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GENOME-BASED CHARACTERIZATION OF STRAIN *ENTEROCCUS FAECIUM* ICIS21 FROM NATURALLY FERMENTED DAIRY PRODUCT

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ABSTRACT

Enterococcus faecium ICIS21 is a facultative anaerobic, gram-positive strain that is important in the food industry. This isolate was selected from a naturally fermented dairy product obtained in the Orenburg region, Russian Federation. Currently, there is increasing evidence supporting the important role of enterococci in human health and their use as a natural method for food preservation. This paper reports a draft genome sequence of *E. faecium* ICIS21, where we studied the strain genome using whole-genome sequencing, assembly, annotation, and subsequent bioinformatic analysis. We characterized its probiotic potential, including the production of bacteriocins (enterocin SE-K4, enterocin A, and enterolysin A), secondary metabolites (cyclic lactone autoinducer and T3pks), vitamins, and genes involved in unsaturated fatty acid biosynthesis. Additionally, an important feature is the presence of a heavy metal resistance gene cluster, determining factors with various mechanisms for binding and removing a complex of heavy metals such as Hg, Co, Zn, Cd, Cu, Mg, Mn, Fe, and Ni. This may be significant for using the strain as a biosorbent for heavy metal ions. The presence of major virulence genes was not detected. The analysis showed the absence of transmissible antibiotic resistance genes (vanA, vanB, ermB, tetM, and aac(6′)-le-aph(2″)-la) and plasmids. The study results will be significant for further analysis of specific elements of the genome of this species and also open up prospects for developing microbial compositions with specified properties to expand the range of functional food products.

Keywords: *Enterococcus faecium*, Whole-genome sequencing, Genome analysis, Probiotic, Heavy metals tolerance, Bacteriocins

INTRODUCTION

Enterococci are non-spore-forming, catalase-negative, gram-positive cocci that occur in pairs or long chains **(Ben Braïek and Smaoui, 2019)**. They are considered human pathogens and serve as indicators of faecal contamination of water **(Cariolato *et al.*, 2008)**. At the same time, some strains are currently used as probiotics **(Giraffa, 2003)**. They belong to the group of lactic acid bacteria and are among the most widespread, with the digestive tract of humans and animals **(Murray, 1990)**, plants, soil, as well as food products — especially dairy products **(Hanchi *et al.*, 2018)** — being their predominant habitats. To date, ample data have been reported on the safety of enterococci isolated from diverse food products. *E. faecium* strains characterized as safe, with no major virulence genes, have been recovered from Fermented Chili, Kimchi, and artisanal Tunisian Meat **(Xiao *et al.*, 2024; Kim *et al.*, 2022; Zommiti *et al.*, 2018)**.

The use of enterococci due to their probiotic capability and ability to survive under adverse conditions (salinity, temperature, pH) establishes them as important industrial components of fermented milk products **(Yerlikaya and Akbulut, 2020)**.

Enterococci play an important role in the food industry due to their beneficial biotechnological properties, such as bacteriocin production **(Santos *et al.*, 2015)**, as well as lipolytic and proteolytic activities that influence the organoleptic parameters of the product **(RASOULI *et al.*, 2012)**.

Naturally fermented dairy products (spontaneously fermented dairy products) are region-specific foods, traditionally prepared using raw or heat-treated milk through spontaneous fermentation or inoculation with starter cultures derived from previously prepared product **(Josephsen and Jespersen, 2004; Tamang *et al.*, 2016)**.

Fermented milk is a natural, environmentally friendly food product containing a relatively high number of viable microorganisms **(Sionek *et al.*, 2023)**. Lactic acid bacteria are used in manufacturing such products due to their long and safe history of use, having received the GRAS (Generally Recognized As Safe) status **(Abriouel *et al.*, 2012)**. Moreover, studies focused on lactic acid strains isolated from fermented products suggest that some of them may possess probiotic properties and can be considered as starter cultures **(Topisirovic *et al.*, 2006)**.

Currently, an approach based on the analysis of whole-genome sequencing data is applied to characterize microbial strains that are promising for use as probiotics and in the food industry. This analysis allows for the assessment of the presence or absence of virulence genes, antibiotic resistance genes, biofilm formation genes of mobile genetic elements, and bacteriocin-associated genes **(Kim *et al.*, 2016; Ghattargi *et al.*, 2018)**, and shows their phylogenetic relationship, or lack thereof, with clinical virulent isolates. Although most probiotic microorganisms have a safe history of use, the emergence of new strains requires a thorough safety assessment for each of them. Thus, genetic analysis can serve as a criterion for the preliminary strain screening for potential probiotic application.

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This study aims to characterize and analyze the genome of *E. faecium* ICIS21, isolated from naturally fermented dairy products, to assess its potential as a production strain for fermented foods and as a basis for probiotic development.

MATERIALS AND METHODS

Bacterial strain characteristics

Milk samples from household farms, randomly obtained from different districts of the Orenburg region, were transported to the laboratory within 24 hours in a refrigerated state for microbiological analysis. These samples were allowed to undergo natural fermentation in a thermostat at 37 °C for 24 hours, after which lactic acid bacteria were isolated and identified.

The fermented milk samples were serially diluted in sterile saline solution and thoroughly dispersed. The obtained dilutions were plated and incubated on de Man, Rogosa and Sharpe (Lactobacillus MRS Agar) (HiMedia, India). Each homogenized fermented sample (20 µL) was plated onto MRS agar and incubated at 37 °C in an atmosphere containing 5% CO_2 for 48 hours **(Corroler *et al.*, 1998)**. *E. faecium* ICIS21 was selected from a naturally fermented dairy product and is part of the microorganism collection of the Institute of Cellular and Intracellular Symbiosis of the Ural Branch of the Russian Academy of Sciences (Orenburg, Russia). The strain was incubated in MRS agar medium at 37 °C in the atmosphere containing 5% CO^2 for 24 hours. The strain morphology was characterized using scanning electron microscopy (Figure 1).

