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Research Paper

Exploring the use of Safety-Assessed Bacteriocin-Producing Enterococci as Starters for Production of Soy Yoghurt Analogues

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ABSTRACT

Plant-based yoghurt analogs have gained significant popularity recently. Key driving factors are lactose intolerance and the perception that plant-based foods are more sustainable than dairy alternatives. When preparing plant-based yoghurt analogs, often conventional yoghurt cultures are used, despite the fact that plant-based substrates differ significantly from milk. Enterococci are known for their broad carbohydrate utilization repertoire, and many strains are considered safe and are used as probiotics. In this study, we explored the potential of enterococci for fermenting soymilk. Out of four strains tested, *Enterococcus faecium* BT0194, *Enterococcus lactis* B0167_2, *E. lactis* BT0173_2, and *E. lactis* CS4674, three strains acidified plain soymilk to a pH below 4.7 using an initial inoculum of 10^6 cells/ml (standard inoculum when preparing yoghurt). *Enterococcus* is renowned for producing bacteriocins; all strains harbored multiple bacteriocin genes. Thus, we investigated whether the four strains could confer a bioprotective effect, and indeed, a strong antimicrobial effect was observed against the tested pathogens. Three strains demonstrated α-galactosidase activity, which is necessary for degrading the indigestible and flatulence−inducing α-galactosides raffinose, stachyose, and verbascose, which are undesirable in soymilk. Additionally, all tested strains had the ability to degrade phytic acid, an unwanted antinutrient found in many plant-based foods, including soymilk. In conclusion, our results demonstrate that the *Enterococcus* strains tested exhibit considerable potential for use in plant-based fermentations, due to efficient acidification capacity and a capacity to degrade phytic acid in soymilk.

Soy-based yoghurt analogs are promising alternatives to traditional dairy yoghurt, which are becoming increasingly popular due to the demand for plant-based dairy alternatives (Kumari et al., 2022). Soymilk is rich in protein and isoflavones and can be effectively fermented using lactic acid bacteria (LAB) to produce a yoghurt-like product with beneficial health properties (Kumari et al., 2022; Lee et al., 1990). However, there are some drawbacks to soymilk; its flavor is often slightly beany, which to a great extent is due to processing, and soymilk contains nondigestible oligosaccharides such as raffinose and stachyose (Scalabrini et al., 1998; Shin et al., 2000) that cause gastrointestinal discomfort and flatulence (Shi et al., 2022; Sumarna, 2008). Soymilk also contains phytic acid, a plant phosphate storage compound that comprises 65%–85% of the total phosphate content in soy seeds. Phytate contains six negatively charged ions and chelates

divalent cations such as Fe^{2+}, Zn^{2+}, Mg^{2+}, and Ca^{2+}, thereby rendering them unavailable for absorption by monogastric animals. In vegans and vegetarians, in particular, this often leads to micronutrient deficiencies since humans do not produce digestive phytases for degrading this compound (Kumar et al., 2021). Of particular concern is that phytic acid can cause iron deficiency and accompanying anemia (Samtiya et al., 2020).

Plants are in close contact with the soil, which contains numerous spore-forming bacteria, and are thus easily contaminated by these. Especially the spore-forming *Bacillus cereus*, a potential pathogen, is a challenge for plant-based foods. *Bacillus* spores tolerate various environmental stresses, including heat and desiccation. They can survive the pasteurization process and can contribute to spoilage or food safety issues if not adequately controlled (Sinnelä et al., 2019; Sinnelä et al.,

2021). Certain variants of this bacterium can produce toxins that lead to gastrointestinal disturbances, making it crucial to control its presence in fermented products (Adam et al., 2021; Gopal et al., 2015; Hassan et al., 2010; Hil et al., 2010). The risk is particularly pronounced in products that utilize raw or minimally processed ingredients, as these may harbor spores that survive fermentation and subsequent storage (Hil et al., 2010). To address this challenge, plant-based beverages are usually ultra-high temperature (UHT) pasteurized (typically between 135 °C and 150 °C, 2–5 s). Other pathogenic bacteria have also been found in soymilk, e.g. *Escherichia coli* and *Staphylococcus aureus* (Elhalis et al., 2024; Sinnelä et al., 2019; Sinnelä et al., 2021; Adam et al., 2021).

One way to address safety concerns is to ferment plant foods with LAB. They not only produce lactic acid that helps preserve foods but also many can produce bacteriocins, which are antimicrobial compounds (Mokoena et al., 2021). These natural compounds exhibit inhibitory effects against a broad spectrum of bacteria, including pathogenic ones (Maurya et al., 2020), making them valuable in various applications. Fermentation can also contribute to increasing the nutritional value of plant-based foods. For soymilk, various strains of LAB, e.g. *Lactobacillus acidophilus* ATCC 4356, *L. delbrueckii* subsp. *bulgaricus* IM025, *L. johnsonii* NCC 533, *L. rhamnosus* ATCC 53103 (GG), and *S. thermophilus* IM 111 have been used (Domínguez-Murillo & Urías-Silvas, 2024). These strains can lower the pH of soymilk to below 4.5, which causes coagulation of the soy proteins and a typical yoghurt texture. Strains of *Lactiplantibacillus plantarum* and *Bifidobacterium longum* have been shown to improve flavor by reducing the unwanted "beany" taste of soymilk (Fang et al., 2024; Xu et al., 2024).

