Downloaded from orbit.dtu.dk on: Apr 20, 2024

DTU Library

Bad to the bone? - Genomic analysis of Enterococcus isolates from diverse environments reveals that most are safe and display potential as food fermentation microorganisms

Tadesse, Belay Tilahun; Svetlicic, Ema; Zhao, Shuangqing; Berhane, Nega; Jers, Carsten; Solem, Christian; Mijakovic, Ivan

Published in:
Microbiological Research

Link to article, DOI:
10.1016/j.micres.2024.127702

Publication date:
2024

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Tadesse, B. T., Svetlicic, E., Zhao, S., Berhane, N., Jers, C., Solem, C., & Mijakovic, I. (2024). Bad to the bone? - Genomic analysis of Enterococcus isolates from diverse environments reveals that most are safe and display potential as food fermentation microorganisms. *Microbiological Research*, *283*, Article 127702. https://doi.org/10.1016/j.micres.2024.127702

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Microbiological Research 283 (2024) 127702

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Bad to the bone? – Genomic analysis of *Enterococcus* isolates from diverse environments reveals that most are safe and display potential as food fermentation microorganisms

Belay Tilahun Tadesse [a,b], Ema Svetlicic [b], Shuangqing Zhao [a], Nega Berhane [c], Carsten Jers [b], Christian Solem [a,*], Ivan Mijakovic [b,d,**]

[a] *National Food Institute, Research Group for Microbial Biotechnology and Biorefining, Technical University of Denmark, Kgs Lyngby DK-2800, Denmark*
[b] *Novo Nordisk Foundation Center for Biosustainability, Kongens Lyngby, Denmark*
[c] *Institute of Biotechnology, University of Gondar, Ethiopia*
[d] *Systems and Synthetic Biology Division, Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden*

ARTICLE INFO

Keywords:
Enterococcus species
Lactic acid bacteria
Whole-genome screening
Virulence genes
Antibiotic resistance genes
Secondary metabolite clusters
Dairy
Bacteriocins

ABSTRACT

Enterococci comprise a group of lactic acid bacteria (LAB) with considerable potential to serve as food fermentation microorganisms. Unfortunately, enterococci have received a lot of negative attention, due to the occurrence of pathogenic and multidrug resistant strains. In this study, we used genomics to select safe candidates among the forty-four studied enterococcal isolates. The genomes of the forty-four strains were fully sequenced and assessed for presence of virulence and antibiotic resistance genes. Nineteen isolates belonging to the species *Enterococcus lactis, Enterococcus faecium, Enterococcus durans*, and *Enterococcus thailandicus*, were deemed safe from the genome analysis. The presence of secondary metabolite gene clusters for bacteriocins was assessed, and twelve candidates were found to secrete antimicrobial compounds effective against *Listeria monocytogenes isolated from cheese* and *Staphylococcus aureus*. Physiological characterization revealed nineteen industrial potentials; all strains grew well at 42 °C and acidified 1.5 hours faster than their mesophilic counterpart *Lactococcus lactis*, with which they share metabolism and flavor forming ability. We conclude that a large fraction of the examined enterococci were safe and could serve as excellent food fermentation microorganisms with inherent bioprotective abilities.

1. Introduction

Enterococcus is a diverse genus of lactic acid bacteria (LAB), mostly known for their association with the gastrointestinal (GI) tracts of animals (Dubin and Pamer, 2017; Ghazisaeedi et al., 2022; Henning et al., 2015; Holzapfel et al., 2018; Kommineni et al., 2015). Of the more than 50 species currently identified (Ben Braïek and Smaoui, 2019; Dubin and Pamer, 2017), only two species are found in significant numbers in the gut, and these are *Enterococcus faecalis* and *Enterococcus faecium*, which together account for approximately 1% of the adult human microbiota (Dubin and Pamer, 2017; Holzapfel et al., 2018). Other niches occupied by enterococci include plants and soil (Ben Braïek and Smaoui, 2019; Ben Said et al., 2016; Valenzuela et al., 2010), which explains why they often end up in various foods (Bhardwaj et al., 2008; Vimont et al., 2017).

The enterococci that are found in the gut are ancient members of the GI tract consortia of invertebrates, insects, and mammals. Commensal enterococci, despite their success in highly competitive environments, have reduced genomes (2.7 Mb) and they possess auxotrophies for amino acids, vitamins, and other micronutrients (Gilmore et al., 2015). In this manner, they resemble LAB used in food fermentations, e.g., dairy lactococci which have adapted to the milk niche (Kelleher et al., 2017).

Enterococci can be found in many fermented foods all over the world, where they play an important role as flavor formers, and contribute with lipolysis, proteolysis, citrate breakdown, and production of volatile compounds such as acetaldehyde, acetoin, diacetyl, and ethanol (Bhardwaj et al., 2008). Enterococci are robust in many ways; they are capable of growing at both low and high pH (4.4–9.6), tolerate

* Corresponding author.
** Corresponding author at: Novo Nordisk Foundation Center for Biosustainability, Kongens Lyngby, Denmark.
E-mail addresses: chso@food.dtu.dk (C. Solem), ivan.mijakovic@chalmers.se (I. Mijakovic).

https://doi.org/10.1016/j.micres.2024.127702
Received 29 November 2023; Received in revised form 9 February 2024; Accepted 20 March 2024
Available online 22 March 2024
0944-5013/© 2024 The Author(s). Published by Elsevier GmbH. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

up to 6.5% NaCl, have a broad temperature tolerance (10–45 °C), and have an exceptional ability to survive a 30-minute exposure to 60 °C (Ben Braïek and Smaoui, 2019; Franz et al., 2003). Because of occurrence of pathogenic and multidrug resistant strains and their involvement in horizontal gene transfer (Palmer et al., 2010), enterococci are not currently used as starter cultures in cheese production in Europe. However, some approved strains, the safety of which have been demonstrated, are used as probiotics for humans and animals (Popovic et al., 2018). In countries like Austria, Italy, and Switzerland, *E. faecium* SF68 is an approved medication that is used for treating and preventing diarrhea (Greuter et al., 2020). European food safety Association (EFSA) has approved SF68 as a 'gut flora stabilizer' for various animal categories (Holzapfel et al., 2018) and this strain has been authorized for use as a probiotic in pharmaceutical preparations and food supplements for humans and animals (Ghazisaeedi et al., 2022). SF68 has also been shown to be able to alleviate symptoms of intestinal inflammation in human clinical trials (Ghazisaeedi et al., 2022).

In addition to their robustness, enterococci can also contribute to the product shelf life and food safety, as they secrete a wide range of antimicrobial peptides and proteins called bacteriocins, which for enterococci are mostly termed enterocins (Henning et al., 2015; Vandera et al., 2017). Enterococci often secrete multiple enterocins, which potentiates their use as antimicrobial agents in food where they efficiently can interfere with the growth of various food-spoiling and pathogenic bacteria such as *Listeria monocytogenes* (Henning et al., 2015; Vandera et al., 2017), *Salmonella* spp., *Shigella* spp., *Escherichia coli*, *Enterobacter* spp., and *Campylobacter* spp. (Fugaban et al., 2021; S. Flint, 2002)

As mentioned above, the genus *Enterococcus* contains some pathogenic culprits that have cast doubts on the safety of its members (Popovic et al., 2018). Enterococci have been implicated in various nosocomial infections, e.g., wound infections, endocarditis, and urinary tract infections (Mohamed and Huang, 2007), and are known for their ability to transfer antibiotic-resistance genes (Popovic et al., 2018). Members of two species, *E. faecalis* and *E. faecium* are most frequently involved in this. Antibiotic resistance and pathogenicity are more prevalent among *E. faecalis* than *E. faecium* and 80–90% of the pathogenic and antibiotic-resistant enterococcal isolates belong to *E. faecalis* (Popovic et al., 2018). Often *E. faecalis* strains are able to form biofilms (Stępień-Pyśniak et al., 2019), which contributes to their virulence (Holman et al., 2021; O'Driscoll and Crank, 2015; Stępień-Pyśniak et al., 2019). Some isolates are resistant to vancomycin, a last-resort antibiotic (Chotinantakul et al., 2020; Kim et al., 2020), and thus today pose a challenge for hospitals and health care facilities around the world.

It is imperative to differentiate between commensal and pathogenic enterococci and enterococci that are not typically associated with the gut environment. As mentioned above, commensal enterococci have smaller genomes, and have peacefully coexisted with animals, perhaps since they diverged from their common ancestor more than 425 million years ago (Lebreton et al., 2017). There is now strong evidence that antibiotic resistance in enterococci has arisen due to excessive use of antibiotics within the agriculture, e.g., drugs like avoparcin, commonly used as growth enhancers in pigs and poultry, have prompted vancomycin resistance in enterococci (Bager et al., 1997). It is also known that pathogenic strains do not spontaneously arise from non-pathogenic or commensal strains but are transferred between patients in hospital environments (Lebreton et al., 2013) or from animals through contaminated meat. Most pathogenic isolates have accumulated genes that enable them to pursue an infectious lifestyle, e.g., genes for metabolizing mucosal carbohydrates (Lebreton et al., 2013), genes encoding tissue degrading enzymes (Panthee et al., 2021; Thurlow et al., 2010) and sophisticated regulatory mechanisms governing the expression of these genes. As much as 20% of the genome of *E. faecalis* V583, one of the first vancomycin resistant clinical isolates, consists of acquired DNA (Gilmore et al., 2015). Carrying an arsenal of virulence genes is not without consequence, and pathogenic strains most likely cannot compete with commensal strains in the absence of selective pressure, due to the metabolic burden associated with being virulent (Gilmore et al., 2015). Pathogenic enterococci, like many other pathogenic microorganisms, becomes a challenge only when they are present in large numbers, and here their antibiotic resistance comes into play. When competing microorganisms are wiped out due to administration of antibiotics, antibiotic resistant enterococci increase in numbers rapidly, which activates the *fsr* quorum-sensing system, that controls expression of several virulence traits (Qin et al., 2001). Virulence factors in enterococci have been categorized into five different groups, which are "Adherence", "Antiphagocytosis", "Biofilm formation", "Exoenzyme", and "Exotoxin" (Jett et al., 1994). The ability to adhere to surfaces is not necessarily associated with virulence (Segers and Lebeer, 2014), but is an essential step in bacterial pathogenesis. In *E. faecalis* the endocarditis and biofilm associated pili (encoded by *ebpABC*) are directly associated with pathogenesis (Nallapareddy et al., 2011). Bacteria and small particles can traverse the gut epithelium, and enter the bloodstream, but are usually caught by the immune system, e.g., phagocytized by macrophages and neutrophils. Having a polysaccharide capsule is one way to prevent or reduce phagocytosis, and enterococci that rely on this approach have been reported (Bottone et al., 1998).

Complement and antibody-mediated killing is one of the most important mechanisms for of the human body to eliminate pathogens that have entered the bloodstream (Harvey et al., 1992). Some enterococci produce a secreted protease, GelE, that cleaves the complement protein C3, thereby reducing the efficiency of this important immune response (Yong Park et al., 2008). Therefore, before using them in food fermentations, it is important to carefully assess the safety of *Enterococcus* strains.

Currently, several molecular (PCR) and physiological-based methods are used for assessing safety (Cui et al., 2020; Medeiros et al., 2014; Özden Tuncer et al., 2013). A complication with these methods is that it is difficult to distinguish between strains using traditional taxonomic approaches (Panthee et al., 2021). Therefore, for effective surveillance, fast and accurate identification, typing and safety assessment of microorganisms, next generation sequencing (NGS), has become a popular method that can provide detailed information about the isolates (Joensen et al., 2014).

The aim of the present study was to investigate the prevalence of *Enterococcus* strains without virulence attributes among forty-four strains obtained from foods from a local supermarket in Denmark, from Ethiopian *Injera* sourdough, and from a fecal sample. For this purpose, we relied on NGS as a tool to find promising candidates which lack the genes associated with virulence and antibiotic resistance. This led to the identification of several strains that both appear safe and maintain key properties relevant for food fermentation.

2. Materials and methods

2.1. Bacterial strains and culture conditions

In total, forty-four *Enterococcus* strains were isolated from *Injera* sour dough (Ethiopia), cheese, fruits and vegetables collected from a Danish supermarket, or a fecal sample derive from a healthy adult male. The strains were grown in M17 agar (Thermo Scientific™ Oxoid™) supplemented with 1% glucose and 6.5% NaCl at 42 °C overnight. Strains were stored in M17 broth supplemented with 15% glycerol at −80 °C.

2.2. Hemolytic and gelatinase assay

The *Enterococcus* isolates were grown on M17 agar (Thermo Scientific™ Oxoid™) at 37 °C, after which single colonies were streaked onto Columbia Blood Agar (Oxoid Limited, Hampshire, UK) containing 5% (v/v) defibrinated horse blood, after 48 h of incubation at 37 °C. Hemolytic activity was observed as a clear yellow zone around the bacterial (Fugaban et al., 2021; Rebecca Buxton, 2016)). On the other hand, gelatinase activity assay were done by using cell culture estimated to

contain 10^5 cells/mL from its exponential phase grown in M17 broth (Thermo Scientific™ Oxoid™) at 37 °C, was inoculated into tubes containing 10 mL of BHI broth (beef heart (infusion from 250 g), 5 g/L, calf brains (infusion from 200 g), 12.5 g/L, disodium hydrogen phosphate, 2.5 g/L, D(+)-glucose, 2 g/L, peptone, 10 g/L, sodium chloride, 5 g/L) supplemented with 4% gelatin. The tubes were then incubated at 37 °C for 24 hours before being cooled at 4 °C for 30 minutes. Cultures remaining liquid after refrigeration were considered to express gelatinase (Fugaban et al., 2021).

2.3. Antimicrobial susceptibility profiling

The antimicrobial susceptibility testing (AST) was conducted using the recommended standards by EFSA (EFSA, 2012) for *Enterococcus* spp. The assay was carried out using the microbroth dilution technique with the antibiotics ampicillin, chloramphenicol, erythromycin, kanamycin, streptomycin, tetracycline, and vancomycin on cation-adjusted Mueller-Hinton broth supplemented with MRS (5.0 g/L). The assay was performed in a 96-well cell culture microtiter plate: ten dilutions (two-fold) for each antibiotic were used and controls were included as well. The inocula were adjusted to an absorbance A of 0.5 and distributed accordingly to attain a final concentration of 10^5 CFU/mL. The microtiter plates were incubated at 35 ± 1 °C for 18 h. The MIC was recorded as the lowest concentration at which complete growth inhibition was observed.