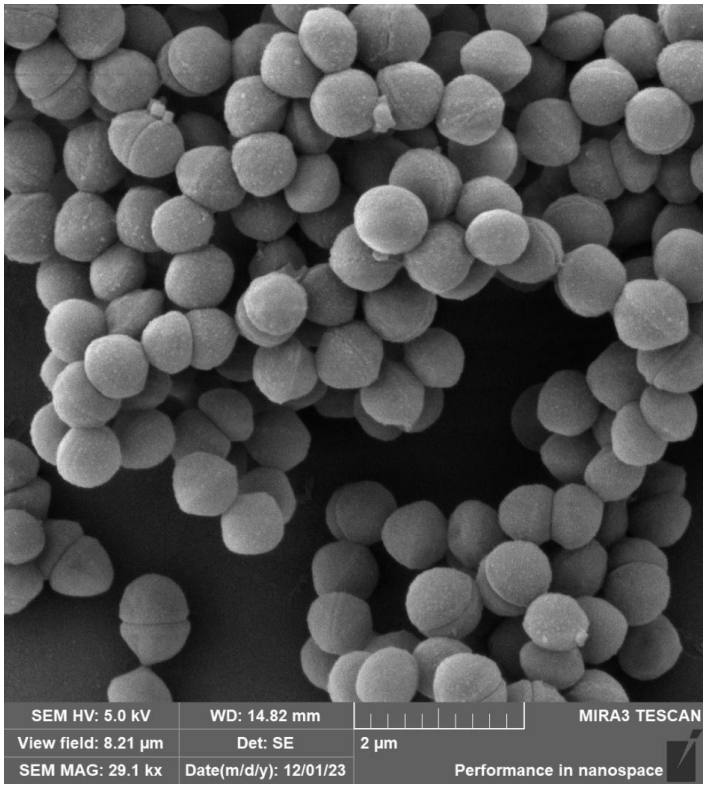

Figure 1 Morphology of the strain *Enterococcus faecium* ICIS21

Genome sequencing, assembly and annotation

DNA isolation, preparation of the DNA library and its whole genome sequencing were carried out at the Center for Collective Use "Persistence of Microorganisms" of the Institute of Cellular and Intracellular Symbiosis of the Ural Branch of the Russian Academy of Sciences (Orenburg, Russia). Genomic DNA was isolated using the Quick DNA Fungal/Bacterial Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. The quality of the isolated DNA was assessed by the A260/280 ratio using a Nanodrop 8000 instrument (Thermo Fisher Scientific, Waltham, MA, USA) and by horizontal electrophoresis in 1% agarose gel. DNA concentration was measured using the dsDNA High Sensitivity Assay Kit on a Qubit 4 Fluorometer (Life Technologies, Carlsbad, CA, USA).
The DNA library for whole genome sequencing was prepared using the NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina® (New England BioLabs, Ipswich, MA, USA). The fragment composition of the library was verified by capillary polyacrylamide gel electrophoresis on the QIAxcel Advanced System platform using the QIAxcel DNA High Resolution Kit (Qiagen, Hilden, Germany). Normalization of the library molar concentration was performed using quantitative PCR on the CFX Connect Real-Time PCR System (Bio Rad, Hercules, CA, USA). Paired-end sequencing (2 × 250 bp) was performed on the MiSeq platform (Illumina, San Diego, CA, USA) using the v.2 reagent kit (Illumina, San Diego, CA, USA).

Bioinformatic Processing of Sequencing Data

A total of 1,042,583 raw reads were obtained. The quality of the obtained reads was assessed using FastQC v. 0.12.1 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).
After assessing the initial sequencing quality, the raw reads were filtered using Trimmomatic v0.39 **(Bolger at al., 2014)** with the following parameters: TRAILING:30, SLIDINGWINDOW:30:30, CROP:295, HEADCROP:12, and MINLEN:30. As a filtration result, 997,468 reads (95.67%) remained.
Using the bacterial genome assembly pipeline Unicycler v0.5.1 **(Wick at al., 2017)**, the genome of the *Enterococcus faecium* ICIS21 strain was assembled. The assembly consists of 93 contigs with a total length of 2,602,346 bp, and an average coverage of 82.7×.
Contigs were analysed for gene prediction using the NCBI Prokaryotic Genome Annotation Pipeline (PGPA) **(Tatusova *et al.*, 2016)** and Rapid Annotation using Subsystems Technology (RAST) **(Aziz *et al.*, 2008)**. Interactive visualization of *E. faecium* ICIS21 genome was performed using GView **(Petkau *et al.*, 2010)**. Average nucleotide identity (ANI) values were calculated using the Orthologous Average Nucleotide Identity tool **(Lee I *et al.*, 2016)**. Nine draft genomes of *E. faecium* strains currently available in the NCBI database were used for comparison: *E. faecium* DME32 (draft WGS: QGHK01, camel milk), *E. faecium* QAUEFNA17 (draft WGS: JAJAOI01, fermented milk product), *E. faecium* P3 116 GL (draft WGS: JAWPCW01, urinary tract), *E. faecium* EF1180 (draft WGS: JAWWRF01, rectum), *E. faecium* P7 56 (draft WGS: JBFSKT01, feces), *E. faecium* M11 (draft WGS: JBEUZQ01, milk), *E. faecium* S6 (draft WGS: JAHCYY01, camel milk), *E. faecium* CV167 (draft WGS: CAXVTA01, milk), *E. faecium* 2227st1 (draft WGS: A8JBCJBA01, feces).
The bioinformatic tool BAGEL4 (http://bagel4.molgenrug.nl/) was used to search for genes involved in the synthesis of potential bacteriocins, and antiSMASH 6.0 (https://antismash.secondarymetabolites.org/) was used to identify biosynthetic gene clusters (BGCs).
The presence of virulence genes was assessed using the Virulence Factors Database (VFDB) (https://www.mgc.ac.cn/VFs/).
Clonal lineages (Sequence type and clonal complex) were determined according to the recommendations (https://pubmlst.org/organisms/enterococcus-faecium).

Probiotic Potential of the Isolated Strains

Antagonistic Activity Against Pathogens

The antagonistic activity of the isolate was evaluated using the direct antagonism cup-plate technique or agar co-cultivation method. Five test strains, including human pathogens—*Staphylococcus aureus* FDA 209P, *Escherichia coli* K-12, *Bacillus cereus* IP 5832, *Pseudomonas aeruginosa* ATCC 27853, and *Salmonella typhimurium* 14028S—were used to assess antimicrobial activity. The optical densities (OD) of these cultures were standardized to 0.6 ± 0.2 at 600 nm using a spectrophotometer (Multiskan, Thermo Scientific, Finland).
Antagonistic activity against pathogens was determined using the spot-on-lawn technique **(Halder *et al.*, 2017)**. All the experiments were repeated three times with three technical replicates each time.

Determination of Antibiotic Susceptibility

The antibiotic susceptibility of *Enterococcus* was determined using the disk diffusion method, as described by **Bauer *et al.* (1966)**, with some modifications. A total of 11 indicator antibiotic discs by NICF LLC (Russia) were used in the study, including azithromycin (AZI) 15 µg, vancomycin (VA) 30 µg, levomycetin (LEV) 30 µg, amoxicillin/clavulanic acid (ACA) 20 µg/10 µg, benzylpenicillin (PEN) 10 U, streptomycin (STR 300) 300 µg, ciprofloxacin (CIP) 5 µg, doxycycline (DOX) 30 µg, ampicillin (AMP) 10 µg, clindamycin (CL) 2 µg and rifampicin (RIF) 5 µg. The EUCAST recommendations were used to interpret the diameters of growth inhibition zones when determining the microorganism susceptibility to antimicrobials by the disk diffusion method.