Of LAB that are naturally found in fermented foods, the enterococci are a particularly interesting group. Species such as *E. lactis*, *Enterococcus durans*, *Enterococcus hirae*, and *Enterococcus thailandicus* are frequently found in fermented foods (Bampidis et al., 2023; Belloso Daza et al., 2021), are safe, and even exhibit probiotic potential (Bampidis et al., 2023; Belloso Daza et al., 2021; Ben Braïek and Smaoui, 2019; Gupta & Tiwari, 2015; Holzapfel et al., 2018; Lucena et al., 2019; Tadesse et al., 2024a, b). Enterococci are capable of inhibiting the growth of several well-known food-borne pathogens, and they have been recommended for use as adjunct cultures in fermented foods (Barbosa et al., 2014). These bacteria are renowned for their ability to secrete multiple antimicrobial peptides or proteins called enterocins (Henning et al., 2015; Vandera et al., 2017), which can enhance shelf life and food safety by inhibiting the growth of spoilage and pathogenic bacteria (Henning et al., 2015). That some enterococci secrete multiple bacteriocins makes them particularly effective against various food-spoiling and pathogenic bacteria, including *Listeria monocytogenes* (Henning et al., 2015; Vandera et al., 2017), *Shigella* spp., *Escherichia coli,* and *Enterobacter* spp. (Cota et al., 2024; Olsen et al., 2024; Flint, 2002).

Previously, we have characterized *Enterococcus* species isolated from different vegetables and plant-based fermented food products and identified several species with multiple bacteriocin gene clusters that lack virulence genes (Tadesse et al., 2024a). Of these isolates, *E. faecium* BT0194, *E. lactis* B0167_2, BT0173_2, and CS4674 lacked virulence factors (e.g., hemolysin and aggregation substance) and clinically important antibiotic resistance genes. Furthermore, they all were found to be sensitive to different clinically important antibiotics including vancomycin, a requirement for use in foods. They also harbored multiple bacteriocin gene clusters and displayed promising antimicrobial activity against *L. monocytogenes.* (Tadesse et al., 2024a, b). Therefore, in this study, we expand the characterization of these strains to determine whether they can be used more widely in food fermentations. The acidification capacity, α-galactosidase activity, and phytase degradation capacity and their antimicrobial activity against selected pathogens are investigated.

Materials and methods

Bacterial strains and culture conditions. Enterococci (*E. faecium* BT0194, *E. lactis* BT0167_2, BT0173_2, and CS4674), were grown overnight at 42 °C in M17 medium (Thermo Scientific™ Oxoid™) supplemented with 1% glucose. The strains were stored in M17 broth supplemented with 1% glucose and 25% glycerol at –80 °C. Before each experiment, strains were streaked on solid M17 (2% (w/v) agar) supplemented with 1% glucose, and colonies were transferred into 100 mL of liquid M17 broth supplemented with 1% glucose and incubated at 42 °C for 16 h.

Acidification of soymilk. To initiate the experiment, a single colony of *E. faecium* BT0194, *E. lactis* BT0167_2, *E. lactis* BT0173_2, and *E. lactis* CS4674, previously cultivated on M17-agar supplemented with 1% glucose at 42 °C, served as the inoculum for subsequent cultures. These cultures were then inoculated in M17 broth supplemented with 1% glucose and incubated at 42 °C until reaching the mid-exponential growth phase. At this point, cells were harvested and washed with 0.9% (w/v) NaCl to remove any residual media components and then resuspended in soymilk (NATURLI, Organic Soy Drink without Sugar from (Mecindo), Sønderborg, Denmark) to an initial cell count of 10^6- cells/ml which is comparable to the inoculum used when preparing conventional yoghurt (Tadesse et al., 2022). Cultures were incubated at 42 °C under static conditions, i.e. with no active aeration. The pH of the soymilk being fermented was monitored using an iCinac (AMS Alliance, Barsanti 17/a, Rome, Italy). For colony−forming unit (CFU) determination, the cells were serially diluted in 0.9% NaCl, plated on GM17 agar, and incubated at 42 °C. Samples were taken at 2 h, 4 h, and 6 h. After 16 h of incubation, the colonies were counted. The experiment was done in triplicates, and error bars were used to indicate the standard deviation.

Antibacterial activity against selected pathogenic microorganisms. The antibacterial activity assay was carried out as described by Yamamoto et al. (2003). The enterococcal colonies were spotted on M17 agar supplemented with 1% glucose, and the plates were incubated at 42 °C. The bacterial strains *Bacillus spores L. monocytogenes* 0107.0513, *S. aureus* CCUG10778, *S. aureus* ATCC29213, *S. epidermidis* ATCC35984, *E. faecalis* V583, *E. faecalis* CS4479, and *E. coli* UTI89 used in the test were grown overnight in brain heart infusion (BHI) broth (Oxoid Ltd, Basingstoke, England) (beef heart, 5 g/L, calf brains, 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) at 37 °C statically. After 16 h incubation, 7 ml of BHI agar medium (soft agar overlay technique, pH 7.0) was cooled to 40 °C and inoculated with 1% (v/v) of actively growing target pathogenic strains (OD600 of 2.0, for *Bacillus* spores, a loop full of spores was taken) and then incubated for 16 h at 37 °C. After 16 h of incubation, the inhibition zones indicated the production of antibacterial compounds. The results were reported as − = negative (no detectable inhibition zone), + = positive (inhibition zone ≤ 5 mm), + + = strongly positive (inhibition zone ≥ 6 mm), and + + + = very strongly positive (inhibition zone ≥10 mm). The assay was performed in duplicate.

