Characterization of a potential probiotic bacterium *Lactococcus raffinolactis* WiKim0068 isolated from fermented vegetable using genomic and *in vitro* analyses

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Abstract

Background: *Lactococcus* members belonging to lactic acid bacteria are distributed in fermented foods and widely used as starter bacteria in the production of fermented dairy products. *Lactococcus raffinolactis* WiKim0068 was isolated from a Korean fermented vegetable (kimchi) and its genome was analyzed.

Results: The complete genome of strain WiKim0068 comprised one chromosome and two plasmids and contained 2,292,235 bp, with a G+C content of 39.7 mol%. Analysis of orthoANI values among Lactococcus genome sequences showed that strain WiKim0068 has > 67% genome sequence similarity to other species and subspecies. In addition, strain WiKim0068 displayed no antibiotic resistance and possessed nicotinate (vitamin B3) and nicotinamide (vitamin B3 amide) metabolism.

Conclusion: These results add to our understanding of the genus *Lactococcus* and suggest that this new strain that is beneficial to human health has potential industrial applications

Keywords: *Lactococcus raffinolactis*, genome sequence, kimchi, probiotics, vitamin B3

Background

Lactic acid bacteria (LAB) activity improves the texture, flavor, and scent of dairy products during fermentation and ripening [1]. In the manufacturing of dairy products, LAB starters contribute to flavor development through the (bio)chemical conversion of milk components, via glycolysis (lactose), lipolysis (fat), and proteolysis (casein) [2]. LAB have been used for food preservation for many years, making their safety an important requirement. In addition, the adhesive ability of LAB in the host intestinal tract is an important property contributing to their effectiveness as probiotic strains [3].

The genus Lactococcus, first established as a separate genus distinct from the genus Streptococcus in 1985, contains gram-positive, catalase-negative, non-motile, non-sporulating, cocci-shaped LAB [4]. Lactococcus members are widely used as starter bacteria in the production of fermented dairy products such as cheese and yogurt [5, 6]. Three strains within the genus Lactococcus—L. raffinolactis, L. lactis subsp. lactis, and L. lactis subsp. Cemoris—are listed in the ‘Inventory of Microbial Food Cultures (MFC)’ of fermented food products as species with demonstrated safety [7].

L. raffinolactis is distributed in a wide range of environments, such as fermented foods (fish, meat, vegetables, and milk) and plant and animal materials [8, 9]. Although the species is used in dairy foods as a starter, little has been reported about this species compared with L. lactis. In this study, we report the isolation, identification, and characterization of the L. raffinolactis WiKim0068 isolated from fermented cabbage (kimchi). We also evaluated the possibility of using strain WiKim0068 in dairy products, the safety of the strain, and analysis of its proteolytic enzymes through complete genome sequence analysis. In vitro assays and predictive gene analysis for antibiotic resistance and adhesion were also performed.

Results And Discussion

Phylogenetic and phenotypic features of the isolated LAB strain

A bacterial strain, designated WiKim0068, was isolated from a Korean fermented food, kimchi. In order to identify the phylogenetic similarity of strain WiKim0068, phylogenetic analysis based on the 16S rRNA gene sequences of strain WiKim0068 and the closely related strains indicated that L. raffinolactis NBRC 100932^T was most closely related to strain WiKim0068 with 99.9% similarity (Fig. 1). This result indicated that strain WiKim0068 belong to the L. raffinolactis species. Positive reactions were observed for sugar assimilation/acid formation from galactose, glucose, fructose, mannose, mannitol, N-acetylglucosamine, esculin, ferric citrate, salicin, cellobiose, maltose, melibiose, saccharose, trehalose, raffinose, and turanose. Enzyme detection with an API ZYM kit was positive for esterase, leucine arylamidase, and naphthol-AS-BI-phosphohydrolase.

General genomic features of strain WiKim0068

The PacBio RS II sequencing system generated 74,558 reads, with an average read length of 8,212 bp. The complete genome of strain WiKim0068 was composed of a circular 2.22 Mb chromosome and two circular plasmids, with a total size of 2.29 Mb. The chromosome contained 2,060 predicted protein-coding genes (CDSs), 13 rRNA genes (5S rRNA, 5; 16S rRNA, 4; 23S rRNA, 4), 55 tRNAs, and 3 other RNAs. The WiKim0068 genome was observed to contain 39.7 mol% G + C content (Table 1), which is within the reported range of 35.5–46.4% for Lactococcus species [10], and similar to the 39.25 mol% observed in two L. raffinolactis strains, 4877 (CALL00000000) and NBRC 100932^T (BCVN00000000). For functional classification, WiKim0068 genome was analyzed using the clusters of orthologous genes (COG) database (http://www.ncbi.nlm.nih.gov/COG/), and 2,000 genes were annotated. The annotated genes were associated with the following categories: general function prediction only (R; 237 genes), carbohydrate transport and metabolism (G; 210 genes), function unknown (S; 190 genes), amino acid transport and metabolism (E; 180 genes), coenzyme transport and metabolism (H; 70 genes), defense mechanisms (V; 63 genes), and secondary metabolites biosynthesis, transport, and catabolism (Q; 15 genes; Supplementary Table S1). In addition, Rapid Annotation using Subsystem Technology analysis revealed categories containing genes related to stress response (2.62%), cofactors, vitamins, prosthetic groups, pigments (5.30%), and virulence, disease and defense (3.39%) (Supplementary Fig. S1). Stress response-related gene categories included: “osmotic stress” (5 genes), “oxidative stress” (17 genes), “cold shock” (1 gene), “heat shock” (15 genes), “detoxification” (9 genes), and “no subcategory” (1 gene). Category of cofactors, vitamins, prosthetic groups, pigments includes major “biotin” (15 genes), “NAD and NADP” (14 genes), “riboflavin, FMN, FAD” (8 genes), and “folate and pterines” (33 genes). Category of virulence, disease and defense includes major “bacteriocins, ribosomally synthesized antibacterial peptides” (8 genes), “resistance to antibiotics and toxic compounds” (29 genes), and “invasion and