Antioxidant Activity

The antioxidant activity was evaluated based on the ability of *E. faecium* ICIS21 to scavenge 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radicals, using the method described by **Düz *et al.* (2020)**. DPPH is a purple free radical that is reduced to pale yellow upon reaction with an antioxidant **(Vougiouklaki *et al.*, 2022)**.
The strain was cultured in Lactobacillus MRS Broth for 24 hours. After incubation, the cells were collected by centrifugation (3,000× g for 10 min), washed three times with PBS (pH 7.4), and resuspended in 500 µL of PBS to a final concentration of 11 log CFU/mL. The cells were then mixed at a 1:1 ratio with 500 µL of freshly prepared DPPH solution (0.05 mmol/L in ethanol) and incubated in the dark for 1 hour. The cell suspension was replaced with PBS for the negative control. A solution of ascorbic acid (1 mg/mL) in distilled water was used as the positive control. The supernatants were obtained by centrifugation (10,000× g for 10 minutes). Absorbance was measured on a spectrophotometer at OD 540 nm. All the experiments were repeated three times with three technical replicates each time.
The inhibition rate was calculated using the following formula:

$$Antioxidant\ Activity\ (\%) = 1 - \frac{OD\ negative\ control - \mathrm{OD}\ sample}{OD\ negative\ control} \times 100\%$$

Acid and Bile Tolerance

The strain ability to survive under adverse conditions of low pH and bile was assessed using a modified method described by **Yu and Tsen (1993)**.
The strain was grown on solid MRS medium at 37 °C for 24 hours, after which a microbial suspension was prepared in MRS broth with OD = 0.5 at λ = 630 nm. Subsequently, Lactobacillus MRS Broth was prepared with different pH levels (5; 4; 3; 2; 1) and bile concentrations (m/V) (0,125%; 0,25%; 0,5%; 1%; 2%), along with Lactobacillus MRS Broth as a control. Control and test samples were dispensed into 96-well plates at 180 µL per well. Further, 20 µL of the test strain suspension was added to each well.
The plate was incubated at 37 °C under microaerophilic conditions for 24 hours, after which the OD of the control and test wells was measured at 630 nm. Strain survival was assessed based on the changes in growth observed in the test wells compared to the control wells. All the experiments were repeated three times with three technical replicates each time.

Accession Number: The genome sequence assembly project has been deposited at GenBank under accession number JBBPHS000000000.2.

RESULTS AND DISCUSSION

General genomic features and genome-based phylogeny of *E. faecium* ICIS21

The genomic characteristics of *E. faecium* strain ICIS21 are shown in Table 1 and Figure 2. Primary data processing made possible to identify 93 contigs, with an average coverage of 82.7x, the length of N50 - 98,203 and the L50 value - 9. It has a total length of 2,602,346 bps. Genome annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (https://www.ncbi.nlm.nih.gov/refseq/annotation_prok/), which identified 2,398 coding sequences, including 2,398 proteins, 147 pseudogenes, and 3 rRNAs partial rRNAs [16S — 1 and 23S — 2], 51 tRNA genes, and 4 non-coding RNA genes.

Table 1 General genome features of *E. faecium* ICIS21

Features	Value
Genome Coverage	82.7x
Number of contigs	93
N50	98,203
L50	9
Genome size (b.p.)	2,602,346
Genes (total)	2,603
CDSs (total)	2,545
Genes (coding)	2,398
CDSs (with protein)	2,398
rRNAs (16S, 23S)	1, 2
tRNAs	51
Pseudo Genes (total)	162
BioProject	PRJNA1095692
BioSample	SAMN40744996
GenBank accession no.	JBBPHS000000000.2

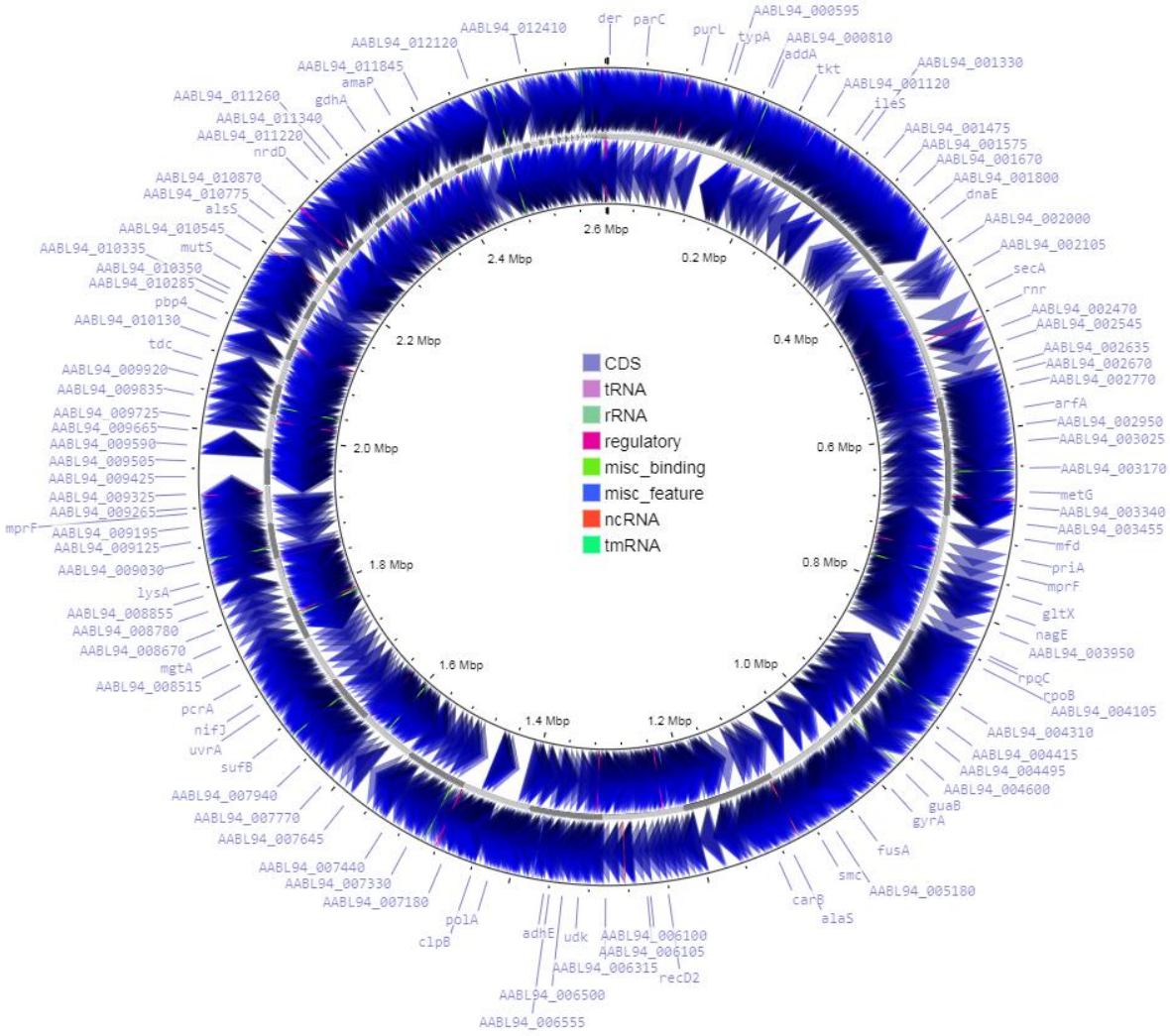

Figure 2 Circular map of *E. faecium* ICIS21 genome

The analysis of *E. faecium* strain ICIS21 using the RAST subsystem identified 23 functional gene subsystems (Figure 3). The predominant ones included genes involved in protein metabolism (22%), carbohydrate metabolism (15%), metabolism of amino acids and their derivatives (13%), nucleoside and nucleotide metabolism (8%), DNA metabolism (7%), metabolism of cofactors, vitamins, prosthetic groups, and pigments (7%), as well as cell wall and capsule formation (5%). Genes involved in the biosynthetic pathways of essential amino acids such as threonine, methionine, lysine, and an intermediate for tryptophan synthesis were identified. Genes responsible for the production of vitamins, including thiamine (vitamin B1), riboflavin (vitamin B2), and biotin (vitamin H), as well as genes involved in unsaturated fatty acid biosynthesis (FASII), were also identified.