Screening for α-galactosidase activity. Screening for α-galactosidase activity was carried out on solid M17 medium supplemented with 1% glucose or on LB agar plates (5 g/L yeast extract, 10 g/L peptone, 10 g/L NaCl, 15 g/L agar, Sigma-Aldrich, Germany), both supplemented with 20 mg/L 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-gal). All plates were cultured at 42 °C for 24 h, and α-galactosidase activity was determined by the appearance of blue colonies (Zhao et al., 2022).

Screening for phytase activity. Phytase activity was assessed by using modified Chalmers broth (Soy peptone 5 g/L, beef extract powder 5 g/L, yeast extract 5 g/L, glucose 20 g/L, agar 15 g/L) supplemented with 1% of sodium phytate (Sigma-Aldrich, Milan, Italy). After streaking the strains on these agar plates, they were incubated

at 42 °C for two days and examined for clearing zones around the colonies. To eliminate false positive results caused by microbial acid production, agar plates were flooded twice with 2% (w/v) aqueous cobalt chloride solution for 20 min. After 20 min of incubation at room temperature, the cobalt chloride solution was removed and phytase activity was evaluated by clear halo formation around the colony (Anastasio et al., 2010; Bae et al., 1999).

Exopolysaccharide formation assay. Screening for exopolysaccharide (EPS) production was carried out by spotting 5 μL of overnight culture suspension on a medium composed of brain heart infusion broth (Oxoid Ltd, Basingstoke, England), 37 g/L, sucrose 50 g/L, agar No 1 (Oxoid) 10 g/L, and Congo red (BDH Ltd) 0.8 g/L and incubated for 24 h at 42 °C. Black colonies with a dry crystalline consistency indicated EPS production, whereas non-EPS producers remained pink. An indeterminate result was indicated by a darkening of the colonies, but with the absence of a dry crystalline colonial morphology (Freeman et al., 1989; Rühmann et al., 2015).

Results and discussion

Growth and acidification of enterococci strains in soymilk. Soymilk is known to contain small amounts of sucrose and other sugars that not all lactic acid bacteria are able to metabolize (Gu et al., 2022). Enterococci, on the other hand, are known to have a broader carbohydrate utilization repertoire (Donald, 2006). The four *Enterococcus* strains tested were able to grow and acidify soymilk effectively. The initial pH of soy milk without added sugar was 6.8, and all strains tested could lower the pH to below 4.7 at 42 °C in 7–10 h (Fig. 1A), and soymilk was coagulated effectively. Acidification and gel formation of soy-based yoghurt alternatives have been reported to be highly sensitive to minor changes in time and temperature, and more than 8 h is usually required to reach a stable pH below 5 at an optimal temperature between 39 and 45 °C (Betancourt et al., 2025). The cell density was monitored during the fermentation and reached a maximum of 1.25 to 3.35×10^8 (CFU/ml) between 4 and 6 h of fermentation, indicating that growth had ceased due to acidification and/or nutrient deprivation (Fig. 1B). When supplemented with 1% sucrose (42 °C), *E. faecium* BT0194, *E. lactis* BT0167_2, and *E. lactis* BT0173_2 lowered the final pH to below 4.5 (Fig. 1C) and the sucrose added markedly enhanced the acidification rate of the strains evaluated in this study. Such rapid acidification is generally considered sufficient for obtaining a soy yoghurt analog that is safe for consumption and that has a desirable texture and microbial stability.

Soy-based yoghurt analog was prepared by adapting the method for producing conventional yoghurt (i.e. 6 h at 43 °C), which has been reported to be well-suited for plant-based milks when the fermentation time is adjusted (Betancourt et al., 2025; Tamime & Robinson, 2007). Yoghurt fermentations are usually carried out at fairly high temperatures, typically 42 °C to 46 °C, using thermotolerant LAB such as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Tamime & Robinson, 2007). A high temperature enables rapid acidification and helps minimize the growth of spoilage microorganisms during the fermentation. Since enterococci in general are thermotolerant, they can also be cultured at high temperatures. For the yoghurt fermentations mentioned above, a fermentation temperature of 42 °C was used. We found, however, that *E. faecium* BT0194, *E. lactis* BT0167_2, and *E. lactis* BT0173_2 could acidify soymilk to below pH 5.5 at 50 °C (Fig. 1D). The benefit of using such a high fermentation temperature is that unwanted microorganisms are strongly inhibited at this high temperature, which could greatly extend shelf life and improve product safety. Using a high temperature, in principle, can promote syneresis; however, this can be avoided by lowering the fermentation temperature before the gelling point is reached.

