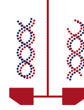
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Genomic, probiotic, and metabolic potentials of *Liquorilactobacillus nagelii* AGA58, a novel bacteriocinogenic motile strain isolated from lactic acid-fermented shalgam

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This study aimed to perform genomic, probiotic, and metabolic characterization of a novel *Liquorilactobacillus nagelii* AGA58 isolated from a lactic acid-fermented shalgam beverage to understand its metabolic potentials and probiotic features. AGA58 is gram-positive, motile, catalase-negative and appears as short rods under the light-microscope. The AGA58 chromosome comprises a single linear chromosome of 2,294,635 bp that is predicted to carry 2135 coding sequences, including 45 tRNA genes, 3 mRNA, and 3 rRNA operons. The genome has a G+C content of 36.9%, including 55 pseudogenes and a single intact prophage. AGA58 is micro-anaerobic due to achieving a shorter doubling time and faster growth rate than micro-aerophilic conditions. It carries flagellar biosynthesis protein-encoding genes predicting motile behavior, which was confirmed with the in vitro motility test. AGA58 is an obligatory homofermentative lactobacillus that can ferment hexose sugars such as galactose, glucose, fructose, sucrose, mannose, *N*-acetyl glucosamine, maltose, and trehalose to lactate through glycolysis. No acid production from pentoses implies that five-carbon sugars are being utilized for purine and pyrimidine synthesis. Putative pyruvate metabolism revealed formate, malate, oxaloacetate, acetate, acetaldehyde, acetoin, and lactate forms from pyruvate. AGA58 is predicted to encode the *LuxS* gene and biosynthesis of class IIa and Blp family class-II bacteriocins suggesting this bacterium's antimicrobial potential, linked to antagonism tests that AGA58 can inhibit *Escherichia coli* ATCC 43895, *Salmonella enterica* serovar Typhimurium ATCC 14028, and *Klebsiella pneumonia* ATCC 13883. Moreover, AGA58 is tolerant to acid and bile concentrations simulating the human gastrointestinal conditions depicting the probiotic potential of the organism as the first report in literature within the same species.

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[**Key words:** *Liquorilactobacillus nagelii*; Comparative genomics; Bacteriocin; Motility; Probiotic; Metabolism]

Lactobacilli are rod-shaped, gram-positive, facultative anaerobe, and catalase-negative microorganisms heavily utilized in the food industry due to their well-established technological features and well-documented beneficial effects on health (1). The lactobacilli are classified as GRAS (Generally Recognized as Safe) by USDA (2). In addition to the starter and probiotic actions, lactic acid bacteria (LAB) are potential bioprotective cultures because of their capability of producing antimicrobials such as nisin, enterocin, pediocin, salivaricin, plantaricin (3,4) which exists in many fermented dairy and vegetable products (5–7).

The lactobacilli are comprehensive and major groups of LAB isolated from various ecological niches (2). Shalgam is a lactic acid-fermented beverage specific to the Southern Anatolia region of Turkey and is known to be a reservoir of lactobacilli such as *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, *Levilactobacillus brevis*, *Levilactobacillus parabrevis*, *Lactobacillus acidophilus*, *Lactobacillus gasseri*, *Lactobacillus helveticus*, *Limosilactobacillus reuteri* with these last two species being predominant organisms (8,9). Until now, no studies on uncovering the shalgam microbiome reported the presence of *Liquorilactobacillus* species such as *Liquorilactobacillus nagelii*. *Liquorilactobacillus* is a lactobacillus from liquids, indicating the isolation of species from liquid environments, including plant sap, water, and alcoholic beverages. Generally, it shows a homofermentative lifestyle with carrying mol % G+C content of 33.9–40.0 (10,11). Except for *Liquorilactobacillus cacaonum*, *Liquorilactobacillus hordei*, and *Liquorilactobacillus mali*, most species in this genus are motile. Before the re-taxonomic structuring of the genus *Lactobacillus* into 23 new genera, *Liquorilactobacillus* species were regarded as part of the *Lactobacillus salivarius* group (12). *L. nagelii* is microaerophilic, mesophilic, and grows in MRS, including 5% (w/v) NaCl at pH 4.5 at room temperature. It uses citrate and malate in the existence of six-carbon sugar such as glucose (13). *L. nagelii* biosynthesizes an exopolysaccharide named dextran, which is produced from sucrose. Previous studies reported that *L. nagelii* had been isolated from water kefir, fermented cassava food, wild cocoa bean fermentation and semi-fermented wine, silage fermentation, and Kombucha (10,14–16).

The source of isolation of a microorganism is one of the primary factors determining the metabolic potential of the strain. For example, *Lactobacillus casei* isolated from silage material has a different carbohydrate utilization pattern compared to *L. casei*

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isolated from cheese microenvironment. Thus, strain-level identification and sequencing of the entire genome of lactobacilli are critical for understanding the strain adaptations to ecologically different conditions (17). Looking into NCBI public database, apart from *L. nagelii* AGA58 reported in the present study, only two strains of *L. nagelii* related to fermented foods have whole genome sequences available as of April 2022. This limits understanding of microbial community dynamics and microbial interactions in fermented foods microbiomes.

Shalgam, at the onset of lactic acid fermentation, contains sucrose, glucose, and fructose (18,19). When the fermentation ceases, a residual amount of sugar remains in the final product meaning fermentable carbohydrates are utilized by the shalgam microbiome (20). Since traditional manufacturing of shalgam relies on spontaneous fermentation, the microbial community is potentially very diverse in harboring unique LAB strains. We isolated a novel *L. nagelii* strain AGA58 from traditional lactic acid-fermented shalgam. The present study aimed to explore the genomic, probiotic, and metabolic potentials of a newly isolated *L. nagelii* AGA58 from shalgam. The whole genome of the *L. nagelii* AGA58 was sequenced, and *in silico* probiotic and metabolic potentials using bioinformatic tools have also been explored. This is the first report describing the genomic, probiotic, and metabolic characteristics of a novel *L. nagelii* AGA58 strain isolated from a plant-based traditional fermented beverage shalgam microbiome.

MATERIALS AND METHODS

Isolation of bacterial strain and growth conditions *L. nagelii* AGA58 was isolated from fermented turnip juice (shalgam) produced in Adana, Turkey. A 10-mL sample of the shalgam was diluted with 90 mL of Maximum Recovery Diluent (Merck, GmbH, Darmstadt, Germany) in a Schott bottle and vortexed for 1 min with a high-speed vortex (MS-3 Basic, IKA-Werke GmbH, Staufen, Germany). A 100-μL sample from serial dilutions was spread on MRS agar (Merck), followed by incubation at 30 °C for 48 h anaerobically. The isolate, later named AGA58, was picked and subjected to colony purification twice. Gram staining and catalase tests were performed to pure isolate *L. nagelii* AGA58. The cryovial stocks of AGA58 were prepared using MRS broth (Merck) with 25% glycerol and were stored at −80 °C.