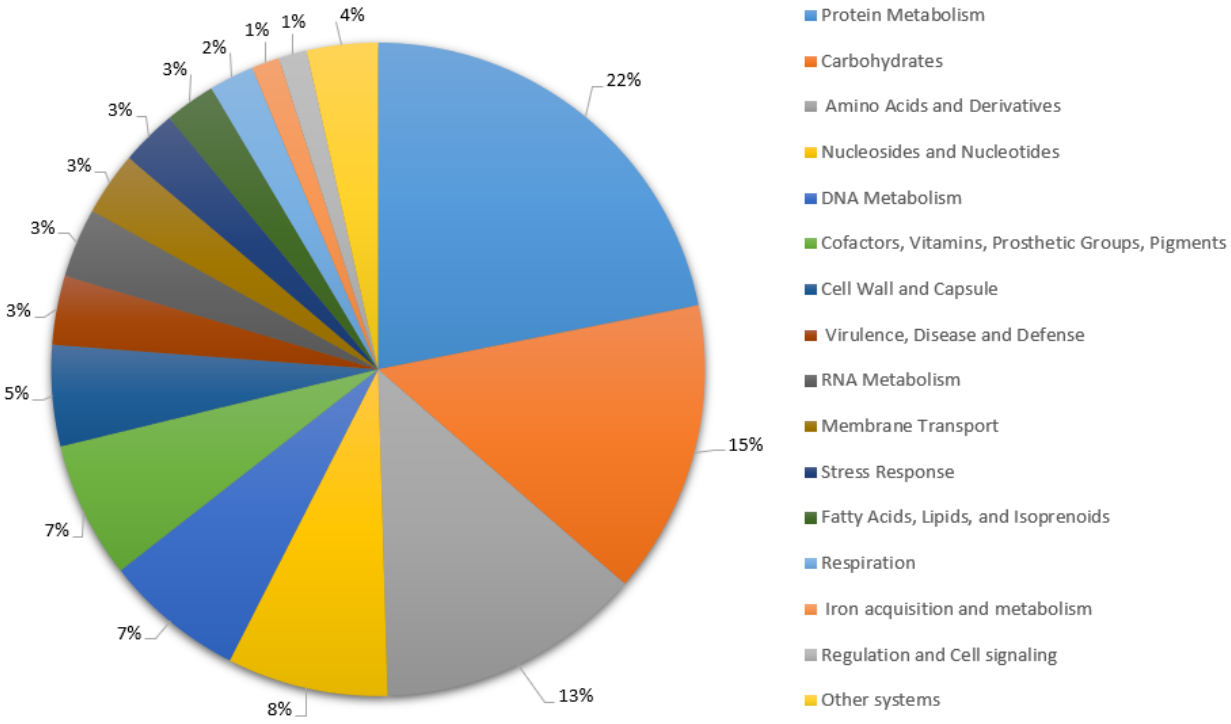

Figure 3 Coding sequences distributed across functional subsystems

ANI and OrthoANI Analysis

The heat map (Figure 4) shows ANI values between *E. faecium* strain ICIS21 and nine other *E. faecium* strains. The analysed strains were isolated from diverse sources, including the human intestine, patient faeces, human rectum, urinary tract, fermented dairy products, and camel milk. Despite originating from different geographical locations, these strains exhibit high similarity. The average nucleotide identity (ANI) analysis shown *E. faecium* ICIS21 was relatively close to other *E. faecium* (98,6-99,65%). The *E. faecium* ICIS21 strain showed 94.69% ANI distance with *E. faecium*-2227st_A8 isolated from feces, while OrthoANI values (Figure 5) ranged from 94.86% to 99.70%.

Safety of *E. faecium* ICIS21

Genome analysis of the *E. faecium* ICIS21 strain revealed the absence of the genes such as *esp*, *hyl*, *cyl*, *asa1*, *gelE*, *efaA*, *ace*, *van*A, *van*B, *hdc*1, and *hdc*2. The *efaA* gene, which is responsible for biofilm formation and found in other *E. faecium* strains **(Urshev and Yungareva, 2021; Inoğlu and Tuncer, 2013)**, used as potential starter cultures or probiotic supplements **(Yuksekdag *et al.*, 2021)**, was detected.

The cytolysin genes such as *cylA*, *cylB*, *cylI*, *cylL-I*, *cylL-S*, *cylR1*, and *cylR2* were absent. Hyaluronidase and gelatinase genes were also not detected. At the same time, it is known that the presence of genes determining capsule formation and adhesion does not characterize a microorganism as pathogenic **(Ahadaf *et al.*, 2025)**. The *E. faecium* ICIS21 strain was found to carry adhesion genes *ebpA*, *efaA*, *slrA* and capsule formation genes *cpsA*, *cpsB*, *eps3*, *rgpG*, *epsE*, which are required for colonization and survival and have previously been identified in probiotics and starter cultures **(El Jeni *et al.*, 2020)**. In addition, resistance to host stress factors **(de Melo Pereira *et al.*, 2018)** and biofilm formation mediated by the quorum-sensing system are key criteria for probiotic selection and important features of the host microbiota, contributing to the formation of a biological barrier and maintaining colonization resistance **(Deng *et al.*, 2020)**. Thus, the *E. faecium* ICIS21 strain was found to carry a biofilm formation gene **(Creti *et al.*, 2006)**.

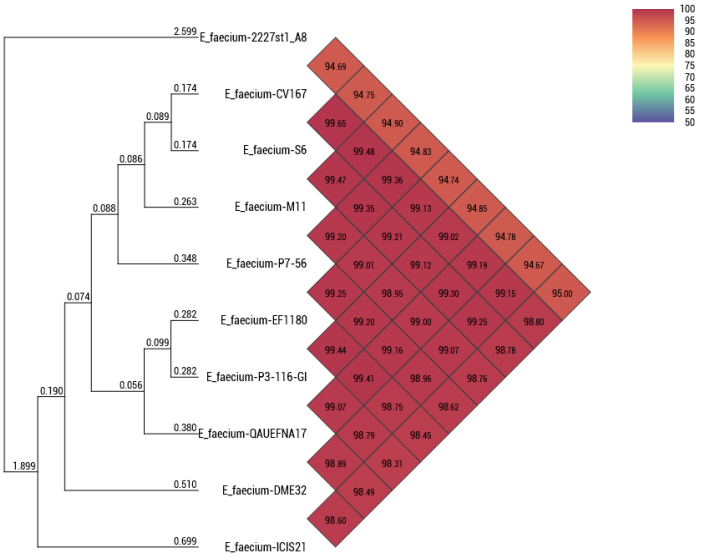

Figure 4 – Heat map of ANI values of *E. faecium* ICIS21

Analysis of genes associated with antimicrobial resistance

Analysis of antibiotic resistance genes revealed the absence of transmissible resistance genes (*vanA, vanB, ermB, tetM, and aac(6')-Ie-aph(2")-Ia*), which is further supported by the absence of plasmids that could harbor these genes. The *E. faecium* ICIS21 genome contained the *addA* gene, which is chromosomal in this strain due to the lack of plasmids.