intracellular resistance" (15 genes). In particular, biotin, riboflavin, and folate are related to human health and digestion and cause various symptoms when deficient [11]. Bacteriocins are antimicrobial peptides produced from bacteria [12] and an alternative to antibiotic resistance bacteria. In addition, bacteriocins have been regarded an important feature in the selection of probiotic strains. These genes were associated with the presence of useful probiotic characteristics, which play important roles in the food and pharmaceutical industries [13–15].

Table 1
Comparative genomic features of Lactococcus raffinolactis WiKim0068, L. raffinolactis 4877, and L. raffinolactis NBRC 100932^T.

	Strain		
	WiKim0068	**4877**	**NBRC 100932^T**
Assembly size (Mb)	2.29	2.28	2.18
DNA G + C content (mol%)	39.7	38.7	39.8
CDSs*	2,187	2,362	2,123
Scaffolds	3	127	114
Genes	2,258	2,409	2,141
Proteins	2,123	2,070	2,030
rRNAs	13	12	2
tRNAs	55	48	29
Finishing quality	Complete	Scaffold	Contig
*CDSs, Coding Sequences			

Comparative genomic analysis

Analysis of the orthologous average nucleotide identity (orthoANI) values among Lactococcus genome sequences showed that strain WiKim0068 had 68.55-98.73% genome sequence similarities with other species and subspecies. The genome was most closely related to that of L. raffinolactis NBRC 100932T (98.73%), followed by L. raffinolactis 4877 (87.02%), L. piscium MKFS47 (76.57%), L. lactis subsp. lactis IL 1403 (69.41%), L. fujiensis JCM 16395 (68.12%), and L. garvieae ATCC 49156 (68.55%; Fig. 2). These results indicated that strain WiKim0068 belongs to the species L. raffinolactis by comparative whole genome sequence analysis [16]. Its similarity to its two closest relative species (L. raffinolactis NBRC 100932T and L. raffinolactis 4877), based on BLAST comparison, is shown in Fig. 3. In addition, we

searched for CRISPRs using the CRISPRFinder platform, but no confirmed CRISPRs were found in the WiKim0068 genome.

Phage and pathogenesis-related genes

PHAST analysis was performed to identify prophage contamination in the WiKim0068 genome. The chromosome contained two intact, one incomplete, and one questionable prophage, while the first plasmid (pWiKim0068-1) contained only one incomplete prophage and the second plasmid (pWiKim0068-2) contained none (Supplementary Fig. S2). Intact prophage regions were located between positions 57,319–90,123 and 1,524,268–1,563,900 bp of the chromosome.

Carbon metabolic pathway

Predicted metabolic pathways in strain WiKim0068 were associated with diverse phosphotransferase (PTS) systems or permeases that transport various carbohydrates, including d-glucose, d-galactose, d-mannose, trehalose, sucrose, cellobiose, N-acetyl-glucosamine, fructose, maltose, mannitol, galactitol, and lactose. The presence of these transport genes indicated the possibility that strain WiKim0068 performs fermentation metabolism using various carbohydrates (Fig. 4). Based on the metabolic pathways in strain WiKim0068, it was confirmed that the strain had heterofermentative pathways. Hexoses (glucose, fructose, and mannose) in strain WiKim0068 can be converted to lactate, ethanol and carbon dioxide. d- and l-Lactate are produced from the reduction of pyruvate by d-lactate dehydrogenase (d-LDH) (EC 1.1.1.28) and l-lactate dehydrogenase (l-LDH) (EC 1.1.1.27), respectively. However, strain WiKim0068 harbors only l-LDH (locus tag: CMV25_RS07125). This is similar to previous studies in which l-LDH was identified in Lactococcus lactis, belonging to the same genus as strain WiKim0068 [17]. Since d-lactate produced by LAB may induce d-lactate acidosis in some individuals [18], it is important to develop LAB for the production of dairy products that produce only l-lactate.

Antibiotic resistance

Recently, interest in foods as mediators of antibiotic resistance has been increasing. LAB, which are widely used in probiotics or starter cultures, have the potential to serve as hosts for antibiotic resistance genes, and carry the risk of transferring genes from various LAB and bacterial pathogens [19]. Strain WiKim0068 showed susceptibility to ampicillin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, penicillin, rifampin, tetracycline, and vancomycin (Table 2).

