Current Research in Microbial Sciences 2 (2021) 100070

Contents lists available at ScienceDirect

Current Research in Microbial Sciences

journal homepage: www.sciencedirect.com/journal/current-research-in-microbial-sciences

Probiotic potential and safety assessment of bacteriocinogenic *Enterococcus faecium* strains with antibacterial activity against *Listeria* and vancomycin-resistant enterococci

Joanna Ivy Irorita Fugaban, Wilhelm Heinrich Holzapfel, Svetoslav Dimitrov Todorov *

ProBacLab Laboratory, Department of Advanced Convergence, Handong Global University, Handong-ro, Heunghae-eup, Pohang, Gyeong-buk, South Korea

ARTICLE INFO

Keywords:
Enterococcus faecium
bacteriocins
probiotics
safety
virulence

ABSTRACT

Enterococcus spp., known for their wide ecological distribution, have been associated with various fermented food products of plant and animal origin. The strains used in this study, bacteriocinogenic *Enterococcus faecium* previously isolated from artisanal soybean paste, have shown strong activity against *Listeria* spp. and vancomycin-resistant enterococci. Although their antimicrobial activity is considered beneficial, the potential application of enterococci is still under debate due to concerns about their safety for human and other animal consumption. Therefore, this study not only focuses on the screening of potential virulence factors, but also the auxiliary beneficial properties of the strains *Ent. faecium* ST651ea, ST7119ea, and ST7319ea. Phenotypic screening for gelatinase, hemolysin, and biogenic amine production showed that the strains were all safe. Furthermore, the antibiogram profiling showed that all the strains were susceptible to the panel of antibiotics used in the assessment except for erythromycin. Yet, *Ent. faecium* ST7319ea was found to carry some of the virulence genes used in the molecular screening for safety including *hyl, esp*, and IS*16*. The probiotic potential and other beneficial properties of the strains were also studied, demonstrating high aggregation and co-aggregation levels compared to previously characterized strains, in addition to high survivability under simulated gastrointestinal conditions, and production of numerous desirable enzymes as evaluated by APIZym, indicating diverse possible biotechnological applications of these strains. Additionally, the strains were found to carry genes coding for γ-aminobutyric acid (GABA) production, an auxiliary characteristic for their probiotic potential. Although these tests showed relatively favorable characteristics, it should be considered that these assays were carried out *in vitro* and should therefore also be assessed under *in vivo* conditions.

1. Introduction

Lactic acid bacteria (LAB) are associated with a wide variety of ecological niches, including different fermented food products based on the plant phyllosphere and animal, dairy origin, and also with the human intestinal tract (Franz et al., 2010; dos Santos et al., 2020; Vizoso Pinto et al., 2006). Although LAB require a nutrient-rich environment, some members of this group are clearly adapted to a broader range of environmental conditions relative to the other members. These include the autochthonous distributions of some species belonging to the genera *Pediococcus, Lactobacillus,* and *Enterococcus*. Even though the applications of enterococci are still under debate, a proposed two-clade classification of *Enterococcus faecium* appears to be feasible to separate pathogenic strains from well-evaluated beneficial strains (Lebreton et al., 2018; Holzapfel et al., 2018). This paved the way to the exploitation and earnest scientific interest towards a better understanding of both the pathogenicity and probiotic properties of this species. The probiotic potential of some representatives of the genus *Enterococcus* has been explored particularly regarding its antibacterial properties for application as biocontrol contaminants in dairy food products (De Vuyst et al., 2003; Franz et al., 2003; Omar et al., 2004) while several strains are applied as starter cultures (Foulquié Moreno et al., 2006), and some are established as probiotics (Holzapfel et al., 2018). On the other hand, reports on some clinically associated enterococci suggest that some strains may pose a serious health risk, especially those specifically harboring virulence factors and carrying antibiotic resistance genes, including resistance to vancomycin. Moreover, some strains of *Ent. faecalis* and *Ent. faecium* isolated from fresh produce were shown to

* Corresponding author.
E-mail address: slavi310570@abv.bg (S.D. Todorov).

https://doi.org/10.1016/j.crmicr.2021.100070
Received 16 July 2021; Received in revised form 17 August 2021; Accepted 28 August 2021
Available online 30 August 2021

harbor antibiotic resistance against ciprofloxacin, tetracycline, and nitrofurantoin (Johnston and Jaykus, 2004). A survey of the prevalence of *Enterococcus* spp. in Brazilian food products conducted by Gomes et al. (2008) showed that the distribution of *Ent. faecium* and *Ent. faecalis* in cheeses, milk, meat, and vegetable food products, alongside with the assessment of associated potential virulence factors, reported that dominantly, although not all, *Ent. faecalis* harbor higher potential virulence genes compared to *Ent. faecium* isolates.

The applications of *Enterococcus* spp. as probiotics have long been debated due to safety concerns. Despite this, some of the *Enterococcus* spp. play major roles in the food production industry, include contribution to cheese ripening, and also fermentation of raw meat and vegetable-based foods such as *kimchi* and sauerkraut, and even fermented milk like *kefir* (Centeno et al., 1996;Giraffa, 2002). Also, some of the known probiotic applications of *Enterococcus* spp. include both human and animal consumption (Franz et al., 2011; Holzapfel et al., 2018).

Enterococci probiotics designed for human consumption were considered advantageous due to their ability to produce multiple beneficial metabolites that also contributes to the stability of microorganisms in the gastrointestinal tract, considering that they are natural gut commensals. *Ent. faecium* SF68, one of the safe enterococcal strains that has long been commercialized and applied as a probiotic, has specifically been used as an alternative to antibiotics for the treatment for diarrhea caused by food-borne pathogens including *Shigella* spp., *Escherichia coli, Enterobacter* spp., *Campylobacter* spp., and some serovars of *Salmonella enterica* (Bellomo et al., 1980; Fugaban et al., 2021b). Additionally, the administration of probiotic preparations, composed of enterococci has been done to patients suffering from antibiotic-associated diarrhea (AAD) and irritable bowel syndrome patients (Wunderlich et al., 1989; Fan et al., 2006). The bile hydrolase enzyme produced by *Enterococcus* spp. allows them to deconjugate bile salts. This feature has been exploited for application in lowering blood cholesterol levels, as exemplified in commercially available probiotic fermented milk called *gaio*, fermented with a human-derived probiotic strain of *Ent. faecium.* The product was prepared with two other *Streptococcus thermophilus* strains and reported to have cholesterol level lowering properties (Agerholm-Larsen et al., 2000). Additionally, applications of *Enterococcus* spp. as probiotics were not only limited to humans but they are also well employed in the swine industry (Taras et al., 2006; Pollmann et al., 2005), the poultry industry (Vahjen and Manner, 2003; Mountzouris et al., 2007), in cattle farming (Ghorbani et al., 2002; Emmanuel et al., 2007; Nocek et al., 2002) and even for domestic pets (Vahjen and Manner, 2003). Although, various evidence for the application of *Enterococcus* strains is promising and encouraging for continuous innovation for the applications of beneficial strains belonging to this group, the debate on their safety as probiotics still continues.

This study aimed to evaluate the safety and beneficial potential of previously characterized bacteriocinogenic *Ent. faecium* strains ST651ea, ST7119ea, and ST7319ea (Fugaban et al., 2021a), found to be active against food-borne pathogens, including *Listeria monocytogenes*, along with emerging-pathogen vancomycin-resistant *Enterococcus* (VRE) strains of clinical origin.

2. Materials and methods

2.1. Bacterial cultures

Ent. faecium strains ST651ea, ST7119ea, and ST7319ea, were previously isolated from locally outsourced *doenjang* (Korean traditional fermented soybean paste) and identified (Fugaban et al., 2021a). These strains were investigated together with other organisms in this study and were stored in presence of glycerol (10% v/v) at -80 °C. The strains were revived under aerobic conditions in de Man, Rogosa and Sharpe (MRS) broth (Difco, Franklin Lakes, NJ, USA), BHI (Difco) and grown at 37 °C for 24–48 h until robust growth was observed.

2.2. Phenotypic evaluation of the safety of Enterococcus faecium strains

2.2.1. Gelatinase enzyme production

The production of the gelatinase enzyme was determined from 18 h old cultures grown in MRS broth at 37 °C. Tubes containing 10 mL BHI broth supplemented with 4% gelatin were inoculated with 100 μL (estimated 10^5 cells/mL) of each strain, and incubated for 24 h at 37 °C, followed by cooling at 4 °C for 30 min. Positive results, indicating production of gelatinase enzyme were associated with retention of the liquid phase after refrigeration. A previously characterized *Bacillus amyloliquefaciens* ST109 (Fugaban et al., 2021b) was used as positive control, while *Lactobacillus plantarum* ATCC14917 and an untreated medium were used as negative controls (dos Santos et al., 2020; Omar et al., 2004). All tests were performed in three independent experiments.

2.2.2. Hemolysin production

Hemolytic activity for the studied strains was evaluated on Columbia Blood Agar (Oxoid LTD, Basingstoke, UK) with 5% defibrinized horse blood according to dos Santos et al. (2020). Strains (18h-old cultures grown in MRS at 37 °C) were spot-plated (10 μL) on the agar surface. Positive hemolytic activity is indicated by clear yellow zones around the bacterial growth. Reference strains used were *Streptococcus pneumoniae* ATCC49619, *B. cereus* ATCC27348, and *Lb. plantarum* ATCC42917 as producers of α-, β-, γ- hemolysins, respectively. All experiments were performed in triplicate.

2.2.3. Biogenic amine production

The detection of amino acid decarboxylase enzyme production was performed as suggested by Bover-Cid and Holzapfel (1999). Previously grown strains were subjected to an induction assay (subsequent sub-culture of strains for 5 days) using MRS broth supplemented with respective amino acid precursors comprising tyrosine (Samchun Chemicals, Daejeon, Republic of Korea), histidine monohydrochloride (Daejung Chemicals, Gyeonggi, Republic of Korea), lysine monohydrochloride (Sigma-Aldrich, St. Louis, MO, USA), and ornithine monohydrochloride (Sigma Aldrich) at concentrations 1% (w/v). The final batch was streaked on MRS agar (1.5%, w/v) supplemented with the corresponding amino acid precursor. Inoculated plates were incubated for at least three days at 37 °C. Biogenic amine production is indicated by purplish coloration around the colonies. Reference strains used for the biogenic amine production were *E. coli* ATCC25922 (positive control) and *Lb.* plantarum ATCC14917 (negative control). All test organisms were tested in at least two independent experiments.

2.2.4. Antimicrobial susceptibility profiling

The antimicrobial susceptibility testing (AST) was performed according to the standards recommended by the Clinical and Laboratory Standards Institute (CLSI) (2012) on Performance Standards for Antimicrobial Susceptibility Testing for *Enterococcus* spp. The assay was performed by microbroth dilution using the antibiotics ampicillin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamycin, kanamycin, streptomycin, tetracycline (all from Sigma-Aldrich), and vancomycin (CheilJedang Pharma Co., Republic of Korea) on cation-adjusted Mueller-Hinton broth supplemented with MRS (5.0 g/L). The assay was performed in a 96-well microplate (SPL Life sciences, Pocheon-si, Gyeonggi-do, Republic of Korea) and comprised 10 antibiotic dilutions in two-fold and controls (growth and sterility controls). The inocula were adjusted to 0.5 McFarland units (approximately 10^7 CFU/mL) and distributed accordingly to obtain a final concentration of 10^5 CFU/mL. The plates were incubated at 35 ± 1 °C for 18 h. The lowest concentration with complete bacterial inhibition was recorded as the MIC and analyzed according to the standards set for *Enterococcus* spp. (Rychen et al., 2017; Wiegand et al., 2008).