2.4. Biofilm formation assay

All selected candidates were assessed for their biofilm formation capacity, using the 96-well microtiter plate. This was done by culturing bacterial isolates in Mueller-Hinton broth with 0.5% glucose and incubating them at 37 °C overnight. The cultures were then diluted in fresh Mueller-Hinton broth with 0.5% glucose and 200 μL of the diluted solution (absorbance A = 0.5, mid log phase) was added to the wells of a 96-well microtiter plate and incubated for 48 h at 37 °C. Biofilms were fixed with 99% (v/v) methanol for 20 min (Dassanayake et al., 2020), dried at room temperature, and then stained with 0.1% crystal violate. The absorbance for each well was measured at 490 nm using a TECAN Infinite M200 pro plate reader. *Enterococcus faecalis V583* was used as the biofilm producer control strain in this study. The results were determined by calculating the absorbance cut-off (ODc), which was defined as the average absorbance A (OD) of the negative control plus three standard deviations (SD) of the negative control (Fallah et al., 2017; Hashem et al., 2017, Dassanayake et al., 2020). The biofilm formation ability was categorized as "non-biofilm formation" (OD ≤ ODc), "weak biofilm formation" (ODc < OD ≤ 2 ODc), "moderate biofilm formation" (2 ODc < OD ≤ 4 ODc) and "strong biofilm formation" (OD > 4 ODc) (Hashem et al., 2017).

2.5. Bacteriocinogenic activity assay

The antibacterial activity assay was done using the method described (Yamamoto et al., 2003). The Enterococci colonies were spotted on M17 agar supplemented with 1% glucose and the plates were incubated at 37 °C. The bacterial strains used in the test were *Micrococcus luteus* ATCC 10240, which was grown overnight in nutrient broth with 200 rpm at 30 °C, and *S. aureus* ATCC29213, *L. monocytogenes* 0107.0111, *L. monocytogenes* 0107.0489, and *L. monocytogenes* 0107.0243, which were grown overnight in BHI broth at 37 °C statically. After overnight incubation 7 mL of autoclaved nutrient agar (0.7% agar, pH 7.0) medium was cooled to 40 °C and inoculated with 1% of actively growing target pathogenic strains (absorbance A = 2.0) and then the plates were transferred to a 30 °C for *Micrococcus luteus* and at 37 °C for the rest test pathogenic strains in the incubator and kept there for 24 h. After incubation at respective temperature overnight, bacterial colonies forming growth inhibition zones indicated production of bacteriocin. The assay was performed in duplicate.

2.6. Species identification using MALDI-TOF mass spectrometry

All isolates were plated on M17 agar (Thermo Scientific™ Oxoid™) supplemented with 1% glucose prior to matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis and a single colony was used for identification (De Bruyne et al., 2011; Huguenin et al., 2023). A single colony was placed in triplicate onto a 96-spot polished steel target plate (Bruker Daltonik, Germany), dried at room temperature. Then 1 μl 70% formic acid was placed on top of the bacterial smear, followed by 1 μl of HCCA matrix (10 mg/mL alpha-4-cyano-4-hydroxycinnamic acid (CHCA, Bruker Daltonik, Germany) in 50% acetonitrile and 2.5% trifluoroacetic acid) and allowed to dry. Spectra were acquired in the positive linear ion mode in the mass range of 2–20 kDa. MBT Compass HT version 5.0 software (Bruker Daltonik) was used to match spectra against a reference database (version 11). The obtained spectra were interpreted against the MBT Comopass® Library Revision H (2021), covering 3893 species/entries. The log score thresholds were considered identification criteria, a log score of 2.00–3.00 indicated high-confidence species identification, a log score of 1.70–1.99 indicated low-confidence species identification, while a score of 0–1.69 was considered unreliable identification.

2.7. DNA extraction and sequencing

Cells grown in GM17 broth were harvested (approx. 2×10^9 cells), by centrifugation (10 min, 5000xg). The pellet was resuspended in PBS and washed three times before the Genomic DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Germany), following the manufacturer's instructions. The quality of the DNA was analyzed using a Nanodrop (Thermo Scientific, USA), its concentration determined using Qubit fluorometric quantification (Thermo Scientific, USA), and the fragment length analyzed using a 5300 Fragment Analyzer System (Agilent Technologies, USA). Whole-genome sequencing was carried out using the Nanopore GridION (Oxford Nanopore Technologies, United Kingdom) platform and the Illumina platform to obtain better coverage of the WGS. The genomic DNA libraries were prepared using Illumina Nextera DNA library prep according to the manufacturer's protocol and sequenced using the Illumina MiSeq system, resulting in paired-end 150 bp reads. To get longer reads, the MinION Nanopore (Oxford Nanopore Technologies, United Kingdom) platform was used, where DNA libraries were prepared using the rapid 96 barcoding kit following the manufacturer's instructions and sequenced using the R9.4.1 flow cell.

2.8. De novo hybrid assembly and annotation

The Illumina raw reads were checked for their quality using fastp (V0.23.2) (S. Chen et al., 2018) with default settings. Adapter trimming of long reads was performed using Filtlong and the read quality was checked with Nanoplot V1.41.0 (De Coster, 2018). The hybrid de novo assembly of the genome was done using the Illumina and Nanopore reads using Unicycler v0.5.0 (Wick et al., 2017) with default settings. Quast; v.5.2.0 ((Gurevich et al., 2013; Mikheenko et al., 2018) with default setting was used to check the assembly quality of all assembled genomes and the reports were summarized using MultiQC V1.11 (Ewels et al., 2016). The de novo assembled genomes were annotated using Prokka (Prokaryotic genome annotation) (Galaxy Version 1.14.6+galaxy1) (Cuccuru et al., 2014; Seemann, 2014). Then the completeness and contamination of genome bins were assessed using lineage-specific marker sets using checkM V1.2.0 (Parks et al., 2015) which checks the quality of genome sequences of isolates, single cells, or genome bins from metagenome assemblies through comparison to an existing database of genomes included in the checkM tool.

2.9. Genome-based species identification

To identify the closest related species from the database, PubMLST (Jolley et al., 2018) was used to initially identify the de novo assembled genomes of the enterococci isolates based on the Ribosomal Multilocus Sequence Typing (rMLST) tool. Genomic similarity was identified through the Average Nucleotide Identity (ANI) by an alignment-free approach using fastANI tool (Jain et al., 2018) compared to assemblies of the *Enterococcus* type strains downloaded from NCBI and genome-to-genome distance were calculated by *in silico* DNA–DNA hybridization (GGDC) against *Enterococcus* type strains.

The genome of the 46 representative type strains of genus *Enterococcus* downloaded from NCBI and the forty-four de novo assembled genomes sequenced in this study was determined. For normalization purposes, all genomes were annotated with Prokka (Galaxy v1.14.6). The core genome was determined using Roary (Galaxy V3.13.0) (Page et al., 2015) using default settings. The pangenome phylogeny tree was produced using the Newick output file of Roary analysis, and the tree was visualized and edited using iTOL V6 and MEGA v7.0.26.

2.10. Comparative analysis

Whole genome-based identification of *Enterococcus* isolates and screening for the presence of virulence genes, antibiotic resistance genes, and useful secondary metabolites like enterocins was performed. Draft genome sequences of the forty-four *Enterococcus* spp. were investigated for the presence of putative virulence genes and antibiotic resistance genes (ARGs), mobile genetic elements (MGEs), and secondary metabolite biosynthetic gene clusters.

ABRicate Mass screening of contigs for antimicrobial and virulence genes (Galaxy Version 1.0.1) (Seemann, 2016) was used to screen for the presence of virulence genes (Table 1) and antibiotic resistance genes, using the Virulence Factors Database (VFDB) (L. Chen et al., 2016; Liu et al., 2022) and Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017), respectively. Virulence Finder 2.0 (Maria et al., 2020) and ResFinder 4.1 (Bortolaia et al., 2020; Clausen et al., 2018; Zankari et al., 2017) services found in Center for Genomic Epidemiology (Technical University of Denmark) were also used to screen for virulence genes and antibiotic resistance genes, respectively. Identification of mobile genetic elements was done using MobileElements Finder V1.0.3 (Johansson et al., 2021).

Since many isolates from this study contained more than one contig, the presence of circular plasmids was suspected. To detect plasmids, the assemblies were screened using ABricate (Galaxy v.1.0.1) (Seemann, 2016) and PlasmidFinder 2.1 (Carattoli et al., 2014; Clausen et al., 2018) with the threshold for minimum percent of identity and 60% minimum percent of coverage as it has indicated in the default setting.

2.11. Secondary metabolite gene cluster analysis

The genome-wide identification, annotation, and analysis of secondary metabolite biosynthesis gene clusters (BGCs) were identified and analyzed using secondary metabolite biosynthetic gene clusters using the Antibiotics and Secondary Metabolite Analysis Shell (antiSMASH, V6.1.1) (Beukers et al., 2017; H. Zhang et al., 2022) and BAGEL4 (Van Heel et al., 2018).

2.12. Carbohydrate metabolism profiling

The carbohydrate utilization profile of the *Enterococcus* isolates was determined using an analytical profile index (API). The API 50 CH and API 50 CHL medium from bioMérieux were used for this purpose, according to the instructions provided by Neamtu *et al.*, (Neamtu et al., 2014). The isolates were inoculated to an absorbance A = 0.5 and mixed with the API50 CHL medium. The cupules of the API strips were covered with mineral oil and incubated at 42°C for 48 hours. After 48 hours of

Table 1
Overview of enterococcal virulence factors arranged by category and function.

Virulence factors	Gene Code	Function	Reference
Adherence			
Adhesion to collagen of *E. faecalis*	*ace*	Plays a key role in the adherence and colonization process	Liu et al., (2022); Ch'ng et al., (2019); Popovic et al., (2018)
adhesion to collagen from *E. faecium*	*acm*	Surface protein expressed due to pheromone induction, which plays an important role in adherence and colonization to host tissues	Oli et al., 2022
Aggregation substance	*prgB/asc10*		Liu et al., (2022); Ch'ng et al., (2019);
	EF0149, EF0485 *asa1*		Liu et al., (2022) Chotinantakul et al., (2020)
Endocarditis- and biofilm-associated pilus	*ebpA, ebpB, ebpC*	Important for biofilm formation, initial attachment, and IE	Chotinantakul et al., (2020); Mohamed and Huang, (2007); Liu et al., (2022)
	ebpR	Transcriptional regulator of *ebpABC*	Mohamed and Huang, (2007)
	srtC	Encodes sortase C, an enzyme that anchors surface proteins to the cell wall	Thomas et al., 2009; Mohamed and Huang, (2007)
E. faecium collagen-binding protein A	*ecbA/fss3*	Involved in biofilm development	Ch'ng et al., (2019); Liu et al., (2022)
E. faecalis endocarditis-associated antigen A	*efaA*	Involved in adhesion	Ch'ng et al., (2019); Liu et al., (2022)
Enterococcal surface protein	*esp*	Promotes primary attachment and biofilm formation	Ch'ng et al., (2019); Nielsen et al., (2012); Popovic et al., (2018)
Second collagen adhesin of *E. faecium*	*scm*	Involved in biofilm development	Liu et al., (2022)
Serine glutamate repeat A	*sgrA*	Involved in biofilm development	Ch'ng et al., (2019); Liu et al., (2022)
Exotoxin			
Cytolysin	*cylR2, cylL-l, cylL-s, cylM*	A secreted toxin expressed in response to pheromones, causing blood hemolysis	Fallah et al., 2017; Fiore et al., 2019; Liu et al., (2022)
Exoenzyme			
Gelatinase	*gelE*	Secreted Zn-metalloprotease that can damage host tissues, promotes the aggregation of cells in microcolonies of initial step of biofilm formation. Expression of *gelE* is induced at a high cell density by the fsr quorum-sensing system.	Popovic et al., (2018); Thomas et al., 2008
Hyaluronidase	EF0818, EF3023	Damages host tissues and causes inflammation	Oli et al., 2022; Liu et al., (2022)
SprE	*sprE*	Hydrolyzes casein, quorum sensing and autolysis (release of eDNA)	Popovic et al., (2018); Oli et al., 2022;

(continued on next page)

Table 1 (*continued*)

Virulence factors	Gene Code	Function	Reference
Immune modulation			
Capsule	*cpsABCDEFGHIK*,		Liu et al., (2022); Thurlow et al., 2009
Biofilm			
BopD	*bopD*	Biofilm on plastic surface/a putative sugar-binding transcriptional regulator	Liu et al., (2022); Oli et al., 2022
Fecal streptococci regulator locus genes	*fsrA*, *fsrB*, *fsrC*	Regulates matrix remodeling by upregulating several biofilm-associated genes and operons (including *bopABCD*, *ebpABC*, *gelE* and *sprE*)	Oli et al., 2022; Liu et al., (2022)

incubation, the color of the media changed from purple to yellow, except for the tube containing the substrate esculin turns to and black indicating acid production which in turn confirms the strain could utilize the target sugar.

3. Results and discussion

3.1. Isolation and preliminary identification using MALDI-TOF

Enterococcus isolates were isolated from cheeses, fruits, plant tubers, and vegetables from local supermarkets in Denmark, Injera sourdough collected from Ethiopia and from a fecal sample from a healthy subject. Enterococci normally grows well at high temperatures (42 °C) and tolerate relatively high salt concentrations, and for this reason, the rich M17 broth supplemented with 6.5% NaCl and 2% glucose was used as a "selective" medium. We also included the pH indicator bromocresol purple in the medium to allow for easy detection of lactic acid-producing microorganisms, which form yellow colonies on this medium. More than 100 candidates were found, which were catalase-negative, and these were further identified using MALDI-TOF. A total of forty-four isolates were identified as Enterococci: *Enterococcus faecium* (20 isolates), *Enterococcus faecalis* (4 isolates), *Enterococcus durans* (8 isolates), *Enterococcus casseliflavus* (9 isolates) and *Enterococcus thailandicus* (3 isolates) (Supplementary Table s1). Further, all isolates were whole genome sequenced to identify the presence or absence of virulence genes, antibiotic resistance genes, and bacteriocin biosynthesis clusters.