Table 2
Antibiotic susceptibility of Lactococcus raffinolactis WiKim0068.

Antibiotic	Antibiotic susceptibility test	
	Amount	Inhibition zone diameter (mm)
Ampicillin	10 μg	30
Chloramphenicol	30 μg	40
Ciprofloxacin	5 μg	28
Erythromycin	15 μg	48
Gentamicin	10 μg	18
Penicillin	10 U	64
Rifampin	5 μg	42
Tetracycline	30 μg	65
Vancomycin	30 μg	23

Amino acid and nicotinate and nicotinamide metabolism

The amino acid metabolism-related genes of strain WiKim0068 were annotated using the KEGG database. Among 163 genes involved in amino acid metabolism, strain WiKim0068 harbors the most genes involved in the amino acid metabolism of cysteine, methionine, alanine, aspartate, and glutamate (Fig. 5), suggesting that the strain possesses the biosynthesis and utilization ability of the various amino acids.

Vitamin B3, one of the eight B vitamins, is also known as nicotinate or niacin. This endogenous metabolite is an effective antioxidant, preventing oxidative damage [20]. In general, nicotinamide and nicotinate metabolites are frequently reported with Lactobacillus strains [21–23], while Lactococcus members were unknown about these metabolites. Interestingly, in silico analysis of WiKim0068 genome was predicted an almost complete complement metabolic pathway from nicotinamide and nicotinate metabolism genes (Fig. 6), and through this metabolic pathway, 0.932 mg L^{-1} vitamin B3 was produced in cultured cells (Supplementary Fig. S3). These results indicated that nicotinate and nicotinamide metabolism occurs in strain WiKim0068.

Conclusions

The complete genome of L. raffinolactis WiKim0068 provided information on its antibiotic susceptibility, antibiotic resistance-related proteins, and nicotinate and nicotinamide metabolic activity. In addition, in

vitro analysis indicated that the strain possesses beneficial health effects such as adhesion ability and vitamin B3 production. These results suggest that L. raffinolactis WiKim0068 could be utilized in comparative genome analysis with other Lactococcus strains and in industrial applications.

Methods

Isolation and characterization of a bacterial strain

Strain WiKim0068 was isolated from a Korean fermented food, kimchi in Gwangju, Korea using the dilution plating method, and incubated on De Man, Rogosa and Sharpe (MRS) agar (MB cell, LA, USA) at 30 °C for 48 h under anaerobic conditions. Physiological characteristics (acid production, carbon-source utilization, enzyme activity, and biochemical feature) were determined using the API 50CH, API ZYM, and API 20E galleries (bioMérieux, France) according to the manufacturer's instructions.

Genome sequencing and annotation

Genomic DNA extraction was performed using the QIAcube system with a QIAamp DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The genome was sequenced using the PacBio RS II sequencing system (Pacific Biosciences, Menlo Park, CA). The reads were assembled de novo using Hierarchical Genome Assembly Process version 3.0 (HGAP 3.0) in PacBio SMRT analysis version 2.3.0., as described by Jang et al. [24]. The complete genome sequence was annotated using the combined results of the automatic National Center for Biotechnology Information (NCBI) Prokaryotic Genomes Annotation Pipeline 4.1 [25] and the RAST server [26]. Phylogenetic tree based on 16S rRNA gene sequences extracted from the genome, were constructed, as described by Ismaeil et al. [27], using the neighbor-joining [28], minimum-evolution [29], and maximum likelihood [30] methods, based on 1,000 randomly generated trees. Protein functions were grouped according to COG using WebMGA on-line tools (for carbohydrate metabolism, antibiotic resistance-related genes, adhesion, proteolytic enzymes, and amino acid metabolism) [31]. Nicotinate and nicotinamide metabolic pathway was mapped using the Kyoto Encyclopedia of Genes and Genomes (KEGG) [32]. The fermentative metabolic pathways were constructed based on predicted KEGG pathways and BLASTP analysis using reference gene sequences. Antimicrobial resistance genes was identified using ResFinder 3.0, available from the Center for Genomic Epidemiology (http://genomicepidemiology.org/). Prophage identification was performed using the PHAge Search Tool (PHAST) [33].

Comparative genomic analysis

For comparative genomic analysis of strain WiKim0068, the genome sequences of two other L. raffinolactis strains: L. raffinolactis 4877 (CALL00000000.1) and L. raffinolactis NBRC 100932^T (BCVN00000000.1) were obtained from GenBank and used as references. To determine the similarity between genome sequences, OrthoANI values of Lactococcus raffinolactis WiKim0068 and related strains

in the genus Lactococcus were calculated using the orthologous average nucleotide identity tool (OAT software, www.ezbiocloud.net/sw/oat; ChunLab) [34]. Circular comparison map of the genomic sequences was created with Blast Ring Image Generator (BRIG) software [35]. Clustered regularly interspaced short palindromic repeats (CRISPRs) were analyzed using CRISPRFinder [36]. When the algorithm was detected exactly three identical (repeated and sequential) repeating regions separated by a variable order, it was considered "confirmed CRISPR".

