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Whole genome sequence analyses-based assessment of virulence potential and antimicrobial susceptibilities and resistance of *Enterococcus faecium* strains isolated from commercial swine and cattle probiotic products

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Abstract

Enterococcus faecium is one of the more commonly used bacterial species as a probiotic in animals. The organism, a common inhabitant of the gut of animals and humans, is a major nosocomial pathogen responsible for a variety infections in humans and sporadic infections in animals. In swine and cattle, *E. faecium*-based probiotic products are used for growth promotion and gut functional and health benefits. The objective of this study was to utilize whole genome sequence-based analysis to assess virulence potential, detect antimicrobial resistance genes, and analyze phylogenetic relationships of *E. faecium* strains from commercial swine and cattle probiotics. Genomic DNA extracted from *E. faecium* strains, isolated from commercial probiotic products of swine ($n = 9$) and cattle ($n = 13$), were sequenced in an Illumina MiSeq platform and analyzed. Seven of the nine swine strains and seven of the 13 cattle strains were identified as *Enterococcus lactis,* and not as *E. faecium.* None of the 22 probiotic strains carried major virulence genes required to initiate infections, but many carried genes involved in adhesion to host cells, which may benefit the probiotic strains to colonize and persist in the gut. Strains also carried genes encoding resistance to a few medically important antibiotics, which included aminoglycosides [*aac(6′)-Ii, aph(3′)-III, ant(6)-Ia*], macrolide, lincosamide and streptogramin B (*msrC*), tetracyclines [*tet*(L) *and tet*(M)], and phenicols [*cat-(pc194)*]. The comparison of the genotypic to phentypic AMR data showed presence of both related and unrelated genes in the probiotic strains. Swine and cattle probiotic *E. faecium* strains belonged to diverse sequence types. Phylogenetic analysis of the probiotic strains, and strains of human ($n = 29$), swine ($n = 4$), and cattle ($n = 4$) origin, downloaded from GenBank, indicated close clustering of strains belonging to the same species and source, but a few swine and cattle probiotic strains clustered closely with other cattle and human fecal strains. In conclusion, the absence of major virulence genes characteristic of the clinical *E. faecium* strains suggests that these probiotic strains are unlikely to initiate opportunistic infection. However, the carriage of AMR genes to medically important antibiotics and close clustering of the probiotic strains with other human and cattle fecal strains suggests that probiotic strains may pose risk to serve as a source of transmitting AMR genes to other gut bacteria.

Lay Summary

Probiotics, also called direct-fed microbials, are widely used in swine and cattle production systems, as an alternative for antibiotics. The benefits of feeding probiotic products include growth promotion and gut functional benefits. One of the more common bacterial species used in swine and cattle commercial probiotic products is *Enterococcus faecium*. The species is also a member of the normal flora of hindgut of humans and animals. In recent years, the species has emerged as a major hospital-acquired infection in humans, mainly because of the propensity to become resistant to antibiotics. In the United States, the species is considered as generally recognized as safe. In this study, the virulence and antimicrobial resistance genes profiles of 9 and 13 *E. faecium* strains isolated from commercial swine and cattle probiotics, respectively, were assessed by sequencing the whole genome DNA. The analysis indicated that 14 of 22 strains were *Enterococcus lactis,* and not *E. faecium*. The absence of major virulence genes characteristic of the clinical *E. faecium* strains suggests that the strains are unlikely to initiate opportunistic infection. However, the carriage of genes that confer resistance to medically important antibiotics suggests that probiotic strains may pose risk as a source of antimicrobial resistance genes to other bacteria.

Key words: antimicrobial resistance genes, cattle, *Enterococcus faecium*, probiotics, swine, virulence genes, whole genome sequencing

Abbreviations: AMR, antimicrobial resistance;CC, clonal complex;DNA, deoxyribonucleic acid;EFSA, European Food Safety Authority;FAO-WHO, Food and Agriculture Organization-World Health Organization;FDA, Food and Drug Administration;GRAS, Generally Recognized as Safe;LB, Luria Bertani;MLST, multilocus sequence typing;NCBI, National Center for Biotechnology Information;PFGE, pulsed-field gel electrophoresis;RAST, Rapid Annotation Using Subsystem Technology;rRNA, ribosomal ribonucleic acid;ST, sequence type;VFDB, virulence factor database;WGS, whole genome sequencing

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Introduction

Enterococcus faecium, a lactic acid producer, is one of the more widely used bacterial species, next only to *Lactobacillus acidophilus*, in the commercial probiotic products of swine and cattle. The reported benefits of feeding *E. faecium*-based probiotic products include growth promotion and other specific gut functional and health benefits (Franz et al., 2011). In pigs, the gut functional benefits include increased intestinal transport and gut barrier functions and competitive exclusion of pathogens, resulting in reduced neonatal and post-weaning diarrhea in piglets (Pollmann et al., 2005; Scharek et al., 2005; Lodemann et al., 2006; Taras et al., 2006). Feeding of *E. faecium*-based probiotics has shown to increase milk production during early lactation in dairy cows (Nocek et al., 2003) and control of diarrhea in milk-fed calves (Masucci et al., 2011). The milk production response is attributed to favorable alterations in ruminal fermentation, such as stimulation of ruminal bacterial activities, specifically of lactate-utilizing bacteria resulting in reduced risk of ruminal subacute acidosis, and reduced methanogens and methane production (Ghorbani et al., 2002; Nocek et al., 2002; Masucci et al., 2011; Pang et al., 2014; Mamuad et al., 2019).