2.3. Biomolecular safety tests

2.3.1. Screening for the presence of potential virulence genes

Bacterial cells grown overnight in MRS at 37 °C were used for the DNA isolation using ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's recommendations. The concentration and purity of the DNA were determined using SPECTROstar Nano nanodrop (BMG LABTECH, Rotenberg, Germany).

Bacterial DNA from the studied strains was screened by PCR assay for the presence of potential virulence genes including *efa*A (endocarditis specific proteins), *cyt* (cytolysin), IS*16* (*Enterococcus* pathogenicity island), *esp* (enterococcal surface protein), *asa*1 (aggregation substance protein), and *hyl* (hyaluronidase) as suggested by EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (Rychen et al., 2017). Oligonucleotide sequences of primers are shown in Table 1. PCR reactions were performed in a Veriti 96 well Thermal Cycler (Applied Biosystems, Thermo Scientific, Waltham, MA, USA), and amplicons were separated on 1.5% (w/v) agarose gel (1x TAE buffer, 100 V, 1 h) and stained with SYBR©Safe DNA gel stain (0.02 μL/mL) (Thermo Scientific) (GH-200 Genera Biosystems, Victoria, Australia; Elite 300 Plus Power Supply, Wealtec Bioscience Co., Ltd., Taiwan) and visualized using an Omega Lum™ G gel documenter (Aplegen, Inc., CA, USA).

2.3.2. Screening for the presence of vancomycin-resistance associated genes

Detection of vancomycin-resistance-related genes was performed by PCR assay using the genes *van*A, *van*B, *van*C, *van*D, *van*E, and *van*G (Fugaban et al., 2021b). The oligonucleotide primer sequences and PCR conditions are mentioned in Table 1. Separation, visualization, and analysis of the PCR products were performed as described before.

2.4. Assessment of the probiotic potential of bacteriocinogenic *Enterococcus faecium* strains

2.4.1. Enzyme production profiling

Enzyme production of the evaluated strains was profiled using API-Zym (bioMérieux, Marcy l'Etoile, France), carried out according to the manufacturer's instructions. Bacterial cells were obtained from an 18 h-old culture grown in MRS agar at 37 °C and were inoculated on the provided strips, followed by incubation at 37 °C for 4 h. Enzyme production was monitored through color changes and interpretation of results was carried out based on the guidelines provided by the manufacturer.

2.4.2. D/L-lactic acid production

Measurement of the D- and L-lactic acid produced by the strains *Ent. faecium* ST651ea, ST7119ea, and ST7319ea was performed using Megazyme D-/L-lactic acid assay kit (Megazyme, Bray, Wicklow, Ireland). The cell-free supernatant of each strain was obtained by centrifugation (12000 x g, 5 min) from 18 h-old culture grown in MRS at 37 °C for 24 h. The assay was performed according to the manufacturer's instructions. Analysis of results was calculated using the corresponding spreadsheet provided by the manufacturer available on their customer assistance page (http://www.megazyme.com). The produced lactic acid by each strain was interpreted in proportion showing the amounts of both D- and L-lactic acid.

2.4.3. Screening for adhesion genes

Bacterial cells in the stationary phase were harvested by centrifugation (8000 x g, 10 min, 4 °C) and the genomic DNA of the evaluated strains was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's suggestion with few modifications. DNA concentration and purity were measured by SPECTROstar Nano nanodrop (BMG LABTECH, Otterberg, Germany). Adhesion genes, *map*A (mucus adhesion associated gene), *mub* (mucus adhesion associated gene), *EF-Tu* (adhesion-like factor), *prg*B (aggregation substance

Table 1
Primers used in the molecular-based screening of safety and beneficial properties.

Genes	Primer Oligonucleotide Sequence (5' – 3')	References
Vancomycin resistance-associated genes		
*van*A	F: GTA GGC TGC GAT ATT CAA AGC R: CGA TTC AAT TGC GTA GTC CAA	
*van*B	F: GTA GGC TGC GAT ATT CAA AGC R: GCC GAC AAT CAA ATC ATC CTC	
*van*C	F: ATC CAA GCT ATT GAC CCG CT R: TGT GGC AGG ATC GTT TTC AT	Fugaban et al. (2021)
*van*D	F: TGT GGG ATG CGA TAT TCA A R: TGC AGC CAA GTA TCC GGT AA	
*van*E	F: TGT GGT ATC GGA GCT GCA G R: GTC GAT TCT CGC TAA TCC	
*van*G	F: GAA GAT GGT ACT TTG CAG GGC A R: AGC CGC TTC TTG TAT CCG TTT T	
Adhesion genes		
*EF*1249	F: GCG GTC GAC AAA CGA GGG ATT TAT TAT G R: CTG GCG GCC GCG TTT AAT ACA ATT AGG AAG CAG A	Todorov et al. (2010), Castilho et al. (2019), dos Santos et al. (2020)
*EF*2380	F: GCG GTC GAC GAC ATC TAT GAA AAC AAT R: TCC GCG CCG CCT TAA ACT TTC TCC TT	
EF2662	F: GGC GTC GAC CAC TTA AAC TGA TAG AGA GGA AT R: CGC GCC GCA ATT AAT TAT TAA CTA GTT TCC	
EF-Tu	F: TTC TGG TCG TAT CGA TCG TG R: CCA CGT AAT AAC GCA CCA AC	
*map*A	F: TGG ATT CTG CTT GAG GTA AG R: GAC TAG TAA TAA CGC GAC CG	
mub	F: GTA GTT ACT CAG TGA CGA TCA ATG R: TAA TTG TAA AGG TAT AAT CGG AGG	
*prg*B	F: GCC GTC GAC TCG AGG AGA ATG ATA CAT GAA T R: CCT GCG GCC GCG TCC TTC TTT TCG TCT TCA A	
Potential Virulence Genes		
hyl	F: ACA GAA GAG CTG CAG GAA ATG R: GAC TGA CGT CCA AGT TTC CAA	Vankerckhoven et al. (2004)
esp	F: AGA TTT CAT CTT TGA TTC TTG G R: AAT TGA TTC TTT AGC ATC TGG	Vankerckhoven et al. (2004)
*efa*A	F: GCCAATTGGGACAGACCCTC R: CGC CTT CTG TTC CTT CTT TGG C	Martín-Platero et al. (2009)
asa1	F: GCA CGC TAT TAC GAA CTA TGA R: TAA GAA AGA ACA TCA CCA CGA	Vankerckhoven et al. (2004)

(*continued on next page*)

Table 1 (*continued*)

Genes	Primer Oligonucleotide Sequence (5' – 3')	References
*cyl*A	F: ACT CGG GGA TTG ATA GGC R: GCT GCT AAA GCT GCG CTT	Vankerckhoven et al. (2004)
IS*16*	F: CAT GTT CCA CGA ACC AGA G R: TCA AAA AGT GGG CTT GGC	Werner et al. (2011)
GABA-operon		
gad	F:CCT CGA GAA GCC GAT CGC TTA GTT CG R: TCA TAT TGA CCG GTA TAA GTG ATG CCC	Bajić et al. (2020)
Folate (Vitamin B9)		
*pab*C	F: CGG ACA AGC ATA ATG AAT ACT CGG AAT R: GGA TTG ATA ACC GCT TCT ATT GCC GA	Meucci et al. (2018)
*pab*B	F: CCT CAA TTC ATA CAA CCC TCT CAC A R: CAG ACA AAT CTT CAC TCA CGC CAT AA	
*fol*K-Q	F: CAC TAG TGT CTA TTG ACT CAA ATA TTT T R: CGT TTT TAT GGC TAT CAC GGG GCT	
*fol*P-E	F: GAG ATA GTC TTA ACG ACA TCA CGA TT R: GCA GTC TAT CAA TTA TTG GAA GCT TT	

gene), *EF*2662 (choline-binding protein gene), *EF*1249 (fibronectin-binding genes), and *EF*2380 (membrane-associated zinc metalloprotease gene) were used in the PCR-based adhesion gene detection assay (Todorov et al., 2010; Castilho et al., 2019; dos Santos et al., 2020). Sequences of the oligonucleotide primers are shown in Table 1. PCR products were separated through gel electrophoresis on agarose gel (1, 2%, w/v), and stained and visualized as described before.

2.4.4. Bacterial cell-surface hydrophobicity

Bacterial adherence was evaluated according to the assay proposed by Rosenberg (1984) using hydrocarbons (*n*-hexadecane, Sigma-Aldrich) with modification as follows. Cells of the evaluated strains were collected (4000 × g, 20 min, 4 °C) and washed twice using 100 mM potassium phosphate buffer (pH 6.5) and re-suspending using the same buffer to achieve an optical density reading ($OD_{560nm} \approx 1.0$, refered as A_0). This was followed by the addition of *n*-hexadecane to the cell suspensions in the ratio 1:5 before emulsification (vortex mixing) for 2 min. Set-ups were incubated for 1 h at 37 °C. After separation of the phases, *n*-hexadecane on the surface of the cell suspension was gradually removed before measuring the optical density for A_1 (after 1 h incubation) of the hydrophilic phase at OD_{560nm}, and % hydrophobicity was quantified using the equation:

$$\%\ hydrophobicity = \left[\frac{A_0 - A_1}{A_0}\right] \times 100$$

The cell surface hydrophobicity quantification assay was performed in at least two independent experiments wherein each set-up was measured in triplicate.

2.4.5. Aggregation properties

Auto-aggregation: The ability of the evaluated strains to auto-aggregate was evaluated according to dos Santos et al. (2020). Each strain was grown in MRS broth for 18 h at 37 °C. The cells were harvested through centrifugation (4000 × g, 20 min, 4 °C) and washed twice and re-suspended in sterile phosphate buffer saline (Lonza, Basel, Switzerland). The suspensions were then readjusted using the same buffer to obtain a final OD_{6_60nm} with reading ≈ 0.3 using a UV-vis spectrophotometer (Optizen, Daejeon, Republic of Korea). Set-ups were incubated for one hour at 37 °C and centrifuged for 20 min at 300 × g before measuring the optical density (OD_{660nm}) of the top phase. Calculations for the % auto-aggregation were carried out using the formula:

$$\%\ auto - aggregation = \left[\frac{(OD_0 - OD_1)}{OD_0}\right] \times 100$$

where OD_o and OD_1 refer to optical density readings before and after incubation, respectively. All experiments were performed in triplicates in at least two independent assays.

Co-aggregation: The evaluation of the strains to co-aggregate with other microorganisms was evaluated using partner strains classified as either pathogenic or beneficial strains. The partner microorganisms *L. monocytogenes* ATCC15313, *Listeria innocua* ATCC33090, *Ent. faecium* VRE19, were cultured in BHI broth and *Lactobacillus rhamnosus* LGG, *Lactobacillus sakei* HEM802, *Ent. faecium* HEM1108, and *Lactobacillus fermentum* HEM792 in MRS broth. All cultures were grown for 24 h at 37 °C before cell harvesting as previously described. Partner organisms adjusted to $OD_{660nm} \approx 0.3$ were individually mixed with strains *Ent. faecium* ST651ea, *Ent. faecium* ST7119ea, and *Ent. faecium* 7319ea ($OD_{660nm} \approx 0.3$) in a 1:1 ratio. The OD_0 (at OD_{660nm}) was recorded for 60 s after mixing and OD_1 (at OD_{660nm}) was measured after one-hour incubation at 37 °C. Calculations for % co-aggregation were carried out as follows:

$$\%\ co - aggregation = \left[\frac{(OD_0 - OD_1)}{OD_0}\right] \times 100$$

where OD_0 and OD_1 are turbidity readings right after mixing of partner strains and after one-hour incubation, respectively (dos Santos et al., 2020). All experiments were performed in at least two independent repetitions comprising assays carried out in triplicates.