Genomic analysis of bacteriocins

The BAGEL4 platform was used to identify genes responsible for the production of potential bacteriocins and other bioactive compounds. According to the study results, the genome of *E. faecium* ICIS21 contained three bacteriocin clusters: enterocin SE-K4, enterocin A, and enterolysin A (Figure 5).

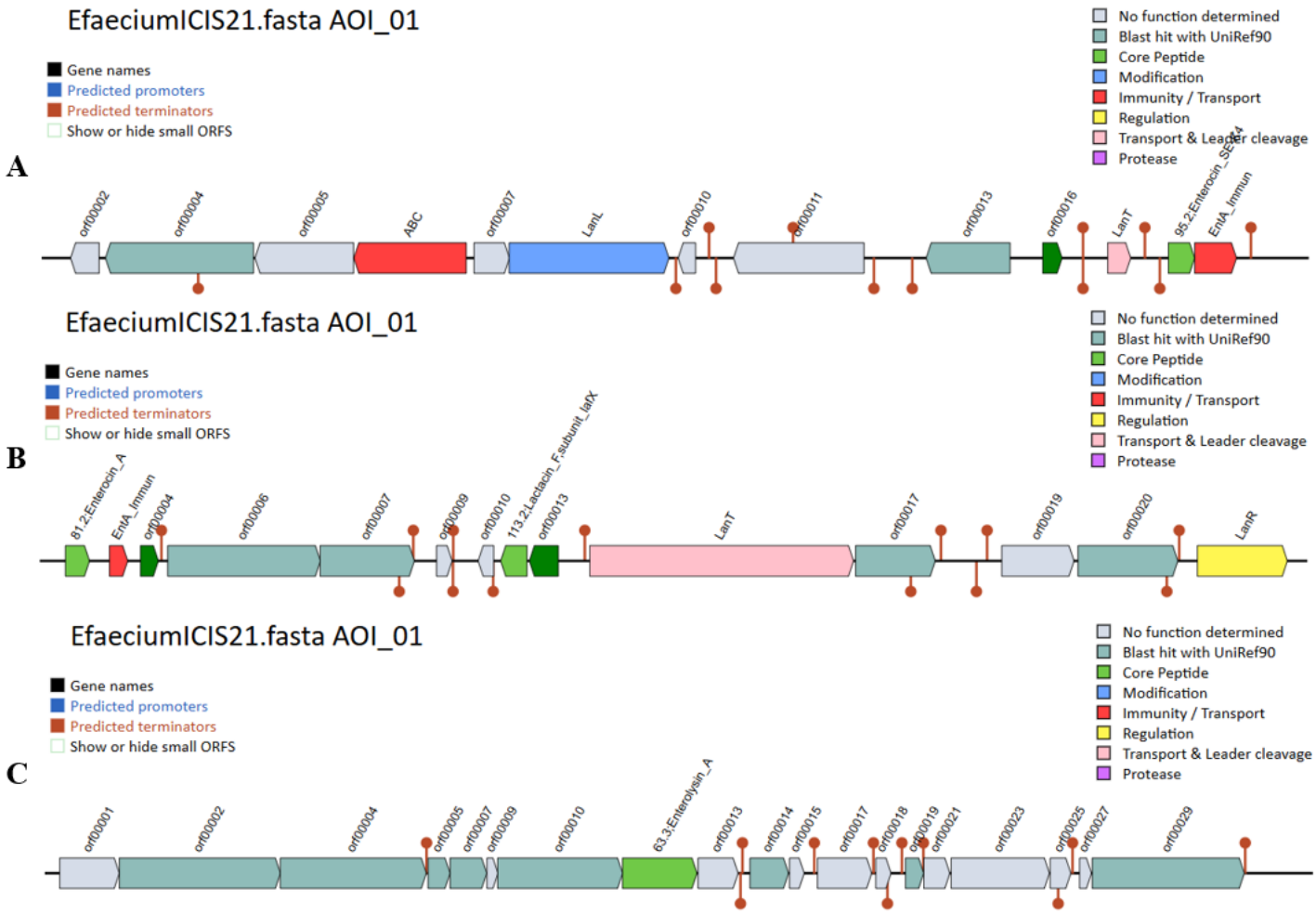

Figure 5 Bacteriocins produced by *E. faecium* strain ICIS21, (a) Enterocin SE-K4, (b) Enterocin A, (c) Enterolysin A

Secondary metabolites

The antiSMASH 6.0 tool was used for the detection and characterization of biosynthetic gene clusters. Two secondary metabolite biosynthetic gene clusters (BGCs) were identified: genes for the synthesis of a cyclic lactone autoinducer (Figure 6a) and type III polyketide synthase (T3pks) genes (Figure 6b).

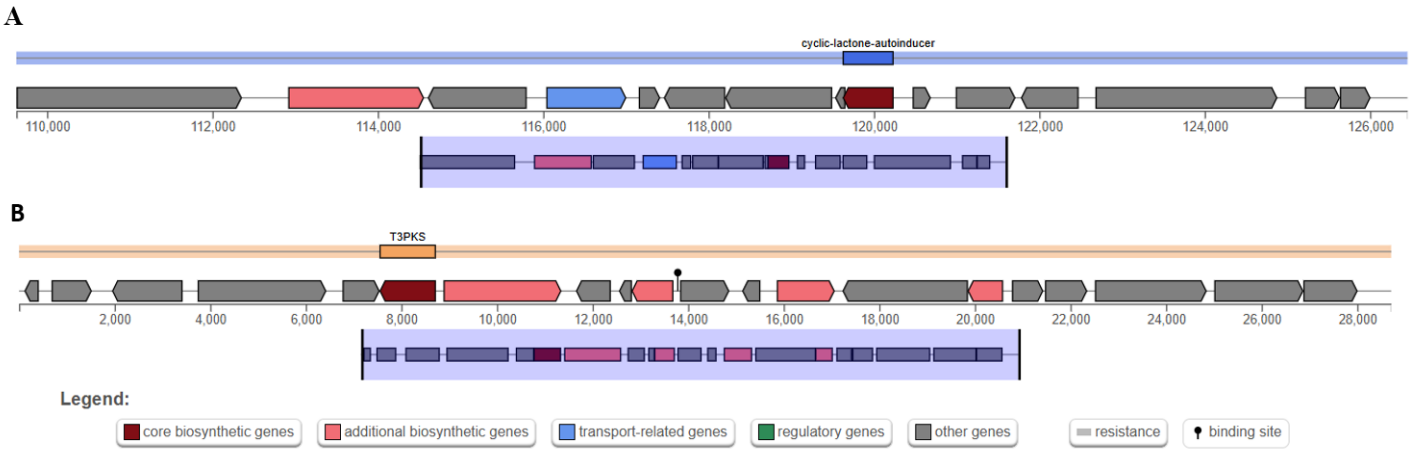

Figure 6 Secondary metabolites produced by *E. faecium* ICIS21. Colours indicate gene types: red – core biosynthetic genes, pink – additional biosynthetic genes, green – regulatory genes, blue – transport genes, grey – other genes. (a) Cyclic lactone autoinducer cluster; (b) T3pks cluster.

Two additional BGCs with low similarity were identified: post-translationally modified peptides (RiPPs) and carnobacteriocin XY (22% similarity).