Table 2
General characteristics of the hybrid assembly sequence, GenBank accession numbers are provided.

Sample ID	PubMLST Typing	Number of contigs	Genome size (Mb)	GC%	CDS	rRNA	tRNA	GenBank accession number
BT0126	*E. faecium*	13	2.8	38.0	2757	15	67	SAMN35878025
BT0194	*E. faecium*	11	2.7	38.0	2623	14	64	SAMN35878026
BT0142	*E. lactis*	6	2.8	38.3	2667	18	68	SAMN35878027
BT0143	*E. lactis*	9	2.8	38.2	2797	18	68	SAMN35878028
BT0144	*E. lactis*	3	2.8	38.2	2699	18	68	SAMN35878029
BT0148	*E. lactis*	3	2.8	38.2	2699	18	68	SAMN35878030
BT0167	*E. lactis*	17	3.6	37.5	3471	22	101	SAMN35878031
BT0167_2	*E. lactis*	28	3.7	37.6	3535	21	95	SAMN35878032
BT0168	*E. lactis*	6	2.9	38.1	2776	18	70	SAMN35878033
BT0169	*E. lactis*	60	3.0	38.3	2897	15	79	SAMN35878034
BT0170	*E. lactis*	5	2.9	38.1	2778	18	70	SAMN35878035
BT0171	*E. lactis*	4	2.9	38.1	2778	18	70	SAMN35878036
BT0172	*E. lactis*	6	2.9	38.1	2777	18	70	SAMN35878037
BT0173_2	*E. lactis*	6	2.9	38.1	2777	18	70	SAMN35878038
BT0174_2	*E. lactis*	19	2.9	38.1	2770	15	55	SAMN35878039
BT0175	*E. lactis*	3	2.7	38.4	2552	18	68	SAMN35878040
BT0176	*E. lactis*	3	2.7	38.4	2551	18	68	SAMN35878041
BT0177	*E. lactis*	3	2.7	38.4	2548	18	68	SAMN35878042
BT0197	*E. lactis*	4	2.7	38.2	2699	18	68	SAMN35878043
CS4674	*E. lactis*	3	2.8	38.2	2700	18	68	SAMN35878044
BT0127	*E. faecalis*	49	2.9	37.5	2838	3	50	JATAAU000000000
BT0220	*E. faecalis*	36	2.9	37.2	2838	3	45	JATAAT000000000
CS4479	*E. faecalis*	14	3.0	37.2	2992	12	65	JATAAS000000000
CS4672	*E. faecalis*	6	2.9	37.6	2743	12	59	JATAAR000000000
BT0139	*E. durans*	1	3.0	38.0	2681	18	66	SAMN35878045
BT0140	*E. durans*	1	2.9	38.0	2646	18	67	SAMN35878046
BT0141	*E. durans*	1	3.0	38.0	2669	18	66	SAMN35878047
BT0145	*E. durans*	1	3.0	38.0	2671	18	66	SAMN35878048
BT0146	*E. durans*	1	3.0	38.0	2681	18	66	SAMN35878049
BT0150	*E. durans*	1	3.1	37.9	2862	18	67	SAMN35878050
BT0152	*E. durans*	31	3.0	38.0	2702	23	69	SAMN35878051
BT0155	*E. durans*	5	3.0	38.0	2665	18	66	SAMN35878052
BT0165	*E.* casseliflavus	12	3.9	42.3	3635	15	63	SAMN35878053
BT0166	*E.* casseliflavus	34	4.1	42.3	3787	7	26	SAMN35878054
BT0173	*E.* casseliflavus	3	3.6	42.8	3332	15	60	SAMN35878055
BT0174	*E.* casseliflavus	41	3.9	42.2	3649	9	58	SAMN35878056
BT0178	*E.* casseliflavus	7	3.5	42.3	3397	15	61	SAMN35878057
BT0180	*E.* casseliflavus	4	3.5	42.3	3326	15	61	SAMN35878058
BT0181	*E.* casseliflavus	8	3.5	42.3	3324	15	61	SAMN35878059
BT0221	*E.* casseliflavus	2	3.7	42.4	3477	15	60	SAMN35878060
BT0222	*E.* casseliflavus	10	3.7	42.4	3479	16	60	SAMN35878061
CS4675	*E. thailandicus*	8	2.9	36.8	2736	18	66	SAMN35878062
CS4676	*E. thailandicus*	15	2.8	36.8	2649	19	65	SAMN35878063
CS4677	*E. thailandicus*	31	3.1	36.7	2946	18	86	SAMN35878064

3.2. Genome sequencing and preliminary analysis

The genomes of all forty-four *Enterococcus* isolates were sequenced and their genome size ranged from 2.7 to 4.1 Mbp. During the assembly process, the N50bp of the genome ranged from 247683 to 3632905. The average content of structural genes, rRNAs, and tRNAs were 2914, 16.2, and 66 respectively. The GC content for the isolates ranged from 36.7% to 42%. The range of coding sequences was between 2548 and 3649. The *Enterococcus* species had 3–23 rRNA and 45–101 tRNAs in their genome. In general, *E. casseliflavous* strains have a relatively larger genome size than that of the other *Enterococcus* species, ranging from 3.5 to 4.1Mbp, and this is also what we found here. The GC content of the genomes ranged from 36.7% to 42%, which also agrees with previous findings (Zhong et al., 2017). A detailed overview of each genome sequence is presented in Table 2.

3.3. Whole genome sequence-based strain typing

Whole genome sequence-based strain typing is a powerful tool in the field of microbiology and epidemiology. It is a genomic approach used to characterize and differentiate bacterial strains at the molecular level. This method utilizes the entire genetic information of an organism for efficient strain typing. Using the genome sequences of *Enterococcus* isolates obtained, we typed the strains and evaluated the similarity with genome sequenced type strains. The closest match of all forty-four isolates were identified as *Enterococcus faecium* (2), *Enterococcus lactis* (12), *Enterococcus durans* (8), *Enterococcus casseliflavus* (9), *Enterococcus thailandicus* (3) and *Enterococcus faecalis* (4) using PubMLST based on Ribosomal Multilocus Sequence Typing (rMLST) tool. The results of the MALDI-TOF identification were consistent with the genome-based typing, apart from *Enterococcus faecium*, which was identified as *Enterococcus lactis*. However, the two species were identical when examined by MALDI-TOF. (Supplementary Table s1**)**. To ensure accuracy and suitability for whole-genome analyses and gain better insight into the relatedness and diversity of microbial genomes, both FastANI and digital DNA–DNA hybridization (dDDH) methods were utilized. Once established, genomic similarity analyses were carried out on the assemblies using these methods and presented in Supplementary Table s1.

Average nucleotide identity (ANI) values were determined for all genomes, where an ANI above 95% was found for all genomes (Supplementary Table s1), except for two *E. casseliflavus* isolates BT0165 and BT0222, where a value of 95% and 94.9% and dDDH values of 66.3% and 67.9 were obtained, respectively. Thus, these isolates were expected to be new species closely related to *E. casseliflavus* (Svetlicic et al., 2023).

The pangenome based phylogenetic tree was constructed using the binary presence and absence of accessory genes. The binary presence and absence of accessory genes was processed using the Roary pangenome pipeline on Galaxy using gff files form the Prokka annotation output files. The newick formate files of Roary output were used to edit the phylogenetic tree in iTol. *E. durans* isolates were clustered in two sub-clades, where all the isolates of *E. durans* isolated in this study were found in the same sub-clade in the phylogenetic tree. Additionally, *E. durans* BT0139 and *E. durans* BT0146 were found to be closely related species, even though they were isolated from different samples originating from different countries (Fig. 1). The pangenome-based phylogenetic tree also revealed that the three *E. thailandicus* CS4675, CS4676, and CS4677 clustered together.

3.4. Identifying virulence and antibiotic resistance genes

As mentioned above, *Enterococcus* as a genus has received a lot of

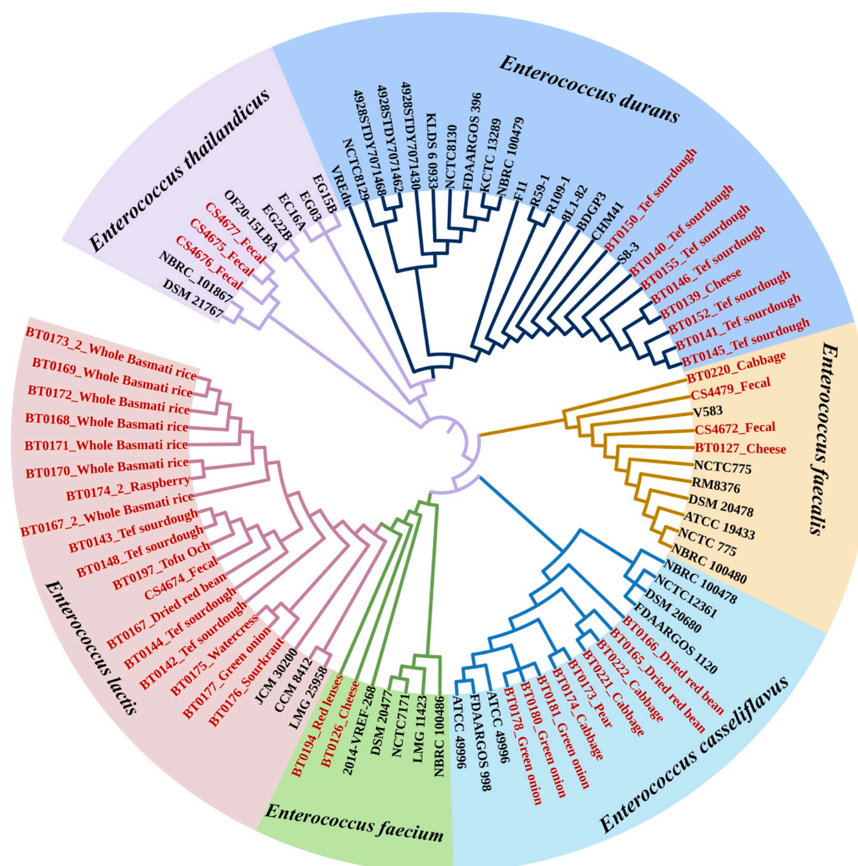

Fig. 1. Pangenome-based phylogenetic tree constructed for all 44 isolates and 46 strains retrieved from NCBI database with the font color in black. The genomes of the enterococci isolates were colored red with their prospective sample source.

negative attention due to the occurrence of pathogenic strains and strains able to transmit antibiotic-resistance genes (Fang, 2020; Valledor et al., 2022). The pathogenic enterococci are known to carry specific virulence genes that encode virulence factors (Panthee et al., 2021). However, isolates from the *Enterococcus* genus are not the only bacteria found in food products harboring virulence genes. Pathogenic streptococci, e.g., *Streptococcus infantarius* strains isolated from goat milk have been shown to harbor *gelE*, *esp*, *efaA*, *ace*, *VanB* and *epfSTR* (dos Santos et al., 2020). It has been reported by Aguirre and Collins (1993) that several lactic acid bacteria have been linked to human infections. According to the literature, *Leuconostoc* sp. are typically responsible for generalized infections in adults, such as bacteremia, but have also been known to cause meningitis in children. Some lactobacilli species have been associated with localized infections and endocarditis. Various *Leuconostoc sp.*, *Lactobacillus sp.*, *Lactobacillus confuses*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, and *Pediococcus acidilactici* have been linked to bacteremia, septic wounds, meningitis, pneumonia, and empyema (Aguirre and Collins, 1993).

In Table 1 we provide a detailed overview of virulence factors identified in enterococci, most of which, for obvious reasons, are unwanted in food microorganisms. As mentioned earlier (Table 1), virulence factors can be grouped into five different categories: "Adherence", "Exotoxin", "Exoenzymes", "Immune modulation", and "Biofilm formation" (L. Chen et al., 2016). Our genome-wide analysis revealed the presence of a total of 38 virulence genes (> 80% identity) in the genomes of the isolates (Full list can be found in Supplementary Table s2), of which most were in the category "Adherence" (Fig. 2). Certain virulence factors, including cytolysin, gelatinase, and proteins required for biofilm formation, are importantly associated with pathogenesis (Panthee et al., 2021). Hemolysis, or cytolysis, since not only blood cells can be affected, is regarded as the most critical virulence trait in *Enterococcus*, and to express this activity the genes *cylM, cylB*, and *cylA* need to be present (Day et al., 2003; Kayser, 2003). The genome of the isolates characterized in this study mostly lacked virulence genes except one of *E. faecalis* isolates that contains full set of cytolysin genes (*cylI*, *cylR1*, *cylR2*, *cylL-1*, *cylL-S*, and *cylM*). Cytolysin activity was assessed for all isolates, and with the exception of *Enterococcus faecalis* CS4479, all were negative (Fig. 2).

Another virulence factor is the enterococcal exoenzyme, called gelatinase (*GelE*), an extracellular zinc-metalloprotease that is directly involved in tissue damage during invasive infections and which can interfere with the complement system, which is an important immune response (Panthee et al., 2021; Thurlow et al., 2010). Furthermore, gelatinase has been shown to be involved in *E. faecalis* translocation, i.e., their passage from the gastrointestinal tract to extraintestinal sites, such as the mesenteric lymph node complex (MLN), liver, spleen, kidney, and bloodstream (Zeng et al., 2005). Except for the *E. faecalis* strains all *Enterococcus* isolates lacked the *gelE* gene. Two of the *E. faecalis* isolates were gut derived, whereas the two other strains originated from cheese and cabbage. Physiological characterization demonstrated that the gene was functionally expressed in three of the *E. faecalis* strains: BT0220, CS4479, and CS4672 (Fig. 2).