2.5. Molecular identification of potential beneficial metabolites

2.5.1. γ-Aminobutyric acid (GABA)

Identification of some of the functional properties of LAB includes detection of putative beneficial metabolites such as GABA. This has been performed by molecular-based detection of the GABA production associated genes. Oligonucleotide sequences of the primers as presented in Table 1, were used to carry out this assay (Bajić et al., 2020). PCR products were determined and analyzed by gel electrophoresis on agarose gel (2%, w/v) and stained and visualized as described before.

2.5.2. Vitamin B12 (folate)

PCR-based detection assay for the genes responsible for bacterial biosynthesis of folate was carried out according to the suggestions of Meucci et al. (2018). Sequences of the primers are shown in Table 1. Gel electrophoresis (agarose gel 1, 2%) visualization and analysis have been carried out as previously described.

2.6. Gastrointestinal tract simulation assay

2.6.1. Gastrointestinal survival assay Enterococcus faecium strains ST651ea, ST7119ea, and ST7319ea

The survivability of the evaluated strains under mimicked gastrointestinal conditions has been evaluated as suggested by dos Santos et al. (2020). For the GIT simulation assay cultures in the mid-stationary phase were inoculated in 100 mL of MRS broth and incubated at 37 °C for 18 h.

The initial viable bacterial counts (t_0) were quantified (CFU/mL) by using one mL of the previously prepared bacterial culture and serial dilution in a sterile saline solution and plated on MRS supplemented with 1.5% (w/v) agar before incubation at 37 °C for 72 h under anaerobic conditions (anaerobic jars, Oxoid, Basingstoke, Hampshire,

United Kingdom).

Simulation of the gastric fluid conditions was performed by preparing electrolyte solutions composed of sodium chloride (NaCl, 6.20 g/L), potassium chloride (KCl, 2.20 g/L), calcium chloride ($CaCl_2$, 0.22 g/L), and sodium bicarbonate ($NaHCO_3$, 1.20 g/L) adjusted to a final pH of 2.5 and supplemented with 0.3% of pepsin (Sigma-Aldrich). Six mL of previously prepared bacterial culture were obtained and added to 10 mL of simulated gastric juice solution and incubated for 1 h at 37 °C with continuous agitation (150 rpm) under anaerobic conditions. Samples were withdrawn, serially diluted, and quantified as previously described to obtain CFU/mL for t_1.

Duodenal pass simulation was performed by taking 2 mL of the sample from the previous set-up and adding to 8 mL of artificial duodenum solution prepared by formulating an electrolyte solution comprising $NaHCO_3$ (6.4 g/L), KCl (1.28 g/L), NaCl (1.28 g/L) adjusted to a final pH of 7.2 and supplemented with 0.5% bile salt (Oxgall, Difco) and 0.1% pancreatin (Sigma-Aldrich). The bacterial suspension was incubated in an anaerobic system for 3 h at 37 °C under continuous agitation (150 rpm) before quantification (CFU/mL, t_2) as previously described. Calculations for the survival rate were determined as:

$$Survival\ rate\ (\%) = \left[\frac{\log \frac{CFU}{mL} N_0}{\log \frac{CFU}{mL} N_1}\right]$$

where N_0 and N_1 are viable cell counts of strains *Ent. faecium* ST651ea, *Ent. faecium* ST7119ea, and *Ent. faecium* ST7319ea before and after the simulation passes. All experiments were performed in at least three independent experiments comprising three replicates for each set-up.

2.6.2. GIT survival of test organisms in competition with the studied Enterococcus faecium strains

The GIT competition assay was performed using the same component assay as previously described. Test organisms *L. monocytogenes* ATCC15313, *L. innocua* ATCC33090, were grown in 100 mL BHI broth at 37 °C for 18 h. Antagonistic strains, *Ent. faecium* strains ST651ea, ST7119ea, and ST7319ea, on the other hand, were grown in MRS broth. Before performing the assays, bacterial cells from all the above strains were all adjusted to obtain $OD_{600} = 0.5$ to standardize the cell concentrations used in the experiments. Each test organism was measured against all bacteriocinogenic strains being evaluated for their safety. Serial dilutions of adjusted bacterial suspensions were plated using respective growth media for determining the initial viable cell population (CFU/mL). The obtained counts are designated as t_0.

Following the same assay for the gastric pass and duodenum pass simulations, all antagonistic set-ups with the test organisms *L. monocytogenes* ATCC15313 and *L. innocua* ATCC33090 were plated in *Listeria* selective medium (RAPID'*L.mono* Medium, BioRad, Hercules, CA, USA). All plates were incubated for 72 h at 37 °C before viable cell counting. Calculations for survival rate were carried out as previously described. All experiments were performed in at least three independent experiments comprising three replicates for each set-up.

2.7. Statistical and data analysis

All quantification was carried out in at least two independent experiments with at least three replicates. The statistical analysis and data visualization were carried out in GraphPad Prism 9.

3. Results and discussions

3.1. Phenotypic evaluation of the safety of previously characterized bacteriocinogenic Enterococcus faecium strains

3.1.1. Production of gelatinase, hemolysin, and biogenic amines

Beneficial LAB have been empirically employed for centuries in the food chain until their existence and functions, including their advantages and disadvantages in the food systems, have gradually been scientifically elucidated. Although several LAB strains are generally recognized as safe (GRAS), some species in this group are considered to be at the crossroads between safety and potential hazards, particularly members of the genus *Enterococcus* (Franz et al., 1999, 2003). The debate on the application of *Enterococcus* spp. in the food chain is on-going for a long time. Even though these bacteria are naturally residing in the gut of humans and animals, relatively higher health risks are associated with this group in comparison with the other members of the LAB. Various putative virulence factors and the production of enzymes and potentially harmful metabolites (e.g., gelatinase, hemolysins, and biogenic amines) should also be considered. Gelatinase, a protease that hydrolyses bioactive peptides, can potentially degrade collagen, hemoglobin, casein, and other bioactive peptides that play a significant role as a defense line for humans and animals during infection (Coque et al., 1995). Additionally, severe cases of endocarditis in murine models and dermal necrosis in rabbit skin were associated with the ability of enterococci to produce hemolysins (Coque et al., 1995; Franz et al., 1999). In this study, the evaluation of *Ent. faecium* strains ST651ea, ST7119ea, and ST7319ea, all of which were isolated from locally produced Korean fermented soybean paste, were evaluated for hemolytic and gelatinase activity with no strain showing positive results as indicated in Table 2, including other virulence factors that may aid the enterococci to evade, invade, and embed in membrane surfaces to successfully cause pathological changes and infections (Coque et al., 1995; Jett et al., 1994). Thus, it is an imperative to evaluate *Enterococcus* spp. strains before any intended application particularly as probiotic strain or starter culture for food and feed industries.

Production of biogenic amines was assessed for all the evaluated strains, showing that no strain produced nitrogenous by-products when supplemented with histidine, lysine, ornithine, or tyrosine (as shown in Table 3). Although biogenic amine (BA) production is not directly associated with the virulence of *Enterococcus* spp., their accumulation from exogenous (food) sources may have toxic effects (BA intoxication i.e., *scombroid poisoning* and tyramine intoxication), when consumed in amounts beyond the safety threshold. According to Smit et al. (2005) and Fernández et al. (2007), some LAB strains are major producers of biogenic amine in fermented food systems. Formerly considered to be strain-specific, Ladero et al. (2012) suggested that this characteristic may be generalized for some species; this has been supported by continuous advancements in the techniques and approaches employed. Among the LAB, *Enterococcus* spp. are known to represent some of the strongest producers of BAs. This group has been particularly associated with the decarboxylation of arginine to putrescine while most strains typically produce tyrosine decarboxylase (of *Ent. faecalis, Ent. faecium,* and *Ent. mundii*) with the formation of tyramine (Bargossi et al., 2015; Fernández et al., 2007; Kučerová et al., 2010; Ladero et al., 2012). Although the BA production of enterococci plays a significant role in the

Table 2
Phenotypic assessment of gelatinase and haemolysin productions of strains *E. faecium* strains ST651ea, ST7119ea, and ST7319ea *in vitro*.

Strains Evaluated	Results
Gelatinase Activity	
E. faecium ST651ea	-
E. faecium ST7119ea	-
E. faecium ST7319ea	-
B. amyloliquefaciens ST109 (positive control)	+
Lb. plantarum ATCC14917 (negative control)	-
Haemolytic Activity	
Ent. faecium ST651ea	-
Ent. faecium ST7119ea	-
Ent. faecium ST7319ea	-
S. pneumoniae ATCC49619 (α-haemolysis control)	+
B. cereus ATCC27348 (β-haemolysis control)	+
Lb. plantarum ATCC14917 (γ-haemolysis control)	+

Table 3
Phenotypic demonstration of biogenic amine decarboxylation of *E. faecium* strains evaluated.

Strain evaluated	Biogenic amine amino acid precursor used			
	Tyrosine	Histidine	Lysine	Ornithine
E. faecium ST651ea	-	-	-	-
E. faecium ST7119ea	-	-	-	-
E. faecium ST7319ea	-	-	-	-
E. coli ATCC25922	+	+	+	+
Lb. plantarum ATCC14917	-	-	-	-

continuous debate on their safety and their involvement in the food chain, the presence and use of *Enterococcus* spp. in fermented food have long been recognized, and their beneficial properties were considered to outweigh this negative aspect. Their specific role is acknowledged in the development of crucial metabolites contributing to desirable sensory characteristics of particular foods such as in cheese ripening and fermentation of meat products and sausages (Smit et al., 2005).

3.1.2. Antimicrobial susceptibility profiling

Antibiotic susceptibility profiles of *Ent. faecium* strains ST651ea, ST7119ea, and ST7319ea (Table 2) were determined against ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, chloramphenicol, and ciprofloxacin through micro-broth dilution and were compared based on the breakpoints from the European Food Safety Authority (EFSA)(2011) as stated in the guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. Results showed (Table 3) that the resistance of all strains evaluated were below the stated breakpoints except erythromycin. It has been noted that most members of enterococci are known to inherently possess resistance to antibiotics especially to ciprofloxacin, penicillin, and erythromycin with occurrence rates of 56.3%, 45.8%, and 27.1%, accordingly (Franz et al., 2003). Thus, it is imperative to identify the antibiogram profile of any candidate strain intended for human and animal use; this is especially important for distinctive antibiotics that may potentially control enterocccal proliferation in aberrant niches. *Ent. faecium* associated infections were previously described to be only associated with <10% of hospital-associated infections worldwide, but the alarming increase in the associated vancomycin-resistant enterococci infections has now reached 30% of nosocomial-acquired infections (Centers of Disease Control and Prevention, 2019). The World Health Organization (WHO) has indeed declared that the search for alternative treatments for VRE-associated infections is now classified as high priority along with AMR pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), clarithromycin-resistant *Helicobacter pylori* (CRHP), and fluoroquinolone-resistant *Salmonella* (WHO, 2018).