Genomic DNA extraction, whole-genome sequencing, and *de novo* assembly *L. nagelii* AGA58 cryo-culture was subcultured twice in MRS broth and anaerobically incubated for 24 h at 30 °C. A 1-mL fresh culture was transferred to a sterile 2-mL microcentrifuge tube and centrifuged at 6000× *g* for 10 min at 4 °C. The supernatant was removed. Following the manufacturer's recommendations, the total genomic DNA extraction was performed in a cell pellet by PureLink Genomic DNA Mini Kit (Invitrogen, Thermo-Fisher Scientific, Carlsbad, CA, USA). The quality and concentration of genomic DNA were checked by a Qubit 3.0 fluorometer (Invitrogen) and agarose gel electrophoresis (1.5%). The sequencing libraries were constructed using Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA), and sequencing was conducted by Illumina Novaseq platform as paired-end (PE) 2 × 250 bases read. The adapter sequences and other contaminants were trimmed with the JGI-RQC Filter pipeline (BBTools v 38.22) by preserving the reads belonging to the strain AGA58 and assembled in the genome assembly service of PATRIC 3.6.12 (https://patricbrc.org/app/Assembly2) using an auto-assembly strategy (21).

Bioinformatic analysis Genome annotation and comprehensive genome analysis were conducted using NCBI Prokaryotic Genome Annotation Pipeline (PGAP) and PATRIC 3.6.12. platform (21,22). The prophage regions on the AGA58 genome were identified and annotated with the PHASTER-Phage Search Tool Enhanced Release (23). The BLAST ring alignment and prophage mapping for *L. nagelii* AGA58 (NZ_CP092367.1), DSM 13675 (NZ_CP049304.1), and TMW 1.1827 (NZ_CP018180.1) were performed via BRIG v 0.95 (24). The calculation of orthologous average nucleotide identity values (OrthoANI) of AGA58 and other *Liquorilactobacillus* strains was performed by OrthoANI tool v 0.93.1 (25). The metabolic pathways of *L. nagelii* AGA58 were predicted using BlastKOALA for screening against the KEGG database (25). The bacteriocin biosynthesis responsible gene cluster prediction and region-to-region MIBiG comparison were executed using the BAGEL4 and antiSMASH v 6.0, respectively (26,27). Then, the NCBI protein BLAST suite confirmed each member of the predicted gene clusters (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The whole-genome sequence of *L. nagelii* strain AGA58 has been submitted to NCBI under accession number NZ_CP092367.1.

Carbohydrate fermentation The carbohydrate fermentation patterns of the AGA58 strain were determined using an API 50 CHL kit (BioMérieux, Marcy l'Etoile, France) consisting of 49 different carbohydrate tests under the manufacturer's protocols.

Determination of antibiotic susceptibility Antibiogram tests were performed to screen resistance or sensitivity of AGA58 against commonly used antibiotics. Commercially available antibiotic disks [methicillin, vancomycin, amikacin, kanamycin, azithromycin, tetracycline, penicillin G (Bioanalyse, Yenimahalle, Ankara, Turkey); ampicillin, oxacillin, carbenicillin, amoxicillin, streptomycin, erythromycin, rifampicin (Oxoid, Basingstoke, Hampshire, UK)] were utilized to determine antibiotic susceptibility of *L. nagelii* AGA58. The disk diffusion assay was performed per the modified method of Kirby–Bauer (28). Interpretation of inhibition zone (mm) results achieved following the Clinical and Laboratory Standards Institute's performance standards for the antimicrobial testing (29). An inhibition zone less than or equal to 14 mm was noted as resistant (R). An inhibition zone greater than 20 mm was considered sensitive (S). Between 15 and 19 mm, zones were recorded as semi-sensitive or intermediate (I).

Motility test The overnight grown cultures of *L. nagelii* AGA58, *Klebsiella pneumonia* ATCC 13883 and *Proteus mirabilis* ATCC 29906 were submerged in motility test medium (3 g/L beef extract, 10 g/L pancreatic digest of casein, 5 g/L NaCl, 4 g/L agar–agar, 10 g/L dextrose, 0.5 g/L 2,3,5-triphenyltetrazolium chloride) using sterile inoculation needle followed by incubation at 37 °C under anaerobic, aerobic, and aerobic conditions, respectively. All ingredients of the motility medium were purchased from Merck. *K. pneumonia* ATCC 13883 was utilized as a negative control, whereas *P. mirabilis* ATCC 29906 was employed as a positive control. Red-colored zones across the TTC motility media represent the motility of the organism. Limited, local-thin growth on the inoculation spot means no motility is observed (30).

β-hemolytic activity test Evaluation of the β-hemolytic activity of the AGA58 strain was performed via 5% sheep blood containing Columbia agar plate (Oxoid) (31). The isolate was plated on the Columbia agar and incubated at 37 °C for 48 h under anaerobic conditions.

Cell surface hydrophobicity and auto-aggregation assays The adherence of bacteria to hydrocarbons was measured according to the modified method reported by Krausova et al. (32). Bacterial cells were cultured in MRS broth (Merck) at 37 °C for 18 h before centrifuging at 8000× *g* per minute for 10 min. The cell pellets were washed twice with pH 7.0 phosphate solution, resuspended in phosphate buffer, and the initial absorbance at 600 nm was adjusted to 0.6 OD (Ab_I). Vortexed cell suspension was combined with n-hexadecane (3:1) and incubated at 37 °C for 10 min. The mixture was vortexed again and maintained at 37 °C for 1 h to separate the phases. At 600 nm wavelength, its absorbance (Ab_F) was measured after the aqueous phase was removed with care.

$$\text{Cell surface hydrophobicity} = 100 \times (Ab_I\text{-}Ab_F)/Ab_I \quad (1)$$

Auto-aggregation test was conducted according to the method described by Krausova et al. (32). A 5-mL sample of culture was correctly mixed and incubated at 37 °C for 24 h. A 1-mL sample of upper suspension was extracted from an undisturbed incubation tube; optical density (OD) was measured at 600 nm, and the percentage of auto-aggregation was estimated as follows:

$$\text{Aggregation (\%)} = 1 - (OD_{\text{upper suspension}}/OD_{\text{total culture}}) \times 100 \quad (2)$$

Antibacterial activity test The antibacterial activity test was performed by the agar well diffusion method according to Mishra and Prasad (33). The supernatant of 18–20 h grown AGA58 was tested against *Escherichia coli* ATCC 43895, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 33019, *Salmonella enterica* serovar Typhimurium ATCC 14028, *Proteus vulgaris* ATCC 8427, and *K. pneumonia* ATCC 13883.