Antimicrobial activity of enterococcal strains. The enterococcal strains used in this study have been characterized and identified previously based on their whole genome sequences. Scrutinizing the
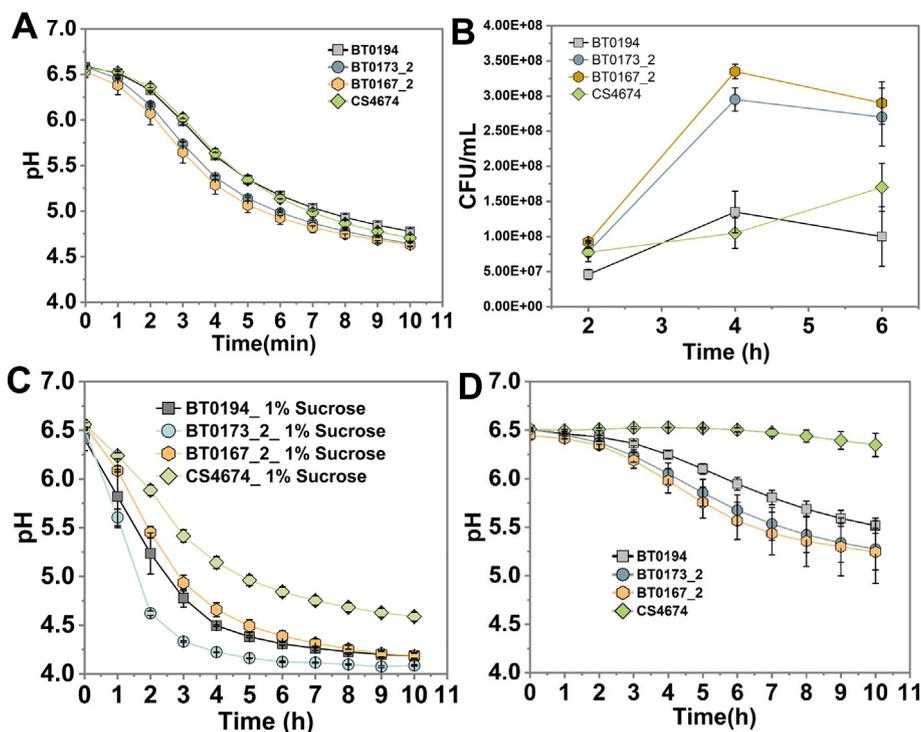

Figure 1. Acidification profiles for four safe *Enterococcus* strains grown in soy milk. **A.** Acidification profile at 42 °C. **B.** Cell density (CFU/ml) at 42 °C. Acidification profiles were recorded using an iCinac. Static conditions were used (no active aeration, slow stirring). C. Acidification profile of four safe *Enterococcus* strains grown in soy milk supplemented with 1% sucrose at 42 °C. **D.** Acidification of four safe *Enterococcus* strains in soymilk at 50 °C recorded using an iCinac. Static conditions were used (no active aeration). Standard deviations are indicated by error bars. Experiments were carried out in triplicate.

genome sequences revealed that they are equipped with multiple bacteriocin gene clusters, and subsequent characterization disclosed a potent antimicrobial activity against three *L. monocytogenes* strains (Tadesse et al., 2024a, b). In this study, we wanted to assess antimicrobial activity of *E. faecium* BT0194, *E. lactis* BT0167_2, *E. lactis* BT0173_2, and CS4674 against different pathogenic bacterial strains, including *S. aureus* CCUG10778, *S. aureus* ATCC29213, *S. epidermidis* ATCC35984, *L. monocytogenes* 0107.0513, *E. faecalis* V583, *E. faecalis* CS4479, and *E. coli* UTI89 (Fig. 2) and against spore−forming *Bacillus* spp. (Table 1, Fig. S1).

It was found that three strains could inhibit the growth of all pathogens tested; however, *E. lactis* CS4674 was not effective (Table 1). *L. monocytogenes* 0107.0513 (Zhao et al., 2022) was effectively inhibited by the enterococcal strains used in this study (Fig. 2). These three strains were able to inhibit the growth of all test pathogens. BT0194 displayed the most potent antibacterial activity against the test pathogens when compared to the other bacteriocin-producing enterococcal strains (inhibition zone > 5 mm for all tested pathogens). The largest inhibition zones were observed for *E. faecium* BT0194, *E. lactis* BT0167_2, and *E. lactis* BT0173_2 against *E. faecalis* V583, *E. faecalis* CS4479 (all above 17 mm), and against *L. monocytogenes* 0107.0513

Figure 2. Antimicrobial activity of enterococci. Diameter of the inhibition of various enterococcal strains against pathogenic bacteria (*L. monocytogenes* 0107.0513, *S. aureus* ATCC29213, *S. epidermidis* ATCC35984, *E. faecalis* V583, *E. faecalis* CS4479, and Bacillus spores 1). Error bars indicate standard deviations from the triplicate experiments.

