lactobacillus reuteri* (BeNa Culture Collection, China, No. 192190), *Lactobacillus acidophilus* (BeNa Culture Collection, China, No. 185342), and *Lactobacillus casei* (BeNa Culture Collection, China, No.134415) were cultured anaerobically in Macconkey broth (Hopebio, China) at 2% (v/v) volume at 37 °C for 24 h. *E. coli O78* (China Veterinary Culture Catalogue, No. 1418) was cultured in Nutrient broth (Hopebio, China) at 1% (v/v) volume at 37 °C for 24 h.

Genomic analysis and bioinformatics

The genomic DNA of *S. alactolyticus* strain FGM was extracted by Bacterial genomic DNA Extraction Kit (OMEGA, USA) following the manufacturer's instructions. The DNA was sent to Beijing Novogene Science and Technology Co., Ltd. for full gene sequencing by Illumina Hiseq and Miseq platform. After assembling and optimizing the reads by SOAPdenovo software, the genome components of strain FGM were predicted using five softwares: GeneMarkS (verison 4.17), RepeatMasker (verison 4.0.5), rRNAmmer(verison 1.2), tRNAscan-SE (verison 1.3.1), and Rfam (verison 11.0). The database of Gene Ontology (GO) was used to predict and assign the function of genes from strain FGM. To further investigate biological properties and safety of relevance, the antibiotic resistance genes were detected using the Comprehensive Antibiotic Resistance Database (CARD) (Jia et al. 2017). The presence of genes associated with bacterial pathogenesis was then checked against the virulence factors database (VFDB) (Liu et al. 2018).

Colony and strain morpologhy

S. alactolyticus strain FGM was inoculated on a blood agar plate (Hopebio, China) at 37 °C for 20~24 h anaerobically, before being analyzed for colony morphology and gram staining. The strain FGM cells were collected

and washed 3 times by sterile PBS (Solarbio, China). They were fixed with 2.5% glutaraldehyde solution (Ameko, USA) at 4 °C for 1 h. After washing 3 times by sterile PBS (pH 7.2–7.4), the cells were dehydrated in sequence by 30%, 50%, 60%, 70%, 80%, 90%, 95%, and 100% ethyl alcohol. The specimens were critical-point dried using liquid CO_2 and sputter-coated with gold before the examination on a scanning electron microscope (SEM, JSM-5600, JEOL, Japan).

Antibiotic susceptibility test

Antibiotic susceptibility test was evaluated by the disk diffusion method using drug-sensitive slips (Hangwei, China). Kanamycin (30 μg/table), ciprofloxacin (5 μg/table), penicillin (10 μg/table), trimoxazole (23.75/1.25 μg/table), erythromycin (15 μg/table), norfloxacin (10 μg/table), cefalotin (30 μg/table), lincomycin (2 μg/table), oxytetracycline (30 μg/table), and amoxicillin (20 μg/table) antibiotics were used in this test. A total of 100 μL 1.0×10^8 CFU/mL FGM suspensions were evenly spread on blood agar plates and the drug-sensitive papers put on them. After incubation for 24 h at 37 °C, the inhibitory zone diameters (IZD) of drugs were determined by vernier calipers.

Hemolytic activity test

Fresh culture of *S. alactolyticus* strain FGM was streaked on blood agar plates, incubated at 37 °C for 48 h, and then evaluated for the presence of hemolytic haloes. *S. gallolyticus* (ATCC9809) was included as a positive control. The experiment was set three replicates.

Acid tolerance

Acid tolerance of *S. alactolyticus* strain FGM was tested as previously described (Li et al. 2016). A 2% (v/v) volume of the overnight cultures of strain FGM were added to MRS broth with the pH adjusted to 2.0 by 2 mol/L HCl. In the control group, the same counts of cells were added to MRS broth, and the pH value of the initial suspension was 6.0. All the groups were incubated for 4 h in an incubator at 37 °C and 100 r/min. Samples were taken at 0, 1, 2, 3, and 4 h, respectively. Three repeats were set up at each observation point. After serial twofold dilutions, the suspensions were counted to calculate the survival rate of live cells.

Bile salt tolerance

The method used to assess bile salt tolerance was referenced from literature (Kaushik et al. 2009). A total of 2% (v/v) volume of the overnight cultures of *S. alactolyticus* strain FGM were inoculated in MRS broth supplemented with 0.15% and 0.3% (w/v) bile salts (Sigma-Aldrich, USA) followed by incubation at 37 °C and 100 r/min for 4 h. Samples were taken at 0, 1, 2, 3, and 4 h, respectively. Three repeats were set up at each observation point. After serial twofold dilutions, the cells were counted to calculate the survival rate of *S. alactolyticus* strain FGM.

Adherence assays to Caco-2 cells

Microscopic inspection of strain FGM's adherence ability was referenced from literature (Zhang 2014). Caco-2 cell lines were routinely cultured in MEM/EBSS full medium (Hyclone, USA) supplemented with 10% (v/v) fetal bovine serum (AusgeneX, Australia) and 1% antibiotic penicillin/streptomycin (Sigma-Aldrich, USA). Aliquots of 6 ml containing 5×10^5 Caco-2 cells/mL were seeded on a 6-well Corning tissue culture plate and incubated at 37 °C in a 5% CO_2 humid atmosphere until a complete monolayer of cells was obtained. After washing 4 times using sterile PBS, the wells were filled with 2 mL 1.0×10^8 CFU/mL FGM suspensions in MEM/EBSS medium (without antibiotics) and incubated at 37 °C in 5% CO_2 humid atmosphere for 2 h. The medium of the wells was discarded, and the wells washed with sterile PBS for 4 times to clean out the unbound bacteria. The cells in wells were then fixed with 4% neutral formalin (Solarbio, China) for 15 min. After removing the fixative, the gram staining procedure was done as described in the protocol of the Gram staining kit (Solarobio, China). Finally, the wells were evaluated under a microscope (LZXS-YQ-D03-003, Olympus, Japan) with a × 100 objective lens.