Growth kinetics in artificial gastric digestion The growth kinetics of AGA58 were evaluated at different pH and bile concentrations to determine this organism's artificial gastric digestion survival based on the methods described by Sun and Griffiths (34) and Yetiman et al. (35). Oxyrase enzyme (Merck) was used to reduce the oxygen level in a microtiter plate. The pH of the MRS medium (Merck) was adjusted to 6.8–8.4, which is optimum for oxyrase. Oxyrase was added to the medium in proportions, according to McMahon et al. (36). MRS medium with five different pH values (pH 2, 3, 4, 5, and 7) was prepared using 3 N HCl and 3 N NaOH, and four different bile concentrations (0% control, 0.3%, 0.5%, and 1%) were prepared using ox-bile extract (Merck) followed by incubation at 36.5 °C for 30 min to activate oxyrase. Growth measurements were performed in a microplate reader (Model Sense, Hidex, Turku, Finland) using 96 well-plates with a lid. Each well was inoculated with 200 μL of overnight grown culture incubated at 30 °C. Each sample was run in quadruplicates. Spectrophotometric measurements were carried out at 30 and 37 °C at 300-rpm orbital shake. The OD_{600} measurement was performed every 20 min at 72 h of post-inoculation. Statistical analysis of doubling time and maximum specific growth rates were performed using one-

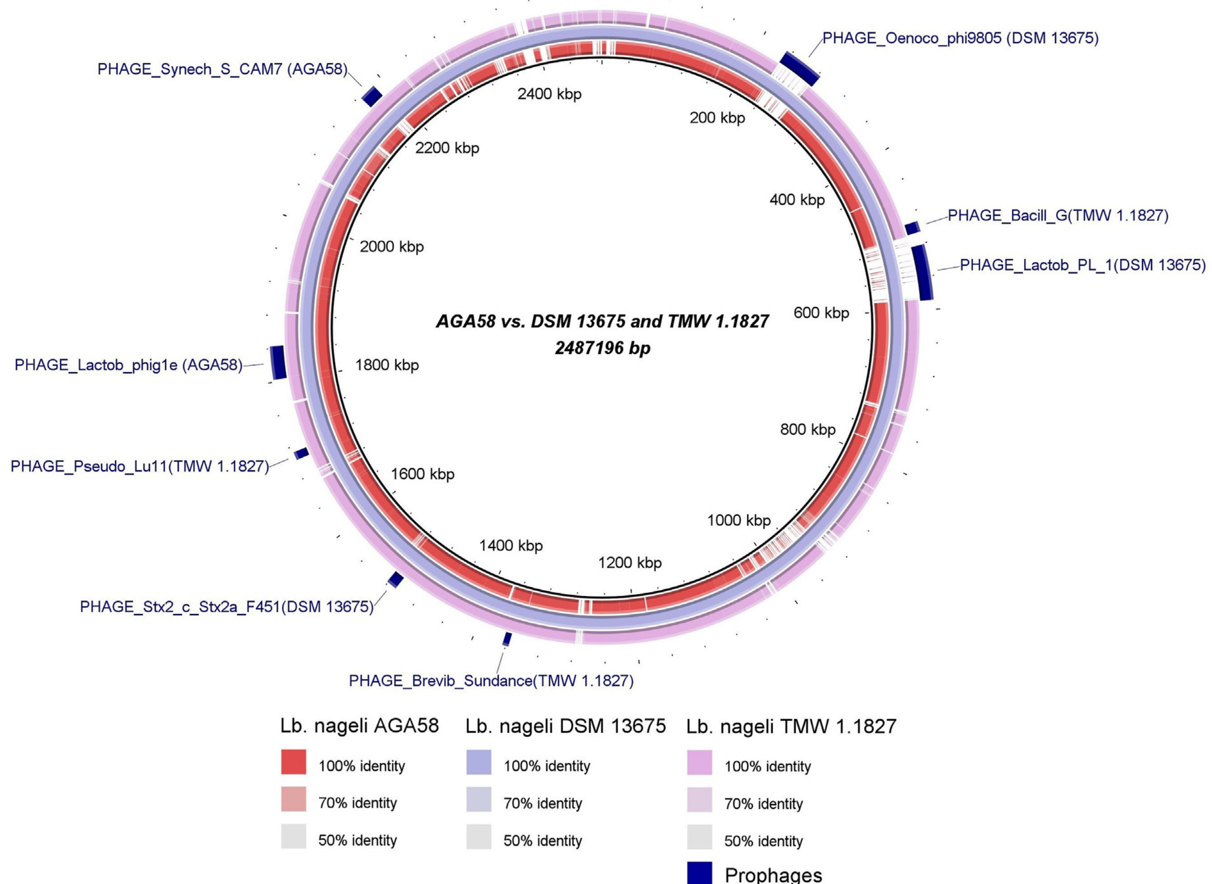

FIG. 1. Blast RING alignment of the genomes of *Liquorilactobacillus nagelii* AGA58, DSM 1367, and TMW 1.1827 strains which were ordered from inside to outside. Phage regions of all *L. nagelii* strains were mapped on aligned genomes.

way ANOVA, and multiple comparisons between means were conducted via Tukey HSD.

RESULTS

Genomics of *L. nagelii* AGA58 *L. nagelii* AGA58 is a gram-positive, rod-shaped, catalase-negative lactobacilli isolated from a traditional lactic acid-fermented turnip and red carrot beverage called shalgam produced in the Southern Anatolia region. The genome of AGA58 consists of a single linear chromosome with a genomic size of 2,294,635 bp, 36.9% G+C content, 2135 protein CDS, 45 tRNA, 3 rRNA (Fig. 1, Table 1), and 2 CRISPR array regions. The genome harbors a single intact prophage, PHAGE_Lactob_phig1e_NC_004305 (Fig. 1, Table S2), and three bacteriocins (class IIa and BLP family Class II bacteriocins carrying two subunits) in the same operon (Fig. 2). The KEGG orthology (KO) functional categories of identified protein-coding sequences in the genome of *L. nagelii* AGA58, DSM 13675, and TMW 1.1827 are shown in Table S1. Most genes involved in genetic information processing, carbohydrate metabolism, and amino acid metabolism indicate that this organism requires various carbon and nitrogen sources to maintain its lifestyle. AGA58 genome is predicted to carry glutamine synthetase type I (EC 6.3.1.2), which could potentially biosynthesize L-glutamine from ammonia (37). Based on the OrthoANI results, it was found that the AGA58 shows 98.72% and 98.55% orthologous average nucleotide identity with TMW 1.1827 and DSM 13675, respectively (Fig. 3). It is generally considered that the orthoANI identity above 95% indicates that compared genomes share the same species (38). This reveals that AGA58 belongs to the *L. nagelii* species. Other closely related neighbors of *L. nagelii* AGA58 were found to be *L. hordei* TMW 1.1822 and *L. mali* LM596, isolated from water kefir and apple juice, respectively. *L. nagelii* AGA58 carries a single intact prophage, whereas *L. nagelii* TMW 1.1827 possesses no intact prophage though another strain of *L. nagelii* DSM 13675 harbors 2 intact prophages. *L. nagelii* TMW 1.1827 has a genomic size of 2.41 Mb and shows a G+C content of 36.68% and 2391 CDS, including three plasmids (Table 1).