Virulence of *Enterococcus* species is greatly affected by their ability to form biofilms (Ali et al., 2020; Ch'ng et al., 2019). Different genes are involved at different stages of biofilm formation (Hall-Stoodley et al., 2004; Ran et al., 2015). Factors such as aggregation substance (*agg*), the endocarditis-associated antigen A (*efaA*), collagen adhesin of *E. faecalis* (*ace*), enterococcus surface protein (*esp*) (Ch'ng et al., 2019; Kafil and

Fig. 2. The whole-genome phylogenetic tree was constructed based on the pangenome of all forty-four isolates' antimicrobial resistance and virulence-associated genes identified using ABRicate Mass screening of contigs for antimicrobial and virulence genes, using the CARD and VFDB respectively. The shape filled with distinct colors indicates presence and the shape without a color filled indicates absence. **SF68** is the *E. faecium* strain EFSA approved as probiotic and **V583** is *E. faecalis* which is known as a pathogenic strain. Ami = aminoglycosides, Trim = trimethoprim, Phen = phenicol, MLS = macrolide–lincosamide –streptogramin B, Tet = tetracycline, and Glyc = Glycopeptide.

Mobarez, 2015; Nallapareddy et al., 2011; Nielsen et al., 2012) either are needed for, or can promote biofilm formation (Chotinantakul et al., 2020). The *E. faecalis* quorum-sensing regulatory systems locus (*fsr*) is responsible for sensing the cell density and regulating virulence (L. Ali et al., 2017; Qin et al., 2000). The *fsr* gene also regulates the production of gelatinase and a serine protease (Qin et al., 2000). Madsen et al., (2017) found that the *EbpA-srtC* gene cluster and the Biofilm-Associated Pili (*Ebp*) of *E. faecalis* both play important roles in endocarditis (Madsen et al., 2017). In this study the *E. faecalis* species were the only ones to contain genes needed for biofilm formation and clustered together with the virulent *E. faecalis* strain V583 in the phylogenetic tree developed using the pangenome (Fig. 1). All *E. faecalis* carried biofilm related genes *bopD* and three *fsr* genes (*fsrABC*). Besides those species, none of the examined species harbored virulence genes related to biofilm, nor *fsr* genes. All isolates were analyzed further for their capacity to produce biofilm, which only three *E. faecalis* BT0220, CS4479, and CS4672 and one *E. faecium* BT0126 were capable of (Fig. 2).

E. faecium SF68 is a safe strain that has been approved by authorities to be used as a probiotic. SF68 contains three genes related to adhesion (*acm* and *sgrA*), and all of these can be found in the genomes of our *E. faecium* and *E. lactis* isolates. The *E. casseliflavus* and *E. durans* isolates did not carry any virulence genes. The only exception was *E. durans* BT0150 which carried one adherence related gene, which is not considered to be a virulence determinant in *E. faecium* (Arias and Murray, 2012; Özden Tuncer et al., 2013), is not involved in collagen adherence and biofilm formation (Kopit et al., 2014), and is found in many *Enterococcus* strains of food origin (Domingos-Lopes et al., 2017; Franz et al., 2003) as well as in commercial *Enterococcus* probiotics (P. Li et al., 2017). Adherence genes are commonly found in different food grade and probiotic strains such as *L. delbrueckii ssp. bulgaricus* and *S. thermophilus* (Guan et al., 2021; Wang et al., 2021; Xu et al., 2023).

Another gene in the “Adherence” group, *ecbA*, was found in the genomes of the *E. thailandicus* isolates, one *E. durans* BT0150 isolate and one *E. faecium* BT0126 isolate. The *ecbA* gene is believed to be involved in biofilm development, however, in general, only few adherence genes have been shown to be involved in biofilm-associated infections *in vivo* and these are *atlA*, *ebpABC*, and *esp* (Ch’ng et al., 2019).

EFSA provides clear guidelines as to the safety of *Enterococcus* species for use in animal nutrition: they must not harbor one of the genetic elements *IS16, hylEfm*, and *esp* (EFSA, 2012). *IS16* is an insertion sequence that is involved in ampicillin resistance, as well as a novel identifier for the emerging multi-drug resistant strains of *E. faecium* (Fugaban et al., 2021). The transfer of virulence factors could greatly facilitate the spreading potential in the nosocomial clinical enterococci species due to the presence of IS*16* (Ghattargi et al., 2018; Werner et al., 2011). ***hylEfm*** encodes hyaluronidase activity, a known virulence factor in pathogenic bacteria such as *Streptococcus pyogenes, Staphylococcus aureus* and *Streptococcus pneumoniae* (Rice et al., 2003). On the other hand, the presence of the *esp* gene is associated with an increased ability to form biofilms (Tendolkar et al., 2005). In this study, no exotoxin, immune modulation, or biofilm formation related genes were present in the genomes of all *E. faecium*, *E. lactis, E. durans*, *E. casseliflavus,* and *E. thailandicus*. Furthermore, all isolates lacked IS16 mobile genetic elements and *hylEfm*.

Enterococcus spp., like many other LAB, are often resistant to different antibiotics. Special attention has been given to vancomycin resistance, as many *Enterococcus* isolates have been reported to be resistant to vancomycin (Zhong et al., 2017).

In the isolates studied, genes involved in resistance to aminoglycosides, were found in 35% of the isolates, mostly the *E. faecium*, *E. lactis E. durans* species. Many of the antibiotic resistance genes (ARGs) found in some of the strains provide resistance to aminoglycoside and MLS (macrolide–lincosamide –streptogramin B) antibiotics (Fig. 2). Most *E. lactis* genomes carried genes encoding resistance to aminoglycosides, erythromycin, macrolide, and streptogramin B.

All *E. durans* strains contained only one resistance gene which provided resistance to aminoglycosides, where *E. faecalis* carried chloramphenicol, trimethoprim, and MLS resistance genes. *Enterococcus faecalis* CS4479 contained a plasmid, which carried three aminoglycoside resistance genes and another plasmid (repUS43 replicon), which carries a tetracycline resistance gene (tet(M)). On the other hand, the two *E. faecium* strains (BT0194 and BT0126), carried resistance genes for both aminoglycosides and erythromycin. *E. faecium* BT0126 also carried a plasmid encoding tetracycline resistance (Supplementary Table s3). Apart from these strains, none of the remaining *Enterococcus* isolates contained plasmids conferring antibiotic resistance.

E. casseliflavus is not a typical pathogen and is rarely found in clinical samples (Yoshino, 2023). Infection often occurs in people that are immunocompromised, e.g., cancer patients or the elderly, or people with diabetes (Yoshino, 2023). *E. casseliflavus* is known to carry the *vanC* gene, which confers resistance to vancomycin (Yoshino, 2023), but infections are easily managed since most isolates are sensitive to common antibiotics such as ampicillin (Iaria et al., 2005). We found that all *E. casseliflavus* isolates carried vancomycin resistance genes, whereas all other isolates did not. According to the studies by Oravcova et al. (2017) and Taskeen Raza et al. (2018), *E. faecium* isolates carried resistance genes to aminoglycosides, while *E. faecalis* isolated from hospitalized patients contained additional *vanA* gene as well as the *erm(B)*, *aph (3′)-IIIa*, and *tet(M)* genes (Oravcova et al., 2017; Taskeen Raza et al., 2018). Additionally, *E. casseliflavus* carried the *tet(M)* gene which confers resistance to tetracycline.

3.5. Antimicrobial susceptibility profiling

The EFSA guideline, 2012 indicates that *Enterococcus* species must be susceptible to ampicillin (MIC$\leq$2 mg/L) (EFSA, 2012). Therefore, the nineteen *Enterococcus* isolates without potential virulence genes and vancomycin resistance gene were characterized further. All the isolates were tested for susceptibility to ampicillin, chloramphenicol, erythromycin, kanamycin, streptomycin, tetracycline, and vancomycin.

Antibiotic susceptibility profiles of selected *Enterococcus* species were determined using the micro-broth dilution method, where sensitivity to ampicillin, chloramphenicol, erythromycin, kanamycin, streptomycin, tetracycline, and vancomycin were tested. The results were compared based on the cutoff point set by EFSA, (2012) that the antimicrobial sensitivity profile of all strains was determined as it has presented in Table 3. All the isolates were sensitive to the tested antibiotics and the minimum inhibitory concentration (MIC) was far below EFSA’s cutoff points, except for erythromycin, where one strain, *E. lactis* CS4674, had a MIC value greater than 4 μg/mL.

The genome of some of the selected isolates carried genes encoding for aminoglycoside, erythromycin, macrolide, and streptogramin B resistance, but phenotypically they were sensitive to these antibiotics. It is important to note that the approved probiotic strain SF68 also carries some antibiotic resistance genes, but their presence did not give rise to resistance.

3.6. Genome mining for bacteriocin and secondary metabolite biosynthetic gene clusters

It is well-known that bacteriocin-producing LAB can help preserve fermented foods, as the bacteriocins can inhibit growth of pathogenic and spoilage microorganisms (Ben Braïek and Smaoui, 2019; Franz et al., 2003), and it has been suggested that *Enterococcus* could be used as bio-protective cultures (Barbosa et al., 2014). A genome-wide search for secondary metabolite biosynthesis gene clusters in these safe *Enterococcus* isolates showed that all of them contained at least two of these secondary metabolite biosynthetic gene clusters. These were Radical *S*-adenosylmethionine (RaS) enzymes that are involved in biosynthesis of ribosomally synthesized and post-translationally modified peptides (RaS-RiPPs), nonribosomal peptide synthetases (NRPS), Ribosomally synthesized and post-translationally modified peptides (RiPPs), and

Table 3
Antimicrobial susceptibility profiles of the selected Enterococcus isolates.

Species	Minimum Inhibitory Concentration (μg/mL)						
	Ampicillin	Vancomycin	Tetracycline	Chloramphenicol	Kanamycin	Streptomycin	Erythromycin
E. faecium BT0194	0.5	2	0.25	1	128	32	0.5
E. lactis BT0168	0.5	0.5	0.25	2	128	128	4
E. lactis BT0169	0.5	2	0.25	2	128	128	2
E. lactis BT0170	0.5	2	0.25	2	128	128	4
E. lactis BT0171	0.5	2	0.25	2	128	128	4
E. lactis BT0173_2	0.25	1	0.25	2	128	128	2
E. lactis BT0167_2	0.25	1	0.25	2	128	128	2
E. lactis BT0142	0.5	2	0.25	4	128	64	4
E. lactis BT0143	0.25	0.5	0.25	2	64	128	0.125
E. lactis BT0144	0.5	2	0.25	2	128	128	4
E. lactis BT0148	0.5	2	0.25	4	128	128	4
E. lactis BT0197	0.25	4	0.125	2	64	16	0.5
E. lactis CS4674	0.25	1	0.25	2	128	128	8
E. durans BT0139	0.25	0.5	0.25	2	32	32	0.125
E. durans BT0140	0.5	1	0.25	2	32	32	0.125
E. durans BT0141	0.5	2	0.25	2	32	32	0.125
E. durans BT0146	0.25	0.5	0.125	1	32	32	0.125
E. durans BT0155	0.25	1	0.25	2	64	128	0.125
E. thailandicus CS4677	0.5	2	0.125	2	128	128	2

EFSA Cutoff; Ampicillin: 2 μg/mL, Vancomycin: 4 μg/mL, Tetracycline: 4 μg/mL, Chloramphenicol: 16 μg/mL, Kanamycin: 1024 μg/mL, Streptomycin: 128 μg/mL, Erythromycin: 4 μg/mL (EFSA, 2012)

terpene. (Fig. 3).

The genome analysis revealed that all the isolates, except for *E. durans* strains BT0155, BT0141, BT0146, BT0139, and BT0140 contained at least one or more bacteriocin biosynthesis clusters (Fig. 3**)**.

Bacteriocins can be organized into three different classes, namely class I, class II, and class III, where most of the identified clusters coded for class II bacteriocins, which is in accord with the study of Wu et al. (2022). These included Enterocin A, Enterocin B, Enterocin P, Enterocin Nkr-5–3B, Enterocin L50a, and Enterocin Q. Class II bacteriocins are unmodified bacteriocins that are thermostable, and these can be sub-divided into four sub-classes, such as pediocin-like (Enterocin_A, Enterocin_P), two-peptide based (Enterocin_L50a, Enterocin_Q), and leaderless single-peptide (Enterocin_B) bacteriocins (Wu et al., 2022). Class II bacteriocins have been shown to inhibit the growth of food born pathogen *L. monocytogenes* (Cintas et al., 1997). In this study most of the bacteriocin gene clusters found in enterococci genome were class II bacteriocins, (Enterocin_A, Enterocin_P, Enterocin_L50a, Enterocin_Q, Enterocin_B) (Fig. 3). Genes encoding Enterocin L50a, a highly active plasmid-encoded broad-spectrum bacteriocin (Wu et al., 2022) were found in the genomes of all *E. lactis* strains. Only one bacteriocin biosynthesis cluster encoded a class III bacteriocin (large molecular weight heat labile class of enterocin), namely Enterolysin A, and genes encoding this bacteriocin were detected in most *Enterococcus* genomes. Enterolysin A has a bacteriolytic mode of action and inhibits growth of selected *enterococci*, *pediococci*, *lactococci*, and *lactobacilli* (Nilsen et al., 2003). All *E. lactis* species harbored Enterocin P, except one of the *E. lactis,* BT0142, and additional Bacteriocin hiracin-JM79 were also found in the *E. lactis* species (BT0197, BT0148, BT0144, CS4677and

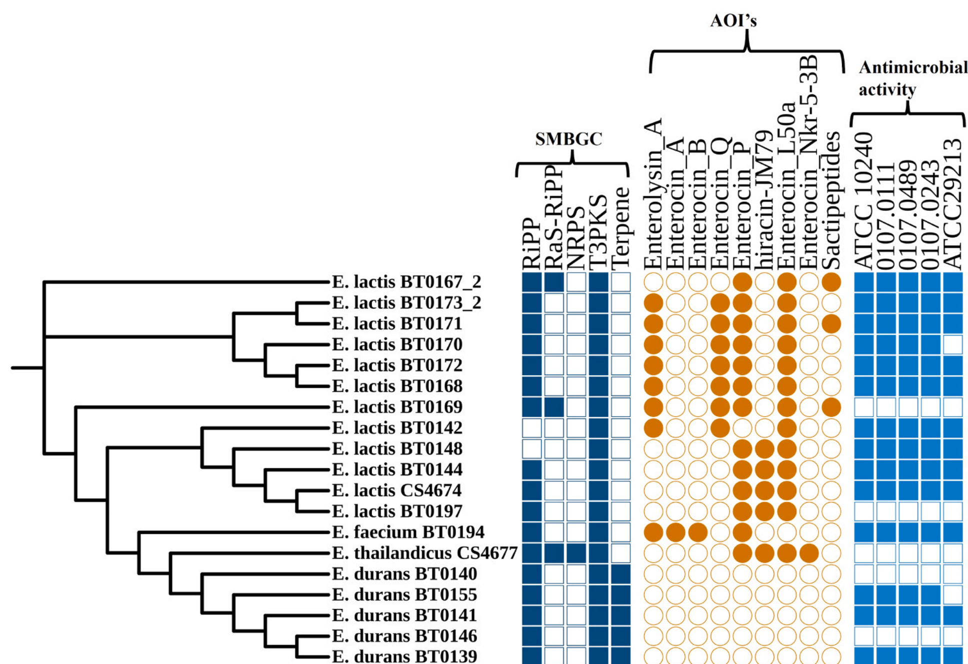

Fig. 3. Enterococci isolate having known secondary metabolite biosynthetic gene cluster (SMBGC) for bacteriocins and area of interest (AOI's) and antimicrobial activity to selected pathogens. The shape filled with distinct colors indicates presence and the shape without a color filled indicates absence.