The adherence ability of strain FGM was assessed more exactly following the method previously described (Bianchi et al. 2010; Li et al. 2016). FGM cells were harvested after overnight cultivation and adjusted to 1.0×10^9 CFU/mL, 6.0×10^8 CFU/mL, and 3.0×10^8 CFU/mL concentrations with sterile PBS. The bacterial solutions were labeled by FITC (Solarbio, China) working solution at 37 °C, 120 r/min, 1 h. After washing with sterile PBS for 4 times, the samples were resuspended with MEM/EBSS full medium without antibiotics. The bacterial solution at each concentration without FITC tagging was prepared as background values.

Caco-2 cells were seeded in each well of a 6-well tissue culture plate as described in section 2.8.1 above. A total of 1 mL of FGM-FITC suspensions of different concentrations and 1 mL MEM/EBSS full medium (without antibiotics) were added to the wells. The mixtures were incubated at 37 °C for 2 h in a 5% CO_2 humid atmosphere. The cells in the wells were washed 4 times with sterile PBS and then digested by 1 mL 0.25% EDTA (Solarbio, China) for 5 min. One milliliter MEM/EBSS full medium (without antibiotics) was then added to each well to stop digestion. The fluorescence intensity of the collected cells was tested by Microplate Reader (SpectraMax M2, USA) at the absorption and emission

wavelength of 485 nm and 530 nm, respectively. The unlabeled FGM at each concentration incubated with Caco-2 cells were prepared as background values. Four replications were set up at each observational point.

Bacterial adhesion rate

The bacterial adhesion rate was calculated using the formula:

$$\text{Adhesion rate}\ (\%) = \frac{(C_{FGM-FITC+Caco-2} - C_{FGM+Caco-2}) \times 100\%}{C_{FGM-FITC} - C_{FGM}}$$

Where: $C_{FGM\text{-}FITC\ +\ Caco\text{-}2}$ means the fluorescence intensity of Caco-2 cells after incubated with FGM tagged by FITC

$C_{FGM\ +\ Caco\text{-}2}$ means the fluorescence intensity of Caco-2 cells after incubated with FGM without tagged by FITC

$C_{FGM\text{-}FITC}$ means the fluorescence intensity of FGM tagged by FITC

C_{FGM} means the fluorescence intensity of FGM without tagged by FITC.

Inhibitory pathogens activity

Inhibitory diffusion of E. coli O78

E. coli O78 solution was cultured in Macconkey broth (Hopebio, China) at 37 °C for 24 h at 2% (v/v) volume. The cells were harvested after overnight cultivation and adjusted to 1.0×10^8 CFU/mL with sterile PBS. The sterile swabs were used to spread *E. coli O78* on Macconkey agar plates (Hopebio, China) evenly. After drying at room temperature for 5 min, the Oxford cups were placed at the marked location on the plates and were filled with 200 μL 1.0×10^9 CFU/mL probiotic suspension (*L. reuteri*, *L. acidophilus* or *L. casei*) or FGM cells suspension, respectively. The Oxford cups in the control group were filled with 200 μL MRS broth. The plates were incubated at 37 °C for 24 h and then IZD of germs were determined by Vernier calipers. The experiment was done in four replicates.

Inhibitory adherence of E. coli O78

E. coli O78 cells were harvested after overnight cultivation and adjusted to 1.0×10^9 CFU/mL with sterile PBS. After labeling with FITC, the bacterial suspension was washed with sterile PBS and resuspended in MEM/EBSS full medium. The bacterial suspension without FITC tagging was prepared as background values. Caco-2 cells were seeded in each well of the 6-well tissues culture plate as described in section 2.8.1 above. A total of 1 mL *E. coli*-FITC suspension was put in each well, and 1 mL 6.0×10^8 CFU/mL probiotic samples (*L. reuteri*, *L. acidophilus* or *L. casei*) or FGM cells suspension added respectively. The mixtures were incubated at 37 °C for 2 h in a 5% CO_2 humid atmosphere. The cells were washed 4 times with sterile PBS before being digested by 1 mL 0.25% EDTA for 5 min. Digestion was stopped by the addition of 1 mL MEM/EBSS full medium (without antibiotics) to the wells. The fluorescence intensity of the collected cells was tested by Microplate Reader at the absorption wavelength is 485 nm and the emission wavelength of 530 nm. The unlabeled *E. coli O78* at each concentration incubated with Caco-2 cells were used as background values. Four repeats were set up at each observational point.

The loss rate of adhering *E. coli* (%)

This was calculated using the formula:

$$\text{Adhesion loss rate}\ (\%) = \frac{[(C_{EC\text{-}FITC+Caco\text{-}2} - C_{EC+Caco\text{-}2}) - (C_{EC\text{-}FITC+CB+Caco\text{-}2} - C_{EC+CB+Caco\text{-}2})] \times 100\%}{(C_{EC\text{-}FITC+Caco\text{-}2} - C_{EC+Caco\text{-}2})}$$

Where: $C_{EC\text{-}FITC\ +\ Caco\text{-}2}$ means the fluorescence intensity of Caco-2 cells after incubated with *E. coli* tagged by FITC.

$C_{EC\ +\ Caco\text{-}2}$ means the fluorescence intensity of Caco-2 cells after incubated with *E. coli* without tagged by FITC.

$C_{EC\text{-}FITC\ +\ CB\ +\ Caco\text{-}2}$ means the fluorescence intensity of Caco-2 cells after incubated with *E. coli* tagged by FITC and the competitive bacteria.

$C_{EC\ +\ CB\ +\ Caco\text{-}2}$ means the fluorescence intensity of Caco-2 cells after incubated with *E. coli* without tagged by FITC and the competitive bacteria.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) and Tukey's test was used to perform post hoc analysis using the GraphPad Prism software (version 7, GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was set at $P < 0.05$.

Results

Genomic analysis and bioinformatics

The genome sequence of *S. alactolyticus* strain FGM was uploaded to DDBJ/EMBL/GenBank (Accession No. JAAXMT000000000). An overview of the genome assemblies and annotations of the strain FGM are summarized in Table 1. The germ was assembled into 17 scaffolds (> 500 bp) and 29 contigs which were predicted from the scaffolds (> 500 bp). The guanine-cytosine (GC) content and core-genome size of strain FGM was 40.38% and 1.7 Mbp, respectively. The length of the longest contig and scaffold were 600 Kb and 972 Kb, respectively, which indicated fine continuity of assembly. The whole-genome component of strain FGM was predicted to consist of 1768 coding genes (which make up 87.7% of the whole genome), 127 scattered repeats, 38 tandem repeats, 21 minisatellite DNA, 36 tRNA, 4 rRNA, and 1 sRNA.