Enterococcus faecium and other enterococcal species are hindgut commensals of humans and animals; however, *E. faecium* has emerged as one of the major nosocomial pathogens in humans (Lee et al., 2019). It is one among the group referred to as "ESKAPE" pathogens (*E. faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), which have the propensity to become resistant to antimicrobial drugs and are responsible for the majority of the nosocomial human infections (Rice, 2008). The *E. faecium* is responsible for the majority of the *Enterococcus* health care-associated infections resistant to vancomycin, a critically important antibiotic used to treat a variety of severe Gram-positive bacterial infections in humans (Lee et al., 2019).

Testing for antimicrobial resistance and virulence characteristics of the bacterial probiotic is one of the guidelines implemented by Food and Agriculture Organization-World Health Organization in order to claim a product as probiotic (FAO/WHO, 2002). In Europe, the Food Safety Authority (EFSA) has established that the nature of any AMR of a microorganism should be determined prior to approval of the product as a probiotic for use in animals (EFSA, 2012). *Enterococcus faecium* contained in human probiotic products has been evaluated for the presence of virulence and antimicrobial resistance genes (Natarajan and Parani, 2015; Ghattargi et al., 2018). In a previous study, we determined the phenotypic susceptibilities and resistance to antimicrobials, detected virulence genes, and assessed genetic diversity based on pulsed-field gel electrophoresis (PFGE) of *E. faecium* strains isolated from commercial probiotic products used in swine and cattle (Amachawadi et al., 2018). *Enterococcus faecium* strains from 15 probiotic products (6 swine and 9 cattle) exhibited phenotypic resistance to at least one antimicrobial and a high proportion of strains was resistant to lincomycin, followed by tetracycline, daptomycin, ciprofloxacin, kanamycin, and penicillin.

Whole genome sequencing has become a routine procedure to characterize and predict AMR in bacteria (Zankari et al., 2012; Tyson et al., 2018) and is currently used by the National Antimicrobial Resistance Monitoring System to characterize pathogens that move through the food supply to cause human illnesses (FDA, 2017). The objective of this study was to utilize whole genome sequence-based analyses to characterize virulence and AMR gene profiles and phylogenetic relationships of *E. faecium* strains isolated from commercial swine and cattle probiotics.

Materials and Methods

Enterococcus faecium strains

Twenty-two *E. faecium* strains, isolated from commercial probiotic products of swine ($n = 9$; identified as A to I) and cattle ($n = 13$: identified as J to V), were used. The isolation, species confirmation, and phenotypic AMR profiles have been reported previously (Amachawadi et al., 2018).

DNA extraction and whole genome sequencing

A single colony of each *E. faecium* strain grown on the blood agar (Remel Inc., Lenexa, KS) was inoculated into Luria Bertani (LB) broth (Becton and Dickinson, Franklin Lakes, NJ) and incubated on a shaker at 37 °C. Genomic DNA was extracted from overnight cultures using the Qiagen DNeasy blood and tissue kit (Qiagen, Inc., Valencia, CA). The purity of the DNA was determined spectrophotometrically using the Nanodrop (Thermo Scientific, Waltham, MA). Genomic libraries of the strains were constructed using Nextera XT DNA Library Preparation kit (Illumina, Inc., San Diego, CA) and whole genome sequencing was performed on an Illumina MiSeq platform (Illumina, Inc., San Diego, CA) using the MiSeq version 2 reagent kit with 2 × 250 cycles. De novo assembly of the quality-controlled trimmed sequenced reads was performed using the SPAdes genome assembler version 3.8.2 (Bankevich et al., 2012).

Sequence analyses

The draft genome sequences were annotated using RAST (Rapid Annotation using Subsystem Technology; https://rast.nmpdr.org/). The number of genes categorized as those associated with virulence, disease and defense, mobile elements (plasmids, phages, prophages, and transposable elements), membrane transport, iron acquisition and metabolism, and stress response in each strain were determined using RAST (Overbeek et al., 2014). Virulence genes and AMR genes of the draft genomes of *E. faecium* strains were determined using Virulence Factor Database (VFDB; http://www.mgc.ac.cn/VFs/main.htm; Chen et al., 2005) and ResFinder 3.2 (https://cge.cbs.dtu.dk/services/ResFinder/) (Zankari et al., 2012), respectively. Plasmid sequences were identified using PlasmidFinder 2.1 (https://cge.cbs.dtu.dk/services/PlasmidFinder/; Joensen et al., 2014). The sequence types (ST) of the strains were determined in silico using MLST 2.0 (https://cge.cbs.dtu.dk/services/MLST/; Wirth et al., 2006; Jaureguy et al., 2008).