In vitro analyses

Antibiotic susceptibility test

Antibiotic susceptibility was determined using diffusion disks (Becton Dickinson Microbiology Systems, Cockeysville, MD). Disks containing ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), penicillin (10 U), rifampin (5 µg), or tetracycline (30 µg), were added to bacteria-inoculated MRS agar. The strain was inoculated in MRS broth at 30 °C for 48 h. The cell density of the cultures was adjusted to approximately 10^8 colony forming units (CFUs) mL^{-1}. Any growth within the 18-mm-diameter zone of each antibiotic was considered indicative of resistance.

Quantitative vitamin B analysis

Vitamin B levels were determined with a NexeraX2 HPLC (Shimadzu, Japan) equipped with an LCMS-2020 LC/MS System (Shimadzu). Vitamin B was separated on an Aegispak C-8 column (150 mm × 2 mm, 3 µm; Young Jin Biochrom, Korea) at 40 °C in 0.1% formic acid in distilled water, and eluted with an acetonitrile gradient (0–100%). Optimal operating conditions for LC-MS/MS analysis were applied according to the method of Wirkus et al. [37].

Abbreviations

CDS:Coding sequences; RAST:Rapid Annotation using Subsystem Technology; PHAST:PHAge Search Tool; KEGG:Kyoto Encyclopedia of Genes and Genomes; orthoANI:orthologous Average Nucleotide Identity; COG:Clusters of Orthologous Genes

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The complete genome sequences have been deposited to the DNA databank of Japan/the European Molecular Biology Laboratory/GenBank under the accession numbers CP023392–CP023394.

Competing interests

The authors declare no competing financial interest.

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Authors' contributions

SWR and SHL designed and coordinated all the experiments. MYJ performed cultivation, DNA extraction and purification. CSL, MYJ, and MJS performed the sequencing, genome assembly, gene prediction, gene annotation, and comparative genomic analysis. CSL, MYJ, SHL, and SWR wrote manuscript. All authors have read and approved the manuscript.

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Not applicable.