3.2. Biomolecular characterization of safety

3.2.1. Screening for the presence of potential virulence genes

The development of rapid techniques to screen the safety of strains has come a long way. The use of various molecular techniques (genomics) has allowed presumptive assessment of the safety of various microorganisms circulating in the food chain. In this study, the presence of various potential virulence factors involved in the attachment, evasion, and translocation was determined by PCR-based assay including genes associated with endocarditis specific proteins (*efaA*), cytolysin (*cyt*), *Enterococcus* pathogenicity island (IS*16*), enterococcal surface protein (*esp*), aggregation substance protein (*asa1*), and hyaluronidase (*hyl*). The detection technique showed that only *Ent. faecium* ST7319ea harbors the genes *hyl, esp,* and IS*16* (Table 4). The screening for these genes was carried out according to the guidelines suggested by the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (Rychen et al., 2017) for the safety assessment of *Ent. faecium* in animal nutrition, whereas it was stated that screening for specific virulence genes IS*16, esp,* and *hyl* should be conducted when all strains are found to be susceptible to the key antibiotics suggested in the guidelines.

Table 4
Antimicrobial susceptibility profiles of the *Enterococcus faecium* strains.

Antibiotics	Experimental Minimum Inhibitory Concentration (μg/mL)			EFSA Cutoff
	Ent. faecium ST651ea	*Ent. faecium* ST7119ea	*Ent. faecium* ST7319ea	
Ampicillin	1	1	1	2
Vancomycin	1	2	1	4
Gentamicin	32	8	16	32
Kanamycin	64	128	256	1024
Streptomycin	64	64	64	128
Erythromycin	64	64	32	4
Clindamycin	0.5	0.5	0.5	4
Tetracycline	0.5	0.5	0.5	4
Chloramphenicol	16	16	16	16
Ciprofloxacin	≤0.25	≤0.25	≤0.25	n.r.

The gene IS*16* (insertion sequence involved in ampicillin resistance), characterized by Leavis et al. (2007) as a novel identifier for the emerging multi-drug resistant strains of *Ent. faecium,* has been a key discriminator for the diversification of *Ent. faecium* into the two-clade subdivision suggested by Palmer et al. (2012), and which was established primarily based on the whole genome sequences of the strains evaluated. In this classification, a subpopulation termed as *Clade A* typically encompasses clinically isolated ampicillin-resistant *Ent. faecium*, while the other subpopulation classified as *Clade B* predominantly includes *Ent. faecium* of both human and animal origin that are susceptible to ampicillin (EFSA, 2011; Leavis et al., 2007; Palmer et al., 2012; Novais et al., 2016).

Another virulence factor associated with one of the largest pathogenicity islands (~ 60 − 100 Kbp) in *Ent. faecium* strains associated with infections (Leavis et al., 2004), the *esp* gene, has been screened for in the evaluated strains; this feature was found to be linked in the ability of members of enterococci to form a biofilm. According to Tendolkar et al. (2004), the enterococcal surface protein enhances biofilm formation by aiding in the initial attachment of planktonic cells on surfaces. In the same study, comparative biofilm formation characterization of both *esp*- and non-*esp*-carrying strains were carried out and showed association of biofilm formation and the presence of *esp* genes could be demonstrated.

According to Rice et al. (2003), the detection of open reading frames of hyaluronidase coding genes (*hyl*) are predominantly characterized in *Ent. faecium* strains isolated from nosocomial settings. Hyaluronidases of Gram-positive bacterial species were found to facilitates initial infection or colonization of pathogenic microorganisms such as *Cl. perfringens, Staph. uberis, Staph. aureus, Str. pyogenes,* and *Str. pneumoniae* (Hynes and Walton, 2000), in which it aids in the breakdown of hyaluronate commonly found in animal skin cells and tissues. The detection of this key potential virulence factor provides an additional level of protection in screening strains that can potentially be introduced into the food chain. Although detection of these genes only signifies possible expression, further characterization of the said virulence genes should be implemented.

3.2.2. Screening for the presence of vancomycin-resistance associated genes

Vancomycin, one of the last resort drugs usually administered against various systemic infections, is a bactericidal antibiotic that acts by interfering in the cell wall synthesis of target microorganisms. The ability of these microorganisms to adapt to the presence of vancomycin has been attributed to modifications in various clusters of genes which are central to the level(s) of resistance of these strains (Faron et al., 2016; Manson et al., 2003). In this study, the PCR-based assay for the detection of vancomycin-resistance (VR) gene clusters in *Ent. faecium* strains ST651ea, ST7119ea, and ST7319ea was carried out for *van A, B, C, D, E,* and *G*. The screening showed that all vancomycin resistance-associated gene clusters were all undetected in the strains

evaluated as indicated in Table 4. The structural genes used were known to be individually or in clustered, responsible for glycopeptide ligase-aided resistance to vancomycin and other related (glycopeptide) antibiotics. Distinct antibiotic resistance characteristics are associated with each cluster; the *van*A operon mediated resistance has been described predominantly for *Ent. faecium* and *Ent. faecalis* with modifications in the terminal peptides of N-acetylmuramic acid (NAM) subunits of the cell wall causing a significant decrease in the affinity for vancomycin; such strains were also found to be phenotypically resistant to teicoplanin– another glycopeptide antibiotic (Faron et al., 2016). The *van*B resistance, on the other hand, causes resistance in variable concentrations of vancomycin (reaching up to ≥250 μg/mL), although this type of resistance is less prevalent than the latter (Coombs et al., 2014; Faron et al., 2016; Walsh et al., 1996). The *van*C-operon mediated resistance is typically known for intrinsic resistance to vancomycin at low levels (2 to 32 μg/mL) relative to the other VR genotypes and is not mobile (chromosomally associated) as compared to the previous two gene clusters (plasmid-associated) (Courvalin, 2006; Reynolds and Courvalin, 2005). Thus, the *vanC*-type of VR resistance is also chromosomally located and should typically be characterized as susceptible but this pathway is rendered malfunctional due to *IS*19 associated insertion in a specific gene in the operon thereby resulting in resistance (Courvalin, 2006; Depardieu et al., 2004). The *vanE*-type, on the other hand, has the same genotypic organization and location as *van*C but differs in the modifications of the target and is typically resistant at levels between 8 and 32 μg/mL of vancomycin (Abadía Patiño et al., 2002; Courvalin, 2006). As for the latter three gene clusters, *van*G has low resistance to vancomycin (16 μg/mL), but is susceptible to teicoplanin (Courvalin, 2006; McKessar et al., 2000; Du et al. 2019). Albeit that the studied strains have been found susceptible to all the screened antibiotics, especially vancomycin, it is still essential to identify the presence of these gene clusters in these strains to assess possible risks for their application as probiotics for both animal and human consumption.

3.3. Assessment of the probiotic/beneficial potential of bacteriocinogenic *Enterococcus faecium* strains

3.3.1. Enzyme production profiling

Enzyme production has been one of the most exploited applications of various beneficial strains in industry. In addition, physiological and functional advantages and properties of the strains are also aided by their capacity to produce enzymes. In this study, the strains were profiled for their ability to produce different enzymes using the APIZym (BioMerieux) assay. Collectively, these *Ent. faecium* strains were shown to produce the following enzymes: esterase (C4), esterase lipase (C8), acid phosphatase, arylamidases (leucine, valine, cysteine), α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, β-galactosidase,) and N-acetyl-B-glycosaminidase (Table 6). These enzymes play various key roles in different industrial applications. According to Ramakrishnan et al. (2012), the production of esterase plays a significant role in the development of flavor, consistency and texture of fermented food products. Additionally, characterization of an intracellular esterase from *Ent. faecium* ACA-CDC 237, isolated from Greek feta cheese, was found to play a synergistic role with lipolytic enzymes for maturation during cheese making (Tsakalidou et al., 1994).

It has been noted that enterococci are dominantly present as compared to the other LAB in artisanal cheeses (51% to 49%), wherein it was observed that the dominant species were *Ent. faecium*. All the strains produced acid phosphatases, an enzyme that is optimally active at pH 5.0, and usually associated with the hydrolysis of most monophosphorylated substrates and phytate, commonly associated with the raw materials used in the fermentation of various products of plant and animal origin (Palacios et al., 2005). Additionally, from a physiological vantage point, the presence of acid phosphatases aids in the adjustment of these microorganisms to stress by sequestering toxic compounds that usually accumulate in an acidic environment during successful fermentation processes. Aside from this, it has been observed that the strains can produce high amounts of various peptidases (leucine, valine, and cysteine arylamidase) that can be advantageous in cheese ripening industries coupled with their low production of proteases as exhibited by the low production of α-chymotrypsin, a ubiquitously available proteolytic enzyme. In their study on the assessment of technological and safety assessment of various beneficial enterococci isolates, Jaouani et al. (2015) suggested this as a desirable trait for good adjunct/starter cultures; this will serve to support cheese ripening and enhance cheese flavor development, also by reduction of bitterness texture improvement the of the product. Furthermore, the presence of these enzymes can also be attributed to the high-protein soybean products from which the strains have been isolated.

Based on positive naphthol-AS-BI-phosphate reactions, all the

Table 5
Molecular-based assay for the detection of adhesion and various virulence genes in strains *Ent. faecium* ST651ea, ST711ea, and ST7319ea.

Genes Evaluated	Strains Evaluated *Ent. faecium* ST651ea	*Ent. faecium* ST7119ea	*Ent. faecium* ST7319ea
Vancomycin-resistance genes			
vanA	-	-	-
vanB	-	-	-
vanC	-	-	-
vanD	-	-	-
vanE	-	-	-
vanG	-	-	-
Adhesion genes			
*EF*1249	-	-	-
*EF*2380	-	-	-
EF2662	-	-	-
EF-Tu	-	-	-
*map*A	-	-	-
mub	-	-	-
*prg*B	-	-	-
Other potential virulence factors			
hyl	-	-	+
esp	-	-	+
*efa*A	-	-	-
asa1	-	-	-
cyt	-	-	-
IS*16*	-	-	+

Table 6
APIZym profiles of the studied *Enterococcus faecium* strains.

Enzyme assayed for	*Ent. faecium* ST651ea	*Ent. faecium* ST7119ea	*Ent. faecium* ST7319ea
Control	0*	0	0
Alkaline phosphatase	0	0	0
Esterase (C 4)	0	3	2
Esterase Lipase (C 8)	1	3	2
Lipase (C 14)	2	2	1
Leucine arylamidase	5	5	4
Valine arylamidase	5	4	3
Cysteine arylamidase	2	4	3
Trypsin	0	0	0
α-chymotrypsin	2	0	0
Acid phosphatase	3	4	3
Naphthol-AS-BI-phosphohydrolase	5	5	5
α-galactosidase	1	0	0
β-galactosidase	1	5	5
ß-glucuronidase	0	0	0
α-glucosidase	0	0	0
β-glucosidase	0	1	0
N-acetyl-ß-glucosaminidase	2	2	2
α-mannosidase	0	0	0
α-fucosidase	0	0	0

* 0 = no acitivity; 1, 2 = weak activity; 3–5 = strong enzymatic activity.

evaluated strains were shown to be producers of hydrolases. This feature was described by Colombo et al. (2018) to be inherent to most LAB; this enzyme supports the antioxidant and anti-inflammatory effects of beneficial strains employed as probiotics in various food products. It was also notably observed that *Ent. faecium* strains ST7119ea and ST7319ea were strong producers of β-galactosidase, the enzyme responsible for the cleavage of lactose to its respective carbohydrate subunits (Saqib et al., 2017). This enzyme has been exploited in the production of commercially available lactose-free fermented milk products.

No evidence of any other enzymes in the evaluation APIZym panel (as shown in Table 6) could be detected for the *Ent. faecium* strains ST651ea, ST7119ea, and ST7319ea.