Phylogenetic analysis

The phylogenetic relationship between sequence types (ST), based on in silico MLST, of the *E. faecium* strains was determined using PHYLOViZ-2.0 (Nascimento et al., 2017), which implements goeBURST algorithm (Global Optimal eBURST; Feil et al., 2004). The algorithm assigns isolates into clonal complexes (CCs) based on the differences in their allelic profiles. Clonal complexes are defined in three levels (SLV, DLV, and TLV) based on the differences in one or

more housekeeping genes. The relationship of the analyzed isolates is represented as an unrooted tree. Additionally, the WGS of *E. faecium* strains of human (*n* = 29), cattle (*n* = 4), and swine (*n* = 4) origin were downloaded from GenBank (Supplementary Table 1), and their STs were determined and included in the goeBURST analysis.

Parsnp v1.2 (http://harvest.readthedocs.io/en/latest/content/parsnp.html) was used to align the core genomes of *E. faecium* strains from the present study (*n* = 22; A to V). Whole genome sequences of *E. faecium* strains of human (*n* = 29), cattle (*n* = 4), and swine (*n* = 4) origin were also included in the analysis. The alignment of core genomes was followed by the construction of maximum likelihood tree. The phylogenetic tree was subsequently imported to FigTree 1.4.3 software (http://tree.bio.ed.ac.uk/software/figtree/; Rambaut, 2012) for better visualization.

Results

Species confirmation

The 22 probiotic strains from 9 swine and 13 cattle products were confirmed as *E. faecium* based on the 16S rRNA gene sequence in the draft genome sequences using SpeciesFinder 2.0 (Larsen et al., 2014). The draft genome sequences of all 9 swine probiotic strains and 9 of 13 cattle probiotic strains matched with that of the *E. faecium* T-110, the reference sequence in the database (a human probiotic strain; GenBank accession no. CP006030). The draft genome sequences of three cattle probiotic strains matched with that of a human *E. faecium* strain (blood of a hospitalized patient; GenBank accession no. CP011281). The draft genome sequence of one cattle strain (probiotic M) matched with that of an *E. faecium* strain (GenBank accession no. JX409651), but confidence of the result was reported as fail. In the National Center for Biotechnology Information (NCBI) database, seven of the nine swine probiotic strains (B, C, D, E, F, G, and H) and seven of the 12 cattle probiotic strains (M, N, P, Q, R, T, and U) were identified as *Enterococcus lactis* (Table 1).

Table 1. Species identification comparison of *Enterococcus faecium* strains isolated from commercial swine (*n* = 9) and cattle (*n* = 13) probiotic products

Probiotic product code	Species identity by the SpeciesFinder[1]	Species identity from the National Center for Biotechnology Information database
Swine probiotics		
A	*Enterococcus faecium*	*Enterococcus faecium*
B	*Enterococcus faecium*	*Enterococcus lactis*
C	*Enterococcus faecium*	*Enterococcus lactis*
D	*Enterococcus faecium*	*Enterococcus lactis*
E	*Enterococcus faecium*	*Enterococcus lactis*
F	*Enterococcus faecium*	*Enterococcus lactis*
G	*Enterococcus faecium*	*Enterococcus lactis*
H	*Enterococcus faecium*	*Enterococcus lactis*
I	*Enterococcus faecium*	*Enterococcus faecium*
Cattle probiotics		
J	*Enterococcus faecium*	*Enterococcus faecium*
K	*Enterococcus faecium*	*Enterococcus faecium*
L	*Enterococcus faecium*	*Enterococcus faecium*
M	*Enterococcus faecium*	*Enterococcus lactis*
N	*Enterococcus faecium*	*Enterococcus lactis*
O	*Enterococcus faecium*	*Enterococcus faecium*
P	*Enterococcus faecium*	*Enterococcus lactis*
Q	*Enterococcus faecium*	*Enterococcus lactis*
R	*Enterococcus faecium*	*Enterococcus lactis*
S	*Enterococcus faecium*	*Enterococcus faecium*
T	*Enterococcus faecium*	*Enterococcus lactis*
U	*Enterococcus faecium*	*Enterococcus lactis*
V	*Enterococcus faecium*	*Enterococcus faecium*

[1]SpeciesFinder at the Center for Genomic Epidemiology (https://www.genomicepidemiology.org/).