(above 22.5 mm) (Fig. 2; Fig. 3D). The antimicrobial activity varied between test pathogens, which reveals species specificity of the bacteriocins produced by the strains. From previous work (Tadesse et al. 2024), it has been found that all strains contain genes encoding enterocin P and enterolysin A (Fig. 3B &C), and in addition, *E. faecium* BT0194 carries genes encoding enterocin A and B (Fig. 3A). By secreting multiple bacteriocins with different modes of action, the strains display a potent synergistic antimicrobial activity towards which target bacteria have difficulties adapting, i.e., the risk of antimicrobial resistance is minimized. Enterocin P interacts with negatively charged cell membranes, causing membrane destabilization and bacterial death (Wu et al., 2022), whereas enterolysin A acts by cleaving specific peptide bonds in the peptidoglycan layer of the bacterial cell wall, leading to cell lysis (Khan et al., 2013). Enterocin A interferes with carbohydrate uptake by binding to the mannose phosphotransferase system (Man-PTS), thus providing a quite specific antibacterial activity (Fimland et al., 2005), while enterocin B exerts its activity through pore formation in the bacterial cell membrane, disrupting membrane integrity and causing cell death (Kjos et al., 2011).

E. faecalis V583, a vancomycin−resistant pathogenic strain, was effectively inhibited by the strains evaluated in this study. Studies have shown that nonvirulent bacteriocin−producing strains can displace pathogenic and antibiotic-resistant strains in the gut (Gilmore et al., 2015). Thus, the strains characterized here, in particular *E. faecium* BT0194 with its potent antimicrobial effect, not only have great potential as food fermentation microorganisms but also could have therapeutic value by displacing unwanted pathogenic enterococci in the gut, although the latter effect remains to be demonstrated.

Using naturally occurring antimicrobials in food for preservation purposes is gaining attention due to increasing consumer preference for natural products and concerns about the development of microbial resistance to conventional preservatives. Research on bacteriocins produced by LAB isolated from milk and dairy products has highlighted their potential as natural food preservatives (Todorov, 2018). Nisin, discovered in 1928 and first marketed in England in 1953, is approved for use in over 50 countries as a natural food preservative (E234) (de Arauz et al., 2009). Nisin is safe, digestible, and does not alter food sensory properties, making it an effective bio-preservative (Younes et al., 2017). However, adding purified nisin is expensive. Consequently, in cases where foods are fermented to achieve a desired product quality, using strains with the ability to simultaneously provide antimicrobial activity would be beneficial. *Enterococcus* with antimicrobial activity shows great potential here.

Removal of indigestible oligosaccharides. Soymilk is rich in indigestible α-galactosides such as raffinose, stachyose, and verbascose (Çelem & Önal, 2022). Since humans lack α-galactosidase activity, these carbohydrates are metabolized by various bacteria in the upper gut, resulting in excessive gas formation, which can lead to discomfort
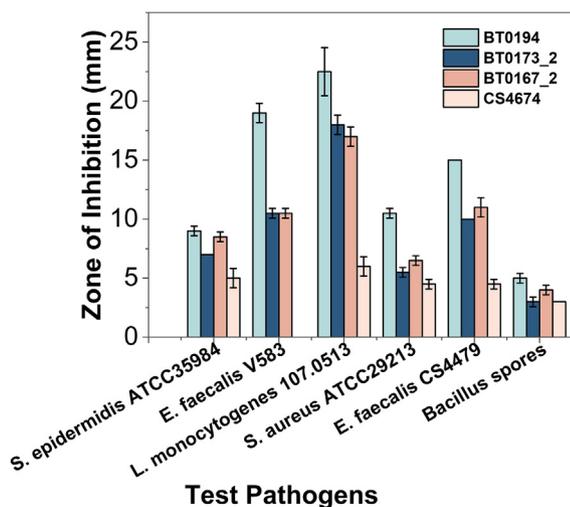
Table 1

Antibacterial activity of strains against different pathogenic species and a spore−forming Bacillus. Bacillus spores 1 = spores of *Bacillus subtilis*, Bacillus spores 2 = spores of *Bacillus thuringiensis*; this species is closely related to *Bacillus anthracis* and *Bacillus cereus*, and is a member of the *Bacillus cereus* group of bacteria (Helgason et al., 2000)

Pathogens	Bacteriocin−producing *Enterococcus* strains			
	BT0194	BT0173_2	BT0167_2	CS4674
Bacillus spores 1	+	+	+	+
Bacillus spores 2	+	+	+	+
L. monocytogenes 0107.0513	+ + +	+ + +	+ + +	+
S. aureus ATCC29213	+	+	+	+
S. aureus CCUG10778	+	+	+	+
S. epidermis ATCC35984	+	+	+	−
E. coli UTI89	+	+	+	−
E. faecalis V583	+ + +	+ +	+ +	−
E. faecalis CS4479	+ + +	+ + +	+ + +	−