3.3.2. D/L-lactic acid production

Named after the primary metabolite of carbohydrate metabolism, the LAB produce either the L(+)-or D(-) isomer of lactic acid, or a combination of both isomers. Although L(+)-lactic acid has been known to be majorly produced during fermentation, traces of D(-)-lactic acid may still be produced. Some species such as *Lactobacillus delbrueckii* subsp. *bulgaricus* and all *Leuconostoc* species, typically produce D(-)-lactic acid. High intake of this isomer, especially by infants, has been associated with acidosis; this may play a role in the etiology of some chronic diseases (Vitetta et al., 2017). Thus, the evaluation of the ratio between these two isomers is one of the criteria used for the evaluation of a good probiotic candidate. In this study, the amounts D(-)- and L(+)-lactic acids produced by the strains were: 0.769 g/L and 14.609 g/L for *Ent. faecium* ST651ea, 0.641 g/L and 10.231 g/L for *Ent. faecium* ST7119ea, 1.025 g/L and 16.677 g/L for *Ent. faecium* ST7319ea. These data are in agreement with the values and observations reported by Bhagwat and Annapure (2019), which showed mean total lactic acid produced by various *Enterococcus* spp. strains isolated from humans ranges between 5 and 12 g/L, while Yoshimune et al., (2017), on the other hand, demonstrated that *Enterococcus* spp. majorly produces L(+)-lactic acid in a fermentation system with trace amounts of D(-)-lactic.

3.3.3. Bacterial cell-surface hydrophobicity

Good probiotic candidates are not only screened for their ability to survive in the gut but also for their ability to attach on mucosal surfaces. This physiological aspect was used as a preliminary indicator for their ability to colonize and persist in the gut (Ouwehand et al., 1999; Rosenberg, 1984, 2006). In this study, the cell surface adherence was measured using the BATH assay, whereby hydrophobicity is measured as indication of potential adherence ability. Low levels with values ranging between 14 and 16% have been measured for these strains. Similar observations were also reported by dos Santos et al. (2015) using the same assay for bacteriocinogenic *Ent. faecium* strains EM485 and EM925 isolated from Brazilian cheeses. Although these results may suggest a relatively low adherence potential, this property should be further evaluated in an *ex vivo* model for further characterization of the strains.

3.3.4. Aggregation properties

Auto-aggregation, also referred to as bacterial flocculation or auto-agglutination, is defined as the formation of multicellular clumps of the same type of bacterial cell. This phenomenon has been linked to various bacterial cell functionalities including their ability to adapt to stressful environments and evade host immune responses during infections. Additionally, auto-aggregation has been linked to the initial step of biofilm formation (Angmo et al., 2016; Collado et al., 2008; Trunk et al., 2018). While these characteristics are associated with pathogenic microorganisms, aggregation has also been considered as an advantage to beneficial strains, including probiotics, considering that it predicts possible adhesion properties *in vitro* to the mucosal surfaces, thereby aiding in successful niche colonization (Collado et al., 2008; García-Cayuela et al., 2014). On the other hand, co-aggregation is defined as the ability of genetically distinct bacterial strains to flocculate or clump together. This phenomenon has been associated with the formation of various multispecies biofilms (Rickard et al., 2003), thus making it an imperative property to be evaluated particularly for strains intended for the food chain.

In this study, auto-aggregation and co-aggregation of the three strains of *Ent. faecium*, compared with previously characterized beneficial strains and pathogenic reference strains, were measured *in vitro* (Fig. 1). High levels of mean auto-aggregation (>50%) were exhibited by *Ent. faecium* ST651ea, ST7119ea, and ST7319ea; these were relatively higher compared to the other microorganisms included in the panel including previously characterized probiotic strains and pathogenic strains (Fig. 1). Furthermore, co-aggregation value wear observed to be higher for the evaluated strains partnered with the strains *Lb. sakei* HEM802, *Ent. faecium* HEM1108, and *Lb. fermentum* HEM792, all of which have previously been characterized as beneficial strains.

Although the generalization and correlation of aggregation and cell surface attachment must still be validated further, we can summarize that the evaluated strains, *Ent. faecium* ST651ea, ST7119ea, and ST7319ea, have strong aggregation capabilities and that correlation with auto-aggregation can only be assumed on strain level and variations can be observed within taxonomic clusters (dos Santos et al., 2015; Zommiti et al., 2018). Based on the co-aggregation rate of the strains being studied, low rates (10–15%) for all the strains paired with *L. innocua* and *L. monocytogenes* strains were noted, while high aggregation was observed between *Ent. faecium* ST651ea, ST7119ea, and ST7319ea and *Ent. faecium* VRE19 with rates ranging between 24 and 54% for all strains. However, this high rate of aggregation with a pathogenic microorganism is considered detrimental especially if the pathogen is innately resistant to the bacteriocins produced by the strains being studied (Del Re et al., 2000). The cell-to-cell contact between the producer and target cell (susceptible to the bacteriocins of the producer cell) would increase exposure of the susceptible pathogenic strain to the antimicrobial peptides, thus, from this angle, it may be considered as an advantageous characteristic for the strains evaluated.

3.4. Molecular identification of potentially beneficial metabolites

Gamma-aminobutyric acid (GABA), a ubiquitous and naturally occurring inhibitory neurotransmitter in humans and other animals, aids in the physiological regulation of various body systems including the cardiovascular and the central nervous system. It is a four-carbon molecule that acts as the primary inhibitory neurotransmitter in the brain. GABA production has long been associated with LAB, some of which were used as starter cultures and are commonly found in fermented food products. Some studies have also banked on this property of LAB as an application for various immunological and physiological mediatory functions (Boonstra et al., 2015; Cui et al., 2020). The efficacy of GABA has been attributed to its ability to pass through the blood-brain barrier, and serving as the intermediate molecule between various beneficial strains that have been found to play a significant role in the gut-brain axis (Bajić et al., 2020). According to Cui et al. (2020), several well-characterized GABA producing strains have been isolated from traditional fermented foods such as *kimchi*, yogurt, fermented soybeans, cheeses, among others; wherein representative(s) from the genus *Lactobacillus, Enterococcus, Leuconostoc, Pediococcus,* and *Weissella* was/were GABA producer(s). In this study, the ability of the *Ent. faecium* strains ST651ea, ST7119ea, and ST7319ea, all isolated from artisanal produced fermented soybean paste, were screened for the presence of GABA-production associated genes. The PCR-based screening assay showed that all the strains were positive for the gene assayed, thereby showing the strains to be potential GABA producers. Their ability to express the gene and the physiological ability to produce this compound must be evaluated further.

Another functional property associated with LAB includes folate production. This has been investigated in strains of *Str. thermophilus* (Iyer et al., 2010; Meucci et al., 2018; Tarrah et al., 2018), *Lb. plantarum,*

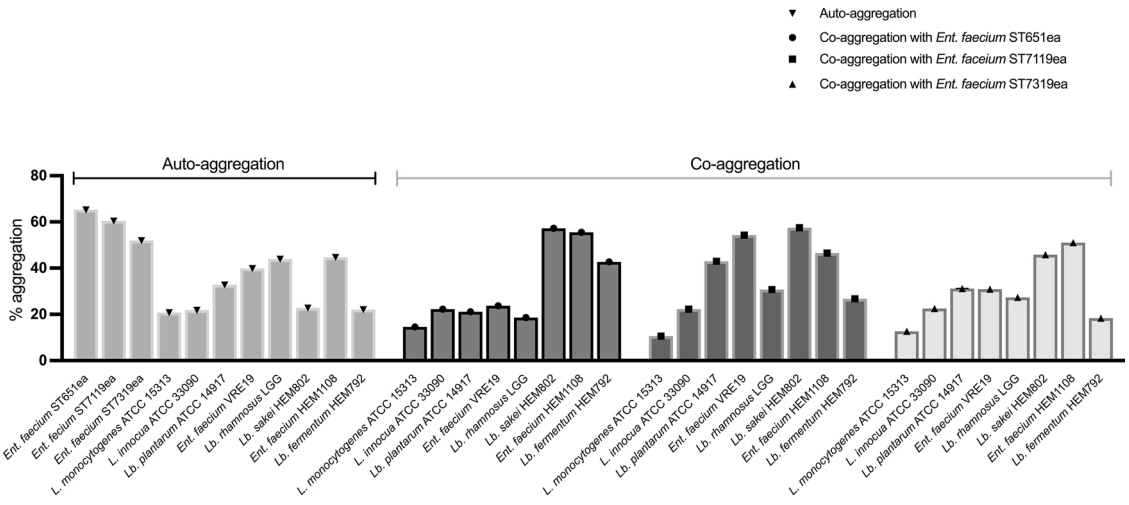

Fig. 1. Rates of aggregation and co-aggregation of *Ent. faecium* ST651ea, *Ent. faecium* ST7119ea, and *Ent. faecium* ST7319ea with their corresponding partner microorganisms.

Bif. adolescentis, and *Bif. pseudocatelinatum* (Rossi et al., 2011). Although results of our screening assay indicated that the strains do not have the complete operon responsible for producing vitamin B12, screening for this beneficial property may reveal an additional benefit. Indeed, some *Ent. faecium* strains have been reported as novel sources of this metabolite (Li et al., 2017). This has also been highlighted by Meucci et al. (2018), also mentioning that the ability of LAB to synthesize this metabolite is highly strain dependent as demonstrated for various strains of *Str. thermophilus, Lb. delbrueckii* subsp. *bulgaricus*, and *Lactococcus* spp.

3.5. Gastrointestinal survival assay Enterococcus faecium strains ST651ea, ST7119ea, and ST7319ea

Enterococcus species are considered as typical members of the lower digestive tract. Their ability to survive in a wide range of environmental pH-values relative to other LAB allows autochthonous enterococci to dominate various niches. One of the criteria that needs to be met to consider a strain as a good probiotic candidate is a high survival under the conditions of the gastrointestinal tract in order to ensure sufficiently high viable dose of the probiotic to reach the target organ as intended for its application. Results showed no significant decrease in population CFU numbers between T0, even after the duodenum simulation passage (T2), as shown in Fig. 2A. This demonstrates high survivability and viability of the evaluated strains.

Similar observations were reported by Amaral et al. (2017) indicating the high tolerance of enterococci to gastrointestinal tract conditions, even in the presence of key active enzymes as demonstrated *in vitro*. It was also stated by Nazzaro et al. (2012) that a high number of cells should be left viable (~log 6 to 7) upon reaching the GIT to facilitate the intended functionality of a probiotic in the host. Although this consideration is of primary importance, the type of vector should also be considered – may it be food products or any other industrial applications – and that may significantly affect their viability. Furthermore, *in vitro* assessment of survivability in the gastrointestinal conditions only provides a prediction of how these strains function in the host system, thus, *in vivo* tests should be conducted to confirm the viability of these strains under practical conditons.

3.6. GIT survival of the test organisms in competition with Enterococcus faecium strains

Aside from high survivability in the GIT, a good probiotic candidate should also be successful in competing and establishing a niche under practical conditions especially in the presence of undesirable members of the gut microbiota. In this study, the same conditions were applied to evaluate the ability of the strains to compete with presentative strains of two known food-contaminant microorganisms, *L. monocytogenes* and *L. innocua.* The survival rates for *L. innocua* ATCC33090 in competition with *Ent. faecium* ST651ea, ST7119ea, and 7319ea were all significant after the gastric simulation passage relative to the controls demonstrated with the rates of 70%, 69%, and 67%, respectively against the control (Fig. 2B). Whereas *L. monocytogenes* ATCC15313 had survival rates after the gastric simulation of 60%, 70%, and 69% when co-incubated in this condition with *Ent. faecium* ST651ea, ST7119ea, and ST7319ea, accordingly (Fig. 2C). Further reduction was observed after duodenum simulation passage for both test organisms with an overall survival rate of around 52 to 53% in all competition set-ups for *L. innocua* ATCC33090, while rates for *L. monocytogenes* ATCC15313 in competition set-ups had the highest decrease observed against *Ent. faecium* ST7119ea and lowest against *Ent. faecium* ST651ea (Fig. 2). These variabilities in the observations can be attributed to the differences among strains (Amaral et al., 2017). Additionally, limitations of the employed methods should also be taken into consideration especially that these observations were made only *in vitro.* Thus, this information, although promising, can only provide possible behavior of the strains when applied to the target hosts and should be evaluated further *in vivo* to assess the ability of the strains to produce their bacteriocins and further assess the extent of efficiency of inhibition by the bacteriocinogenic strains.