Rapid annotation using subsystem technology

Average genome size, no. of contigs, and N50 of swine probiotic strains were 2.72 Mb (2.54–2.92 Mb), 247 (176–423), and 75644 (51101–97268), respectively. For cattle probiotic strains, average genome size, number of contigs, and N50 of cattle probiotic strains are 2.91 Mb (2.52–3.86 Mb), 917 (155–4686), and 33548 (11508–50978), respectively. The functional categories of genes detected in the swine and cattle probiotic strains are shown in Table 2. Based on the RAST subsystem annotation, average number of genes associated with mobile genetic elements (plasmids, phages, prophages, and transposable elements) were 16 (Range = 5–30) and 14 (Range = 5–27) in swine and cattle probiotic strains, respectively (Table 2). Among the plasmid sequences detected, pAMbeta and pIP816 belonging to rep_1 family of plasmids were the most common and were present in swine (*n* = 5) and cattle (*n* = 6) probiotic strains. Other plasmid sequences detected included pGL (rep_{29} family), p200B (rep_{18a} family), pRE25 (rep_2 family), pKL0018 (rep_1 family), DOp2 (rep_{US1} family), and DOp1 (rep_{US43} family; data not shown).

Virulence genes

All 22 strains isolated from swine and cattle probiotic products carried *bopD* (biofilm on plastic surface), *upps* (encodes for undecaprenyl pyrophosphate synthase), and *cdsA* (encodes for phosphatidate cytidylyltransferase) genes. The strains also carried *acm* (collagen adhesion; seven swine and eight cattle probiotic strains), *efa* (*Enterococcus faecalis* antigen A; all swine and 12 cattle strains), *ebp* (endocarditis- and biofilm-associated pili; *ebpA*, *B*, *C* present only in a few swine and cattle strains), and *srtC* (sortase C; four swine and 12 cattle strains; Table 3). The prevalence of virulence genes in each of the 22 *E. faecium* strains isolated from the commercial probiotics is shown in Supplementary Table 2.

Antimicrobial resistance genes

The two most prevalent AMR genes were *aac(6′)-Ii* (21/22 strains), which encodes for acetyl transferase that confers resistance by enzymatic modification of aminoglycoside, and *msrC* (22/22 strains), which encodes for a ribosomal protection protein and confers resistance to macrolide, lincosamide and streptogramin B (MLS) (Table 4). The other AMR genes detected were *aph(3′)-III* and *ant(6)-Ia*) (2/22 strains) that encode for phosphotransferase and nucleotidyltransferase, respectively, and confer resistance by enzymatic modification of aminoglycosides, *tet(L)* and *tet(M)* that encode for efflux pump and ribosomal protection protein, respectively, to confer tetracycline resistance (3/22 strains), and

Table 2. Average number of functional categories of genes, based on Rapid Annotation using Subsystem Technology analysis of whole genome sequences of *Enterococcus faecium* strains isolated from commercial swine (*n* = 9) and cattle (*n* = 13) probiotic products

Source	Functional categories of genes, Mean (Range)				
	Virulence, disease, and defense	Phages, prophages, transposable elements, and plasmids	Membrane transport	Iron acquisition and metabolism	Stress response
Swine (*n* = 9)	56 (52–64)	16 (5–30)	56 (48–65)	22 (21–25)	72 (70–74)
Cattle (*n* = 13)	62 (52–78)	14 (5–27)	57 (49–67)	25 (21–40)	72 (68–78)

Table 3. Virulence genes identified from whole genome sequencing of *Enterococcus faecium* strains isolated from commercial swine (*n* = 9) and cattle (*n* = 13) probiotic products

Virulence genes	Product	Function	Swine (*n* = 9)	Cattle (*n* = 13)
Adherence				
acm	Collagen adhesin	Adherence to collagen	7	8
efaA	*Enterococcus faecalis* antigen A	Adherence to biotic and abiotic surfaces	9	12
ebpA	Endocarditis- and biofilm-associated pili	Adherence to host extracellular matrix proteins	4	8
ebpB			3	9
ebpC			3	8
srtC	Sortase C	Involved in biofilm formation by polymerizing *ebp* pili	4	12
Biofilm formation				
bopD	Biofilm on plastic	Sugar binding transcriptional regulator	9	13
Genes for evasion of immune system				
upps	Undecaprenyl pyrophosphate synthase	Evasion of host immune system	9	13
cdsA	Phosphatidate cytidylyltransferase	Evasion of host immune system	9	13

Table 4. Antimicrobial resistance genes identified from whole genome sequencing of *Enterococcus faecium* strains isolated from commercial swine (*n* = 9) and cattle (*n* = 13) probiotic products