Table 1 The statistics of the genome assembly and components of *S. alactolyticus* strain FGM

Assembly		Number	Genome size (bp)	N50 (bp)	Max length(bp)	GC content %
Contigs		29	1,698,167	141,685	600,007	40.38
Scaffolds		17	1,701,811	972,409	972,409	40.38
Annotation		Number	Gene total length (bp)	Gene average length (bp)		Gene length/genome (%)
Coding genes		1768	1,492,563	844		87.7
Scattered repeat		127	8323	69		0.49
Tandem repeat		38	19,253	–		1.13
Minisatellite DNA		21	889	–		0.05
nRNA		36	2734	75		0.16
rRNA	5s	1	114	114		0.33
	16s	2	2740	1370		
	23s	1	2791	2791		
sRNA		1	165	165		0.01

"–" means undetected

The database of the Gene Ontology system (GO) was used to annotate the predicted genes of strain FGM (Fig. 1). A total of 2655 genes were describing molecular function, 1359 genes describing cellular component, and 1902 genes describing biological processes in the strain.

Putative resistance-related genes of strain FGM are identified and listed in Table 2. Thirty amino acid sequences were predicted to aid in resisting some antibiotics in the strain FGM including β-lactam, macrolide, lincomycin, polypeptide, aminoglycoside, quinolone, streptogramin, tetracycline, and sulfonamide. Besides, the strain was potentially tolerant to phenicol which promotes the bile secretion and thus, might be related to the bile salt tolerance of the strain.

Finally, we used the VFanalyzer software to predict potential virulence factors (VF) and filtered the comparison results according to identity and *E*-value. As shown in Table 3, 43 amino acid sequences were forecasted to be VF in the strain FGM. These amino acids could be contributing to the overall adaptability of bacteria to harsh environments and various stresses. The most related genes were attributed to the capsule which has been proved to inhibit the binding of the activated complement factor C3b to the surface of germ, prevent the activation of the alternative complement pathway, and

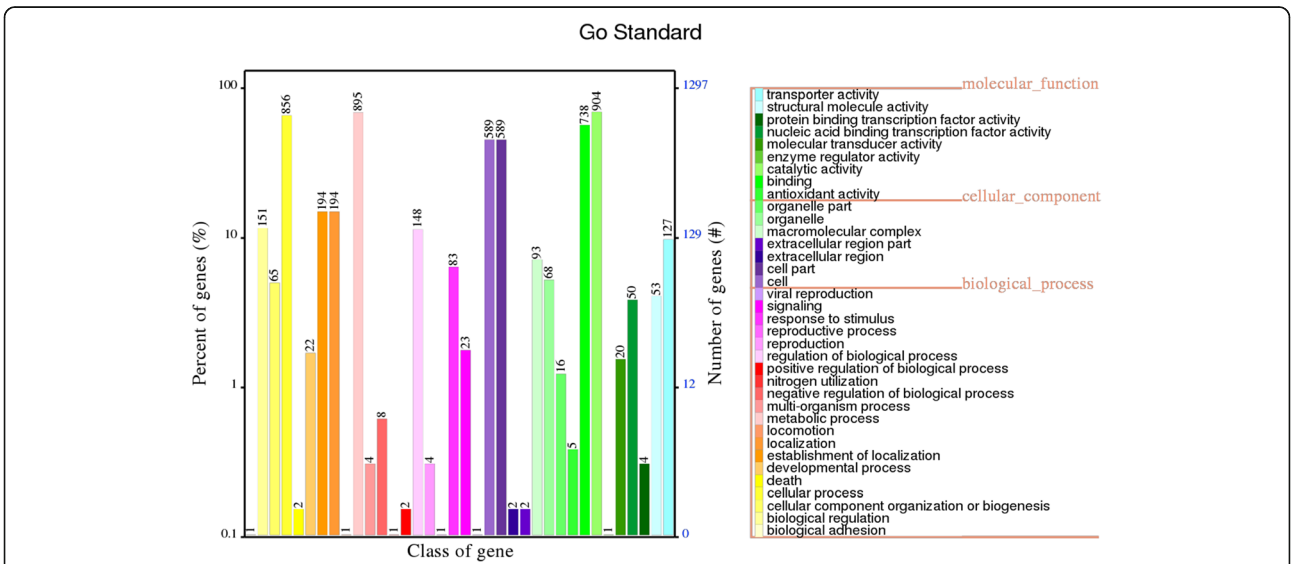

Fig. 1 Gene annotation of *S. alactolyticus* strain FGM. A total of 5916 genes could be assigned to cellular component, biological process, and molecular function by the Gene Ontology classification system

Table 2 Distribution of antibiotic resistance-related proteins in *S. alactolyticus* strain FGM

ARO_type/No.	AR0_category	Antibiotics_category
lnuC/1	Antibiotic inactivation enzyme,	Lincosamide
mprF/1, Tsnr/1, bacA/1	Antibiotic target modifying enzyme, gene altering cell wall charge	Peptide
parY/3	Antibiotic-resistant gene variant or mutant, gene involved in self-resistance to antibiotic	Aminocoumarin
arlR/2, baeR/1, vanRM/1, arlS/3, vanRC/1, vane/1, vanTG/1	Antibiotic resistance gene cluster, cassette, or operon, efflux pump complex or subunit conferring antibiotic resistance, protein(s) and two-component regulatory system modulating antibiotic efflux, protein(s) conferring antibiotic resistance via molecular bypass	Aminoglycoside glycopeptide
PBP1a/1, PBP2x/1	Antibiotic-resistant gene variant or mutant	Beta-lactam
mfd/2	Antibiotic target protection protein	Fluoroquinolone
cfrA/1	Antibiotic target modifying enzyme	Macrolide, phenicol streptogramin
pgsA/1	Antibiotic-resistant gene variant or mutant	Lipopeptide
PmrE/2, arnA/1	Gene altering cell wall charge	Polymyxin
sul3	Antibiotic target replacement protein	Sulfonamide
tet44/1, tetB(P)/1, tetT/1	Antibiotic target protection protein	Tetracycline

inhibit complement-mediated opsonophagocytosis. According to the result, strain FGM had 10 genes that were properly involved in the biological process of adhering host cells. The strain also had several other genes that proved to participate in multi-biological functions. However, the hemolytic gene was missing in the strain.