4. Conclusions

Previously identified and characterized bacteriocinogenic *Ent. faecium* strains ST651ea, ST7119ea, and ST7319ea showed to be safe according to phenotypic evaluation of various potential virulence factors including production of gelatinase, hemolysin, and biogenic amine production. Furthermore, all the strains were sensitive to key therapeutic antibiotics and did not have genes associated with vancomycin resistance. Yet, strain *Ent. faecium* ST7319ea was found to harbor some of the virulence genes screened including *hyl*, IS*16*, and *esp*, thus, as suggested in the EFSA guidelines on the use of *Enterococcus* spp. as feed additives, this strain may not be considered safe. On the other hand, both safe strains may serve as good adjunct, starter cultures, or probiotic candidates. This is supported by promising enzyme production profiles, high GIT survival and aggregation, and ability to outcompete harmful/undesirable microorganisms such as *L. monocytogenes* and *L. innocua, in vitro.*

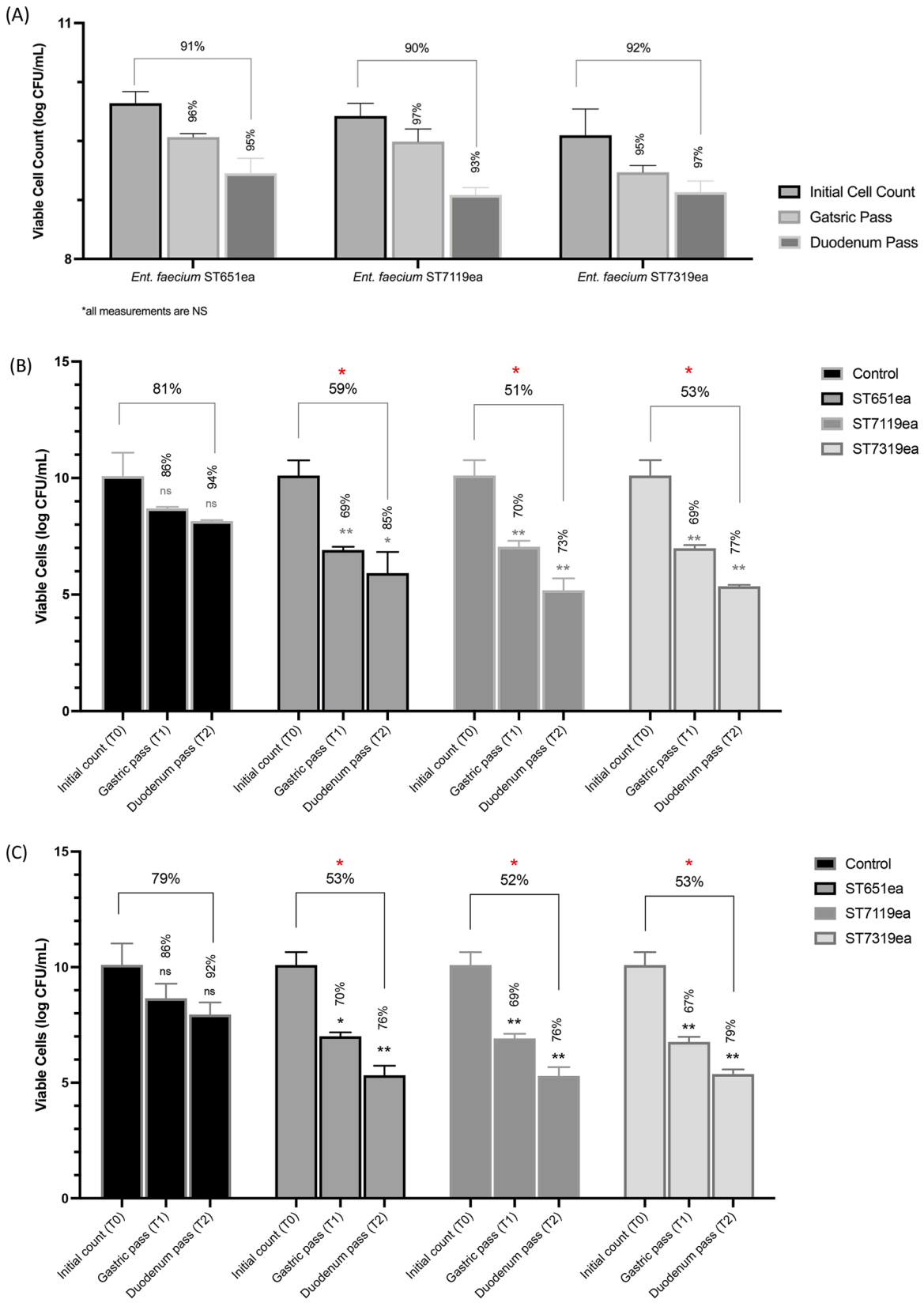

Fig. 2. Survival rates of (A) *Ent. faecium* strains ST651ea, ST7119ea, ST7319ea; and test organisms (B) *L. monocytogenes* ATCC15313 and (C) *L. innocua* ATCC33090 in competition with *Ent. faecium* strains in the gastrointestinal simulation model. Survival rates in comparison with the previous timepoint are indicated on the top of the correspond boxplots. Asterisks above box plots demonstrates statistical significance as determined by Welch's test by comparing the mean log values of each viable bacterial count from the corresponding passage with that of the control. Significance as demonstrated by each asterisk is as follows: $^{*}p \leq 0.05$, $^{**}p \leq 0.001$. Statistical significance for overall passage were quantified by two-way ANOVA and Turkey's test post-hoc analysis of $^{*}p \leq 0.05$.

CRediT authorship contribution statement

Joanna Ivy Irorita Fugaban: Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. **Wilhelm Heinrich Holzapfel:** Writing – review & editing, Funding acquisition. **Svetoslav Dimitrov Todorov:** Formal analysis, Conceptualization, Funding acquisition, Writing – review & editing, Supervision.

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.

References

Abadía Patiño, L., Courvalin, P., Perichon, B., 2002. VanE gene cluster of vancomycin-resistant Enterococcus faecalis BM4405. J. Bacteriol. 184 (23), 6457–6464. https://doi.org/10.1128/jb.184.23.6457-6464.2002.

Agerholm-Larsen, L., Bell, M.L., Grunwald, G.K., Astrup, A., 2000. The effect of a probiotic milk product on plasma cholesterol: a meta-analysis of short-term intervention studies. Eur. J. Clin. Nutr. 54 (11), 856–860. https://doi.org/10.1038/sj.ejcn.1601104.

Angmo, K., Kumari, A., Savitri, T.C.B., 2016. Probiotic characterization of lactic acid bacteria isolated from fermented foods and beverage of Ladakh. LWT Food Sci. Technol. 66, 428–435. https://doi.org/10.1016/j.lwt.2015.10.057.

Amaral, D.M.F., Silva, L.F., Casarotti, S.N., Nascimento, L.C.S., Penna, A.L.B., 2017. Enterococcus faecium and Enterococcus durans isolated from cheese: Survival in the presence of medications under simulated gastrointestinal conditions and adhesion properties. J. Dairy Sci. 100 (2), 933–949. https://doi.org/10.3168/jds.2016-11513.

Bajić, S.S., Đokić, J., Dinić, M., Tomić, S., Popović, N., Brdarić, E., Golić, N., Tolinački, M., 2020. GABA potentiate the immunoregulatory effects of Lactobacillus brevis BGZLS10-17 via ATG5-dependent autophagy *in vitro*. Sci. Rep. 10 (1), 1347. https://doi.org/10.1038/s41598-020-58177-2.

Bargossi, E., Gardini, F., Gatto, V., Montanari, C., Torriani, S., Tabanelli, G., 2015. The capability of tyramine production and correlation between phenotypic and genetic characteristics of Enterococcus faecium and Enterococcus faecalis strains. Front. Microbiol. 6 https://doi.org/10.3389/fmicb.2015.01371.

Bellomo, G., Mangiagli, A., Nicastro, L., Frigerio, G., 1980. A controlled double-blind study of SF68 strain as a new biological preparation for the treatment of diarrhoea in pediatrics. Curr. Ther. Res. 28, 927–936 http://doi.org/10.101/0168-1605(95)00029-j.

Bhagwat, A., Annapure, U.S., 2019. *In vitro* assessment of metabolic profile of Enterococcus strains of human origin. J. Genet. Eng. Biotechnol. 17, 11. https://doi.org/10.1186/s43141-019-0009-0.

Boonstra, E., de Kleijn, R., Colzato, L.S., Alkemade, A., Forstmann, B.U., Nieuwenhuis, S., 2015. Neurotransmitters as food supplements: the effects of GABA on brain and behavior. Front. Psychol. 6 https://doi.org/10.3389/fpsyg.2015.01520.

Bover-Cid, S., Holzapfel, W.H., 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. Int. J. Food Microbiol. 53 (1), 33–41. https://doi.org/10.1016/s0168-1605(99)00152-x, 9. h 1999 Dec 1.

Castilho, N.P.A., Colombo, M., Oliveira, L.L., Todorov, S.D., Nero, L.A., 2019. Lactobacillus curvatus UFV-NPAC1 and other lactic acid bacteria isolated from calabresa, a fermented meat product, present high bacteriocinogenic activity against Listeria monocytogenes. BMC Microbiol. 19 (1), 63. https://doi.org/10.1186/s12866-019-1436-4.

Centeno, J.A., Menéndez, S., Rodríguez-Otero, J.L., 1996. Main microbial flora present as natural starters in Cebreiro raw cow's-milk cheese (Northwest Spain). Int. J. Food Microbiol. 33 (2), 307–313. https://doi.org/10.1016/0168-1605(96)01165-8.

Centers of Disease and Control (CDC). (2019). Antibiotic resistance threats and reports. Extracted from https://www.cdc.gov/DrugResistance/Biggest-Threats.html, accessed: October, 2020.

Clinical and Laboratory Standards Institute (CLSI), 2012. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—9th Edition. Clinical and Laboratory Standards Institute, Wayne, PA. CLSI document M07-A9.

Collado, M.C., Meriluoto, J., Salminen, S., 2008. Adhesion and aggregation properties of probiotic and pathogen strains. Eur. Food Res. Technol. 226 (5), 1065–1073. https://doi.org/10.1007/s00217-007-0632-x.

Colombo, M., Castilho, N.P.A., Todorov, S.D., Nero, L.A., 2018. Beneficial properties of lactic acid bacteria naturally present in dairy production. BMC Microbiol. 18 (1), 219. https://doi.org/10.1186/s12866-018-1356-8.

Coombs, G.W., Pearson, J.C., Daley, D.A., Le, T., Robinson, O.J., Gottlieb, T., Howden, B. P., Johnson, P.D.R., Bennett, C.M., Stinear, T.P., Turnidge, J.D., 2014. Molecular epidemiology of enterococcal bacteremia in Australia. J. Clin. Microbiol. 52 (3), 897–905. https://doi.org/10.1128/JCM.03286-13.

Coque, T.M., Patterson, J.E., Steckelberg, J.M., Murray, B.E., 1995. Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from feces of hospitalized and community-based persons. J. Infect. Dis. 171 (5), 1223–1229. https://doi.org/10.1093/infdis/171.5.1223.