Antimicrobial resistance gene	Product/resistance mechanism	Gene function	Swine (*n* = 9)	Cattle (*n* = 13)
aac(6′)-Ii	6′-N-aminoglycoside acetyltransferase/ Enzymatic modification	Aminoglycoside resistance	9	12
aph(3′)-III	3′-aminoglycoside O-phosphotransferase type IIIa/Enzymatic modification	Aminoglycoside resistance	0	2
ant(6)-Ia	aminoglycoside nucleotidyltransferases/ Enzymatic modification	Aminoglycoside resistance	0	2
tet(L)	Efflux pump	Tetracycline resistance	1	2
tet(M)	Ribosomal protection protein/Ribosomal protection	Tetracycline resistance	1	1
msrC	ABC-F ATP-binding protein/Ribosomal protection	Macrolide, lincosamide, and streptogramin B resistance	9	13
cat-(pc194)	Chloramphenicol acetyl transferase/Enzymatic modification	Phenicol resistance	0	2

cat-(pc194) (2/22 strains) that encode for acetyl transferase, which confers resistance by enzymatic modification of chloramphenicol.

Genotypic to phenotypic resistance (Amachawadi et al., 2018) concordance is shown in Table 5. The three swine strains (C, E, and H) and four cattle strains (L, N, S, and U) that were pan susceptible to antimicrobials in NARMS Gram-positive panel (CMV3AGPF) carried an aminoglycoside resistance gene, *aac(6′)-Ii*, and a MLS resistance gene, *msrC*. Strains that showed phenotypic resistance to kanamycin, an aminoglycoside (J, K, and V), and lincomycin (B, D, F, G, I, J, K, M, O, T, and V) carried genes related to the resistant antibiotic, *aac(6′)-Ii* and *msrC*, respectively. The two strains (J and K) that were phenotypically resistant to erythromycin, a macrolide, carried the *msrC* gene. Of the three strains phenotypically resistant to tetracycline, only one strain (A) carried the *tet(L)* and *tet(M)*genes and the other two did not carry any *tet* genes. Strains phenotypically resistant to

Table 5. Phenotypic and genotypic resistance concordance in *Enterococcus faecium* strains isolated from commercial swine (*n* = 9) and cattle (*n* = 13) probiotic products

Phenotypic resistance	Product code	Resistance breakpoint (µg/mL)[1,2]	Minimum inhibitory concentration (µg/mL)[2]	Genotypic resistance	
				Related	Unrelated
Swine probiotics					
None	C, E, H				*aac(6′)-Ii*, *msrC*
Ciprofloxacin	A	≥ 4	4		*aac(6′)-Ii*, *msrC*
Daptomycin	A, B, I	NA[3]	16, 16, 8		*aac(6′)-Ii*, *msrC*
Lincomycin	B, D, F, G, I	≥ 8	8, 8, 8, 8, 8,	*msrC*	*aac(6′)-Ii*
Tetracycline	A	≥16	32	*tet(L)*, *tet(M)*	*aac(6′)-Ii*, *msrC*
Cattle probiotics					
None	L, N, S, U				*aac(6′)-Ii*, *msrC*
Chloram-phenicol	J, K, V	≥ 32	32, 32, 32		*aac(6′)-Ii*, *msrC*
Ciprofloxacin	P	≥ 4	4		*aac(6′)-Ii*, *aph(3′)-III*, *ant(6)-Ia*,*cat-(pc194*, *msrC*, *tet(L)*, *tet(M)*
Ciprofloxacin	Q	≥ 4	4		*aac(6′)-Ii*, *aph(3′)-III*, *ant(6)-Ia*,*cat-(pc194)*,*msrC*, *tet(L)*
Ciprofloxacin	R	≥ 4	4		*aac(6′)-Ii*, *msrC*
Daptomycin	O	NA[2]	8		*aac(6′)-Ii*, *msrC*
Erythromycin	J, K	≥ 8	8, 8	*msrC*	*aac(6′)-Ii*, *msrC*
Kanamycin	J, K, V	≥ 1,024	1,024, 1,024, 1,024	*aac(6′)-Ii*	*msrC*
Lincomycin	J, K, M, O, T, V	≥ 8	8, 8, 8, 8, 8, 8	*msrC*	*aac(6′)-Ii*, *msrC*
Penicillin	J	≥ 16	16		*aac(6′)-Ii*, *msrC*
Tetracycline	J, K, V	≥ 16	32		*aac(6′)-Ii*, *msrC*

[1]Breakpoints established by the Clinical Laboratory Standards Institute.
[2] Amachawadi et al., 2018.
[3] NA, Not applicable. A susceptibility breakpoint of ≥ 4 µg/mL for daptomycin exists but no resistant breakpoint has been established. In this study, isolates with minimum inhibitory concentration of ≥ 8 µg/mL were considered as resistant.