Morphological features

As shown in Fig. 2a, the strain FGM formed opaque, white, circular, and flat glistening colonies with neat edges on the MRS agar plate. The microscopic and SEM observation of strain FGM in Fig. 2b, c showed that the cells were gram-positive, oval-shaped, always in pairs or chains, and without spores or flagella. The Feret's diameter of strain FGM was 1.40 ± 0.11 μm.

Antibiotic susceptibility test

The antibiotic susceptibility of *S. alactolyticus* strain FGM was tested using ten antibiotics which were classified into β-lactam, aminoglycosides, quinolone,

Table 3 Distribution of virulence factors prediction for *S. alactolyticus* strain FGM

No.	VF_name	Related_genes	Functions
1	Bile-salt hydrolase	bsh cholylglycine hydrolase	Bile salt tolerance
24	Capsule	oppF, cpsM, cpsB, STER_1071, STER_1069, STER_1068, STER_1068, uppS, rgpB, eps3, rgpA, epsB, SGO_1024, rgpG, rgpF, cps2K, cpsY, rgpC, SMU.322c, STER_1444, STER_1223, eps2, rmlA, STER_1222	Immune evasion
1	Trigger factor	tig/ropA trigger factor	Immune evasion
1	MOMP	CT396 molecular chaperone DnaK	Anti-phagocytosis
3	Polysaccharide capsule	manA, epsE, gALE	Anti-phagocytosis
1	EF-Tu	EF-Tu	Adherence
2	Fibronectin-binding protein	scpB, pavA	Adherence
1	GbpC	gbpB putative peptidoglycan hydrolase	Adherence
1	GroEL	groEL chaperonin GroEL	Adherence
2	Sortase A	srtA Sortase surface protein transp-eptidase	Adherence
1	lipoprotein rotamase A	slrA peptidyl-prolyl cis-trans isom- erase, cyclophilin-type	Adherence
1	plasmin receptor/GAPDH	plr/gapA	Adherence
1	enolase	eno	Adherence
1	Serine protease	htrA/degP Endopeptidase degP	Antioxidant
1	ClpE	clpE	Cell division
1	ClpP	clpP	Stress tolerance, virulence, and capsule forming

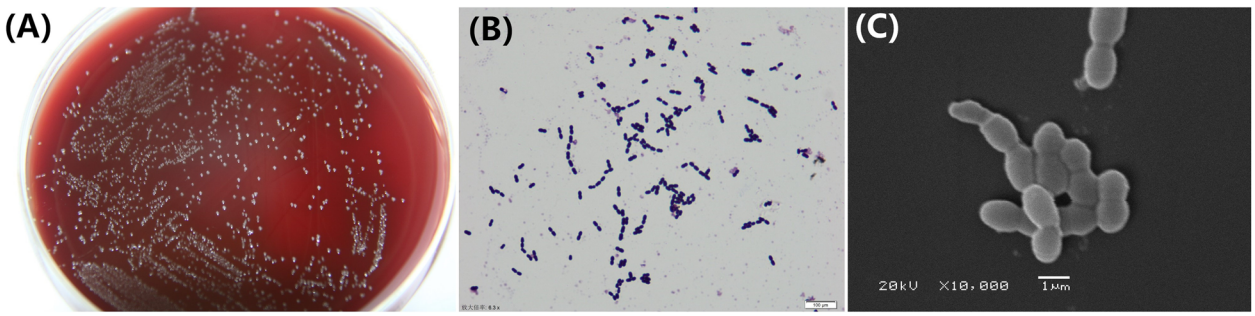

Fig. 2 The morphology of FGM colonies and strain. **a** An image of FGM colonies cultured on blood agar. **b** Light micrograph of a gram-stained for FGM with the bar was of 100 μm. **c** SEM micrographs of FGM stain with the bar was of 1 μm

sulfonamides, macrolides, lincosamides, and tetracyclines (Table 4). It was found that the strain FGM is susceptible to Ciprofloxacin, Penicillin, Trimoxazole, Cephalothin Lincomycin, Erythromycin, Erythromycin, and Amoxicillin, and resistant to Trimoxazole (sulfonamides) and Oxytetracycline (tetracyclines). Furthermore, Kanamycin (aminoglycosides) showed intermediate inhibition on the strain FGM.

Hemolysin detection

Based on hemolytic activity, streptococcus is divided into α-hemolytic streptococcus, β-hemolytic streptococcus, and γ-streptococcus (without hemolytic and pathogenic). In this study, no hemolysis ring was observed on the blood plates of *S. alactolyticus* strain FGM after 48 h of culturing (Fig. 3a). Contrastingly, the obvious hemolysis rings were shown on the blood plates with *S. gallolyticus* ATCC9809 (Fig. 3b). From the VF and phenotypic predictions at the gene level, it could be concluded that *S. alactolyticus* strain FGM belonged to γ-streptococcus.

Acid tolerance

Gastric acid tolerance (pH 2–3) is one of the barriers that probiotic bacteria must overcome to survive (Tulumoglu et al. 2018). Normally, the liquid food stays in the stomach of monogastric animals for 1~2 h (Shen et al. 2007). As shown in Fig. 4, the bacterial concentration in the control group increased with time (0 h~4 h). Compared to the control group (CG), the growth of FGM cells in the acid-treated group was extremely inhibited at 3 h and 4 h ($P < 0.01$). The survival rate of live cells rates in pH 2 group were 91.18% (1 h), 86.51% (2 h), 58.85% (3 h), and 17.73% (4 h). However, it should be noted that there were still about 3.10×10^8 CFU/mL alive cells in pH 2 broth at 4 h.