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Figures

Fig. 1

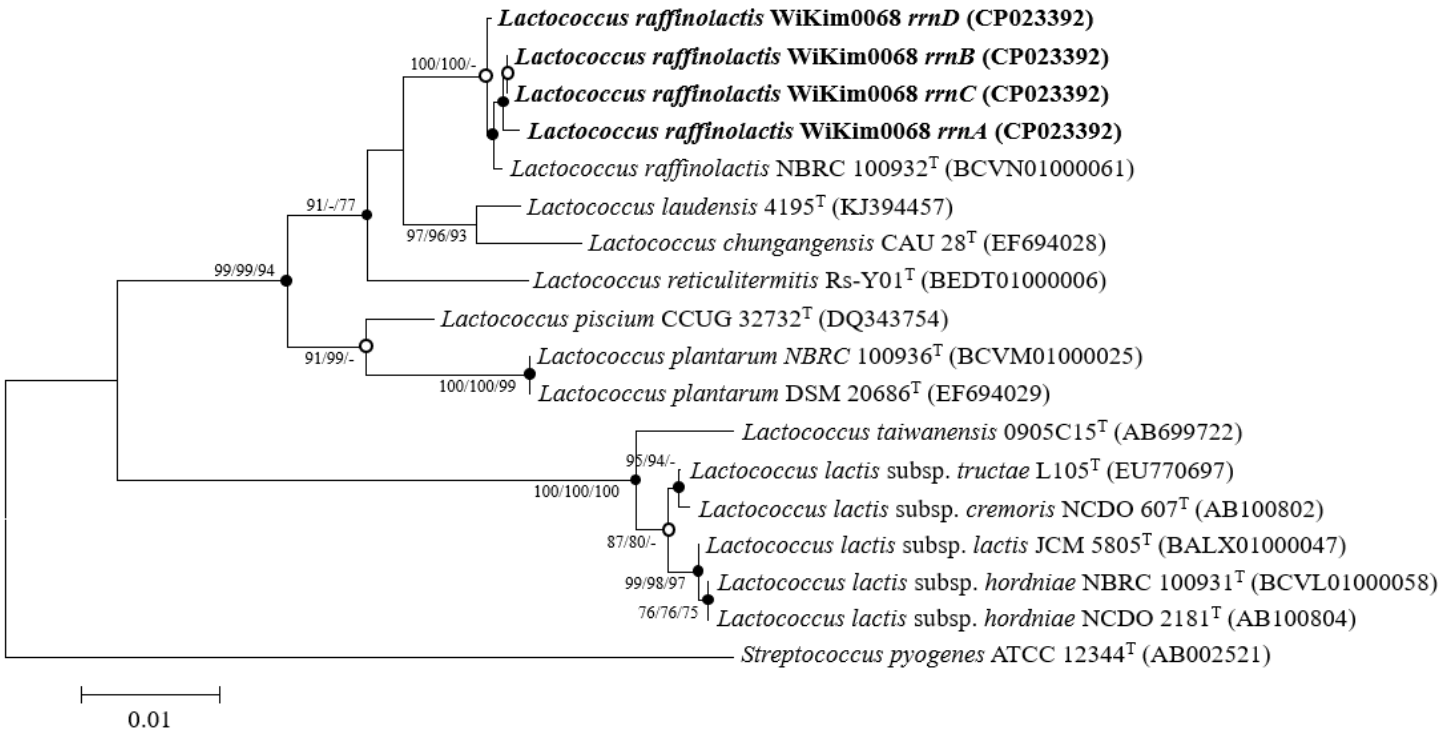

Figure 1

Phylogenetic tree based on 16S rRNA gene sequences showing the taxonomic position of strain WiKim0068. Numbers at the nodes represent bootstrap values (> 70%) and were calculated using neighbor-joining/minimum-evolution/maximum likelihood probabilities based on 1,000 replicates. Streptococcus pyogenes ATCC 12344T was used as an out-group. Bar, 0.01 accumulated changes per nucleotides.

Fig. 2

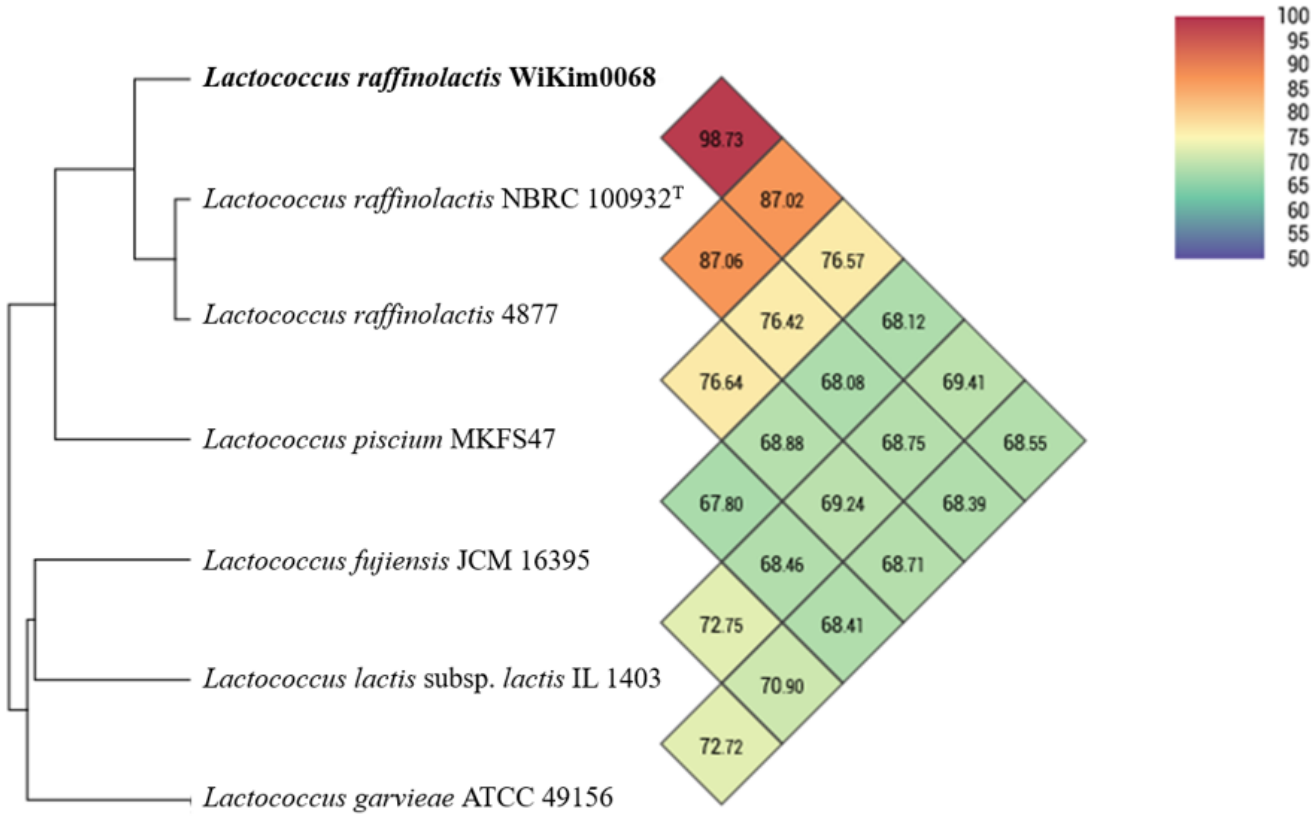

Figure 2

OrthoANI values between Lactococcus raffinolactis WiKim0068 and the closely related strains: L. raffinolactis NBRC 100932T, L. raffinolactis 4877, L. piscium MKFS47, L. fujiensis JCM 16395, L. lacitis subsp. lactis IL 1403, and L. garvieae ATCC 49156.