Courvalin, P., 2006. Vancomycin resistance in gram-positive cocci. Clin. Infect. Dis. 42, S25–S34.. https://doi.org/10.1086/491711. Supplement_1.

Cui, Y., Miao, K., Niyaphorn, S., Qu, X., 2020. Production of gamma-aminobutyric acid from lactic acid bacteria: a systematic review. Int. J. Mol. Sci. 21 (3), 995. https://doi.org/10.3390/ijms21030995.

Del Re, B., Sgorbati, B., Miglioli, M., Palenzona, D., 2000. Adhesion, autoaggregation and hydrophobicity of 13 strains of Bifidobacterium longum. Lett. Appl. Microbiol. 31 (6), 438–442. https://doi.org/10.1046/j.1365-2672.2000.00845.x.

Depardieu, F., Perichon, B., Courvalin, P., 2004. Detection of the van alphabet and identification of enterococci and staphylococci at the species level by multiplex PCR. J. Clin. Microbiol. 42 (12), 5857–5860. https://doi.org/10.1128/JCM.42.12.5857-5860.2004.

De Vuyst, L., Foulquié Moreno, M.R., Revets, H., 2003. Screening for enterocins and detection of hemolysin and vancomycin resistance in enterococci of different origins. Int. J. Food Microbiol. 84 (3), 299–318. https://doi.org/10.1016/s0168-1605(02)00425-7.

dos Santos, K.M.O., Vieira, A.D.S., Salles, H.O., Oliveira, J., da, S., Rocha, C.R.C., Borges, M., de, F., Bruno, L.M., Franco, B.D.G.M., Todorov, S.D., 2015. Safety, beneficial and technological properties of Enterococcus faecium isolated from Brazilian cheeses. Braz. J. Microbiol. 46 (1), 237–249. https://doi.org/10.1590/S1517-838246120131245.

dos Santos, K.M.O., de Matos, C.R., Salles, H.O., Franco, B.D.G.M., 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.

Du, F., Lv, X., Duan, D., Wang, L., Huang, J., 2019. Characterization of a linezolid- and vancomycin-resistant streptococcus suis isolate that harbors optrA and vanG operons. Front. Microbiol. 10 http://doi.org/10.3389/fmicb.2019.02026.

Rychen, G., Aquilina, G., Azimonti, G., Bampidis, V., Bastos, M., de, L., Bories, G., Chesson, A., Cocconcelli, P.S., Flachowsky, G., Gropp, J., Kolar, B., Kouba, M., López-Alonso, M., López Puente, S., Mantovani, A., Mayo, B., Ramos, F., Saarela, M., de Villa, R., Wallace, R., Wester, P., Anguita, M., Galobart, J., Innocenti, M., Martino, L., EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2017. Guidance on the assessment of the safety of feed additives for the target species. EFSA J. 15 (10) https://doi.org/10.2903/j.efsa.2017.5021.

Emmanuel, D.G.V., Jafari, A., Beauchemin, K.A., Leedle, J.A.Z., Ametaj, B.N., 2007. Feeding live cultures of Enterococcus faecium and Saccharomyces cerevisiae induces an inflammatory response in feedlot steers. J. Anim. Sci. 85 (1), 233–239. https://doi.org/10.2527/jas.2006-216.

European Food Safety Authority (EFSA), 2011. EFSA approaches to risk assessment in the area of antimicrobial resistance. EFSA Suppor. Publ. 8 (10) https://doi.org/10.2903/j.efsa.2011.196.

European Food Safety Authority (EFSA), 2011. Guidance on the safety assessment of Enterococcus faecium in animal nutrition. EFSA J. 10 (5), 2682. https://doi.org/10.2903/j.efsa.2012.2682.

Fan, Y., Chen, S., Yu, Y., Si, J., Liu, B., 2006. A probiotic treatment containing Lactobacillus, Bifidobacterium and Enterococcus improves IBS symptoms in an open label trial. J. Zhejiang Univ. Science. B 7 (12), 987–991. https://doi.org/10.1631/jzus.2006.B0987.

Faron, M.L., Ledeboer, N.A., Buchan, B.W., 2016. Resistance mechanisms, epidemiology, and approaches to screening for vancomycin-resistant Enterococcus in the health care setting. J. Clin. Microbiol. 54 (10), 2436–2447. https://doi.org/10.1128/JCM.00211-16.

Fernández, M., Linares, D.M., Rodríguez, A., Alvarez, M.A., 2007. Factors affecting tyramine production in Enterococcus durans IPLA 655. Appl. Microbiol. Biotechnol. 73 (6), 1400–1406. https://doi.org/10.1007/s00253-006-0596-y.

Foulquié Moreno, M.R., Sarantinopoulos, P., Tsakalidou, E., De Vuyst, L., 2006. The role and application of enterococci in food and health. Int. J. Food Microbiol. 106 (1), 1–24. https://doi.org/10.1016/j.ijfoodmicro.2005.06.026.

Franz, C., 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.

Franz, C.M.A.P., Holzapfel, W.H., Stiles, M.E., 1999. Enterococci at the crossroads of food safety? Int. J. Food Microbiol. 47 (1–2), 1–24. https://doi.org/10.1016/S0168-1605(99)00007-0.

Franz, C.M.A.P., Cho, G.S., Holzapfel, W.H., Gálvez, A., 2010. Safety of lactic acid bacteria. In: Mozzi, F., Raya, R., Vignolo. (Eds.), Biotechnology of Lactic Acid Bacteria. John Wiley & Sons, Ltd, pp. 341–359. https://doi.org/10.1002/9780813820866.ch19.

Franz, C.M.A.P., Huch, M., Abriouel, H., Holzapfel, W., Gálvez, A., 2011. Enterococci as probiotics and their implications in food safety. Int. J. Food Microbiol. 151 (2), 125–140. https://doi.org/10.1016/j.ijfoodmicro.2011.08.014.

Fugaban, J.I.I., Vazquez Bucheli, J.E., Holzapfel, W.H., Todorov, S.D., 2021a. Characterization of partially purified bacteriocins produced by Enterococcus faecium strains isolated from soybean paste active against Listeria spp. and vancomycin-resistant enterococci. Microorganisms 9 (5), 1085. https://doi.org/10.3390/microorganisms9051085.

Fugaban, J.I.I., Vazquez Bucheli, J.E., Holzapfel, W.H., Todorov, S.D., 2021b. Bacteriocinogenic Bacillus spp. isolated from Korean fermented cabbage (Kimchi)—beneficial or hazardous? Fermentation 7 (2), 56. https://doi.org/10.3390/fermentation7020056.

García-Cayuela, T., Korany, A.M., Bustos, I., Gómez de Cadiñanos, P., Requena, L., Peláez, T., C., Martínez-Cuesta, M.C, 2014. Adhesion abilities of dairy Lactobacillus plantarum strains showing an aggregation phenotype. Food Res. Int. 57, 44–50. https://doi.org/10.1016/j.foodres.2014.01.010.