Bile salt tolerance

Bile plays a fundamental role in the specific and nonspecific defense mechanisms of the gut. The magnitude of its inhibitory effects is determined primarily by the concentration of bile salts (Charteris et al. 1998). Normally, relevant physiological concentrations of animal bile range from 0.03 to 0.30% (Chang et al. 2001). As shown

Table 4 The result of disc diffusion test of *S. alactolyticus* FGM strain

Drugs	Abbreviation	Dose per piece (μg)	Inhibitory zone diameter (mm)	Sensibility Degree
Kanamycin	KAN	30	14.60 ± 1.18	Intermediary
Ciprofloxacin	CIP	5	22.96 ± 0.41	Sensitive
Penicillin	PEN	10U	38.70 ± 3.28	Sensitive
Trimoxazole	TRI	23.75/1.25	0	Resistant
Erythromycin	ERY	15	27.70 ± 1.40	Sensitive
Enrofloxacin	ENR	10	24.13 ± 0.40	Sensitive
Cephalothin	CEP	30	26.27 ± 0.49	Sensitive
Lincomycin	LC	2	17.32 ± 2.04	Sensitive
Oxytetracycline	OXY	30	13.11 ± 0.25	Resistant
Amoxicillin	AMX	10	29.67 ± 0.87	Sensitive

The judgement criterion of antibiotics susceptibility was referenced from the interpretation file from Hangzhou microbial reagent co. Ltd. Inhibitory zone diameter is represented as the means ± SD from the 4 repeats

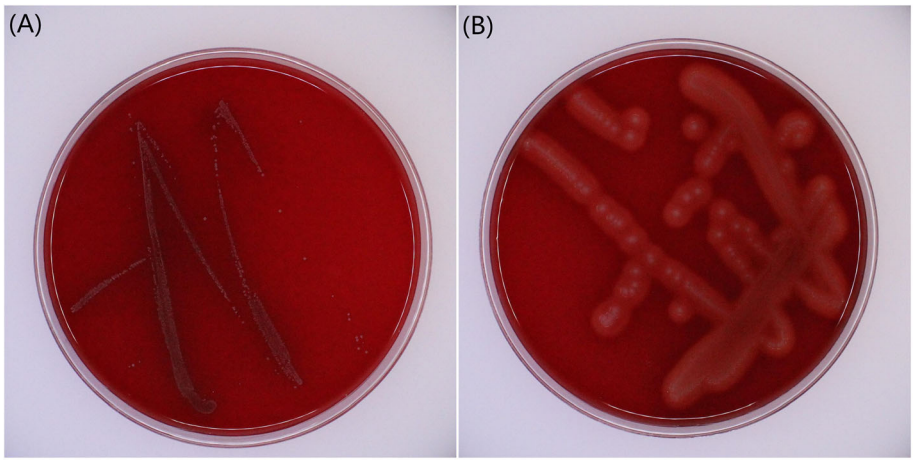

Fig. 3 The hemolytic evaluation of FGM strain. The Hemolysis rings on the blood agars cultured with **a** *S. alactolyticus* strain FGM and **b** *S. gallolyticus* ATCC9809 judged by naked eye observation

in Fig. 5, compared with the control group, the growth of FGM cells in the treated groups were both inhibited obviously at 1 h~4 h ($P < 0.05$). The germ survival rates in 0.15% BS group were 77.44% (1 h), 77.08% (2 h), 71.04% (3 h), and 26.93% (4 h). The germ survival rates in 0.30% BS group were 65.10% (1 h), 60.06% (2 h), 46.06% (3 h), and 16.35% (4 h). The status of bacterial proliferation in the 0.30% BS group was worse than in the 0.15% BS group. However, at 4 h, there were still 4.67×10^8 CFU/mL alive cells in the broth with 0.15% bile salts and 2.83×10^8 CFU/mL alive cells in the broth with 0.30% bile salts.

Adherence assays to Caco-2 cells

As shown in Fig. 6b, a certain amount of gram-positive, oval-shaped, paired or chained strain FGM cells adhered

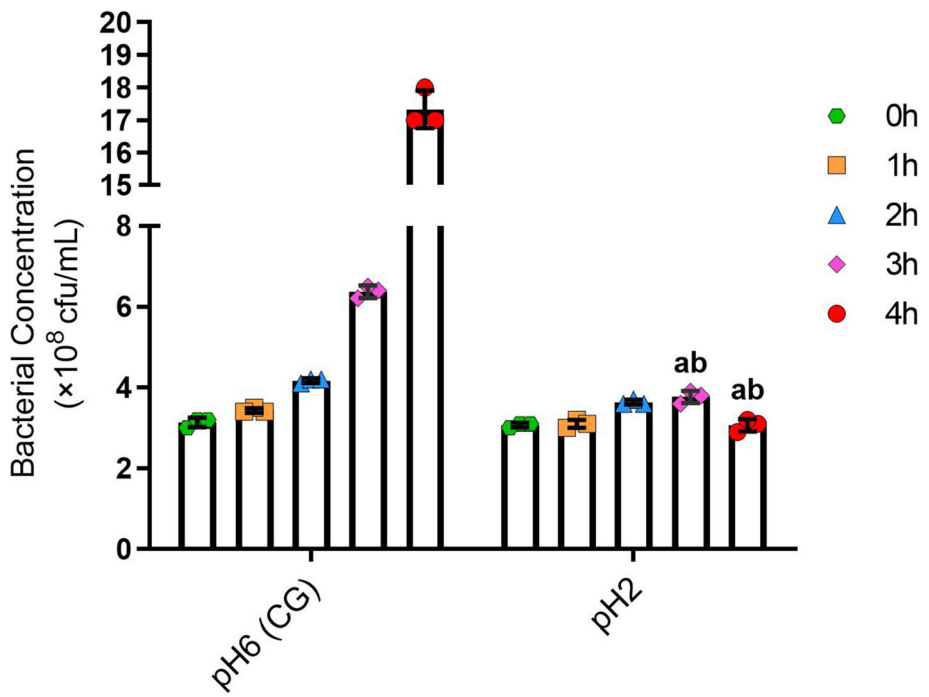

Fig. 4 The bacterial concentration of FGM cells in different acid-treated groups. Results are expressed as means ± SD (***n*** = 3) of viable cells. The pH value of the control group (CG) was 6.0. The data of treated groups were compared with the data of CG at the same time point. Mean values with a letter (a) differ significantly (***P*** < 0.05), and with different letters (ab) differ extremely significantly (***P*** < 0.01).