Fig. 3

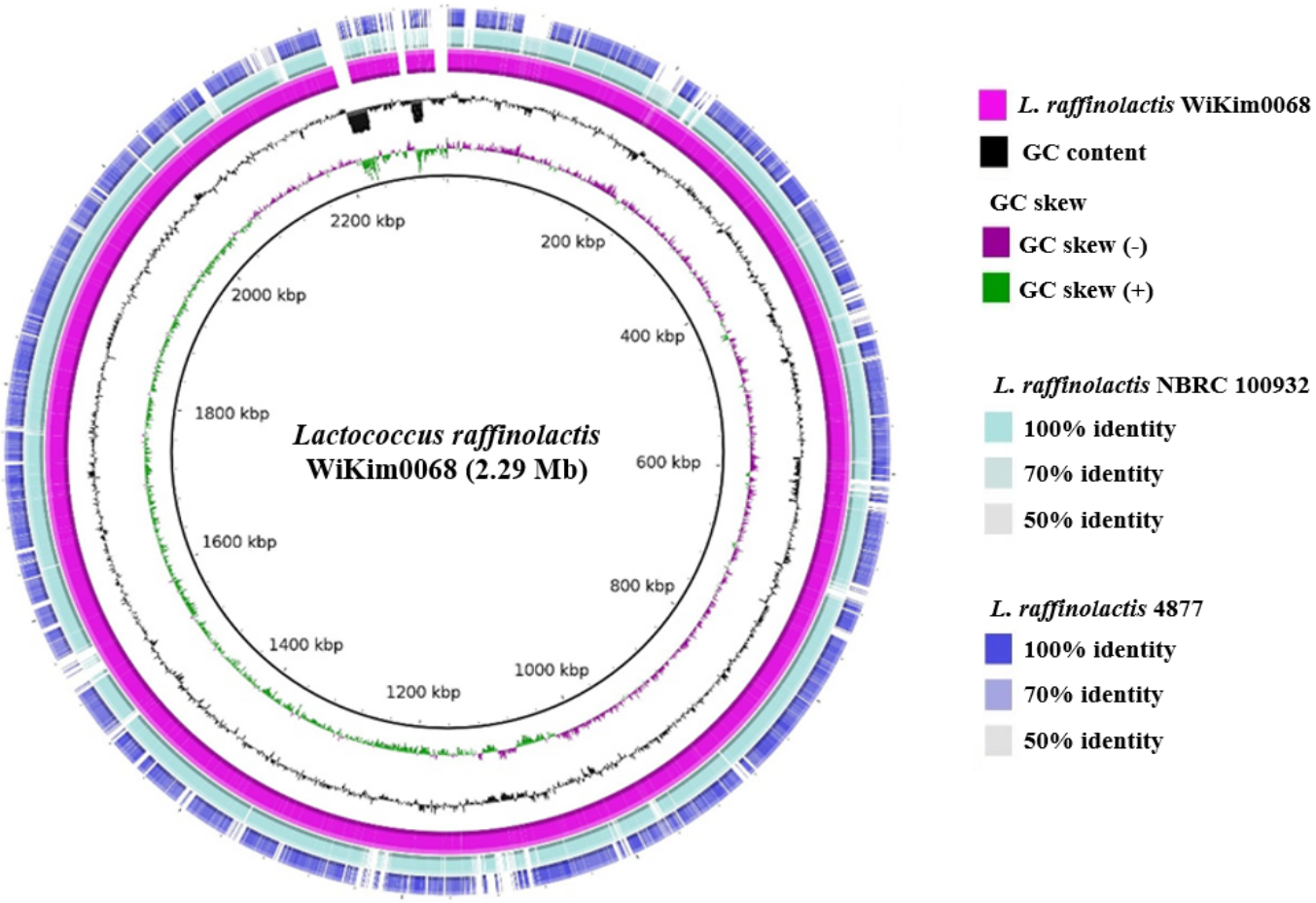

Figure 3

Circular comparison of the genomes of Lactococcus raffinolactis WiKim0068 and reference strains, L. raffinolactis NBRC 100932T and L. raffinolactis 4877. Similarity between species is represented by color intensity.