TABLE 1. Genomic characteristics of *Liquorilactobacillus nagelii* AGA 58, DSM 13675 and TMW 1.1827.

Feature	AGA58	DSM 13675	TMW 1.1827
Size (bp)	2,294,635	2,487,196	2,406,166
GC content (%)	36.9	36.8	36.7
Genes (total)	2242	2549	2470
Protein coding sequences	2135	2387	2325
tRNA	45	57	57
rRNA	3	18	18
Non-coding RNA	4	4	4
Pseudogenes	55	83	66
CRISPR-arrays	2	1	2
Plasmid	None	1	3

Motility The flagellar biosynthesis responsible gene cluster of *L. nagelii* strain AGA58 is shown in Table S3. A total of 32 genes

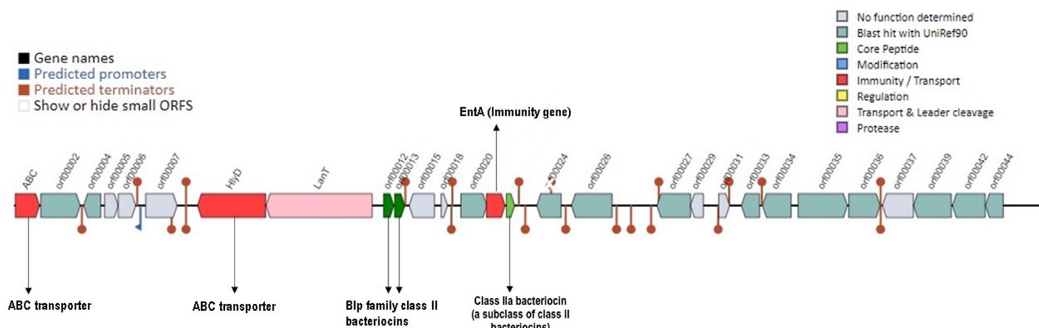

FIG. 2. The predicted gene cluster responsible for the biosynthesis of bacteriocins by using BAGEL4 webserver. The dark green genes encode blp II class bacteriocins, and the light green gene encodes class IIa bacteriocin.

TABLE 2. Comparison of carbohydrate fermentation patterns of *Liquorilactobacillus nagelii* AGA58 and DSM 13675 strains[a].

Sugar	Strain	
	AGA58[b]	DSM 13675[c]
Control	–	–
Glycerol	–	–
Erythritol	–	–
D-Arabinose	–	–
L-Arabinose	–	–
D-Ribose	–	–
D-Xylose	–	–
L-Xylose	–	–
Adonitol	–	–
Methyl-β-D-xylopyranoside	–	–
D-Galactose	+	+
D-Glucose	+	+
D-Fructose	+	+
D-Mannose	+	+
D-Sorbose	–	+
D-Rhamnose	–	+
Dulcitol	–	–
Inositol	–	–
D-Mannitol	–	+
D-Sorbitol	–	+
Methyl-α-D-mannopyranoside	–	–
Methyl-α-D-glucopyranoside	–	+
N-Acetylglucosamine	+	+
Amygdalin	–	+
Arbutin	–	–
Esculin ferric citrate	+	+
Salicin	+	+
D-Cellobiose	+	+
D-Maltose	+	+
D-Lactose	–	–
D-Melibiose	–	–
D-Sucrose	+	+
D-Trehalose	+	+
Inulin	–	–
D-Melezitose	–	–
D-Raffinose	–	–
Amidon (starch)	–	–
Glycogen	–	–
Xylitol	–	–
Gentiobiose	+	+
D-Turanose	–	–
D-Lyxose	–	–
D-Tagatose	–	+
D-Fucose	–	–
L-Fucose	–	–
D-Arabitol	–	–
L-Arabitol	–	–
Gluconate	–	–
2-Keto-gluconate	–	–
5-Keto-gluconate	–	–

[a] Fermentation results are expressed as follows: (+) positive, (–) negative.
[b] AGA58 strain has been isolated first in this study.
[c] DSM13675 strain was previously studied by Buron-Moles et al. (1).

responsible for flagellar biosynthesis were found in the genome of AGA58. The in vitro motility test results are shown in Fig. S1. It was observed that AGA58 created a red color gradient across the motility medium, which implies that *L. nagelii* AGA58 is a motile organism.

Probiotic characterization When novel probiotic strains are identified, specific characterization tests must be conducted to prove their probiotic capabilities, such as antimicrobial activity, bacteriocin biosynthesis, antibiotic resistance, auto-aggregation, cell surface hydrophobicity, acid and bile tolerance, and probiogenomics.

Bacteriocins Bacteriocin screening via BAGEL4 and anti-SMASH v 6.0 revealed that AGA58 was predicted to carry three core bacteriocin biosynthesis genes in the same operon (Fig. 2). BAGEL4 predicted two Blp family class II bacteriocins and a single class IIa bacteriocin. Region-to-region MIBiG comparison via AntiSMASH v 6.0 predicting bacteriocinogenic gene cluster revealed that the closest gene clusters were found to be gassericins of *Lactobacillus gasseri* (Tables S4–S6).

Antibiotic resistance In vitro antibiotic sensitivity test results revealed that AGA58 was sensitive to ampicillin (10 μg), carbenicillin (100 μg), erythromycin (10 μg), penicillin G (10 U), tetracycline (30 mcg), rifampicin (5 μg), and amoxicillin (25 μg) with the following zones of inhibition achieved: 15.75 mm, 18.55 mm, 21.25 mm, 15.53 mm, 25.78 mm, 24.12 mm, and 17.71 mm, respectively. On the other hand, azithromycin, oxacillin, streptomycin, vancomycin, methicillin, amikacin, and kanamycin provided lower than 15 mm inhibition zones, indicating AGA58 is resistant to those antibiotics. However, looking into the genome, only vancomycin and penicillin resistance genes were found in AGA58 (Table S7), which reveals that phenotype and genotype do not overlap completely (39).