CS4674). It was discovered that these gene clusters were encoded in a plasmid Fig. 5B&D.

Listeria monocytogenes is a widely distributed food-borne pathogen, and a great challenge for dairies (Ribeiro et al., 2014). For selected *Enterococcus* isolates, we tested their activity against pathogenic strains *of* L. *monocytogenes* (*L. monocytogenes* 0107.0111, *L. monocytogenes* 0107.0489 and *L. monocytogenes* 0107.0243), *S. aureus* ATCC29213 and bacteriocin sensitive indicator *Micrococcus luteus* ATCC 10240. It was found that most candidates had the ability to inhibit the growth of *M. luteus* ATCC 10240, *S. aureus* ATCC29213 and three strains of *L. monocytogenes* (*L. monocytogenes* 0107.0111, *L. monocytogenes* 0107.0489 and *L. monocytogenes* 0107.0243) isolated from cheese (Fig. 3; Supplementary Table s4; Supplementary Figure s1**)**. It was observed that some isolates possessed bacteriocin genes but failed to exhibit anti-bacterial activity against the tested pathogens. This could be attributed to the fact that the genes might not have been expressed.

Until now, a role for terpenes in enterococci has not been assigned (Beukers et al., 2017). Some of the *E. durans* isolates displayed antimicrobial activity against *Listeria*, despite lacking genes encoding bacteriocins. These isolates, however, carried genes for terpene production. Plant derived terpenes are known for their potent antimicrobial effects (Yamada et al., 2015), and it is possible that terpenes produced by the *E. durans* isolates could have the same effect. In the past it was believed that terpenes were mainly produced by plants, but now it is known that terpene synthases are widely distributed in bacteria as well (Yamada et al., 2015).

3.7. Genomic and phenotypic characterization of carbohydrate metabolism

Enterococci can thrive in a variety of environments, including the gastrointestinal tracts of various animals, due to their ability to metabolize a wide range of carbohydrates. These carbohydrates include not only simple sugars, but also complex polymers, as noted by Ramsey et al. (Ramsey et al., 2014). Enterococci are facultative anaerobes with a homofermentative metabolism, i.e., lactic acid is the predominant end-product of carbohydrate fermentation (Švec and Franz, 2014). Like other LAB, enterococci generate ATP through fermentation and substrate-level phosphorylation, rather than by oxidative phosphorylation (Ghazisaeedi et al., 2022). On slowly metabolized carbohydrates, enterococci can also display a mixed-acid fermentation profile, similar to that of *L. lactis*, and uniquely possess a pyruvate dehydrogenase that is active under strongly reducing conditions. $NADH/NAD^+$ ratios that completely inhibit *E. coli* PDHc have little effect on *E. faecalis* PDHc (SNOEP et al., 1992). Overall, enterococci produce the same end-products as its mesophilic cousin *L. lactis*, including lactate, formate, ethanol, and acetate, acetoin, and diacetyl (Ramsey et al., 2014).

To get an overview of the capacity of the isolates to ferment different carbohydrates, the API 50 CH kit was applied, and the result is shown in Fig. 4 below. From this analysis it is apparent that *Enterococcus* has great potential for using sugars like raffinose, maltose, trehalose, sugars that are found in larger amounts in different food crops. The trisaccharide raffinose, which has anti-nutritional properties and causes flatulence (Elango et al., 2022), is found in beans, various vegetables, and whole grains and cannot be digested by humans (Zhang et al., 2011). Several of the *E. lactis* and *E. durans* isolates could utilize raffinose (Fig. 4), and all these strains carried two copies of genes encoding α-galactosidase, an enzyme needed for degrading raffinose (Lafond et al., 2020). Thus, enterococci appear to be excellent candidates for fermenting plant-based foods as they have the potential to both grow on as well as selectively remove undesirable carbohydrates.

Most of the isolates were able to utilize L-arabinose and carried genes encoding the necessary enzymes involved in L-arabinose metabolism (Zhong et al., 2017). Many strains were able to utilize lactose (Fig. 4**)**, and harbored a lactose operon, either chromosomally integrated or on a plasmid. This is relevant as enterococci are frequently found in fermented dairy products, especially cheese, where they play an important role in ripening. The utilization of lactose is regulated by the lac repressor (LacR), encoded by *lacR*, where LacR acts as a transcriptional repressor of the lactose operon (Kowalczyk and Bardowski, 2007). The lac operon comprises 8 genes (*lacABCDFEGX*). In *E. faecium* BT0194 this operon was on a plasmid (Fig. 5**C)** whereas it was located on the chromosome in the *E. lactis* isolates. The lac operon in *Enterococcus* is arranged as in *L. lactis* (Kowalczyk and Bardowski, 2007).

Sucrose is often found in large amounts in plant tissues. Many lactic acid bacteria possess enzymes like sucrose-6-phosphate hydrolases and sucrose phosphorylases that enable them to metabolize this

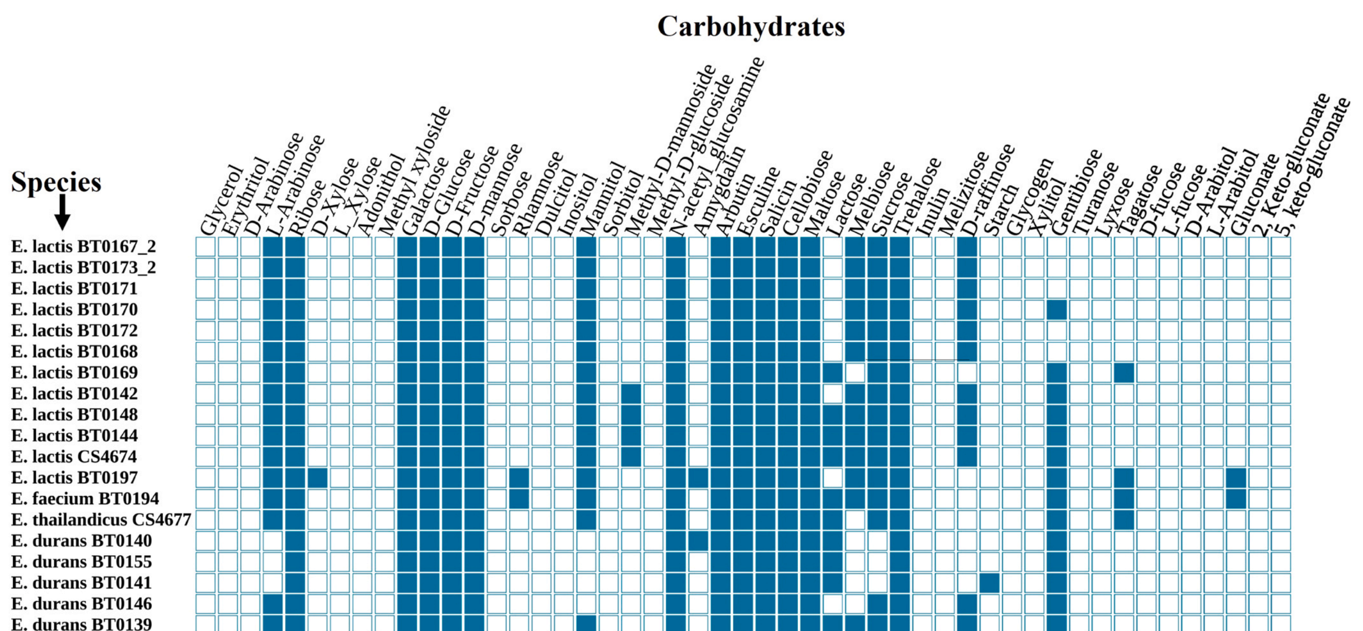

Fig. 4. Carbohydrate metabolism profile of selected *Enterococcus* species. The shape filled with distinct colors indicates presence and the shape without a color filled indicates absence.

Fig. 5. Organization of the sucrose regulon and Bacteriocin Enterocin_P gene cluster, **(A),** *E. faecium*, **(B)** *E. lactis*. ***degA*** —repressor of the sucrose operon; ***scrB***—sucrose-6-P hydrolase; ***scrA***—PTS system, sucrose specific IIBC; ***gmuE***—fructokinase; **(C)**, Organization of the lactose regulon in *E. faecium*. **lacR** _ Lactose phosphotransferase system repressor, lacABCD _ tagatose pathway enzymes **(lacA** _ Galactose-6-phosphate isomerase subunit, **lacB** _ Galactose-6-phosphate isomerase subunit, **lacC** _Tagatose-6-phosphate kinase, **lacD** _ Tagatose 1,6-diphosphate aldolase), lacFE _ lactose PEP: PTS **(lacF** _ PTS system lactose-specific EIIA component, **lacE** = PTS system lactose-specific EIICB component), **lacG** _6-phospho-β-galactosidase. (**D**), Organization of bacteriocin enterocin_P and hiracin-JM79 gene cluster.

carbohydrate (Reid and Abratt, 2005). The genes encoding the enzymes for utilizing sucrose are arranged in an operon. In the utilization of sucrose in *L. lactis* two divergently transcribed operons are involved—*sacBK* and *sacAR* (Kowalczyk and Bardowski, 2007; Reid and Abratt, 2005). In *Enterococcus* the genes are arranged in a similar manner (Fig. 5**A & B)**. All strains of *Enterococcus*, except *E. durans* BT0140, *E. durans* BT0141 and *E. durans* BT0155, were found to be capable of utilizing sucrose. In *E. faecium* and *E. lactis* sucrose metabolism is plasmid encoded. In *E. faecium*, the sucrose genes, together with the Enterocin_P encoding genes, are flanked by IS mobile genetic elements, whereas in *E. lactis*, the Enterocin_P encoding genes and the sucrose genes are present on the same plasmid but in a different location (Fig. 5**A & B**).

Certain LABs are able to metabolize citrate, which enhances acid stress tolerance. Pyruvate generated from citrate can be converted into the butter aroma compounds acetoin and diacetyl, which are important flavor compounds in butter and many cheeses (Jamaly et al., 2010; Serio et al., 2010). Only some of the *Enterococcus* isolates were able to utilize citrate, although all isolates harbored all the genes coding for enzymes responsible for citrate metabolism: α-acetolactate synthase (ALS) and α-acetolactate decarboxylase (ALDC).

Although enterococci and lactococci have a similar central metabolism, enterococci have the ability to grow at higher temperatures. The latter is of industrial relevance as a high growth temperature is a precondition for rapid acidification. Rapid acidification is beneficial as it reduces fermentation time and enhances food safety (Martinussen et al., 2013). The latter is especially important for fermentation of plant substrates, in particular the ones that cannot be easily pasteurized/heat sterilized. It was found that all of the isolates, except one, acidified faster than *Lactococcus lactis* (Supplementary Fig. s2). Overall, the results reveal a great unrealized potential of enterococci as food fermentation microorganisms, especially for plant-food applications.

3.8. Bacteriophage resistance measures – presence of CRISPR-Cas

When certain bacteria face the threat of invasion by genetic elements such as phages, they rely on CRISPR-Cas defense mechanisms, a target directed nuclease system that cleaves invading DNA (Beukers et al., 2017). CRISPR is short for "clustered regularly interspaced short palindromic repeats" and Cas represents "CRISPR associated" (Beukers et al., 2017). The CRISPR spacers and repeats get transcribed and processed into small CRISPR RNAs (crRNAs), which specify acquired immunity against bacteriophage infection through a mechanism that depends on the precise match between CRISPR spacers and phage targets (Luciano A. Marraffini et al., 2008; Makarova et al., 2011; Palmer and Gilmore, 2010). A CRISPR locus consists of an array of short direct repeats and variable DNA sequences called 'spacers', surrounded by various Cas genes. The presence of different Cas genes characterizes the three types of CRISPR-Cas systems, namely Cas3 for type I, Cas9 for type II, and Cas10 for type III (Beukers et al., 2017; Makarova et al., 2011). Recently, two more types have been proposed, type IV and type V. Type II CRISPR-Cas systems are typically found in enterococci, but a recent report revealed that *Enterococcus cecorum* has a type I system (Beukers et al., 2017).

In this study all the isolates contained CRISPR arrays, where *E. durans* contained CRISPR arrays in their genomes flanked by Cas genes, including *cas9*, *cas1*, *cas2*, and *csn2* (Supplementary Table s5). Besides *E. durans*, all strains of *E. lactis* and *E. faecium* contained two CRISPR arrays not accompanied by Cas genes. Bhaya et al., (Bhaya et al., 2011) reported that in situations where Cas genes are not present, it is speculated that these arrays could be inactive or that Cas genes from other comparable arrays could be capable of facilitating their function. The CRISPR found in the genome of *E. durans* was classified as a Cas-type IIA as it has been reported by Beukers et al., (2017) and *E. hirae*, *E. thailandicus*, *E. villorum*, and *E. durans* contained CRISPR arrays in their genomes flanked by Cas genes, including cas9, cas1, cas2, and csn2, except for *E. villorum* which lacked the csn2 gene.