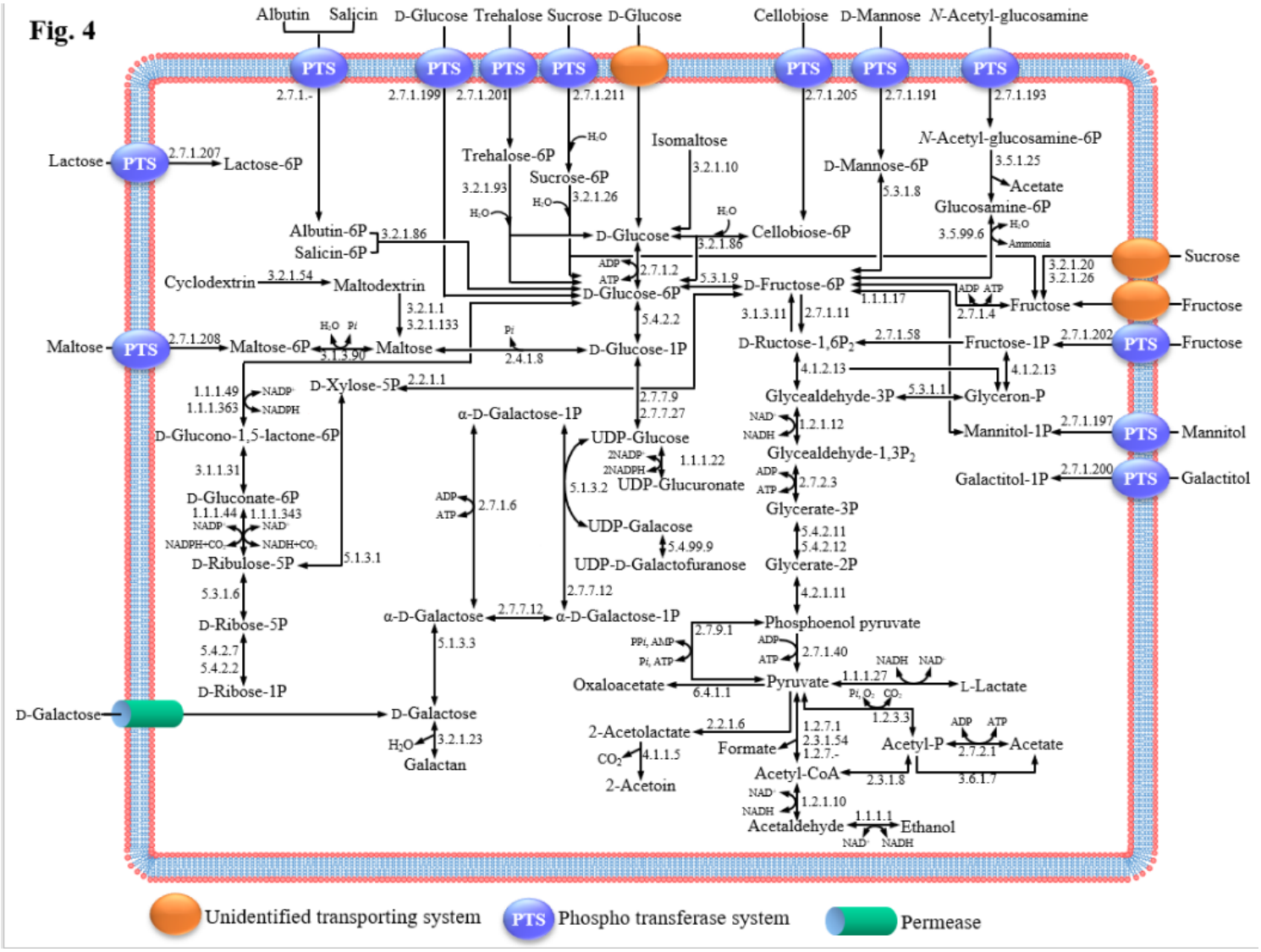

Figure 4

Predicted fermentative metabolic pathways of various carbon compounds in Lactococcus raffinolactis WiKim0068 during fermentation. PTS, phosphotransferase systems; UDP, uridine diphosphate.