Giraffa, G., 2002. Enterococci from foods. FEMS Microbiol. Rev. 26 (2), 163–171. https://doi.org/10.1111/j.1574-6976.2002.tb00608.x.
Ghorbani, G.R., Morgavi, D.P., Beauchemin, K.A., Leedle, J.A.Z., 2002. Effects of bacterial direct-fed microbials on ruminal fermentation, blood variables, and the microbial populations of feedlot cattle. J. Anim. Sci. 80 (7), 1977–1985. https://doi.org/10.2527/2002.8071977x.
Gomes, B.C., Esteves, C.T., Palazzo, I.C.V., Darini, A.L.C., Felis, G.E., Sechi, L.A., Franco, B.D.G.M., De Martinis, E.C.P., 2008. Prevalence and characterization of Enterococcus spp. isolated from Brazilian foods. Food Microbiol. 25 (5), 668–675. https://doi.org/10.1016/j.fm.2008.03.008.
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.
Hynes, W.L., Walton, S.L., 2000. Hyaluronidases of gram-positive bacteria. FEMS Microbiol. Lett. 183 (2), 201–207. https://doi.org/10.1111/j.1574-6968.2000.tb08958.x.
Iyer, R., Tomar, S.K., Kapila, S., Mani, J., Singh, R., 2010. Probiotic properties of folate producing Streptococcus thermophilus strains. Food Res. Int. 43 (1), 103–110. https://doi.org/10.1016/j.foodres.2009.09.011.
Jaouani, I., Abbassi, M.S., Ribeiro, S.C., Khemiri, M., Mansouri, R., Messadi, L., Silva, C. C.G., 2015. Safety and technological properties of bacteriocinogenic enterococci isolates from Tunisia. J. Appl. Microbiol. 119 (4), 1089–1100. https://doi.org/10.1111/jam.12916.
Jett, B.D., Huycke, M.M., Gilmore, M.S., 1994. Virulence of enterococci. Clin. Microbiol. Rev. 7 (4), 462–478. https://doi.org/10.1128/cmr.7.4.462.
Johnston, L.M., Jaykus, L.A., 2004. Antimicrobial resistance of Enterococcus species isolated from produce. Appl. Environ. Microbiol. 70 (5), 3133–3137. https://doi.org/10.1128/AEM.70.5.3133-3137.2004.
Kučerová, K., Svobodová, H., Tůma, Š., Ondráčková, I., Plocková, M., 2010. Production of biogenic amines by enterococci. Czech J. Food Sci. 27 (Special Issue 2), 50–55. https://doi.org/10.17221/673-CJFS.
Ladero, V., Fernández, M., Calles-Enríquez, M., Sánchez-Llana, E., Cañedo, E., Martín, M. C., Alvarez, M.A., 2012. Is the production of the biogenic amines tyramine and putrescine a species-level trait in enterococci? Food Microbiol. 30 (1), 132–138. https://doi.org/10.1016/j.fm.2011.12.016.
Leavis, H., Top, J., Shankar, N., Borgen, K., Bonten, M., van Embden, J., Willems, R.J.L., 2004. A novel putative enterococcal pathogenicity island linked to the esp virulence gene of Enterococcus faecium and associated with epidemicity. J. Bacteriol. 186 (3), 672–682. https://doi.org/10.1128/JB.186.3.672-682.2004.
Leavis, H.L., Willems, R.J.L., Wamel, W.J.B., Schuren, F.H., Caspers, M.P.M., Bonten, M. J.M., 2007. Insertion sequence–driven diversification creates a globally dispersed emerging multiresistant subspecies of E. faecium. PLoS Pathog. 3 (1), e7. https://doi.org/10.1371/journal.ppat.0030007.
Lebreton, F., Valentino, M.D., Schaufler, K., Earl, A.M., Cattoir, V., Gilmore, M.S., 2018. Transferable vancomycin resistance in clade B commensal-type Enterococcus faecium. J. Antimicrob. Chemother. 73 (6), 1479–1486. https://doi.org/10.1093/jac/dky039.
Li, P., Gu, Q., Wang, Y., Yu, Y., Yang, L., Chen, J.V., 2017. Novel vitamin B12-producing Enterococcus spp. and preliminary *in vitro* evaluation of probiotic potentials. Appl. Microbiol. Biotechnol. 101 (15), 6155–6164. https://doi.org/10.1007/s00253-017-8373-7.
Manson, J.M., Keis, S., Smith, J.M.B., Cook, G.M., 2003. A clonal lineage of VanA-type enterococcus faecalis predominates in vancomycin-resistant Enterococci isolated in New Zealand. Antimicrob. Agents Chemother. 47 (1), 204–210. https://doi.org/10.1128/aac.47.1.204-210.2003.
Martín-Platero, A.M., Valdivia, E., Maqueda, M., Martínez-Bueno, M., 2009. Characterization and safety evaluation of enterococci isolated from Spanish goats' milk cheeses. Int. J. Food Microbiol. 132 (1), 24–32. https://doi.org/10.1016/j.ijfoodmicro.2009.03.010.
McKessar, S.J., Berry, A.M., Bell, J.M., Turnidge, J.D., Paton, J.C., 2000. Genetic characterization of vanG, a novel vancomycin resistance locus of Enterococcus faecalis. Antimicrob. Agents Chemother. 44 (11), 3224–3228. https://doi.org/10.1128/AAC.44.11.3224-3228.2000.
Meucci, A., Rossetti, L., Zago, M., Monti, L., Giraffa, G., Carminati, D., Tidona, F., 2018. Folates biosynthesis by Streptococcus thermophilus during growth in milk. Food Microbiol. 69, 116–122. https://doi.org/10.1016/j.fm.2017.08.001.
Mountzouris, K.C., Tsirtsikos, P., Kalamara, E., Nitsch, S., Schatzmayr, G., Fegeros, K., 2007. Evaluation of the efficacy of a probiotic containing Lactobacillus, Bifidobacterium, Enterococcus, and Pediococcus strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. Poult. Sci. 86 (2), 309–317. https://doi.org/10.1093/ps/86.2.309.
Nazzaro, F., Fratianni, F., Nicolaus, B., Poli, A., Orlando, P., 2012. The prebiotic source influences the growth, biochemical features, and survival under simulated gastrointestinal conditions of the probiotic Lactobacillus acidophilus. Anaerobe 18 (3), 280–285. https://doi.org/10.1016/j.anaerobe.2012.03.002.
Nocek, J.E., Kautz, W.P., Leedle, J.A.Z., Allman, J.G., 2002. Ruminal supplementation of direct-fed microbials on diurnal pH variation and *in situ* digestion in dairy cattle. J. Dairy Sci. 85 (2), 429–433. https://doi.org/10.3168/jds.S0022-0302(02)74091-5.
Novais, C., Tedim, A.P., Lanza, V.F., Freitas, A.R., Silveira, E., Escada, R., Roberts, A.P., Al-Haroni, M., Baquero, F., Peixe, L., Coque, T.M., 2016. Co-diversification of Enterococcus faecium core genomes and PBP5: evidences of pbp5 horizontal transfer. Front. Microbiol. 7, 1581. https://doi.org/10.3389/fmicb.2016.01581.
Omar, N.B., Castro, A., Lucas, R., Abriouel, H., Yousif, N.M.K., Franz, C.M.A.P., Holzapfel, W.H., Rubén, P.P., Martínez-Canãmero, M., Gálvez, A., 2004. Functional and safety aspects of Enterococci isolated from different Spanish foods. Syst. Appl. Microbiol. 27 (1), 118–130. https://doi.org/10.1078/0723-2020-00248.
Ouwehand, A.C., Kirjavainen, P.V., Grönlund, M.M., Isolauri, E., Salminen, S.J., 1999. Adhesion of probiotic micro-organisms to intestinal mucus. Int. Dairy J. 9 (9), 623–630. https://doi.org/10.1016/S0958-6946(99)00132-6.
Palacios, M.C., Haros, M., Rosell, C.M., Sanz, Y., 2005. Characterization of an acid phosphatase from Lactobacillus pentosus: regulation and biochemical properties. J. Appl. Microbiol. 98 (1), 229–237. https://doi.org/10.1111/j.1365-2672.2004.02447.x.
Palmer, K.L., Godfrey, P., Griggs, A., Kos, V.N., Zucker, J., Desjardins, C., Cerqueira, G., Gevers, D., Walker, S., Wortman, J., Feldgarden, M., Haas, B., Birren, B., Gilmore, M. S., 2012. Comparative genomics of Enterococci: variation in Enterococcus faecalis, clade structure in E. faecium, and defining characteristics of E. gallinarum and E. casseliflavus. MBio 3 (1). https://doi.org/10.1128/mBio.00318-11.
Pollmann, M., Nordhoff, M., Pospischil, A., Tedin, K., Wieler, L.H., 2005. Effects of a probiotic strain of Enterococcus faecium on the rate of natural chlamydia infection in swine. Infect. Immun. 73 (7), 4346–4353. https://doi.org/10.1128/IAI.73.7.4346-4353.2005.
Ramakrishnan, V., Balakrishnan, B., Rai, A.K., Narayan, B., Halami, P.M., 2012. Concomitant production of lipase, protease and enterocin by Enterococcus faecium NCIM5363 and Enterococcus durans NCIM5427 isolated from fish processing waste. Int. Aquat. Res. 4 (1), 14. https://doi.org/10.1186/2008-6970-4-14.
Reynolds, P.E., Courvalin, P., 2005. Vancomycin resistance in Enterococci due to synthesis of precursors terminating in d-alanyl-d-serine. Antimicrob. Agents Chemother. 49 (1), 21–25. https://doi.org/10.1128/AAC.49.1.21-25.2005.
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, hylEfm, predominates in Enterococcus faecium of clinical origin. J. Infect. Dis. 187 (3), 508–512. https://doi.org/10.1086/367711.
Rickard, A.H., Gilbert, P., High, N.J., Kolenbrander, P.E., Handley, P.S., 2003. Bacterial coaggregation: an integral process in the development of multi-species biofilms. Trends Microbiol. 11 (2), 94–100. https://doi.org/10.1016/s0966-842x(02)00034-3.
Rosenberg, M., 1984. Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. FEMS Microbiol. Lett. 22 (3), 289–295. https://doi.org/10.1111/j.1574-6968.1984.tb00743.x.
Rosenberg, M., 2006. Microbial adhesion to hydrocarbons: twenty-five years of doing MATH. FEMS Microbiol. Lett. 262 (2), 129–134. https://doi.org/10.1111/j.1574-6968.2006.00291.x.
Rossi, M., Amaretti, A., Raimondi, S., 2011. Folate production by probiotic bacteria. Nutrients 3 (1), 118–134. https://doi.org/10.3390/nu3010118.
Saqib, S., Akram, A., Halim, S.A., Tassaduq, R., 2017. Sources of β-galactosidase and its applications in food industry. 3 Biotech 7 (1). https://doi.org/10.1007/s13205-017-0645-5.
Smit, G., Smit, B.A., Engels, W.J.M., 2005. Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. FEMS Microbiol. Rev. 29 (3), 591–610. https://doi.org/10.1016/j.femsre.2005.04.002.
Taras, D., Vahjen, W., Macha, M., Simon, O., 2006. Performance, diarrhea incidence, and occurrence of Escherichia coli virulence genes during long-term administration of a probiotic Enterococcus faecium strain to sows and piglets. J. Anim. Sci. 84 (3), 608–617. https://doi.org/10.2527/2006.843608x.
Tarrah, A., Castilhos, J., Rossi, R.C., Duarte, V., da, S., Ziegler, D.R., Corich, V., Giacomini, A., 2018. *In vitro* probiotic potential and anti-cancer activity of newly isolated folate-producing streptococcus thermophilus strains. Front. Microbiol. 9 https://doi.org/10.3389/fmicb.2018.02214.
Tendolkar, P.M., Baghdayan, A.S., Gilmore, M.S., Shankar, N., 2004. Enterococcal surface protein, esp, enhances biofilm formation by Enterococcus faecalis. Infect. Immun. 72 (10), 6032–6039. https://doi.org/10.1128/IAI.72.10.6032-6039.2004.
Todorov, S.D., Wachsman, M., Tomé, E., Dousset, X., Destro, M.T., Dicks, L.M.T., Franco, B.D.G.M., Vaz-Velho, M., Drider, D., 2010. Characterisation of an antiviral pediocin-like bacteriocin produced by Enterococcus faecium. Food Microbiol. 27 (7), 869–879. https://doi.org/10.1016/j.fm.2010.05.001.
Trunk, T., Khalil, H.S., Leo, J.C., 2018. Bacterial auto aggregation. AIMS Microbiol. 4 (1), 140–164. https://doi.org/10.3934/microbiol.2018.1.140.
Tsakalidou, E., Dalezios, I., Kalantzopoulos, G., 1994. Isolation and partial characterization of an intracellular esterase from Enterococcus faecium ACA-DC 237. J. Biotechnol. 37 (3), 201–208. https://doi.org/10.1016/0168-1656(94)90127-9.
Vahjen, W., Männer, K., 2003. The effect of a probiotic Enterococcus faecium product in diets of healthy dogs on bacteriological counts of Salmonella spp., Campylobacter spp. and Clostridium spp. in faeces. Arch. Tierernahr. 57 (3), 229–233. https://doi.org/10.1080/0003942031000136657.
Vankerckhoven, V., Van Autgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R., Jabes, D., Goossens, H., 2004. Development of a multiplex PCR for the detection of asa1, gelE, cylA, esp, and hyl genes in Enterococci and survey for virulence determinants among European hospital isolates of Enterococcus faecium. J. Clin. Microbiol. 42 (10), 4473–4479. https://doi.org/10.1128/JCM.42.10.4473-4479.2004.
Vizoso Pinto, M.G., Franz, C.M.A.P., Schillinger, U., Holzapfel, W.H., 2006. Lactobacillus spp. with *in vitro* probiotic properties from human faeces and traditional fermented products. Int. J. Food Microbiol. 109 (3), 205–214. https://doi.org/10.1016/j.ijfoodmicro.2006.01.029.
Vitetta, L., Coulson, S., Thomsen, M., Nguyen, T., Hall, S., 2017. Probiotics, D–Lactic acidosis, oxidative stress and strain specificity. Gut Microbes 8 (4), 311–322. https://doi.org/10.1080/19490976.2017.1279379.
Walsh, C.T., Fisher, S.L., Park, I.S., Prahalad, M., Wu, Z., 1996. Bacterial resistance to vancomycin: five genes and one missing hydrogen bond tell the story. Chem. Biol. 3 (1), 21–28. https://doi.org/10.1016/S1074-5521(96)90079-4.

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, 80. https://doi.org/10.1186/1471-2334-11-80.

Wiegand, I., Hilpert, K., Hancock, R.E.W., 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat. Protoc. 3 (2), 163–175. https://doi.org/10.1038/nprot.2007.521.

World Health Organization, 2018. Antimicrobial Resistance and Primary Health Care. World Health Organization. https://apps.who.int/iris/handle/10665/326454. License: CC BY-NC-SA 3.0 IGO.

Wunderlich, P.F., Braun, L., Fumagalli, I., D'Apuzzo, V., Heim, F., Karly, M., Lodi, R., Politta, G., Vonbank, F., Zeltner, L., 1989. Double-blind report on the efficacy of lactic acid-producing Enterococcus SF68 in the prevention of antibiotic-associated diarrhoea and in the treatment of acute diarrhoea. J. Int. Med. Res. 17 (4), 333–338. https://doi.org/10.1177/030006058901700405.

Yoshimune, K., Yamamoto, M., AoyagIi, T., Yumoto, I., 2017. High and rapid L-lactic acid production by Alkaliphilic Enterococcus sp. by adding wheat bran hydrolysate. Ferment. Technol. 6, 138. https://doi.org/10.4172/2167-7972.1000138.

Zommiti, M., Cambronel, M., Maillot, O., Barreau, M., Sebei, K., Feuilloley, M., Ferchichi, M., Connil, N., 2018. Evaluation of probiotic properties and safety of Enterococcus faecium isolated from artisanal tunisian meat "dried ossban. Front. Microbiol. 9 https://doi.org/10.3389/fmicb.2018.01685.