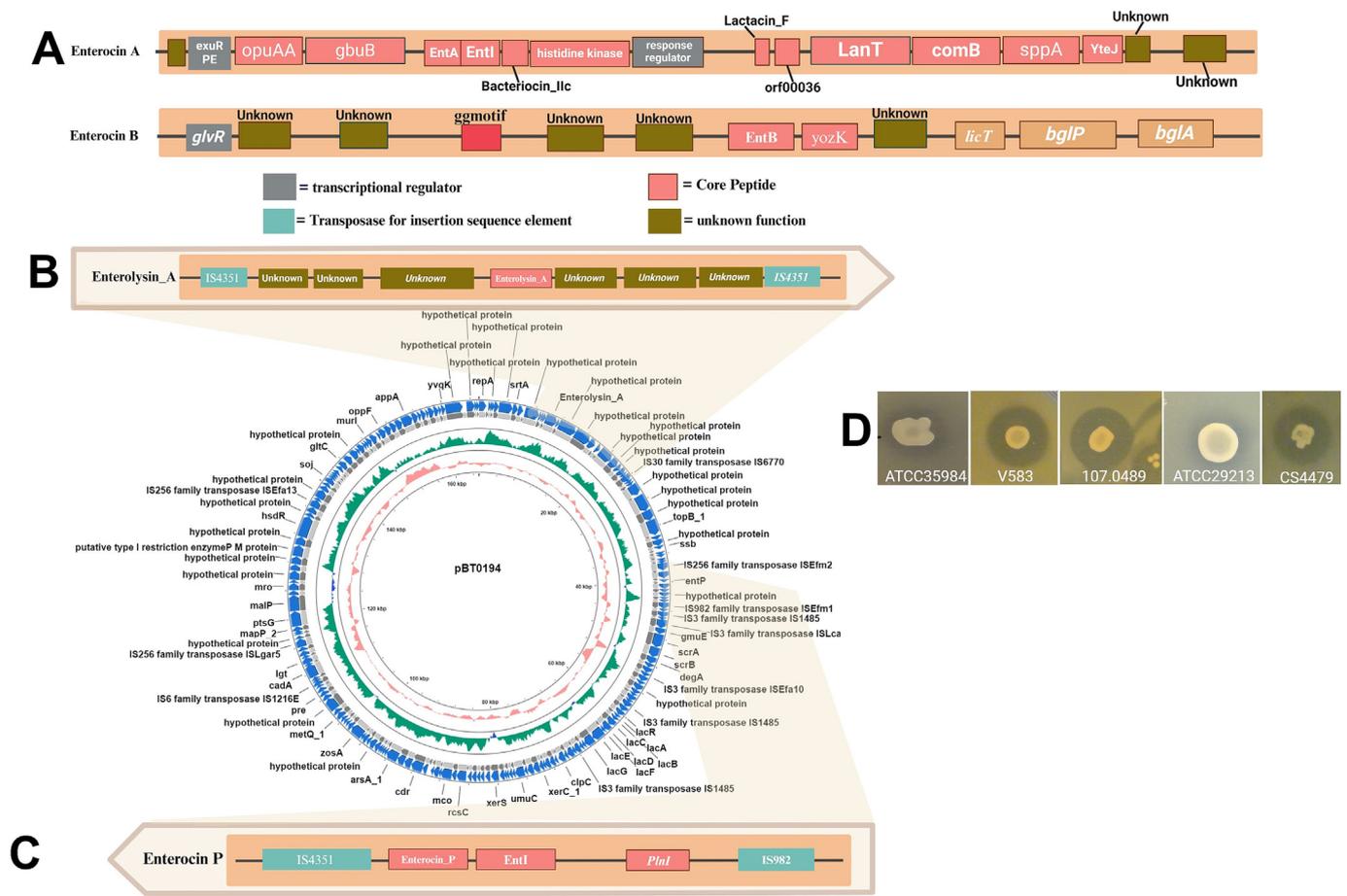i.e., − = Negative, + = Positive, + + = strongly positive and + + + = very strongly positive.

Figure 3. The enterocin gene cluster in *E. faecium* BT0194. **A.** Enterocin A and Enterocin B gene cluster. Exu regulon transcriptional regulator, Glycine betaine transport ATP-binding protein = OpuAA, GbuB = Glycine betaine/carnitine transport permease protein, EntA = Enterocin_A, EntI = Bacteriocin_IIc, histidine kinase, response regulator. **B&C.** Circular plasmid of *E. faecium* with two enterocin gene clusters. From outside to center, the 1st and 2nd circles represent genes on the forward and reverse strand; the 3rd circle represents GC content, the 4th circle represents GC-skew, which can be used to measure the relative amounts of guanine and cytosine; the 5th circle represents the size of the plasmid (167kbp bp). **D.** Zones of inhibition on agar plate for various pathogenic bacteria.

and flatulence (Arunraj et al., 2020; Falkoski et al., 2024; Singh & Vij, 2018).

The genome sequences of the four strains were evaluated, and the presence of α-galactosidase-encoding genes was demonstrated in all of them (Fig. 4A). We also evaluated whether the strains expressed an α-galactosidase activity using the chromogenic substrate X-α-Gal. Including α-galactosidase−positive strains in starter cultures used for making soymilk yoghurt would be an advantage, as these could help eliminate the indigestible α-galactosides. Of the four strains tested, three strains were positive for α-galactosidase (Fig. 4B). In the assay utilized, there seemed to be a higher activity for the strains BT0173_2 and BT0167_2. From our previous characterization using the API50 kit, we also demonstrated their ability to metabolize the α-galactoside raffinose, and thus, these strains are promising candidates for fermenting plant substrates containing such unwanted carbohydrates (Tadesse et al., 2024b). Just to compare, we also assessed the α-galactosidase activity of selected commercially available dairy (YoFlex® mild 1.0), and vegan yoghurt starter cultures (Veggi (Bioferment, Lot: 41210757), Jo Vegan (Bioferment, Lot: 41210157)), and cheese starter culture, Choozit (TA 52 LYO 25 DCU, Lot: 449 459 2912), and found only one starter culture, Veggi (Bioferment, Lot: 41210757), to be positive for α-galactosidase activity (Fig. S2).