Auto-aggregation, cell surface hydrophobicity, and β-hemolytic activity In silico probiotic features of AGA58 and other *L. nagelii* strains are shown in Table S8. The AGA58 is predicted to carry mucin binding capability, encoded in the *srtA* gene encoding Sortase A, a mucus specific LPXTG surface adhesion. The in vitro assay results revealed that *L. nagelii* AGA58 had 80.5% and 10% autoaggregation capacity and cell surface hydrophobicity, respectively. The β-hemolytic activity test showed that *L. nagelii* AGA58 does not have β-hemolytic activity.

Acid and bile tolerance Acid challenge tests of AGA58 performed at pH = 2, 3, 4, 5, and 7 are shown as growth curves in Fig. S2. Obviously, no or poor growth is seen at pH = 2 and pH = 3. However, the AGA58 growth rate gradually increased when grown at pH = 4, pH = 5, or pH = 7. The maximum specific growth rates (μ_{max}) achieved were pH = 7 >, pH = 5 >, and pH = 4. Although pH = 7

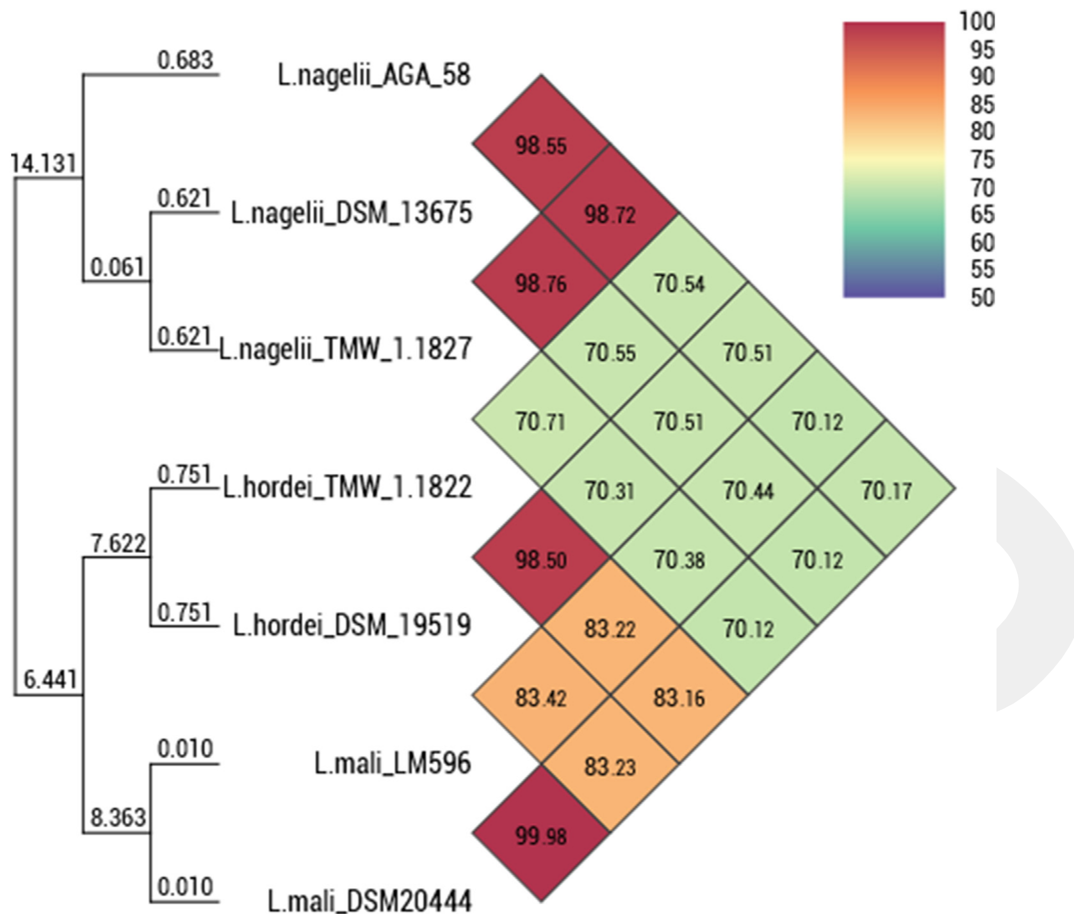

FIG. 3. Orthologous average nucleotide identity dendrogram between *Liquorilactobacillus nagelii* strain AGA58 vs other *Liquorilactobacillus* species.

provided faster μ_{max} compared to the pH = 5 condition, final biomass concentrations were similar. On the other hand, the pH = 4 condition achieved final biomass concentration was remarkably lower than both pH = 5 and pH = 7. Bile salt challenge tests performed across 0.3%, 0.5%, and 1% bile salt treatments provided no significantly different doubling times for AGA58 ($P > 0.05$). Control treatment (i.e., no bile) yielded a significantly shorter doubling time than all the others ($P < 0.05$). Similarly, μ_{max} values obtained across all bile challenges were not significantly different. Again, the control treatment revealed the highest maximum specific growth rate (Figs. S3 and S4).

Probiogenomics Probiotic function-related genes found in the genome of *L. nagelii* AGA58 are shown in Table S8. Stress resistance-related genes such as *dltD*, *dltA*, and *xylA* were found in addition to DNA and protein protection and repair-related genes. Moreover, the *cbh* gene encoding choloylglycine hydrolase as an active removal of stressors, the *LuxS* gene encoding ribosylhomocysteine lyase as an anti-pathogenic effect, the *DltB* gene encoding D-alanyl transfer protein as an immunomodulator, enolase gene functional in collagen binding, and *srtA* encoding sortase A as mucus specific LPXTG surface adhesin were found in the genome of *L. nagelii* AGA58.

Antimicrobial activity The antimicrobial activity results of the supernate of *L. nagelii* AGA58 against seven pathogens are shown in Table S9. The highest inhibition zone was achieved against *B. cereus* ATCC 33019 with an inhibition zone of 9.84 mm, whereas the lowest inhibition zone was reached against *S. enterica* serovar Typhimurium ATCC 14028 with an inhibition zone of 8.35 mm. The inhibition zone of 9.16 mm was achieved against *K. pneumonia* ATCC 13883. No significant inhibition was achieved (i.e., <5 mm) against *E. coli* ATCC 43895, *S. aureus* ATCC 25923, and *P. vulgaris* ATCC 8427.

Carbohydrate and pyruvate metabolism The genomic analysis and in vitro carbohydrate fermentation profiles of *L. nagelii* AGA58 revealed that this strain could metabolize galactose, glucose, fructose, sucrose, mannose, N-acetyl glucosamine, maltose, trehalose, esculin, ferric citrate, salicin, D-cellobiose, D-maltose, gentiobiose sugars (Table 2). Figs. S5–S8 shows the genome-predicted carbohydrate metabolism pathways for *L. nagelii* AGA58 (40). For example, dextrose, one of the easier carbon source, fluxes into the glycolysis pathway when *L. nagelii* AGA58 is grown with this 6-carbon sugar (Fig. 4). D-glucose molecules in the medium enter the cytoplasm through PTS, and they were being phosphorylated into Glucose-6P, followed by conversion into Fructose 1,6 biphosphate via *pfk* gene encoding phosphofructokinase (Fig. 4). Later, it feeds into the lower half of the glycolysis pathway by the *fba* gene encoding fructose biphosphate aldolase. Since AGA58 possesses those key enzymes for the glycolytic pathway, this strain ferments the abovementioned six-carbon sugars to 2 moles of ATP and 2 moles of lactate per each mole of sugar consumed (Tables S10 and S11) (41).