chloramphenicol, ciprofloxacin, daptomycin, and penicillin did not have any relevant AMR genes. The two cattle strains phenotypically resistant to ciprofloxacin were positive for all the AMR genes detected in the probiotic strains, including phenicol resistance gene, *cat-(pc194)*. However, the swine strain A, phenotypically resistant to ciprofloxacin, contained only *aac(6′)-Ii*) and *msrC* genes. Three *E. faecium* strains of cattle probiotics (J, K, and V) that were categorized as MDR because of phenotypic resistance to ≥ 3 classes (phenicols, macrolides, aminoglycosides, lincosamides, Beta-lactams, and tetracyclines) of antimicrobials (chloramphenicol, erythromycin, kanamycin, lincomycin, penicillin, and tetracycline) contained resistance genes for aminoglycoside [*aac(6′)-Ii*] and MLS (*msrC*) resistance. The prevalence of AMR genes in all 22 *E. faecium* strains is shown in Supplementary Table 3.

Sequence types

Enterococcus faecium strains isolated from swine probiotics belonged to six sequence types (ST1, 94, 160, 178, 296, 1513) and those isolated from cattle probiotics belonged to seven STs (94, 160, 178, 296, 611, 696, 1433; Table 6). The genomes of six strains carried novel alleles; hence, the nearest ST assigned by the MLST database (MLST 2.0) is reported (Table 6).

Table 6. In silico multi-locus sequence typing of *Enterococcus faecium* strains isolated from commercial swine (*n* = 9) and cattle (*n* = 13) probiotic products

Sequence type (ST)	Swine (*n* = 9)	Cattle (*n* = 13)
1	11	0
94	1	2
160	1	3
178	21	11
296	2	2
611	0	21
696	0	2
1433	0	11
1513	2	0

[1]Contains novel alleles, hence the nearest STs were reported.

Phylogenetic relationship

The goeBURST analysis of the 59 *E. faecium* strains representing 36 STs revealed 18 clonal complexes (CC). Three major CCs were identified, which included the majority of the STs (19/36), with CC0 being the most common CC with

11 ST. *Enterococcus faecium* strains isolated from cattle probiotics with STs 160 and 611 belonged to CC1, which also clustered with strains of human (ST21, ST1054) and cattle origin (ST32). *Enterococcus faecium* strains isolated from cattle and swine probiotics with STs 94 and 178 belonged to CC3, which also clustered with a human strain (ST361). The remaining *E. faecium* strains of swine and cattle probiotics (ST1, ST296, ST696, ST1433, and ST1513) existed as singletons; they were clonally different and did not share their allelic profiles with other strains. Similarly, other strains of human, cattle, and swine origin belonging to different STs (ST19, ST54, ST156, ST214, ST218, ST430, ST736, ST904, and ST955) also existed as singletons (Figure 1).

Phylogenetic analysis of *E. faecium* strains, based on core genome, revealed close clustering of the strains of the same source, with a few exceptions. One of the strains isolated from cattle probiotics (Probiotic S) clustered with a strain isolated from human feces (Accession no. LN999844). The seven swine probiotic strains and seven cattle probiotic strains identified as *E. lactis,* based on NCBI database, two human fecal strains (Accession nos. CP025685 and CP040878) and a cattle strain (MJDY01) clustered separately (Figure 2).

Data availability

The whole genome sequences of the 8 strains of *E. faecium* and 14 strains of *E. lactis* have been deposited in NCBI GenBank under the BioProject number PRJNA746973 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA746973).

Discussion

In this study, we determined the virulence and AMR genes profiles and phylogenetic relationships of 22 strains isolated from *E. faecium*-based commercial swine and cattle probiotics. Although Species-Finder database, based on 16S rRNA gene sequence, confirmed all 22 as *E. faecium,* the NCBI, which is a more updated database, identified seven swine (B, C, D, E, F, G, and H strains) and seven cattle strains (M, N, P, Q, R, T, and U) as *E. lactis,* and not as *E. faecium. Enterococcus lactis* was first isolated from Italian raw milk cheeses and was identified as a novel species based on 16S rRNA gene sequence analysis (Morandi et al., 2012). The isolates were closely related to *E. hirae, E. durans,* and *E. faecium* with 98.8%, 98.9%, and 99.4% similarity in 16S rRNA sequence, respectively. In addition to 16S rRNA sequence differences, the two species, *E. faecium* and *E. lactis,* can be differentiated by 16S-23S internal transcriber speacer analysis and phenotypically by sugar fermentations (Morandi et al., 2012). Historically, the known strains of *E. faecium* are divided into two clades, clade A, containing the hospital-associated strains, and clade B, containing the community-associated strains. The clade A is further divided into two sub-clades A1 and A2 to include clinical isolates in A1 and animal-associated strains in A2 (Belloso Daza et al., 2021). It is suggested that the split into two sub-clades likely happened after the introduction of antibiotics in human and animal settings, approximately 75 yr ago (Lebreton et al., 2013). Although 16S rRNA gene sequence is considered