Fig. 5

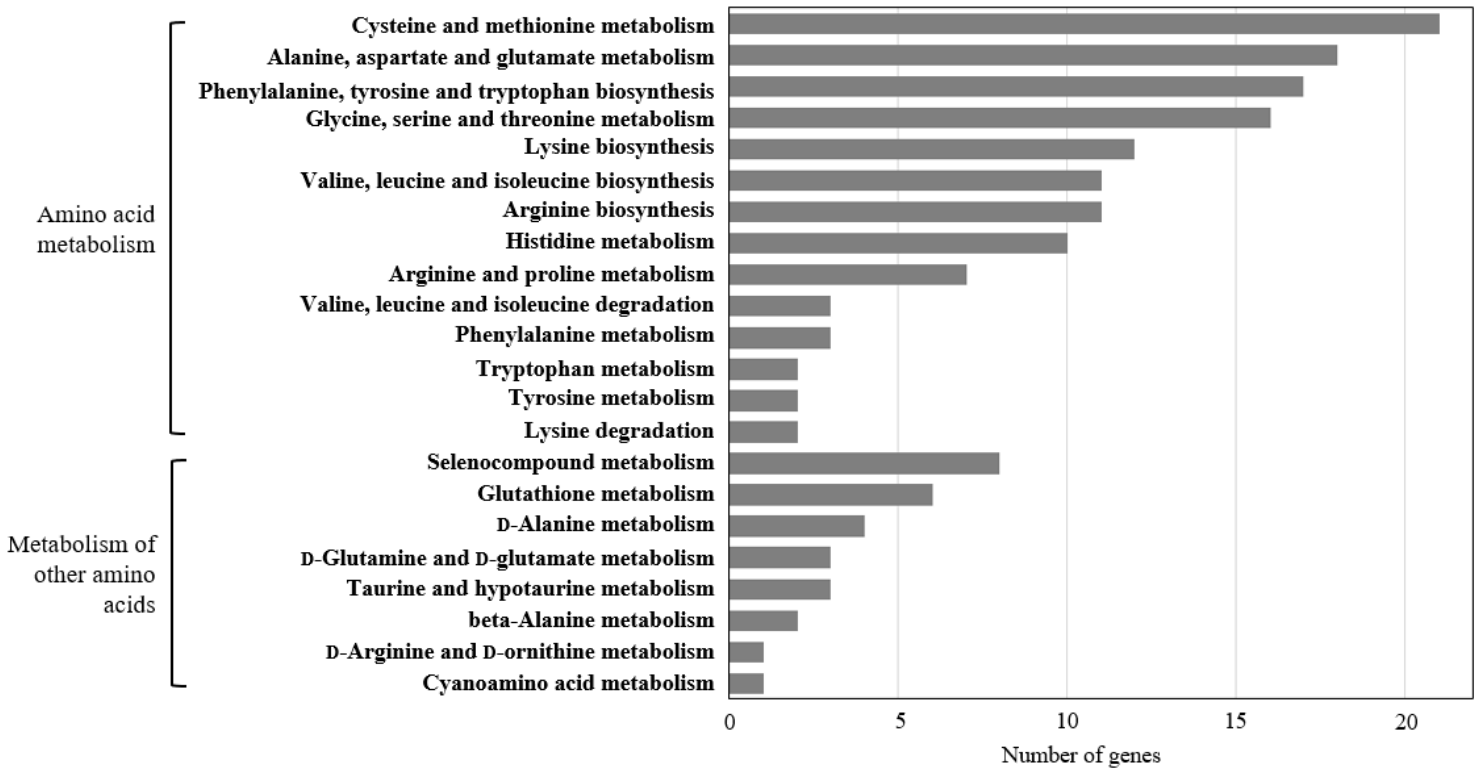

Figure 5

Amino acid metabolism-related genes of Lactococcus raffinolactis WiKim0068.

Fig. 6

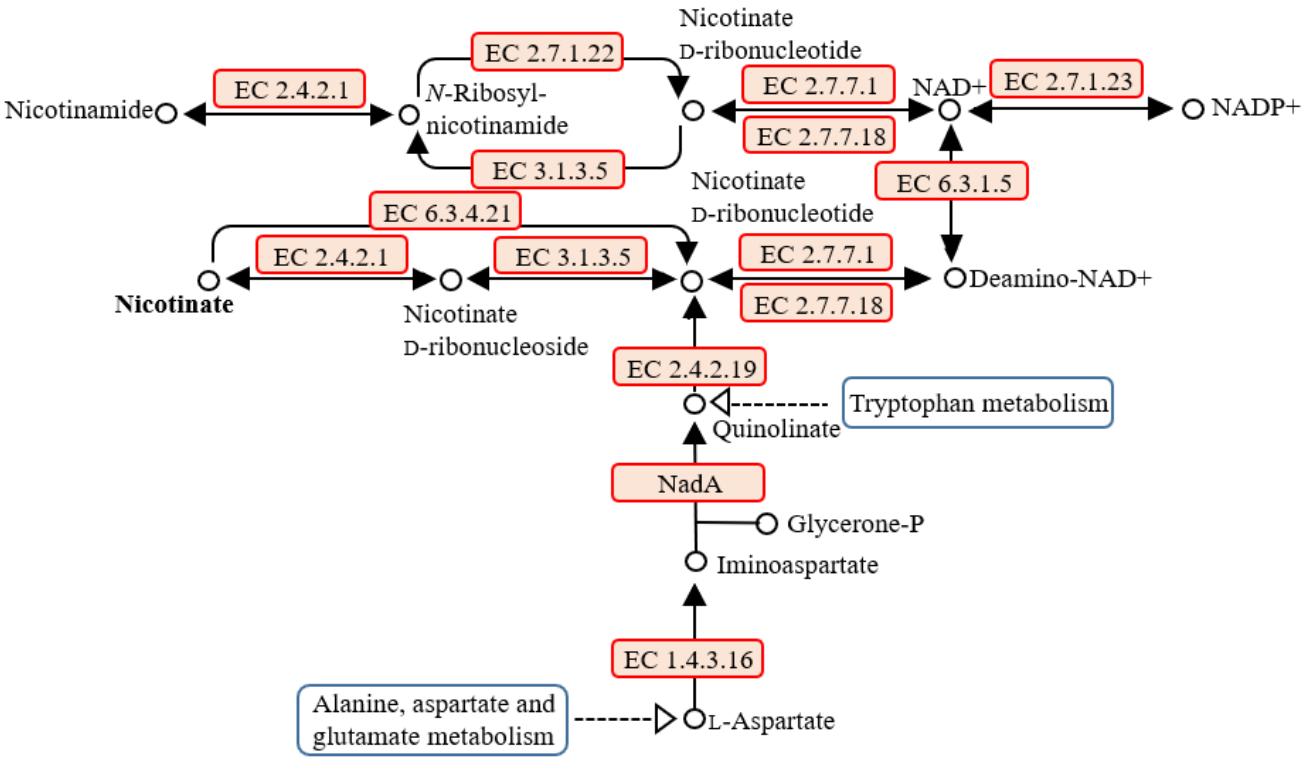

Figure 6

Nicotinate and nicotinamide metabolism in Lactococcus raffinolactis WiKim0068. Red boxes indicate enzymes in the nicotinate and nicotinamide metabolic pathway present in strain WiKim0068.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydata.docx