Heavy metal tolerance genes in *E. faecium* ICIS21

Lactic acid bacteria exhibiting tolerance to heavy metals are currently widely used in bioadsorption methods. The genome of *E. faecium* strain ICIS21 was found to contain numerous genes associated with this capability (Table 2). The genes determining heavy metal resistance facilitate various mechanisms, including ATPase-driven heavy metal transport, heavy metal binding, oxidative or reductive reactions, as well as transcriptional regulation of genes.

Table 2 Heavy metal tolerance genes identified in the genome of *E. faecium* strain ICIS21

Genes	Function	Reference
merA	Enzymatic reduction of Hg^{2+} to Hg^0 by mercury reductase (merA)	**Zscheck and Murray (1990); Foster (1983)**
merR	MerR family protein, is a transcriptional activator, regulates the expression of mercury resistance genes	**Brown *et al.* (2003); Inoue *et al.* (1991); Fang *et al.* (2021)**
czcD	A protein of the cation diffusion facilitator (CDF) subfamily, confers tolerance to cobalt, zinc, and cadmium in *Ralstonia* by reducing cation accumulation	**Anton *et al.* (1999)**
cadA	PCT (probable cadmium-transporting) factor ATPase that transports cadmium	**Silver *et al.* (1993); Lebrun *et al.* (1992)**
cutC	Control of copper homeostasis in *E. faecalis*. Involved in copper uptake, intracellular storage, delivery and efflux in *E. coli*.	**Latorre *et al.* (2011); Rensing and Grass (2003)**
mgtA	Mg^{2+} transport, tolerance to cobalt, manganese, zinc and nickel in *S. aureus*	**Moncrief and Maguire (1999); Schürmann *et al.* (2024)**
mgtE	Magnesium transporting P-type ATPase	**Moncrief and Maguire (1999); Schürmann *et al.* (2024)**
copA	Copper transporting P-type ATPase	**Rensing *et al.* (2000); Fan and Rosen (2002)**
copZ	Metallochaperone, protection against copper action in the cytoplasm	**Lu and Solioz (2001); Corbett *et al.* (2011)**

MLST (Multilocus Sequence Typing) of *E. faecium* ICIS21

Similarity analysis using the "Identify species" algorithm confirmed that the analyzed strain belongs to the species *E. faecium* with 100% support.
MLST analysis was performed using both the classical scheme and a new scheme based on the database by **Bezdicek *et al.* (2023)**. It was established that the *E. faecium* ICIS21 strain showed the highest level of similarity to *E. faecium* ST89 (87.5%) and belonged to the clonal complex CC89. Seven out of eight loci (*dnaE, pbp2B, HP2027, mdlA, narB, uvrA, rpoD*) were found to be identical, which is typical for the species *E. faecium*, while a substitution was observed in one locus *(copA*).

According to the classical scheme, all 7 loci (*purK, gdh, atpA, gyd, adk, ddl, pstS*) showed a perfect match, and the closest strain according to the analysis was *E. faecium* ST286 (100%).

Key probiotic genes of *E. faecium* ICIS21

Probiotic strains and industrial strains used in the production of functional food products must possess a range of characteristics that ensure their adaptation to the host environment and their beneficial health effects. We identified genes responsible for low pH resistance, heat shock proteins, biofilm formation, amino acid synthesis, transfer RNAs involved in protein synthesis, and purine and pyrimidine metabolism (Table 3).

Table 3 *E. faecium* ICIS21 genes encoding probiotic properties

Genes	Function	Reference
mpr	Broad-range (glutamate-specific) endopeptidase is intended for obtaining amino acids for growth by degrading extracellular proteins	**Barbieri *et al.* (2015)**
ABC-F	Proteins function as translation factors that modulate the conformation of the peptidyl transferase center upon binding to the ribosomal tRNA exit site	**Fostier *et al.* (2021)**
typA	Factor crucial for survival under stressful conditions such as growth at low temperature or low pH	**Kiss *et al.* (2004)**
clpB	Chaperone, heat shock protein	**Singh *et al.* (2010)**
uvrA	Resistance factor to ultraviolet radiation and low pH	**Hanna *et al.* (2001)**
clpL	Protein is induced by both heat and cold shock	**Varcamonti *et al.* (2006)**
metG	Cystathionine β-lyase - an enzyme in the major pathway of methionine synthesis	**Sieńko and Paszewski (1999)**
nagE	Enzyme of N-acetylglucosamine uptake and metabolism	**Rogers *et al.* (1988)**
gltX	Glutamyl-tRNA synthetase	**Moore *et al.* (1996)**
adhE	Multifunctional protein physiologically catalyzes the sequential reduction of acetyl-CoA to acetaldehyde and then to ethanol under fermentation conditions	**Membrillo-Hernández *et al.* (2000)**
tdc	The tryptophan decarboxylase enzyme converts tryptophan to tryptamine	**Goddijn *et al.* (1994)**
nrdD	Class III anaerobic ribonucleotide reductases. Class III RNR consists of the catalytic subunit NrdD	**Ofer *et al.* (2011)**
purL	Formylglycinamide ribonucleotide amidotransferase (FGAR-AT), which is an enzyme that catalyzes the fourth step of the purine biosynthetic pathway, is required for biofilm formation in *E. coli*	**Cepas *et al.* (2020)**
guaB	Inosine monophosphate dehydrogenase (IMPDH) is an important enzyme in purine biosynthesis	**Ashbaugh and Wessels (1995)**
carB	Carbamoyl phosphate synthetase, carbamoyl phosphate is a precursor in the biosynthesis of pyrimidine nucleotides and arginine	**Martinussen and Hammer (1988)**
alaS	Alanyl-tRNA synthetase	**Guiliani *et al.* (1997)**
leuS	Leucyl-tRNA synthetase	**Wasmuth and Chu (1980)**
nifJ	Pyruvate ferredoxin oxidoreductase, reduces ferredoxin during the enzymatic catabolism of pyruvate to acetyl coenzyme A	**Schmitz *et al.* (2001)**
yfcC	A factor involved in pyruvate metabolism, indirectly promoting the glyoxylate shunt	**Wang *et al.* (2014)**

DNA repair and synthesis

Probiotic and industrial microbial strains may be exposed to numerous endogenous and exogenous factors that cause DNA damage. To survive under various conditions, bacteria have developed multiple DNA repair mechanisms that maintain genome integrity. Whole-genome sequencing and analysis enable the identification of genes involved in DNA repair pathways, thereby allowing for an overall assessment of the repair potential of a bacterial population. The DNA repair and synthesis genes of *E. faecium* strain ICIS21 are given in Table 4.

Phenotypic Characterization

The *E. faecium* ICIS21 strain was resistant to azithromycin and susceptible to streptomycin, amoxicillin/clavulanic acid, ciprofloxacin, clindamycin, ampicillin, rifampicin, doxycycline, penicillin, and chloramphenicol. It exhibited antagonistic activity against *S. aureus* and *P. aeruginosa* and showed slight inhibition of *E. coli*, *B. cereus*, and *S. typhimurium*. It retained the ability to grow at pH 2 and in bile at a concentration of 1%. The antioxidant activity was 39,48% (Table 5).