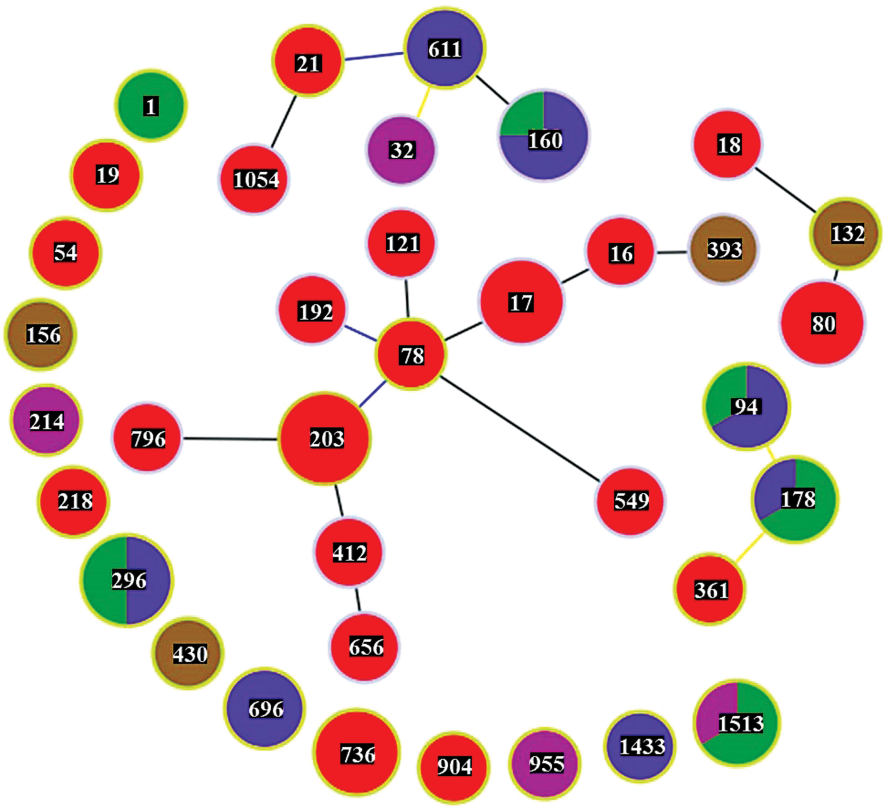

Figure 1. geoBURST analysis of 67 *Enterococcus faecium* strains representing 36 Sequence Types (ST). Numbers within the circles represent STs, size of each circle is proportional to the number of isolates within each ST. Different colors represent the source of *E. faecium* strains; Red: Human strains; Blue: Cattle probiotic strains, Green: Swine probiotic strains; Purple: Cattle strains, Brown: Swine strains. Black link—drawn without recourse to tiebreak rules; Blue link—drawn using tiebreak rule 1 (number of single locus variants (SLV); Yellow link—drawn using tiebreak rule 4 or 5 (frequency found on the data set and ST number, respectively).