4. Conclusions

Whole-genome sequencing combined with the use of bio-informatics tools and the phenotypic characterization allows for safety assessment of microorganisms. In this study 19 safe *Enterococcus* strains could be identified among 44 environmental isolates, and the strains were demonstrated to have many useful properties such as an ability to grow rapidly at elevated temperatures and to produce antimicrobial compounds that can prevent pathogens and spoilage microorganisms. Several of the isolates contain genes encoding proteins involved in adherence, genes that are recognized as virulence genes. The presence of these, however, is not sufficient to cause virulence these genes are frequently found in the genomes of food-grade lactic acid bacteria, such as *S. thermophilus* and *L. delbrueckii ssp. bulgaricus*. All of the 19 strains lacked genes encoding resistance to clinically important antibiotics and were also sensitive towards these. Some contained antibiotic resistance genes found in food-grade LAB, however, these appeared not to be expressed as resistance was not observed. Overall, our data indicates that the strains could be safe for food applications. With respect to food fermentation applications, for several key characteristics they are on par or better than the traditional workhorse *L. lactis*. They have similar flavor forming abilities but are superior in terms of important technological properties such as acidification rate, stress tolerance and have a high inherent capacity to interfere with unwanted microbial growth. The latter is due to production of antimicrobial compounds and their ability to grow at high temperatures that inhibit or prevent growth of most pathogenic and spoilage microorganisms. Overall, this study highlights the remarkable potential of *Enterococcus* spp. in food fermentation and the versatility of this bacterial genus, exhibiting its application in both dairy and plant material fermentation.

CRediT authorship contribution statement

Carsten Jers: Writing – review & editing, Supervision, Methodology. **Christian Solem:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Shuangqing Zhao:** Writing – review & editing, Methodology. **Nega Berhane:** Writing – review & editing. **Ivan Mijakovic:** Writing – review & editing, Supervision, Methodology, Funding acquisition. **Belay Tilahun Tadesse:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Ema Svetlicic:** Writing – review & editing, Software, Methodology, Formal analysis.

Data Availability

Data will be made available on request.

Acknowledgements

This project has received funding from the DTU alliance stipendium grant number 110833 to CS and the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 955626 and the Novo Nordisk Foundation, grant number NNF20CC0035580 to IM.

Conflicts of interest

There are no conflicts to declare.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2024.127702.

References

Aguirre, M., Collins, M.D., 1993. Lactic acid bacteria and human clinical infection. J. Appl. Bacteriol. Vol. 75 (Issue 2), 95–107. https://doi.org/10.1111/j.1365-2672.1993.tb02753.x.

Ali, I.A.A., Cheung, B.P.K., Matinlinna, J.P., Lévesque, C.M., Neelakantan, P., 2020. Trans-cinnamaldehyde potently kills *Enterococcus faecalis* biofilm cells and prevents biofilm recovery. Microb. Pathog. *149* https://doi.org/10.1016/j.micpath.2020.104482.

Ali, L., Goraya, M.U., Arafat, Y., Ajmal, M., Chen, J.L., Yu, D., 2017. Molecular mechanism of quorum-sensing in *Enterococcus faecalis*: Its role in virulence and therapeutic approaches. Int. J. Mol. Sci. Vol. 18 (Issue 5) https://doi.org/10.3390/ijms18050960.

Arias, C.A., Murray, B.E., 2012. The rise of the Enterococcus: Beyond vancomycin resistance. Nat. Rev. Microbiol. Vol. 10 (Issue 4), 266–278. https://doi.org/10.1038/nrmicro2761.

Bager, F., Madsen, M., Christensen, J., Aarestrup, F.M., 1997. Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms. Prev. Vet. Med. Vol. 3.

Barbosa, J., Borges, S., Teixeira, P., 2014. Selection of potential probiotic *Enterococcus faecium* isolated from Portuguese fermented food. Int. J. Food Microbiol. *191*, 144–148. https://doi.org/10.1016/j.ijfoodmicro.2014.09.009.

Ben Braïek, O., Smaoui, S., 2019. Enterococci: between emerging pathogens and potential probiotics. BioMed. Res. Int. Vol. 2019, 5938210 https://doi.org/10.1155/2019/5938210.

Ben Said, L., Klibi, N., Dziri, R., Borgo, F., Boudabous, A., Ben Slama, K., Torres, C., 2016. Prevalence, antimicrobial resistance and genetic lineages of *Enterococcus* spp. from vegetable food, soil and irrigation water in farm environments in Tunisia. J. Sci. Food Agric. *96* (5), 1627–1633. https://doi.org/10.1002/JSFA.7264.

Beukers, A.G., Zaheer, R., Goji, N., Amoako, K.K., Chaves, A.V., Ward, M.P., McAllister, T.A., 2017. Comparative genomics of *Enterococcus* spp. isolated from bovine feces. BMC Microbiol. *17* (1) https://doi.org/10.1186/s12866-017-0962-1.

Bhardwaj, A., Malik, · R K, Chauhan, Prashant, 2008. Functional and safety aspects of enterococci in dairy foods. Indian J. Microbiol Vol. 48.

Bhaya, D., Davison, M., Barrangou, R., 2011. CRISPR-cas systems in bacteria and archaea: Versatile small RNAs for adaptive defense and regulation. Annu. Rev. Genet. *45*, 273–297. https://doi.org/10.1146/annurev-genet-110410-132430.

Bortolaia, V., Kaas, R.S., Ruppe, E., Roberts, M.C., Schwarz, S., Cattoir, V., Philippon, A., Allesoe, R.L., Rebelo, A.R., Florensa, A.F., Fagelhauer, L., Chakraborty, T., Neumann, B., Werner, G., Bender, J.K., Stingl, K., Nguyen, M., Coppens, J., Xavier, B. B., Aarestrup, F.M., 2020. ResFinder 4.0 for predictions of phenotypes from genotypes. J. Antimicrob. Chemother. *75* (12), 3491–3500. https://doi.org/10.1093/jac/dkaa345.

Bottone, E.J., Patel, L., Patel, P., & Robin, T. (1998). Mucoid Encapsulated Enterococcus faecalis: An Emerging Morphotype Isolated from Patients with Urinary Tract Infections.

Carattoli, A., Zankari, E., Garciá-Fernández, A., Larsen, M.V., Lund, O., Villa, L., Aarestrup, F.M., Hasman, H., 2014. In Silico detection and typing of plasmids using plasmidfinder and plasmid multilocus sequence typing. Antimicrob. Agents Chemother. *58* (7), 3895–3903. https://doi.org/10.1128/AAC.02412-14.

Ch'ng, J.H., Chong, K.K.L., Lam, L.N., Wong, J.J., Kline, K.A., 2019. Biofilm-associated infection by enterococci. In: In *Nature Reviews Microbiology*, Vol. 17. Nature Publishing Group, pp. 82–94. https://doi.org/10.1038/s41579-018-0107-z.

Chen, L., Zheng, D., Liu, B., Yang, J., Jin, Q., 2016. VFDB 2016: Hierarchical and refined dataset for big data analysis - 10 years on. Nucleic Acids Res. *44* (D1), D694–D697. https://doi.org/10.1093/nar/gkv1239.

Chen, S., Zhou, Y., Chen, Y., Gu, J., Biotechnology, H., 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. https://doi.org/10.1101/274100.

Chotinantakul, K., Chansiw, N., Okada, S., 2020. Biofilm formation and transfer of a streptomycin resistance gene in enterococci from fermented pork. J. Glob. Antimicrob. Resist. *22*, 434–440. https://doi.org/10.1016/j.jgar.2020.04.016.

Cintas, L.M., Casaus, P., Leiv, †, Håvarstein, S., Hernańdez, P.E., Hernańdez, H., Nes, I.F., 1997. Biochemical and genetic characterization of enterocin p, a novel sec-dependent bacteriocin from enterococcus faecium P13 with a broad antimicrobial spectrum. Appl. Environ. Microbiol. Vol. 63 (Issue 11).

Clausen, P.T.L.C., Aarestrup, F.M., Lund, O., 2018. Rapid and precise alignment of raw reads against redundant databases with KMA. BMC Bioinforma. *19* (1) https://doi.org/10.1186/s12859-018-2336-6.

Cuccuru, G., Orsini, M., Pinna, A., Sbardellati, A., Soranzo, N., Travaglione, A., Uva, P., Zanetti, G., Fotia, G., 2014. Orione, a web-based framework for NGS analysis in microbiology. Bioinformatics *30* (13), 1928–1929. https://doi.org/10.1093/bioinformatics/btu135.

Cui, P., Feng, L., Zhang, L., He, J., An, T., Fu, X., Li, C., Zhao, X., Zhai, Y., Li, H., Yan, W., Li, H., Luo, X., Lei, C., Wang, H., Yang, X., 2020. Antimicrobial resistance, virulence genes, and biofilm formation capacity among *Enterococcus* species From Yaks in Aba Tibetan Autonomous Prefecture, China. Front. Microbiol. *11* https://doi.org/10.3389/fmicb.2020.01250.

Dassanayake, R.P., Falkenberg, S.M., Stasko, J.A., Shircliff, A.L., Lippolis, J.D., Briggs, R. E., 2020. Identification of a reliable fixative solution to preserve the complex architecture of bacterial biofilms for scanning electron microscopy evaluation. PLoS ONE *15* (5). https://doi.org/10.1371/journal.pone.0233973.

Day, A.M., Cove, J.H., Phillips-Jones, M.K., 2003. Cytolysin gene expression in *Enterococcus faecalis* is regulated in response to aerobiosis conditions. Mol. Genet. Genom. *269* (1), 31–39. https://doi.org/10.1007/S00438-003-0819-1.

De Bruyne, K., Slabbinck, B., Waegeman, W., Vauterin, P., De Baets, B., Vandamme, P., 2011. Bacterial species identification from MALDI-TOF mass spectra through data analysis and machine learning. Syst. Appl. Microbiol. *34* (1), 20–29. https://doi.org/10.1016/j.syapm.2010.11.003.

De Coster, W., 2018. NanoPlot. In GitHub repository.

Domingos-Lopes, M.F.P., Stanton, C., Ross, P.R., Dapkevicius, M.L.E., Silva, C.C.G., 2017. Genetic diversity, safety and technological characterization of lactic acid bacteria isolated from artisanal Pico cheese. Food Microbiol. *63*, 178–190. https://doi.org/10.1016/j.fm.2016.11.014.

Dubin, K., Pamer, E.G., 2017. Enterococci and their interactions with the intestinal microbiome. Microbiol. Spectr. *5* (6) https://doi.org/10.1128/microbiolspec.bad-0014-2016.

EFSA, 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J. *10* (6) https://doi.org/10.2903/j.efsa.2012.2740.

Elango, D., Rajendran, K., Van der Laan, L., Sebastiar, S., Raigne, J., Thaiparambil, N.A., El Haddad, N., Raja, B., Wang, W., Ferela, A., Chiteri, K.O., Thudi, M., Varshney, R. K., Chopra, S., Singh, A., Singh, A.K., 2022. Raffinose Family Oligosaccharides: Friend or Foe for Human and Plant Health?. In: In Frontiers in Plant Science, Vol. 13 Frontiers Media S.A. https://doi.org/10.3389/fpls.2022.829118.

Ewels, P., Magnusson, M., Lundin, S., Käller, M., 2016. MultiQC: Summarize analysis results for multiple tools and samples in a single report. Bioinformatics *32* (19), 3047–3048. https://doi.org/10.1093/bioinformatics/btw354.

Fallah, F., Yousefi, M., Pourmand, M.R., Hashemi, A., Nazari Alam, A., Afshar, D., 2017. Phenotypic and genotypic study of biofilm formation in Enterococci isolated from urinary tract infections. Microbial Pathogenesis 108, 85–90. https://doi.org/10.1016/j.micpath.2017.05.014.

Fang, S.Bin, 2020. Enterococci and food safety – Are all probiotics beneficial?. In: In *Pediatrics and Neonatology*, Vol. 61 Elsevier (Singapore) Pte Ltd., pp. 359–360. https://doi.org/10.1016/j.pedneo.2020.01.004

Fiore, E., Van Tyne, D., Gilmore, M.S., 2019. Pathogenicity of Enterococci. Microbiology Spectrum 7 (4). https://doi.org/10.1128/microbiolspec.gpp3-0053-2018.

Flint, S., 2002. Enterococcus faecalis and Enterococcus faecium. In: London: Elsevier Applied Science. D'Aoust JY, Vol. 39. Elsevier Applied Science, Rubin HE, pp. *203–284*. London: Elsevier Applied Science. D'Aoust JY.

Franz, C.M.A.P., Stiles, M.E., Schleifer, K.H., Holzapfel, W.H., 2003. Enterococci in foods - A conundrum for food safety. Int. J. Food Microbiol. *88* (2–3), 105–122. https://doi.org/10.1016/S0168-1605(03)00174-0.

Fugaban, J.I.I., Holzapfel, W.H., Todorov, S.D., 2021. Probiotic potential and safety assessment of bacteriocinogenic *Enterococcus faecium* strains with antibacterial activity against Listeria and vancomycin-resistant enterococci. Curr. Res. Microb. Sci. *2* https://doi.org/10.1016/j.crmicr.2021.100070.

Ghattargi, V.C., Gaikwad, M.A., Meti, B.S., Nimonkar, Y.S., Dixit, K., Prakash, O., Shouche, Y.S., Pawar, S.P., Dhotre, D.P., 2018. Comparative genome analysis reveals key genetic factors associated with probiotic property in *Enterococcus faecium* strains. BMC Genom. *19* (1) https://doi.org/10.1186/s12864-018-5043-9.

Ghazisaeedi, F., Meens, J., Hansche, B., Maurischat, S., Schwerk, P., Goethe, R., Wieler, L.H., Fulde, M., Tedin, K., 2022. A virulence factor as a therapeutic: the probiotic *Enterococcus faecium* SF68 arginine deiminase inhibits innate immune signaling pathways. Gut Microbes *14* (1). https://doi.org/10.1080/19490976.2022.2106105.

Gilmore, M.S., Rauch, M., Ramsey, M.M., Himes, P.R., Varahan, S., Manson, J.M., Lebreton, F., Hancock, L.E., 2015. Pheromone killing of multidrug-resistant *Enterococcus faecalis* V583 by native commensal strains. Proc. Natl. Acad. Sci. USA *112* (23), 7273–7278. https://doi.org/10.1073/pnas.1500553112.

Greuter, T., Michel, M.C., Thomann, D., Weigmann, H., Vavricka, S.R., 2020. Randomized, Placebo-Controlled, Double-Blind and Open-Label Studies in the Treatment and Prevention of Acute Diarrhea With *Enterococcus faecium* SF68. Front. Med. *7* https://doi.org/10.3389/fmed.2020.00276.