Potential of the isolates to degrade antinutrients. As the interest in plant-based diets continues to rise, the use of LAB with phytase activity presents a promising strategy for optimizing the nutritional value of fermented plant-derived products (Kumar et al., 2021). In this study, we found that all the strains (*E. faecium* BT0194, *E. lactis* BT0167_2, and *E. lactis* BT0173_2 against *E. faecalis* V583, *E. faecalis* CS4479) were able to degrade phytic acid (Fig. 4C). We also assessed the phytic acid degradation activity of selected commercially available dairy (YoFlex® mild 1.0), and vegan yoghurt starter cultures (Veggi Bioferment Lot: 41210757 (composed of *Lp. plantarum* and *L. lactis*), Jo Vegan Bioferment Lot: 41210157 (composed of *Streptococcus thermophilus*, *Lactobacillus delbrukii* ssp, *bulgaricus*, *L. acidophilus* and *Bifidobacterium*), and a cheese starter culture, Choozit TA 52 LYO 25 DCU, Lot: 449 459 2912), where only one vegan starter culture, Veggi, was able to degrade phytate (Fig. S3). Phytate-degrading LAB play a crucial role in enhancing the nutritional quality of plant-based fermented foods, by improving mineral absorption and reducing the risk of deficiencies in plant-based diets (Anastasio et al., 2010; Priyodip et al., 2017; Sharma et al., 2020).

Exopolysaccharide formation. Fermenting soymilk with EPS producing LAB can help increase viscosity and improve texture, an effect that often is harnessed in fermented dairy products. In plant-based yoghurt analogs, this effect can be harnessed as well to obtain a product that is more similar to regular yoghurt (Aboulfazli et al., 2015). We therefore characterized the ability of the isolates to produce exopolysaccharides and found that all were able to do so. Besides texture, EPS can contribute to the preservation of fermented foods by inhibiting the growth of spoilage microorganisms and thereby extending shelf life (Kavitake et al., 2023). Furthermore, EPS can promote the growth of beneficial gut microorganisms, thus offering potential health
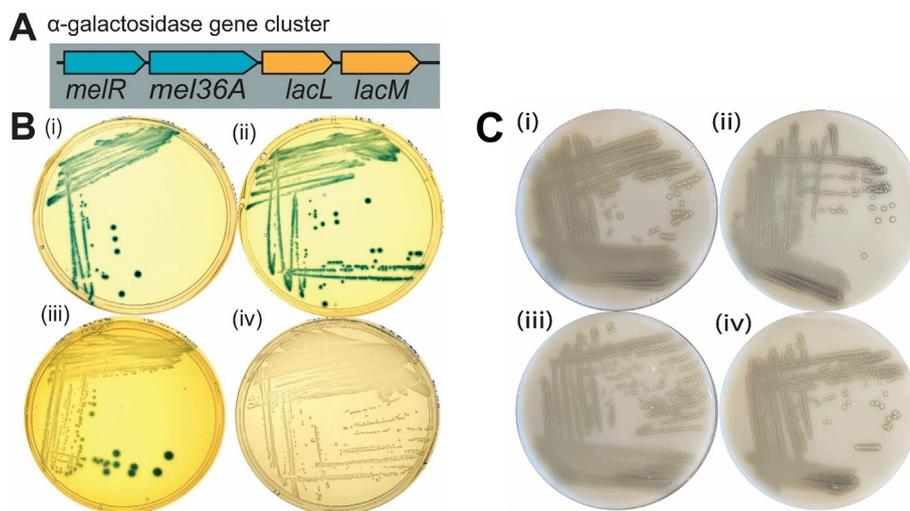
Figure 4. Testing for the presence of α-galactosidase activity using agar plates supplemented with the chromogenic compound X-α-Gal. **A.** The α-galactosidase gene cluster was found in the genomes of all four strains, encoding an HTH-type transcriptional repressor (MelR) and α-galactosidase (*mel36A*). **B.** α-galactosidase test result on agar plate containing chromogenic substrate 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside (X-α-Gal); (i) *E. lactis* BT0173_2, (ii) *E. lactis* BT0167_2, (iii) *E. faecium* BT0194, and (iv) *E. lactis* CS4674. **C.** Phytase plate assay of the strains on a modified Chalmers agar plate; (i) *E. lactis* BT0173_2, (ii) *E. lactis* BT0167_2, (iii) *E. faecium* BT0194, and (iv) *E. lactis* CS4674.
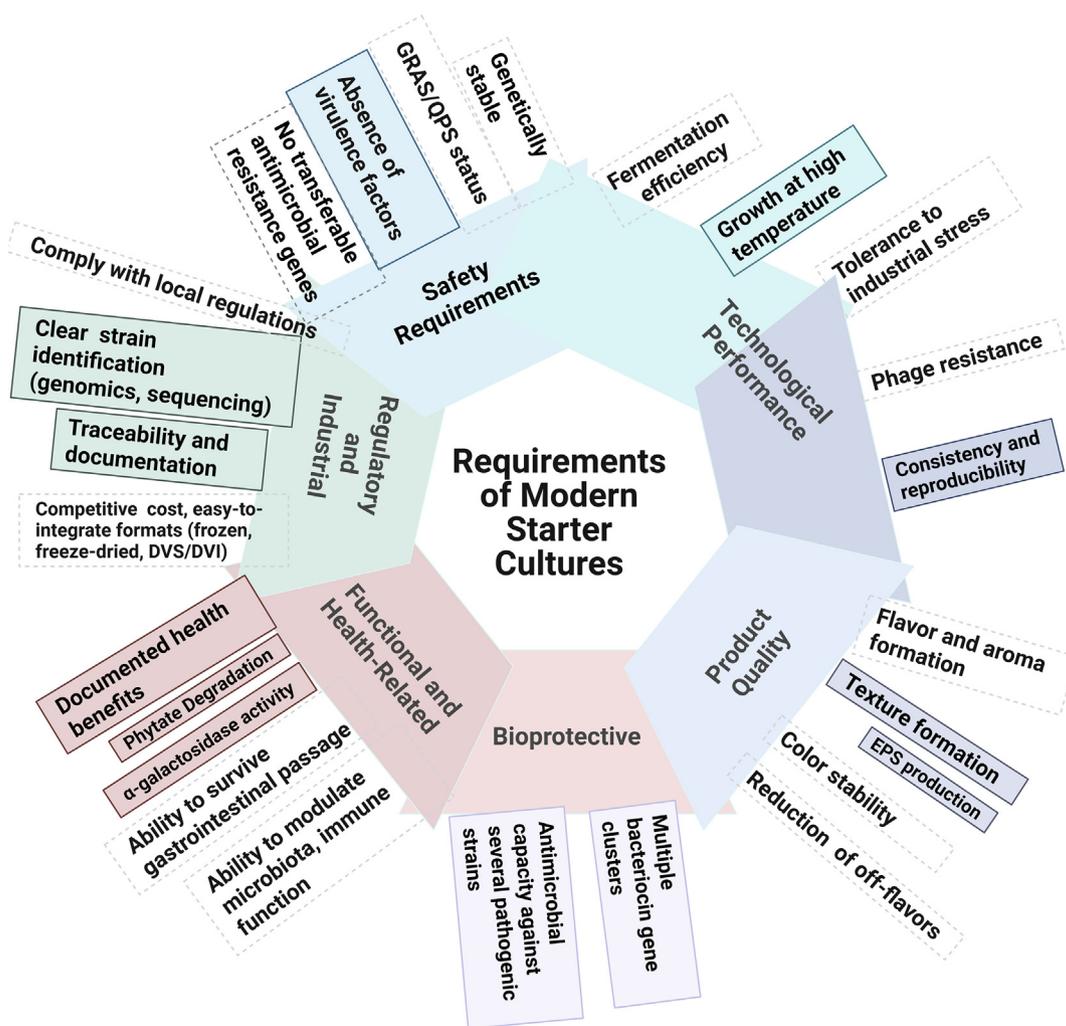