Table 4 Genes of DNA synthesis and repair of the *E. faecium* strain ICIS21

Genes	Function	Reference
dnaE	Encodes the alpha subunit of DNA polymerase III	**Welch and McHENRY (1982)**
SMC	Structural maintenance of chromosomes	**Strunnikov *et al.* (1995)**
secA	Self-regulates its translation by binding to the translation initiation region of mRNA	**Salavati and Oliver (1997)**
rpoB, rpoC	Coding subunits β and β′ of DNA-dependent RNA polymerase (RNAP)	**Zakharova *et al.* (1999)**
priA	Plays a central role in restarting chromosomal replication when replication fork progression is disrupted, and is also involved in homologous recombination and DNA repair	**Kline and Seifert (2005)**
RRF	Ribosome recycling factor	**Raj *et al.* (2005)**
polA	Encodes DNA polymerase I	**Rodes *et al.* (2000)**
pcrA	DNA helicase that functions in both repair and rolling circle replication	**Petit *et al.* (1998)**
mutS	DNA mismatch-binding protein shows promise for mutation detection	**Oliver *et al.* (2002)**

Table 5 Phenotypic characteristics of *E. faecium* strain ICIS21

Property	Characteristics
Antibiotic resistance	Resistant: AZI Intermediate: VA Sensitive: STR 300, ACA, CIP, CL, AMP, RIF, DOX, PEN, LEV
Antagonism Inhibition zone diameter values (mm)	*S. aureus – 12,* *E. coli – 2,* *B. cereus – 4,* *P. aeruginosa – 10,* *S. typhimurium - 4*
Bile resistance (% Bile – Growth (OD630))	Control 0%– 0,576 0,125% – 0,547 0,25% – 0,516 0,5% – 0,471 1% – 0,390 2%– 0,138
pH stability (pH- Growth (OD630))	Control – 0,576 5 – 0,575 4 – 0,577 3 – 0,541 2 – 0,441 1 – 0,099
Antioxidant activity (%)	39, 48

Genes Beneficial for Probiotics

To reach their site of action and exert beneficial effects on the organism, probiotics must survive the harsh conditions of the gastrointestinal tract **(Sanchez *et al.*, 2009)**. Acid resistance is the main criterion for the probiotic selection **(Saad, 2006)**. The genome of *E. faecium* ICIS21 was found to contain the *uvrA* and *typA* genes, which may be involved in the adaptive response to low pH. Stress-inducible proteins also contribute to the survival of probiotic bacteria under adverse conditions **(Suokko *et al.*, 2008)**. The genome of *E. faecium* ICIS21 includes two heat shock proteins, *clpB* and *clpL*. This suggests that, possessing genes for acid and temperature tolerance, the isolate may serve as a potential probiotic.

For the growth of lactic acid bacteria, their cells require nitrogenous compounds, which are obtained through the activity of proteolytic enzymes **(Kieliszek *et al.*, 2021)**. The protease gene *mpr* was identified during the analysis of the *E. faecium* ICIS21 genome. Proteolytic enzymes enhance bacterial growth and viability and may potentially participate in the fermentation of dietary proteins, which is also an important probiotic property **(Shihata and Shah, 2000)**.

Genomic characterization revealed genes that can produce nutrients for normal microflora functioning, such as *ABC-F* (proteins that function as translation factors), *alaS* (alanyl-tRNA synthetase), *leuS* (leucyl-tRNA synthetase), *gltX* (glutamyl-tRNA synthetase), and *nrdD* (anaerobic ribonucleotide reductases).

Gut microbiota plays an important role in maintaining the body health **(Ma *et al.*, 2023)**. The production of vitamins by probiotic microorganisms can become an alternative to costly chemical industry **(Gu and Li, 2016)**. The synthesis of metabolites produced by probiotics is crucial in the regulation of host homeostasis **(Agus *et al.*, 2021)**. We identified the genes *purL*, *guaB* (involved in purine biosynthesis) and *carB* (involved in pyrimidine biosynthesis). The methionine synthesis gene *metG*, encoding an essential amino acid, was found in the genome of *E. faecium* ICIS21. Biogenic amines produced by the gut microbiota play a significant role in maintaining human health **(Krautkramer *et al.*, 2021)**. These small molecules derived from amino acids exhibit diverse biological activities, including hormonal, immune, neuromodulatory, and neurotransmitter functions **(Erdag *et al.*, 2018)**. Tryptamine is a potent neuromodulator **(Khan and Nawaz, 2016)** that is produced through the catabolism of tryptophan by the tryptophan decarboxylase (also known as aromatic L-amino acid decarboxylase) enzyme, and the *tdc* gene encoding this enzyme was identified in *E. faecium* ICIS21 in this study **(Williams *et al.*, 2014)**. According to the literature, bacterially derived tryptamine reduces weight loss, colitis severity, and prevents barrier dysfunction in the murine model of dextran sulfate sodium-induced colitis **(Bhattarai *et al.*, 2020)**. Tryptamine also reduces the production of proinflammatory cytokines in cultured macrophages through activation of AhR receptors and suppresses neuroinflammation in the murine model for multiple sclerosis **(Dopkins *et al.*, 2021)**. The identified *nagE* gene (associated with N-acetylglucosamine metabolism) may be involved in stimulating the growth of gut microbiota (**Choi *et al.*, 2023).**

Probiotic lactic acid microorganisms possess a complex metabolism comprising multiple cycles. Several genes responsible for acetyl-coenzyme A regeneration (*adhE*, *nifJ*) were identified in the genome of our strain **(Piveteau, 1999)**.

DNA repair and synthesis

A vast number of repair proteins function to maintain DNA integrity. The genome of *E. faecium* ICIS21 includes the following genes: *fusA* (encoding elongation factor G, EF-G); *dnaE* (encoding the alpha subunit of DNA polymerase III); SMC (structural maintenance of chromosomes); *secA* (which autoregulates its own translation by binding to the translation initiation region in mRNA); *rpoB* and *rpoC* (encoding the β and β′ subunits of DNA-dependent RNA polymerase, RNAP); *priA* (playing a central role in restarting chromosomal replication upon replication fork arrest, and also involved in homologous recombination and DNA repair); RRF (ribosome recycling factor); *polA* (encoding DNA polymerase I); *pcrA* (a DNA helicase involved in both repair and rolling-circle replication); and *mutS* (a DNA mismatch-binding protein, considered a promising tool for mutation detection).

MLST of *E. faecium* ICIS21

The *E. faecium* ICIS21 strain belongs to sequence type 89 (ST89) and clonal complex 89 (CC89) and, accordingly, it differs from the specific genetic lineages of *E. faecium* strains belonging to the CC17 clonal complex. The latter includes sequence types ST17, ST64, ST78, and ST80, which are adapted to hospital environments and represent high-risk strains **(Franz *et al.*, 2011)**.

Antibiotic resistance

A critical issue in the safety assessment of enterococci is their antibiotic resistance. A key factor in selecting enterococci for probiotic use is the absence of transmissible antibiotic resistance genes (**Cui *et al.*, 2025; Bagci *et al.*, 2019**), which was confirmed for the *E. faecium* ICIS21 strain. In addition, many probiotic and potentially probiotic *E. faecium* strains may exhibit phenotypic antibiotic resistance **(Zaghloul *et al.*, 2023; Rajput *et al.*, 2022).** According to **Rajput *et al.* (2022),** antibiotic resistance is a probiotic trait that facilitates the colonization of desirable microorganisms in patients.