Fructose, another hexose sugar available in the shalgam microenvironment, is also metabolized by AGA58 (Table 2, Fig. 4). D-Fructose is first phosphorylated to beta-D-fructose-6P by the *fk* gene encoding fructokinase, which is further phosphorylated to beta-D-fructose-1,6 P_2 and converted to Glceraldehyde-3P via *fba*. Eventually, D-fructose is metabolized thru the glycolysis shunt and generates both ATP and lactate as a result of the homofermentative

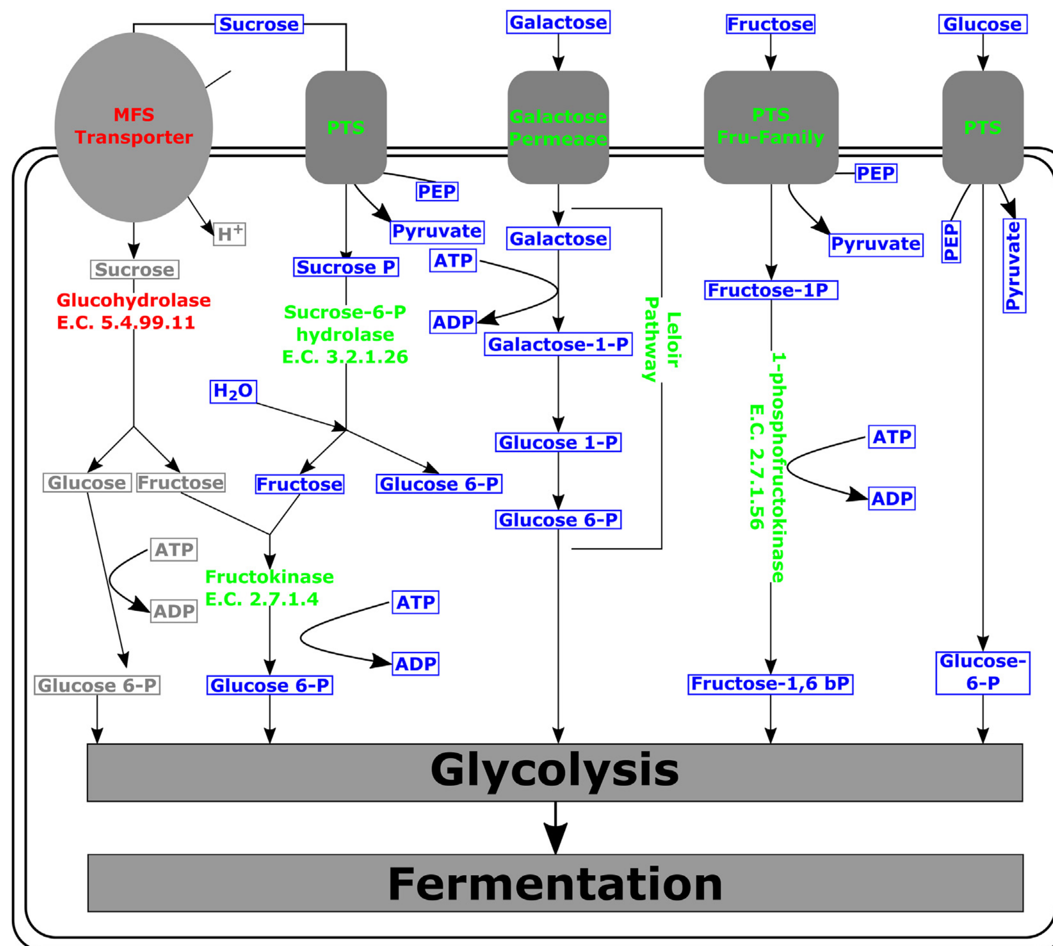

FIG. 4. Putative carbohydrate metabolism overview of *Liquorilactobacillus nagelii* AGA58 per genomic analysis. The green font shows the presence of the enzyme, the red font depicts the absence of the enzyme in the genome of AGA58.

lifestyle (41). Genome analysis of *L. nagelii* AGA58 revealed that galactose is fermented through the Leloir pathway, where it enters the cell via galactose-specific permease, after which it is phosphorylated to Galactose-1-P at the expense of 1 ATP followed by transforming to Glucose-1-P then Glucose-6-P which feeds into the glycolysis shunt resulting in lactate biosynthesis (Fig. 4) (41). The putative genes encoding key enzymes functional in the pentose phosphate pathway are shown in Table S12. *L. nagelii* AGA58 genome is predicted to carry xylose isomerase and phosphoketolase encoding genes, although transketolase, xylulokinase, and transaldolase genes are missing. Pyruvate is a critical metabolite in many fermentations, serving as an electron acceptor for the NADH to NAD^+ regeneration step to maintain redox balance. Under certain conditions, LAB utilizes alternate pathways for using pyruvate than conversion to lactate. The alternative ways in which *L. nagelii* AGA58 is predicted to utilize pyruvate are shown in Fig. 5. Putative pyruvate metabolism of AGA58 revealed formate, malate, oxaloacetate, acetate, acetaldehyde, acetoin, and lactate could form from pyruvate.

DISCUSSION

This study assessed genomic, probiotic, and metabolic features of a newly isolated *L. nagelii* AGA58 strain. We also performed comparative genomics against closely related *L. nagelii* strains of TMW1.1827 and DSM13675, isolated from water kefir and semi-fermented wine, respectively (Table 1, Fig. 3). The G+C contents achieved between all *L. nagelii* strains evaluated in the present study were similar (i.e., 36.7–36.9%). The G+C content of AGA58 was in line with the low-G+C lactobacilli. This perhaps indicates that *L. nagelii* had experienced genomic drift. It is usually thought that lactobacilli are adapted to their environmental conditions by undergoing gene loss or gene decay (42). The proportion of unknown/hypothetical genes found in *L. nagelii* AGA58 was calculated to be at ∼35%, implying that there is still more to discover about *L. nagelii*.