Figure 5. Desirable attributes of candidate bacteria to be included in starter cultures. The *Enterococcus* strains characterized in this study possess several desirable attributes, e.g., safety, technological performance, product quality, bioprotective activity, and functional and health-related attributes. Generated using biorinder.

benefits such as improved digestion and immune function (Caggianiello et al., 2016).

Overall, the strains displayed several key characteristics essential for modern starter cultures. Their acidification capacity, coupled with desirable growth at high temperature, EPS production, and their ability to degrade phytate, all support the development of healthy end products. In addition, safety attributes, including the absence of virulence factors and antibiotic resistance markers, further support their suitability (Fig. 5).

Conclusion

The characterization of the four enterococcal isolates revealed a strong potential for use in multifunctional starter cultures for soymilk. Beneficial attributes include rapid acidification, antimicrobial activity against various food pathogens, enzymatic activities relevant to digestibility and nutritional value, and EPS production that can affect the structural and sensory properties of fermented foods in a beneficial manner. Overall, three of the isolates, *E. faecium* BT0194, *E. lactis* BT0173_2, and *E. lactis* BT0167, were shown to be valuable candidates for the development of innovative, functional plant-based foods with desirable sensory characteristics and enhanced nutritional value. Although the great potential for use of these isolates in plant fermentations has been revealed, further characterization is warranted. For instance, it would be relevant to look into behavior in mixed–culture fermentations, to determine performance in other plant substrates besides soymilk, investigating their effect on bioavailability of micronutrients and reduction of antinutritional factors, and determine the effect on organoleptic properties. Overall, this study demonstrates the significant potential of these enterococcal strains as multifunctional starters for developing next-generation plant-based fermented foods with enhanced nutritional value, safety, and sensory quality.

Data availability

Data will be made available on request.

CRediT authorship contribution statement

Belay Tilahun Tadesse: Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Jennifer C. Molloy:** Writing – review & editing, Supervision, Methodology. **Shuangqing Zhao:** Writing – review & editing, Methodology, Investigation. **Liuyan Gu:** Writing – review & editing, Methodology, Investigation. **Carsten Jers:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization. **Ivan Mijakovic:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Formal analysis, Conceptualization. **Christian Solem:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.jfp.2026.100715.

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