Production of antagonist factors

Lactic acid strains synthesize many antagonistic factors, including bacteriocins **(Klaenhammer, 1988)**. These are antimicrobial peptides formed through post-translational modification by enzymes and released into the extracellular environment **(Alvarez-Sieiro *et al.*, 2016)**. According to the literature, *E. faecium* strains isolated from fermented products have enterocins **(Daza Prieto *et al.*, 2024)**. Such proteins exhibit antimicrobial activity against food-borne pathogens **(Gontijo *et al.*, 2020)**. The genome of *E. faecium* strain ICIS21 included three bacteriocins: enterocin A, enterocin SE-K4, and enterolysin.

Enterocin A is known to belong to class IIa and exhibits antimicrobial activity against certain strains of *Listeria*, *Staphylococcus*, *Pediococcus* and *Enterococcus* **(Escamilla-Martínez *et al.*, 2017)**. It also demonstrates inhibitory effects against *E. faecium* and *E. faecalis*, which account for 86% of vancomycin-resistant enterococcal infections **(McClintock *et al.*, 2016)**.

Moreover, some studies have reported that antimicrobial films based on enterocin A exhibit strong antilisterial activity in sliced dry-cured ham **(Aymerich at el., 2022)**.

The results of another study demonstrated that enterocin A in combination with colicin E1 acts against human AGS cancer cells. Taken together, enterocin A–colicin E1 (ent A–col E1) can be considered a promising candidate for anticancer therapy **(Fathizadeh *et al.*, 2021)**.

The bacteriocin designated enterocin SE-K4 was isolated from the *E. faecalis* strain K-4, with its amino acid sequence demonstrating clear similarity to class IIa bacteriocin. Enterocin SE-K4 is heat-stable and exhibits antimicrobial activity against *E. faecium*, *E. faecalis*, *Bacillus subtilis*, *Clostridium beijerinckii* and *Listeria monocytogenes* **(Eguchi *et al.*, 2001)**.

According to literature data, enterolysin A has antibacterial activity against vancomycin-resistant *E. faecium* **(Fujii *et al.*, 2025)**.

Secondary metabolites

The genes involved in the biosynthesis of secondary metabolites, such as T3pks (a polyketide family) and a cyclic lactone autoinducer were found in the genome of *E. faecium* ICIS21.

The bacteria employ the quorum sensing (QS) system to regulate population density and gene expression **(Irie and Parsek, 2008)**. The QS system utilises autoinducing peptides as signalling molecules, and cyclic lactone peptides are among them **(Geisinger *et al.*, 2009)**.

Polyketides (T3pks) are characterised by antitumour, anticholesterolaemic, and antimicrobial effects **(Yu *et al.*, 2012)**, and are also involved in the biosynthesis of diverse compounds, including lipids—ranging from signalling molecules to biologically active natural products **(Katsuyama and Ohnishi, 2012)**.

According to the literature, T3pks and the cyclic lactone autoinducer have been identified in *E. faecium* and *E. lactis* strains isolated from traditional Montenegrin brine-ripened cheeses and salami **(Daza Prieto *et al.*, 2024)**.

The presence of secondary metabolite gene clusters suggests that strain ICIS21 may serve as a producer of novel natural bioactive compounds.

Resistance to heavy metal salts

Heavy metals are some of the most common pollutants released into the environment. The use of biomarkers for environmental monitoring of chemical stress in organisms is gaining growing attention, offering rapid and sensitive detection. The bacteria have numerous metal resistance genes that can be used as biomarkers, including cation diffusion facilitators and metal ion transporters. In addition, bacteria can be effectively used as biosorbents for the purification of wastewater containing heavy metals. The search for bacterial biosorbents capable of binding hazardous heavy metals, such as cadmium, is crucial for remediation efforts.

Mercury contamination is a significant issue and poses a serious threat to both the environment and human health. The mer operon is efficient and widely distributed due to horizontal and vertical gene transfer in its various forms across diverse bacterial communities, reflecting its essential role and interrelationship in the nature. This operon is one of the widely studied systems of microbial resistance to metals with a high transformation capacity to convert toxic ions into volatile non-toxic forms **(Mathema *et al.*, 2011)**.

Mercury reductase, a flavin oxidoreductase **(Summers and Sugarman, 1974)**, is primarily responsible for the reduction of the highly toxic ionic Hg^{2+} to the less toxic and volatile Hg^0 in an NAD(P)H-dependent reaction. Subsequently, the volatile Hg^0 is transported from the cytosolic region into the periplasmic space **(Mathema *et al.*, 2011)**.

The *czcD* gene encodes a metal ion transporter that functions as an efflux/influx pump for cobalt, zinc, cadmium, nickel, and iron protons **(Fierros-Romero *et al.*, 2020)**.

The well-characterised gene *cadA* mediates cadmium resistance via an ATP-dependent transport mechanism **(Naz *et al.*, 2006)**.

The *cutC* factor, a member of the cut family, has been reported to play a potential role in copper homeostasis. It has been demonstrated that *cutC* expression is induced in response to increased copper concentrations at a late stage. The transcriptional response exhibits a direct correlation with a marked increase in intracellular copper levels in the absence of the *cutC* protein in bacteria, suggesting its role in metal efflux mechanisms **(Latorre *et al.*, 2011; Reyes *et al.*, 2006)**.

The P-type ATPase proteins — *corA*, *mgtE*, and *mgtA*/*mgtB* have been identified in bacteria as three families of magnesium transporters **(Shin *et al.*, 2014)**. Metal regulatory proteins have been identified as sensors of transition metals. One of the mechanisms regulating magnesium homeostasis is known, linking the intracellular magnesium requirement with transport activity through the cytoplasmic gating domains of *corA* and *mgtE* **(Solioz and Stoyanov, 2003)**. According to the literature, the primary element regulating intracellular copper concentration is an operon consisting of four genes: *copY*, *copZ*, *copA*, and *copB*. *CopZ* encodes a copper chaperone, *copY* encodes a copper-sensitive repressor, and *copA* and *copB* encode ATPases involved in copper transport. The cop operon enables *E. hirae* to grow under copper concentrations up to 8 mM as well as under copper-limited conditions **(Solioz *et al.*, 2011)**.

However, the potential for using *E. faecium* ICIS21 as a heavy metal biosorbent based on the identification of a complex of heavy metal resistance genes requires further experimental validation.

CONCLUSION

We analyzed the genome of *E. faecium* ICIS21, which is largely similar to the genome of strains isolated from food and differs from clinical isolates. This strain has a wide range of genes that provide its resistance to various stress factors, which may determine its survival in the conditions of the gastrointestinal tract. The strain is characterised by the presence of genes encoding various antimicrobial factors — enterocin A, enterocin SE-K4, and enterolysin; genes involved in the biosynthesis of secondary metabolites such as T3pks and cyclic lactone autoinducers; and genes related to the production of vitamins and unsaturated fatty acids. Additionally, an important feature is the presence of a heavy metal resistance gene cluster. The absence of true pathogenicity genes is a fundamental criterion for classifying *E. faecium* ICIS21 as a microorganism suitable for the production of fermented dairy products and may be significant for using the strain as a biosorbent for heavy metal ions. However, the obtained genetic analysis data require an experimental assessment of gene expression levels and further studies using *in vivo* models.

Availability of Data and Materials: The genome sequence assembly project has been deposited at GenBank under accession number JBBPHS000000000.2.

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