Motility in lactobacilli was described earlier (43,44), and a motile strain of *L. nagelii* was reported by Endo and Okada (45). However, no flagellated lactobacilli have been reported in the shalgam microbiome until the present study. Several investigations have shown that pili in gram-positive and gram-negative bacteria mediate host–microbe interactions (46–49). It was reported that flagellins and flagellates of lactobacilli expressed interleukin-8 production in human intestinal epithelial cell culture against Toll-Like Receptor 5 (43,50). *L. nagelii* AGA58's flagella might allow it to penetrate the mucus barrier, which could help it colonize and mediate the interaction with the host. These colonizing traits could also confer competitive advantages to AGA58 against other microorganisms that exist in the gut and perhaps imply the probiotic potential of AGA58.

Lactobacilli suppress pathogen growth by secreting antimicrobial substances (46). Several lactobacilli isolated from diverse origins (51–54) limit the growth of pathogens, which aligns with the current study. *L. nagelii* AGA58 was the only bacteriocinogenic *L.*

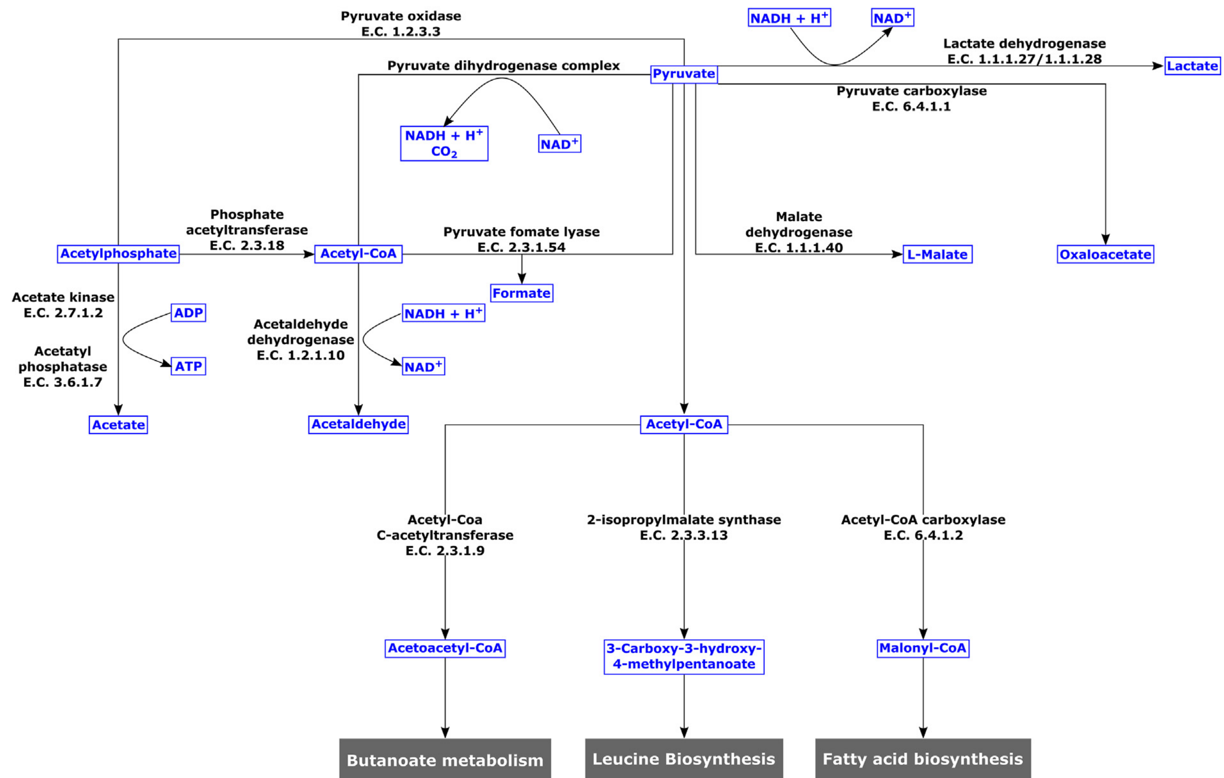

FIG. 5. Putative pyruvate metabolism of *Liquorilactobacillus nagelii* AGA58 revealed from genome analysis.

nagelii whose genomes were deposited to NCBI. The AGA58 was predicted to encode a single class IIa (i.e., a subclass of class II bacteriocins) and two Blp family class II bacteriocins (Fig. 2). Amino acid sequence of class IIa bacteriocin [*MKKEIELSEKELVRIIGGKY-YGNGVSCTKKHGCKVNWGQAFTCSVNRFANFGHGNC*] (Table S4) shows ~80% similarity to Plantaricin_423 peptide, which is known to harbor anti-listerial activity (55). Amino acid sequence diversity could exist between class II bacteriocins. For example, the YGNGV motif is conserved in class IIa (56), which implies that the novel bacteriocin found in *L. nagelii* AGA58 belongs to class IIa. Even though class IIa bacteriocin gene clusters usually reside in the plasmid, in some cases, it is located in chromosome such as for sakacin P, divercin V41, and enterocin A production (56). *L. nagelii* AGA58 perhaps encodes bacteriocin biosynthesis in chromosome since no plasmid was found in its genome. The AGA58 class IIa bacteriocin biosynthesis gene clusters included an ABC-transporter *LanT* and its accessory protein *HlyD*. Moreover, the bacteriocin structural gene resides in three genes-long small operon containing class IIa bacteriocin, immunity protein, and a hypothetical protein (Fig. 2, Table S4) which supports this unique bacteriocin belonging to class IIa subgroup (56). The putative amino acid sequence of Blp family class II bacteriocins in *L. nagelii* AGA58 revealed a GXXXG-like motif which proposes a class IIb subgroup. However, the bacteriocin-encoding gene set is not typical for class IIb as two bacteriocin-encoding genes are not closely adjacent to the immunity protein-encoding gene. In addition, most class IIb gene cluster harbor two genes encoding one ABC transporter complex (56), which doesn not exist in *L. nagelii* AGA58. Blp family class II bacteriocins are extracellularly secreted to combat gram-positive bacteria that are closely related (57). AGA58 possessing such a bacteriocidal gene cluster allows us to link the genomic evidence of bacteriocin presence with in vitro antibacterial activity test results showing the antagonistic effect of supernate of AGA58 grown media against *B. cereus* ATCC 33019, *S. enterica* serovar Typhimurium ATCC 14028, and *K. pneumonia* ATCC 13883. However, a confounding factor remains whether the inhibition achieved with the supernates of *L. nagelii* AGA58 was solely related to bacteriocins and/or organic acids produced during growth. The presence of the *LuxS* gene functional in autoinduction supports the antipathogenic potential of *L. nagelii* AGA58 (Table S8).