Guan, Y., Cui, Y., Qu, X., Jing, K., 2021. Safety and robustness aspects analysis of *Lactobacillus delbrueckii ssp. bulgaricus* LDB-C1 based on the genome analysis and biological tests. Arch. Microbiol. *203* (7), 3955–3964. https://doi.org/10.1007/s00203-021-02383-7.

Gurevich, A., Saveliev, V., Vyahhi, N., Tesler, G., 2013. QUAST: Quality assessment tool for genome assemblies. Bioinformatics *29* (8), 1072–1075. https://doi.org/10.1093/bioinformatics/btt086.

Hall-Stoodley, L., Costerton, J.W., Stoodley, P., 2004. Bacterial biofilms: From the natural environment to infectious diseases. *Nat. Rev. Microbiol.* Vol. 2 (Issue 2), 95–108. https://doi.org/10.1038/nrmicro821.

Harvey, B.S., Baker, C.J., Edwards, M.S., 1992. Contributions of Complement and Immunoglobulin to Neutrophil-Mediated Killing of Enterococci. INFECrION Immun. Vol. 60 (Issue 9).

Hashem, Y.A., Amin, H.M., Essam, T.M., Yassin, A.S., Aziz, R.K., 2017. Biofilm formation in enterococci: Genotype-phenotype correlations and inhibition by vancomycin. Sci. Rep. *7* (1) https://doi.org/10.1038/s41598-017-05901-0.

Henning, C., Gautam, D., Muriana, P., 2015. Identification of multiple bacteriocins in enterococcus spp. Using an enterococcus-specific bacteriocin pcr array. Microorganisms *3* (1), 1–16. https://doi.org/10.3390/microorganisms3010001.

Holman, D.B., Klima, C.L., Gzyl, K.E., Zaheer, R., Service, C., Jones, T.H., Mcallister, T.A., 2021. Antimicrob. Resist. Enterococcus Spp. Isol. a Beef Process. Plant Retail Ground Beef. ⟨https://journals.asm.org/journal/spectrum⟩.

Holzapfel, W., Arini, A., Aeschbacher, M., Coppolecchia, R., Pot, B., 2018. *Enterococcus faecium* SF68 as a model for efficacy and safety evaluation of pharmaceutical probiotics. Benef. Microbes *9* (3), 375–388. https://doi.org/10.3920/BM2017.0148.

Huguenin, A., Kincaid-Smith, J., Depaquit, J., Boissier, J., Ferté, H., 2023. MALDI-TOF: a new tool for the identification of Schistosoma cercariae and detection of hybrids. PLOS Negl. Trop. Dis. *17* (3), e0010577 https://doi.org/10.1371/journal.pntd.0010577.

Iaria, C., Stassi, G., Costa, G.B., Di Leo, R., Toscano, A., Cascio, A., 2005. Enterococcal meningitis caused by *Enterococcus casseliflavus*. First case report. BMC Infect. Dis. *5* https://doi.org/10.1186/1471-2334-5-3.

Jain, C., Rodriguez-R, L.M., Phillippy, A.M., Konstantinidis, K.T., Aluru, S., 2018. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. Nat. Commun. *9* (1) https://doi.org/10.1038/s41467-018-07641-9.

Jamaly, N., Benjouad, A., Comunian, R., Daga, E., Bouksaim, M., 2010. Characterization of Enterococci isolated from Moroccan dairy products. Afr. J. Microbiol. Res. *4* (16), 1768–1774. 〈http://www.academicjournals.org/ajmr〉.

Jett, B.D., Huycke, M.M., Gilmore3, M.S., 1994. Virulence of Enterococci. Clin. Microbiol. Rev. 〈https://journals.asm.org/journal/cmr〉.

Jia, B., Raphenya, A.R., Alcock, B., Waglechner, N., Guo, P., Tsang, K.K., Lago, B.A., Dave, B.M., Pereira, S., Sharma, A.N., Doshi, S., Courtot, M., Lo, R., Williams, L.E., Frye, J.G., Elsayegh, T., Sardar, D., Westman, E.L., Pawlowski, A.C., McArthur, A.G., 2017. CARD 2017: Expansion and model-centric curation of the comprehensive antibiotic resistance database. Nucleic Acids Res. *45* (D1), D566–D573. https://doi.org/10.1093/nar/gkw1004.

Joensen, K.G., Scheutz, F., Lund, O., Hasman, H., Kaas, R.S., Nielsen, E.M., Aarestrup, F. M., 2014. Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. J. Clin. Microbiol. *52* (5), 1501–1510. https://doi.org/10.1128/JCM.03617-13.

Johansson, M.H.K., Bortolaia, V., Tansirichaiya, S., Aarestrup, F.M., Roberts, A.P., Petersen, T.N., 2021. Detection of mobile genetic elements associated with antibiotic resistance in *Salmonella enterica* using a newly developed web tool: MobileElementFinder. J. Antimicrob. Chemother. *76* (1), 101–109. https://doi.org/10.1093/JAC/DKAA390.

Jolley, K.A., Bray, J.E., Maiden, M.C.J., 2018. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications [version 1; referees: 2 approved]. Wellcome Open Res. *3* https://doi.org/10.12688/wellcomeopenres.14826.1.

Kafil, H.S., Mobarez, A.M., 2015. Assessment of biofilm formation by enterococci isolates from urinary tract infections with different virulence profiles. J. King Saud. Univ. - Sci. *27* (4), 312–317. https://doi.org/10.1016/j.jksus.2014.12.007.

Kayser, F.H., 2003. Safety aspects of enterococci from the medical point of view. Int. J. Food Microbiol. *88* (2–3), 255–262. https://doi.org/10.1016/S0168-1605(03)00188-0.

Kelleher, P., Bottacini, F., Mahony, J., Kilcawley, K.N., van Sinderen, D., 2017. Comparative and functional genomics of the *Lactococcus lactis* taxon; insights into evolution and niche adaptation. BMC Genom. *18* (1) https://doi.org/10.1186/s12864-017-3650-5.

Kim, M.A., Rosa, V., Min, K.S., 2020. Characterization of *Enterococcus faecalis* in different culture conditions. Sci. Rep. *10* (1) https://doi.org/10.1038/s41598-020-78998-5.

Kommineni, S., Bretl, D.J., Lam, V., Chakraborty, R., Hayward, M., Simpson, P., Cao, Y., Bousounis, P., Kristich, C.J., Salzman, N.H., 2015. Bacteriocin production augments niche competition by enterococci in the mammalian gastrointestinal tract. Nature *526* (7575), 719–722. https://doi.org/10.1038/nature15524.

Kopit, L.M., Kim, E.B., Siezen, R.J., Harris, L.J., Marco, M.L., 2014. Safety of the surrogate microorganism *Enterococcus faecium* NRRL B-2354 for use in thermal process validation. Appl. Environ. Microbiol. *80* (6), 1899–1909. https://doi.org/10.1128/AEM.03859-13.

Kowalczyk, M., Bardowski, J., 2007. Regulation of sugar catabolism in *Lactococcus lactis*. Crit. Rev. Microbiol. Vol. 33 (Issue 1), 1–13. https://doi.org/10.1080/10408410601172164.

Lafond, M., Tauzin, A.S., Bruel, L., Laville, E., Lombard, V., Esque, J., André, I., Vidal, N., Pompeo, F., Quinson, N., Perrier, J., Fons, M., Potocki-Veronese, G., Giardina, T., 2020. α-Galactosidase and Sucrose-Kinase Relationships in a Bi-functional AgaSK Enzyme Produced by the Human Gut Symbiont *Ruminococcus gnavus* E1. Front. Microbiol. *11* https://doi.org/10.3389/fmicb.2020.579521.

Lebreton, F., van Schaik, W., McGuire, A.M., Godfrey, P., Griggs, A., Mazumdar, V., Corander, J., Cheng, L., Saif, S., Young, S., Zeng, Q., Wortman, J., Birren, B., Willems, R.J.L., Earl, A.M., Gilmore, M.S., 2013. Emergence of epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains. MBio *4* (4). https://doi.org/10.1128/mBio.00534-13.

Lebreton, F., Manson, A.L., Saavedra, J.T., Straub, T.J., Earl, A.M., Gilmore, M.S., 2017. Tracing the Enterococci from Paleozoic Origins to the Hospital. Cell *169* (5), 849–861.e13. https://doi.org/10.1016/j.cell.2017.04.027.

Liu, B., Zheng, D., Zhou, S., Chen, L., Yang, J., 2022. VFDB 2022: A general classification scheme for bacterial virulence factors. Nucleic Acids Res. *50* (D1), D912–D917. https://doi.org/10.1093/nar/gkab1107.

Luciano A, Marraffini, Erik J, Sontheimer, Terns, R.M., 2008. CRISPR interference limits horizontalgene transfer in staphylococci bytargeting DNA. RNA *14* (12), 2572–2579. https://doi.org/10.1261/rna.1246808.

Madsen, K.T., Skov, M.N., Gill, S., Kemp, M., 2017. Virulence factors associated with *Enterococcus Faecalis* infective endocarditis: a mini review. Open Microbiol. J. *11* (1), 1–11. https://doi.org/10.2174/1874285801711010001.

Makarova, K.S., Haft, D.H., Barrangou, R., Brouns, S.J.J., Charpentier, E., Horvath, P., Moineau, S., Mojica, F.J.M., Wolf, Y.I., Yakunin, A.F., Van Der Oost, J., Koonin, E.V., 2011. Evolution and classification of the CRISPR-Cas systems. Nat. Rev. Microbiol. Vol. 9 (Issue 6), 467–477. https://doi.org/10.1038/nrmicro2577.

Maria, A., Tetzschner, M., Johnson, J.R., Johnston, B.D., Lund, O., Scheutz, F., 2020. In Silico Genotyping of Escherichia coli Isolates for Extraintestinal Virulence Genes by Use of Whole-Genome Sequencing Data. J. Clin. Microbiol. 〈https://journals.asm.org/journal/jcm〉.

Martinussen, J., Solem, C., Holm, A.K., Jensen, P.R., 2013. Engineering strategies aimed at control of acidification rate of lactic acid bacteria. Curr. Opin. Biotechnol. Vol. 24 (Issue 2), 124–129. https://doi.org/10.1016/j.copbio.2012.11.009.

Medeiros, A.W., Pereira, R.I., Oliveira, D.V., Martins, P.D., D'azevedo, P.A., Van Der Sand, S., Frazzon, J., Frazzon, A.P.G., 2014. Molecular detection of virulence factors among food and clinical Enterococcus faecalis strains in South Brazil. Braz. j. Microbiol. 〈www.sbmicrobiologia.org.br〉.

Mikheenko, A., Prjibelski, A., Saveliev, V., Antipov, D., Gurevich, A., 2018. Versatile genome assembly evaluation with QUAST-LG. Bioinformatics *34* (13), i142–i150. https://doi.org/10.1093/bioinformatics/bty266.

Mohamed, J.A., Huang, D.B., 2007. Biofilm formation by enterococci. J. Med. Microbiol. Vol. 56 (Issue 12), 1581–1588. https://doi.org/10.1099/jmm.0.47331-0.

Nallapareddy, S.R., Sillanpää, J., Mitchel, J., Singh, K.V., Chowdhury, S.A., Weinstock, G.M., Sullam, P.M., Murray, B.E., 2011. Conservation of Ebp-type pilus genes among enterococci and demonstration of their role in adherence of *Enterococcus faecalis* to human platelets. Infect. Immun. *79* (7), 2911–2920. https://doi.org/10.1128/IAI.00039-11.

Neamtu, B., Tita, O., Neamtu, M., Tita, M., Hila, M., Maniu, I., 2014. Identification of probiotic strains from human milk in breastfed infants with respiratory infections. Acta Univ. Cibiniensis. Ser. E: Food Technol. *18* (2), 73–84. https://doi.org/10.2478/aucft-2014-0016.

Nielsen, H.V., Guiton, P.S., Kline, K.A., Port, G.C., Pinkner, J.S., Neiers, F., Normark, S., Henriques-Normark, B., Caparon, M.G., Hultgren, S.J., 2012. The metal ion-dependent adhesion site motif of the Enterococcus faecalis EbpA pilin mediates pilus function in catheter-associated urinary tract infection. MBio *3* (4). https://doi.org/10.1128/mBio.00177-12.

Nilsen, T., Nes, I.F., Holo, H., 2003. Enterolysin A, a cell wall-degrading bacteriocin from *Enterococcus faecalis* LMG 2333. Appl. Environ. Microbiol. *69* (5), 2975–2984. https://doi.org/10.1128/AEM.69.5.2975-2984.2003.

O'Driscoll, T., Crank, C.W., 2015. Vancomycin-resistant enterococcal infections: epidemiology, clinical manifestations, and optimal management. Infect. Drug Resist. *8*, 217–230. https://doi.org/10.2147/IDR.S54125.

Oli, A. K., Javaregowda, P. K., Jain, A., Kelmani, C. R. *Mechanism Involved in Biofilm Formation of Enterococcus faecalis*.

Oravcova, V., Mihalcin, M., Zakova, J., Pospisilova, L., Masarikova, M., Literak, I., 2017. Vancomycin-resistant enterococci with vanA gene in treated municipal wastewater and their association with human hospital strains. Sci. Total Environ. *609*, 633–643. https://doi.org/10.1016/j.scitotenv.2017.07.121.

Özden Tuncer, B., Ay, Z., Tuncer, Y., 2013. Occurrence of enterocin genes, virulence factors, and antibiotic resistance in 3 bacteriocin-producer *Enterococcus faecium* strains isolated from Turkish tulum cheese. Turk. J. Biol. *37* (4)), 443–449. https://doi.org/10.3906/biy-1209-26.

Page, A.J., Cummins, C.A., Hunt, M., Wong, V.K., Reuter, S., Holden, M.T.G., Fookes, M., Falush, D., Keane, J.A., Parkhill, J., 2015. Roary: Rapid large-scale prokaryote pan genome analysis. Bioinformatics *31* (22), 3691–3693. https://doi.org/10.1093/bioinformatics/btv421.

Palmer, K.L., Gilmore, M.S., 2010. Multidrug-resistant enterococci lack CRISPR-cas. MBio *1* (4). https://doi.org/10.1128/mBio.00227-10.