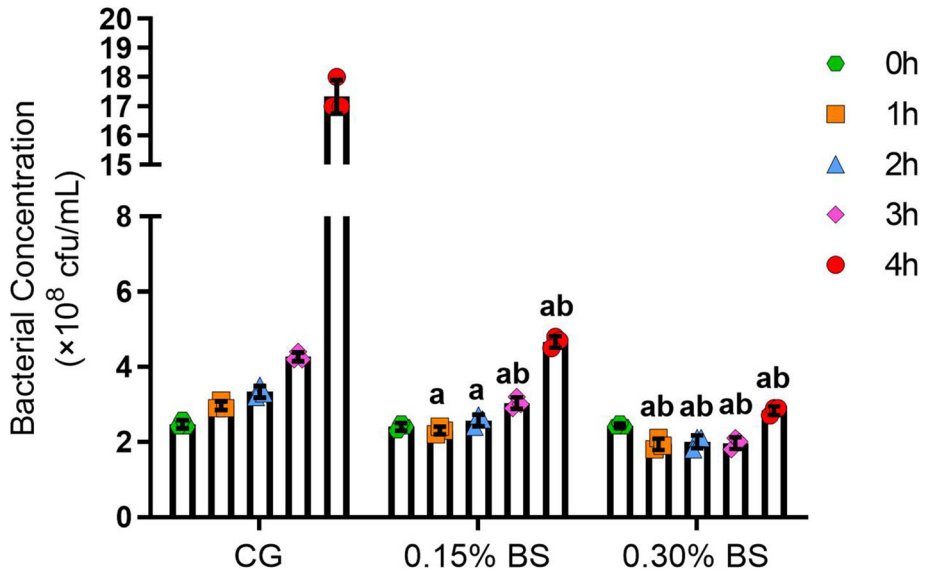

Fig. 5 The bacterial concentration of FGM cells in different bile treated groups with bile salts. Results are expressed as means ± SD (***n*** = 3) of viable cells. The data of treated groups were compared with the data of CG at the same time point. Mean values with a letter (a) differ significantly (***P*** < 0.05), and with different letters (ab) differ extremely significantly (***P*** < 0.01)

to caco-2 cells, and attached mainly at the outside of the cell membrane. On the contrary, no such observation was made in Caco-2 cells in Fig. 6a. To evaluate the adherence ability of bacteria to Caco-2 cells easily and accurately, an improved fluorescence tagging assay and a designed formula were used to calculate the bacterial adhesion rate. Figure 7 showed that *S. alactolyticus* strain FGM was capable of adhering to the intestinal epithelial cells. There was a positive correlation between the concentration of germ and the adherence rate. The adherence rate of FGM cells with 1.0×10^9 CFU/mL was 37.51 ± 3.18% and was the highest among groups which ($P < 0.05$).

Pathogens inhibitory activity

In our test, two methods, anti-*E. coli* growth test and competition test with *E. coli* were used to compare the pathogens inhibitory ability of *S. alactolyticus* strain FGM with other 3 recognized probiotics, and *E. coli O78* was selected as the indicator bacteria. The diameter of the inhibition zone against *E. coli O78* in 4 strains is shown in Fig. 8. The inhibition spectrum of strain FGM was significantly bigger than those of *L. acidophilus*, *L. reuteri*, and *L. casei* ($P < 0.05$). This indicated that FGM pellets have a strong feature to inhibit the growth of *E. coli O78*.

A pathogenic bacterium first attaches to the intestinal epithelium when it enters into the host intestines.

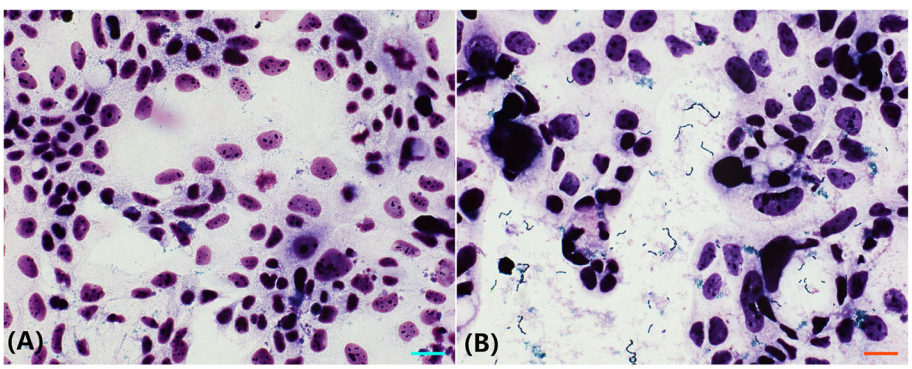

Fig. 6 Adherence ability of FGM strain on Caco-2 cell by microscopic inspection. **a** The Gram-stained Caco-2 cells were not incubated with strain FGM suspension (control group). **b** The morphologies of gram-stained Caco-2 cells were incubated with 1.0×10^8 CFU/mL FGM suspension. The bars were 20 μm

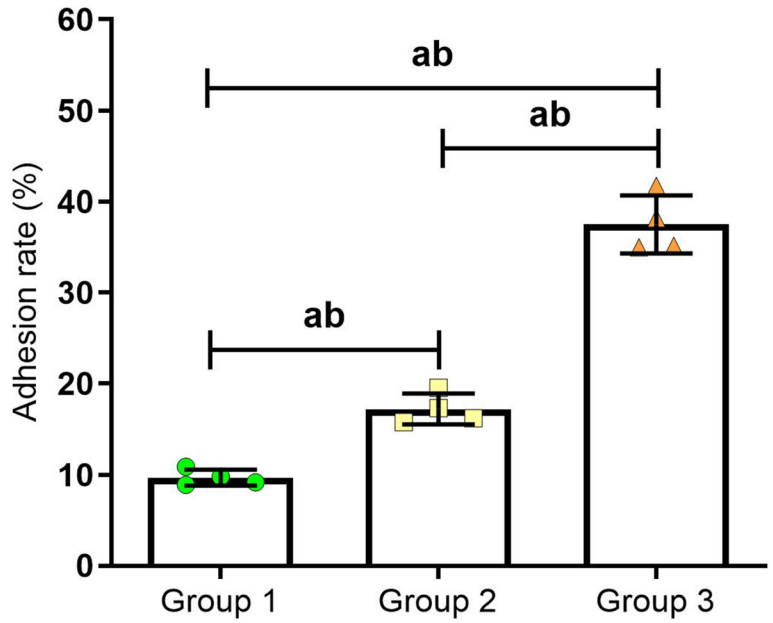

Fig. 7 Adherence rate of different concentrations of strain FGM on Caco-2 cell by fluorescence detection. The concentrations of strain FGM in group 1, group 2, group 3 were 3.0×10^8 CFU/mL, 6.0×10^8 CFU/mL and 1.0×10^9 CFU/mL, respectively. Results are expressed as means ± SD (***n*** = 4) of viable cells. Mean values with a letter (a) differ significantly (***P*** < 0.05)