Bile salt tolerance tests indicate the persistence of *L. nagelii* AGA58 against bile salt levels that mimic human intestinal conditions. A slowdown in doubling time and μ_{max} at increasing concentrations of ox-bile might be attributed to the absence of the *bshA* gene encoding bile salt hydrolase; however, the *cbh* gene encoding bile salt hydrolase like bile resistance was found in the genome of *L. nagelii* AGA58 (Table S8). Moreover, a considerable amount of biomass growth achieved even at 1% bile salt treatment indicates that AGA58 persists in resilience to bile concentrations comparable to that exist in the intestinal juice. Similar results were also reported for another probiotic organism, *Lactobacillus acidophilus* ATCC 4356, which showed a slowdown in growth rate in the presence of acid conditions and stability under similar bile salt conditions (58).

The existence of surface proteins, for example, those embedded into the cell wall, was found to boost adhesion and hydrophobic interactions in several LAB (59). The enolase encoding gene found in the *L. nagelii* AGA58 genome was reported to bind collagen protein in the extracellular matrix (60). A bacterial organism possessing efficient adhesion capacity can outcompete pathogens against the host binding sites (61,62). Several cross-functional proteins could function as adhesion elements, for instance, *GroEL* chaperone, translation elongation tu, and glyceraldehyde 3-phosphate dehydrogenase (61,63). Those adhesion genes are readily available in the genome of AGA58, implying potential adhesion capacity to gut epithelial cells (data not shown). In addition to *L. nagelii* AGA58, both DSM 13675 and TMW 1.1827 carried the essential genes conferring probiotic capacity except the

xylA gene encoding xylose isomerase. *xylA* gene was only encoded in AGA58, which is predicted to interconvert D-xylose and D-xylulose for gut persistence (Table S8).

Hydrophobicity measures the microbial adhesion against hydrocarbons considered as a crucial bacterial surface feature (64) for colonization against mucosal surfaces (65). Cell surface hydrophobicity of AGA58 was twice as much compared to another potential probiotic strain *L. plantarum* DY46 isolated from shalgam (35). However, it was generally lower than *L. rhamnosus* GG (62%) and several commercial probiotic *L. paracasei* (28%–50%) and *L. acidophilus* (2%–88%) strains with the exception of *L. acidophilus* BFE719 which had a 2% cell surface hydrophobicity (66,67). Although *L. acidophilus* BFE 719 showed a low-end hydrophobicity, its adhesion to HT29 MTX cells was 40% as opposed to 25% for *L. rhamnosus* GG. Thus, hydrophobicity might help the adhesion capacity of the strain, but it is not a prerequisite for robust adherence capability (67). Noteworthy, remarkable changes in cell surface hydrophobicity might also be caused by deviations in the expression level of cell surface proteins, which varies based on environmental circumstances and bacterial strain type (68). Further in vitro cell culture studies are needed to better understand the adhesion capacity of *L. nagelii* AGA58 against various cell lines.

L. nagelii AGA58 was isolated from the fermented turnip microenvironment and associated with a different ecosystem than semi-fermented wine isolate DSM 13675, thus coming across different conditions. The distinctions in the adaptation to discrete microenvironments were also explored in their genomes. For example, the annotated differences between AGA58 and DSM 13675 might be explained by genes pertaining to carbohydrate metabolism, particularly enzymes of citrate and accompanying acetolactate metabolism. These enzyme-encoding genes only existed in the shalgam and water kefir isolate of TMW 1.1827. The AGA58 is distinguished from DSM 13675 and TMW 1.1827 with galactose metabolism with AGA58 using the Leloir pathway for galactose uptake by specific galactose permeases versus the latter two utilizing Tagatose shunt (Fig. 4, Tables S13-S16). This is also evident in API 50 CHL carbohydrate fermentation test that AGA58 did not ferment D-tagatose, although DSM13675 did (Table 2). Since galactose sugar is not readily available in respective environments, a challenge remains in explaining specific adaptations based solely on isolation sources. The genomic evidence predicting environmental adaptations seen in malted barley isolate *L. hordeii* DSM 19519 (69) or water kefir isolate *L. hordeii* TMW 1.1822 (70) is more pronounced and decisive in sucrose metabolism.

In homofermentative lactic acid fermentation, lactobacilli utilize dextrose as a 6-carbon source to make pyruvate throughout glycolysis, and then lactic acid is formed by lactate dehydrogenase (71). Several reactions in pyruvate metabolism could occur even with regular glucose fermentation. Those reactions might serve an anabolic role; for example, acetyl-CoA production could be necessary for the biosynthesis of lipids (Fig. 5) (41). Genomic analysis indicated the existence of genes-encoding key enzymes participating in various downstream pyruvate transformations. AGA58 could generate ATP upon acetate formation via acetate kinase and maintain the redox balance using L/D-lactate. Since no alcohol dehydrogenase enzyme encoding gene was found in the AGA58 genome, other avenues for NAD^+ re-cycling could be more favorable, such as transforming pyruvate to acetoin. Acetolactate synthase is the key enzyme for the catabolism of pyruvate to α-acetolactate, which is predicted to be encoded in the genome of AG58, suggesting it might synthesize these compounds. Since α-acetolactate is not a stable molecule, it is reduced to acetoin via α-acetolactate decarboxylase or decarboxylated to diacetyl nonenzymatically. Finally, the diacetyl/acetoin reductase enzyme reduces diacetyl to acetoin, which is then transformed to 2,3-butanediol upon concurrent NAD^+ regeneration (72). Since AGA58 was found to carry all relevant enzyme-encoding genes for the biosynthesis of acetoin, acetaldehyde, and acetate, it might contribute to the flavor profile of shalgam. Although AGA58 has a citrate synthase, it lacks the remaining key enzymes completing the TCA cycle, which aligns with *L. hordei* TMW 1.1822 isolated from water kefir (70,72).

In conclusion, we found that *L. nagelii* AGA58 is a motile strain that carries fundamental probiotic features such as bacteriocin biosynthesis, auto-aggregation, cell surface hydrophobicity, acid-bile tolerance, mucin binding, adhesion, and gut persistence. It can utilize hexose sugars via glycolysis due to its obligatory homofermentative nature, which is also confirmed by genomic evidence. A single intact prophage and transposases found in AGA58 are indicators of the plasticity of the genome. The presence of putative class II type bacteriocin encoding genes reveals that this strain is potentially biosynthesizing antimicrobial peptides, which could be associated with the antimicrobial activity of AGA58. The flagellated *L. nagelii* AGA58 isolated and characterized in the present study exhibited inhibition of pathogens, perhaps via bacteriocin and/or acid production. The ability to biosynthesize flagellar proteins might provide unique probiotic features to AGA58 such as epithelial barrier function maintenance and immune response modulation, which might contribute to the environmental adaptation of *L. nagelii* AGA58 in the host. Future in vitro and in vivo studies are needed to evaluate and confirm the probiotic potential of *L. nagelii* AGA58 in various cell lines and hosts.

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