Palmer, K.L., Kos, V.N., Gilmore, M.S., 2010. Horizontal gene transfer and the genomics of enterococcal antibiotic resistance. Curr. Opin. Microbiol. *13* (5), 632–639. https://doi.org/10.1016/j.mib.2010.08.004.

Panthee, S., Paudel, A., Hamamoto, H., Ogasawara, A.A., Iwasa, T., Blom, J., Sekimizu, K., 2021. Complete genome sequence and comparative genomic analysis of *Enterococcus faecalis* EF-2001, a probiotic bacterium. Genomics *113* (3), 1534–1542. https://doi.org/10.1016/j.ygeno.2021.03.021.

Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., Tyson, G.W., 2015. CheckM: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. *25* (7), 1043–1055. https://doi.org/10.1101/gr.186072.114.

Popovic, N., Dinic, M., Tolinacki, M., Mihajlovic, S., Terzic-Vidojevic, A., Bojic, S., Djokic, J., Golic, N., Veljovic, K., 2018. New insight into biofilm formation ability, the presence of virulence genes and probiotic potential of *Enterococcus* sp. dairy isolates. Front. Microbiol. *9* (JAN)) https://doi.org/10.3389/fmicb.2018.00078.

Qin, X., Singh, K.V., Weinstock, G.M., Murray, B.E., 2000. Effects of *Enterococcus faecalis* fsr Genes on Production of Gelatinase and a Serine Protease and Virulence. Infect. Immun. Vol. 68 (Issue 5). 〈https://journals.asm.org/journal/iai〉.

Qin, X., Singh, K.V., Weinstock, G.M., Murray, B.E., 2001. Characterization of fsr, a regulator controlling expression of gelatinase and serine protease in *Enterococcus faecalis* OG1RF. J. Bacteriol. *183* (11), 3372–3382. https://doi.org/10.1128/JB.183.11.3372-3382.2001.

Ramsey, M., Hartke, A., & Huycke, M. (2014). The Physiology and Metabolism of Enterococci.

Ran, S.J., Jiang, W., Zhu, C.L., Liang, J.P., 2015. Exploration of the mechanisms of biofilm formation by *Enterococcus faecalis* in glucose starvation environments. Aust. Dent. J. *60* (2), 143–153. https://doi.org/10.1111/adj.12324.

Rebecca Buxton, 2016〈www.asmscience.org〉 Blood Agar Plates and Hemolysis Protocols. www.asmscience.org.

Reid, S.J., Abratt, V.R., 2005. Sucrose utilisation in bacteria: genetic organisation and regulation. Appl. Microbiol. Biotechnol. Vol. 67 (Issue 3), 312–321. https://doi.org/10.1007/s00253-004-1885-y.

Ribeiro, S.C., Coelho, M.C., Todorov, S.D., Franco, B.D.G.M., Dapkevicius, M.L.E., Silva, C.C.G., 2014. Technological properties of bacteriocin-producing lactic acid bacteria isolated from Pico cheese an artisanal cow's milk cheese. J. Appl. Microbiol. *116* (3), 573–585. https://doi.org/10.1111/jam.12388.

Rice, L.B., Carias, L., Rudin, S., Vael, C., Goossens, H., Konstabel, C., Klare, I., Nallapareddy, S.R., Huang, W., Murray, B.E., 2003. A potential virulence gene, hyl Efm, predominates in *Enterococcus faecium* of clinical origin. J. Infect. Dis. Vol. 187. 〈http://www.cdc.gov/〉.

dos Santos, K.M.O., de Matos, C.R., Salles, H.O., de Melo Franco, B.D.G., Arellano, K., Holzapfel, W.H., Todorov, S.D., 2020. Exploring Beneficial/Virulence Properties of Two Dairy-Related Strains of *Streptococcus infantarius subsp. infantarius*. Probiotics Antimicrob. Proteins *12* (4), 1524–1541. https://doi.org/10.1007/s12602-020-09637-8.

Seemann, T., 2014. Prokka: Rapid prokaryotic genome annotation. Bioinformatics *30* (14), 2068–2069. https://doi.org/10.1093/bioinformatics/btu153.

Seemann, T., 2016⟨https://github.com/tseemann/abricate⟩ABRicate: mass screening of contigs for antibiotic resistance genes.

Segers, M.E., Lebeer, S., 2014. Towards a better understanding of *Lactobacillus rhamnosus* GG - host interactions. Microb. Cell Factor. *13* https://doi.org/10.1186/1475-2859-13-S1-S7.

Serio, A., Chaves-López, C., Paparella, A., Suzzi, G., 2010. Evaluation of metabolic activities of enterococci isolated from Pecorino Abruzzese cheese. Int. Dairy J. *20* (7), 459–464. https://doi.org/10.1016/j.idairyj.2010.02.005.

Snoep, J.L., Westphal, A.H., Benen, J.A.E., Teixeira de Mattos, M.J., Neussel, O.M., DE KOK, A., 1992. Isolation and characterisation of the pyruvate dehydrogenase complex of anaerobically grown *Enterococcus faecalis* NCTC 775. Eur. J. Biochem. *203* (1–2), 245–250. https://doi.org/10.1111/j.1432-1033.1992.tb19853.x.

Stępień-Pyśniak, D., Hauschild, T., Kosikowska, U., Dec, M., Urban-Chmiel, R., 2019. Biofilm formation capacity and presence of virulence factors among commensal Enterococcus spp. from wild birds. Sci. Rep. *9* (1) https://doi.org/10.1038/s41598-019-47602-w.

Švec, P., Franz, C.M.A.P., 2014. The family Enterococcaceae. In: Lactic Acid Bacteria: Biodiversity and Taxonomy, Vol. 9781444333831. Wiley Blackwell, pp. 171–173. https://doi.org/10.1002/9781118655252.part3.

Svetlicic, E., Jaén-Luchoro, D., Klobucar, R.S., Jers, C., Kazazic, S., Franjevic, D., Klobucar, G., Shelton, B.G., Mijakovic, I., 2023. Genomic characterization and assessment of pathogenic potential of Legionella spp. isolates from environmental monitoring. Front. Microbiol. *13* https://doi.org/10.3389/fmicb.2022.1091964.

Taskeen, Raza, Sidra Rahmat, Ullah, Khalid, Mehmood, Saadia, Andleeb, 2018. Vancomycin Resist. Enterococci.

Tendolkar, P.M., Baghdayan, A.S., Shankar, N., 2005. The N-terminal domain of enterococcal surface protein, Esp, is sufficient for Esp-mediated biofilm enhancement in *Enterococcus faecalis*. J. Bacteriol. *187* (17), 6213–6222. https://doi.org/10.1128/JB.187.17.6213-6222.2005.

Thomas, V.C., Hiromasa, Y., Harms, N., Thurlow, L., Tomich, J., Hancock, L.E., 2009. A fratricidal mechanism is responsible for eDNA release and contributes to biofilm development of Enterococcus faecalis. Molecular Microbiology 72 (4), 1022–1036. https://doi.org/10.1111/j.1365-2958.2009.06703.x.

Thomas, V.C., Thurlow, L.R., Boyle, D., Hancock, L.E., 2008. Regulation of autolysis-dependent extracellular DNA release by Enterococcus faecalis extracellular proteases influences biofilm development. Journal of Bacteriology 190 (16), 5690–5698. https://doi.org/10.1128/JB.00314-08.

Thurlow, L.R., Thomas, V.C., Hancock, L.E., 2009. Capsular polysaccharide production in Enterococcus faecalis and contribution of CpsF to capsule serospecificity. Journal of Bacteriology 191 (20), 6203–6210. https://doi.org/10.1128/JB.00592-09.

Thurlow, L.R., Thomas, V.C., Narayanan, S., Olson, S., Fleming, S.D., Hancock, L.E., 2010. Gelatinase contributes to the pathogenesis of endocarditis caused by *Enterococcus faecalis*. Infect. Immun. *78* (11), 4936–4943. https://doi.org/10.1128/IAI.01118-09.

Valenzuela, A.S., Benomar, N., Abriouel, H., Cañamero, M.M., Gálvez, A., 2010. Isolation and identification of *Enterococcus faecium* from seafoods: antimicrobial resistance and production of bacteriocin-like substances. Food Microbiol. *27* (7), 955–961. https://doi.org/10.1016/j.fm.2010.05.033.

Valledor, S.J.D., Dioso, C.M., Bucheli, J.E.V., Park, Y.J., Suh, D.H., Jung, E.S., Kim, B., Holzapfel, W.H., Todorov, S.D., 2022. Characterization and safety evaluation of two beneficial, enterocin-producing *Enterococcus faecium* strains isolated from kimchi, a Korean fermented cabbage. Food Microbiol. *102* https://doi.org/10.1016/j.fm.2021.103886.

Van Heel, A.J., De Jong, A., Song, C., Viel, J.H., Kok, J., Kuipers, O.P., 2018. BAGEL4: a user-friendly web server to thoroughly mine RiPPs and bacteriocins. Nucleic Acids Res. *46* (W1), W278–W281. https://doi.org/10.1093/nar/gky383.

Vandera, E., Lianou, A., Kakouri, A., Feng, J., Koukkou, A.I., Samelis, J., 2017. Enhanced control of *Listeria monocytogenes* by *Enterococcus faecium* KE82, a multiple enterocin-producing strain, in different milk environments. J. Food Prot. *80* (1), 74–85. https://doi.org/10.4315/0362-028X.JFP-16-082.

Vimont, A., Fernandez, B., Hammami, R., Ababsa, A., Daba, H., Fliss, I., 2017. Bacteriocin-producing Enterococcus faecium LCW 44: A high potential probiotic candidate from raw camel milk. Front. Microbiol. *8* (MAY)) https://doi.org/10.3389/fmicb.2017.00865.

Wang, Y., Liang, Q., Lu, B., Shen, H., Liu, S., Shi, Y., Leptihn, S., Li, H., Wei, J., Liu, C., Xiao, H., Zheng, X., Liu, C., Chen, H., 2021. Whole-genome analysis of probiotic product isolates reveals the presence of genes related to antimicrobial resistance, virulence factors, and toxic metabolites, posing potential health risks. BMC Genom. *22* (1) https://doi.org/10.1186/s12864-021-07539-9.

Werner, G., Fleige, C., Geringer, U., van Schaik, W., Klare, I., Witte, W., 2011. IS element IS16 as a molecular screening tool to identify hospital-associated strains of *Enterococcus faecium*. BMC Infect. Dis. *11* https://doi.org/10.1186/1471-2334-11-80.

Wick, R.R., Judd, L.M., Gorrie, C.L., Holt, K.E., 2017. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput. Biol. *13* (6) https://doi.org/10.1371/journal.pcbi.1005595.

Wu, Y., Pang, X., Wu, Y., Liu, X., Zhang, X., 2022. Enterocins: Classification, Synthesis, Antibacterial Mechanisms and Food Applications. In: In *Molecules*, Vol. 27. MDPI. https://doi.org/10.3390/molecules27072258.

Xu, Y., Hu, J., Liu, D., Tang, J., Liang, M., Wu, J., Xiong, J., 2023. Assessment of the safety and metabolism characteristics of *Streptococcus thermophilus* DMST-H2 based on complete genome and phenotype analysis. LWT, 114907. https://doi.org/10.1016/j.lwt.2023.114907.

Yamada, Y., Kuzuyama, T., Komatsu, M., Shin-ya, K., Omura, S., Cane, D.E., Ikeda, H., 2015. Terpene synthases are widely distributed in bacteria. Proc. Natl. Acad. Sci. USA *112* (3), 857–862. https://doi.org/10.1073/pnas.1422108112.

Yamamoto, Y., Togawa, Y., Shimosaka, M., Okazaki, M., 2003. Purification and characterization of a novel bacteriocin produced by *Enterococcus faecalis* Strain RJ-11. Appl. Environ. Microbiol. *69* (10), 5746–5753. https://doi.org/10.1128/AEM.69.10.5746-5753.2003.

Yong Park, S., Pyo Shin, Y., Han Kim, C., Jin Park, H., Sun Seong, Y., Sam Kim, B., Jae Seo, S., Hee Lee, I., 2008. Immune Evasion of *Enterococcus faecalis* by an extracellular gelatinase that cleaves C3 and iC3b 1. J. Immunol. Vol. 181. ⟨www.jimmunol.org⟩.

Yoshino, Y., 2023. Enterococcus casseliflavus Infection: A Review of Clinical Features and Treatment. In: In Infection and Drug Resistance, Vol. 16. Dove Medical Press Ltd., pp. 363–368. https://doi.org/10.2147/IDR.S398739

Zankari, E., Allesøe, R., Joensen, K.G., Cavaco, L.M., Lund, O., Aarestrup, F.M., 2017. PointFinder: A novel web tool for WGS-based detection of antimicrobial resistance associated with chromosomal point mutations in bacterial pathogens. J. Antimicrob. Chemother. *72* (10), 2764–2768. https://doi.org/10.1093/jac/dkx217.

Zeng, J., Teng, F., Murray, B.E., 2005. Gelatinase is important for translocation of *Enterococcus faecalis* across polarized human enterocyte-like T84 cells. Infect. Immun. *73* (3), 1606–1612. https://doi.org/10.1128/IAI.73.3.1606-1612.2005.

Zhang, H., Chen, Y., Li, Y., Song, Y., Ma, J., Ju, J., 2022. Secondary Metabolites and biosynthetic gene clusters analysis of deep-sea hydrothermal vent-derived Streptomyces sp. SCSIO ZS0520. Mar. Drugs *20* (6). https://doi.org/10.3390/md20060393.

Zhang, X., Vrijenhoek, J.E.P., Bonten, M.J.M., Willems, R.J.L., Van Schaik, W., 2011. A genetic element present on megaplasmids allows *Enterococcus faecium* to use raffinose as carbon source. Environ. Microbiol. *13* (2), 518–528. https://doi.org/10.1111/j.1462-2920.2010.02355.x.

Zhong, Z., Zhang, W., Song, Y., Liu, W., Xu, H., Xi, X., Menghe, B., Zhang, H., Sun, Z., 2017. Comparative genomic analysis of the genus *Enterococcus*. Microbiol. Res. 196, 95–105. https://doi.org/10.1016/j.micres.2016.12.009.