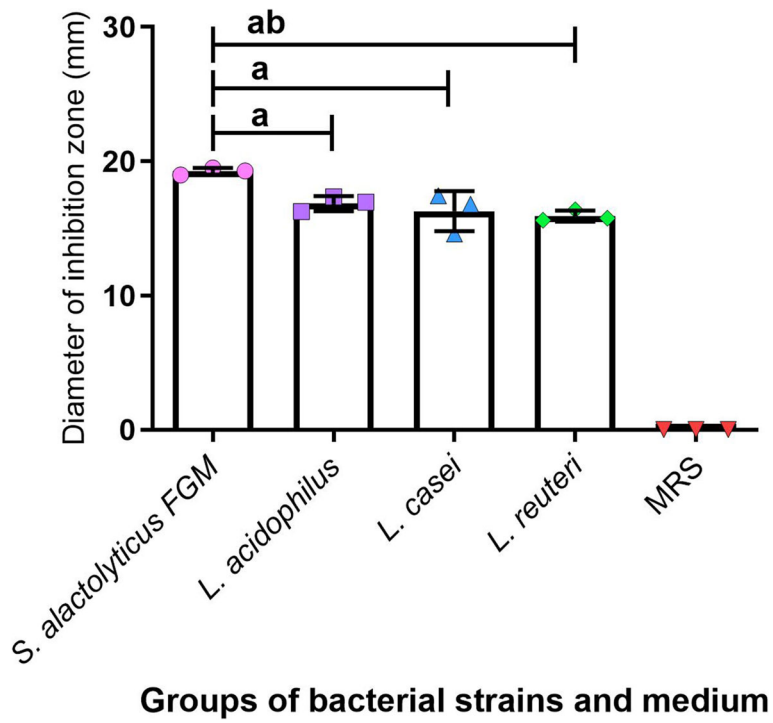

Fig. 8 Inhibition zone size against *E. coli O78* of 4 strains and their respective medium. Results are expressed as means ± SD (***n*** = 4) of viable cells. Mean values with a letter (a) differ significantly (***P*** < 0.05)

Hence, inhibiting its adherence to the intestinal epithelium can decrease the infection rate in animals. As shown in Fig. 9, *S. alactolyticus* strain FGM and other regarded probiotics (*L. acidophilus*, *L. casei*, and *L. reuteri*), all prevented *E. coli O78* from adhering to Caco-2 cells. The adherence loss rate of *E. coli O78* in the strain FGM group was significantly higher than in the group of *L. acidophilus* ($P < 0.05$), and significantly lower than in the group of *L. reuteri* ($P < 0.05$). There was no obvious difference in the loss of adherence rate of *E. coli O78* between the strain FGM group and the *L. casei* group ($P > 0.05$). The result proved that *S. alactolyticus* strain FGM can inhibit the adhesion of *E. coli O78* to the epithelium.

Discussion

With increasing knowledge about their essential role in the host health, the gut microbiota is now considered an important ally, interacting with most cells (Cani 2018). Recent studies showed that probiotics in the gut microbiome play a very important role in the health of humans and animals (Vitetta et al. 2017). *S. alactolyticus* strain FGM isolated from the chicken cecum has a good ability to ferment *A. membranaceus*, which improves the concentration of polysaccharides, and the fermented products are more beneficial to the health of broilers. It is reported that the health benefits of fermented food is very associated with the probiotic lactic acid bacteria (Chen et al. 2020). To indicate the safety and benefits of application in animal feed in future, this study sequenced the genome of *S. alactolyticus* strain FGM and subjected it to genomic analyses and probiotic properties analysis.

Undoubtedly, genome sequencing and analysis of potential probiotic candidates have become mandatory in the last years, to gain information on their safety aspects and functional properties (Reid et al. 2019; Esther et al. 2020). However, no literature had reported on the genome information of *S.* alactolyticus so far. The sequencing analysis showed that the GC content of strain FGM is 40.38% which is similar to the other species of the genus *Streptococcus* (Lifu et al. 2013). Based on the assembly sequence of genes, 2655 genes were annotated according to the GO database. It is noticeable that the strain FGM had a high number (151) of genes annotated to biological adhesion. The capability to adhere to animal intestinal cells is an important characteristic for probiotic bacteria to favor their colonization of the host gut. Using 1.0×10^8 CFU/mL bacterial concentration incubated with Caco-2 cells, Li et al. found that the highest adherence rate among *S. alactolyticus* strains isolated from swine was 9.713% (Li et al. 2016). In this study, we used image observation and fluorescent quantitation to evaluate the capability of *S. alactolyticus* strain FGM to adhere to intestinal epithelial cells, and the adherence

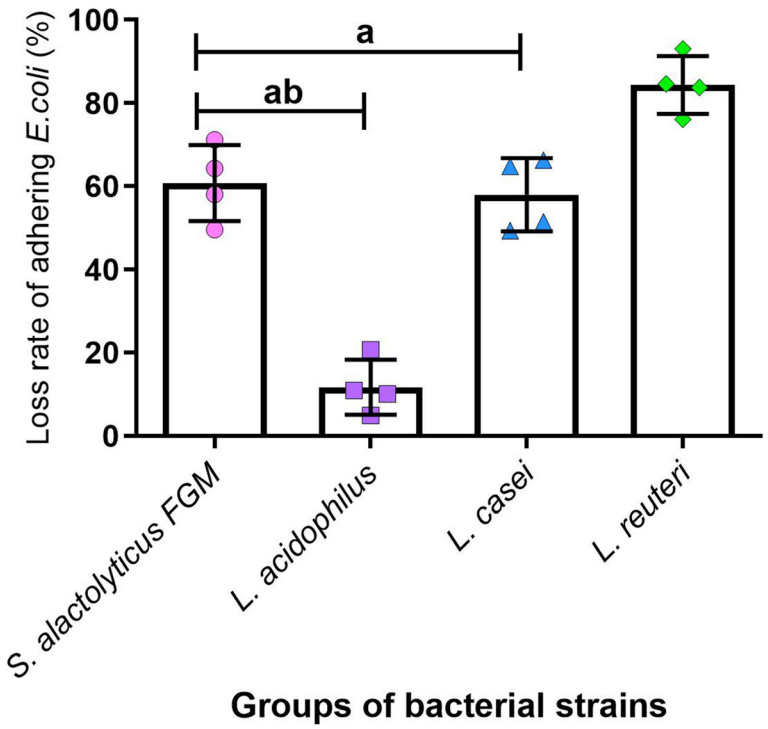

Fig. 9 The adherence loss rate of *E. coli O78* on Caco-2 cell by fluorescence detection. Results are expressed as means ± SD (***n*** = 4) of viable cells. Mean values with a letter (a) differ significantly (***P*** < 0.05)

rate of 1.0×10^9 CFU/mL FGM cells was 37.51 ± 3.18%. The result indicates the strain FGM has capability to adhere to intestinal epithelial cell.

Because of the increasing concern on the diffusion of antibiotic-resistant bacteria, probiotic bacteria must be checked for the presence of antibiotic resistance genes that could be transferred to other bacteria, in particular to pathogens (Curragh and Collins 1992). Contrary to transmissible antibiotic resistance, intrinsic resistance could be quietly favorable, giving a chance to probiotics to survive an antibiotic treatment on the host (Charteris et al. 1998). The susceptibility result of *S. alactolyticus* strain FGM was not strictly consistent with the detection of antibiotic resistance genes conducted using CARD, and just showed the resistance to trimoxazole and oxytetracycline. *S. alactolyticus* strains from the respiratory and genitourinary of porcine clinical specimens in Brazil were reported to have high resistance rates to tetracyclines and macrolides (Moreno et al. 2016). The resistance to kanamycin is also considered intrinsic in all streptococci strains (Farrow et al. 1984). However, *S. alactolyticus* strain FGM did not show resistance to kanamycin (Aminoglycosides) and Erythromycin (Macrolides) in the test. β-Hemolytic activity is one of the main safety concerns besides antibiotics susceptibility and should therefore be evaluated even for strains belonging to bacterial species that possess the QPS or GRAS status (Armin et al. 2019). In our study, *S. alactolyticus* strain FGM did not show the presence of hemolytic gene and activity which reflected its safety. This was similar to the previous findings (Farrow et al. 1984)

As potential probiotics for animals, the bacteria have to adapt to the environments with acid and bile salt to survive in the gastrointestinal tract. It was also reported that chyme can decrease the damage degree of probiotics by acid when passing through the gastrointestinal tracts. Once the bacteria pass through the stomach and duodenum, they enter into the ileum and cecum with the chyme and increase drastically (Kos et al. 2000). Our results indicated that *S. alactolyticus* strain FGM could rem*ain* a certain amount cells in pH 2 broth and 0.15~0.30% bile salt broth in 1~4 h. It showed a similar property to *S. alactolyticus* strains (FB027, FB034, FB018) for bile salt tolerance and acid tolerance (Li et al. 2016). Besides, the functions of amino acids predicted by VFanalyzer and CARD such as bile-salt hydrolase, capsule, MOMP, and cfrA contribute to the survival and colonization of the strain in the harsh gastrointestinal environment.

Research has shown that lactic acid bacteria with high adhesion can compete with pathogenic bacteria for adhesion receptors of the intestinal epithelium and get priority for colonization, to effectively inhibit colonization of pathogenic bacteria and eliminate them (Sun et al. 2007). Meanwhile, competition from lactic acid bacteria for nutrition also limits the excessive multiplication of pathogenic bacteria and maintains the balance of intestinal microecology (Tallon et al. 2007). *Lactobacillus* known as probiotics showed strong adherence and inhibition to pathogens adhesion and growth (Ma et al. 2006; Fernández et al. 2018). In this paper, we compared *S. alactolyticus* strain FGM with three *lactobacillus* strains on pathogens inhibition. The result indicated that *S. alactolyticus* strain FGM was similar with the *Lactobacillus* strains, not only inhibited significantly the growth of *E. coli O78* on adapted medium but also induced competitively the loss of adhering *E. coli O78* amount on Caco-2 cells. This probiotic property provides a reasonable basis for why the strain FGM could improve the health of broilers infected by *E. coli O78* (Wu et al. 2020).

Conclusions

The genomic and phenotypic assessments of safety and probiotic features were studied for *Streptococcus alactolyticus* strain FGM isolated from the chicken cecum in China that already had indicated some technological potential on herb fermentation. Our results demonstrated the absence of adverse effects in the strain regarding hemolytic activity, hemolytic gene, susceptibility to most commonly used antibiotics, putative resistance-related genes and survive-related genes. We also got promising results regarding probiotic potential, such as resistance to acid and bile salts, adhesion ability to Caco-2 cells and inhibition activity to *E. coli*, thus contributing to increase our knowledge of *S. alactolyticus*. However, further studies are needed in order to exclude any possible safety concern and supplement other benefits for animal health prior to use the strain FGM in animal feed.

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Not applicable.

Authors' contributions

Jingyan Zhang conducted research on correlation analysis and drafted a manuscript. Hong Zhang and Kang Zhang participated in the experiments. Lei Wang participated in its design, coordination, and modification. Zhengying Qiu participated in the data analysis. Kai Zhang and Yong Zhang improved the writing of the manuscript. Cong Yue participated in the modification of manuscript. Jianxi Li and Xingxu Zhao designed the experimental scheme, and carried out the overall planning and improvement of the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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