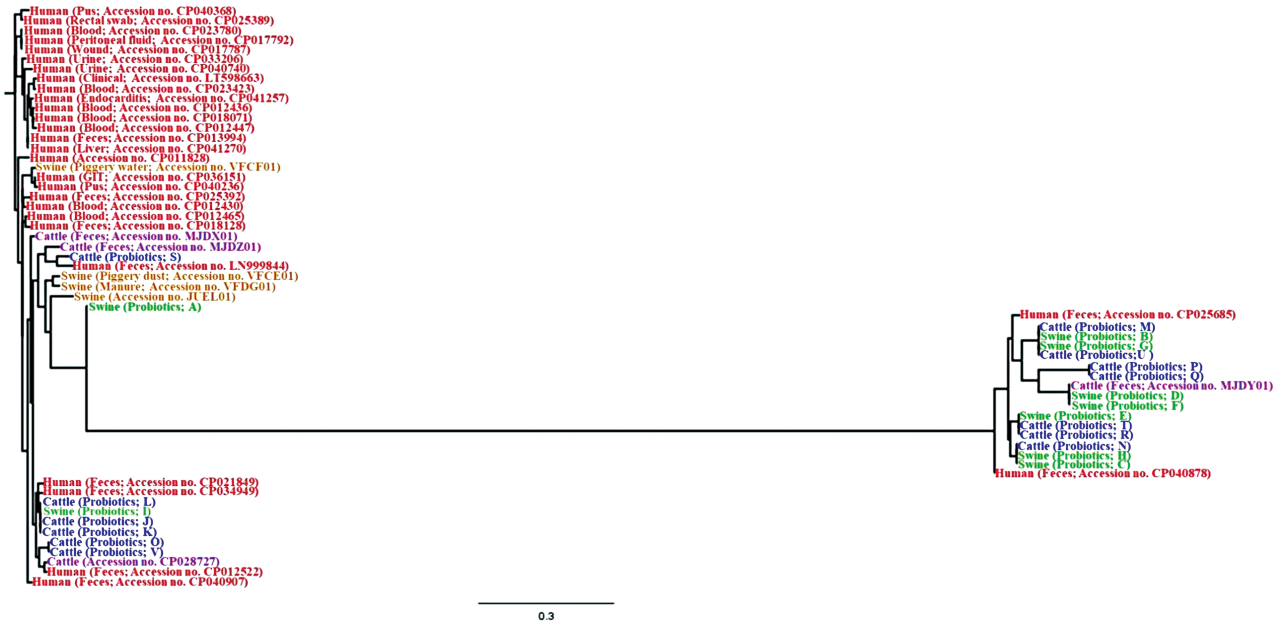

Figure 2. Phylogenetic tree of *E. faecium* strains isolated from cattle and swine probiotic products (strains from the present study—A to V), and whole genome sequences downloaded from GenBank, using Parsnip v1.2 and visualized using FigTree 1.4.3. The colors of the branch tip labels indicate the source of the strains (Red: Human strains; Blue: Cattle probiotic strains; Green: Swine probiotic strains; Purple: Cattle strains; Brown: Swine strains).

generally as a standard for bacterial taxonomy, variability in the number of copies and variability within the many copies of the 16S rRNA gene sequences, which may preclude accurate species identification, are not uncommon phenomenon in bacteria (Ibal et al., 2019; Belloso Daza et al., 2021). However, WGS analysis, which is becoming a more widely used tool, pangenome analysis, provides a more definitive method for microbial taxonomy. Belloso Daza et al. (2021) investigated the taxonomic relationship among strains of *E. faecium* of different origins and *E. lactis* using WGS and concluded that clade B strains differed from *E. faecium* clades A1 and A2 and should be reamed as *E. lactis* (Belloso Daza et al., 2021).

In the United States, the list of microbial species used as probiotics in animal feeds, more commonly called direct-fed microbials, is published in the Association of American Feed Control Official Manual and the list includes six species of *Enterococcus* (Feedstuffs.com., 2021). The six species, including *E. faecium*, have a "Generally Recognized as Safe" (GRAS) status. However, in Europe, according to the Qualified Presumption of Safety list from the European Food safety Authority (EFSA), the genus *Enterococcus* does not have the "GRAS" status (Ogier and Serror, 2008; Hanchi et al., 2018) because of the safety concerns of potential virulence and the propensity to acquire and transfer AMR.

Species of *Enterococcus* are ubiquitously distributed in the environment, primarily inhabiting human and animal gastrointestinal tracts, but also occur in soil, water, variety of foods, and on plants and insects (Franz et al., 2011). *Enterococcus faecium* is also a widely used bacterial species in probiotic products of humans to promote health, and in animals to promote performance (growth in swine and milk production in dairy cows) and health. The documented health benefits in animals include reduced risk of ruminal subacute acidosis in cattle and reduced incidence of diarrhea in piglets and baby calves (Franz et al., 2011). In children, clinical studies have shown that *E. faecium*-based probiotic products reduced the severity of diarrhea and length of the hospital stay (Chen et al., 2010).

Enterococcus species, particularly *faecium* and *faecalis*, are nosocomial pathogens that cause bacteremia, endocarditis, urinary tract infections, intra-abdominal and pelvic infections, and even death, more often in persons with serious underlying diseases and or are immunocompromised (Murray, 1990; Hoge et al., 1991). The emergence of *E. faecium* as a nosocomial pathogen was initially thought to be due to acquisition of AMR; however, AMR alone does not explain virulence. The major virulence factors of *E. faecium*, which contribute to the pathogenicity, include adhesins to mediate adherence and colonization, promote cell clumping and biofilm formation, proteolytic enzymes to degarde host proteins, and a cytolysin, which enhances virulence of pathogenic strains (Franz et al., 2011; Arias and Murray, 2012).

Enterococcus faecium contained in human probiotic products has been evaluated for their safety and efficacy with regard to health (Natarajan and Parani, 2015; Ghattargi et al., 2018). A couple of strains of *E. faecium,* T-110 and LBB.B1, which are contained in several commercial products, have been whole genome sequenced to assess safety. The analysis of T-110 has revealed the absence of most of the genes related to virulence and AMR and presence of a few adhesion genes (Natarajan and Parani, 2015). Noguchi et al. (2011) compared virulence genes and antimicrobial susceptibilities of clinical *E. faecium* strains with strains isolated from six human probiotic products and concluded that none of the probiotic strains contained any of 13 virulence genes assayed and clinical strains were resistant to levofloxacin, a fluorquinolone, whereas all probiotic strains were phenotypically susceptible (Noguchi et al., 2011).

None of the 22 probiotic strains carried major virulence genes, *asa1*, *gelE*, *cylA*, *esp*, and *hyl,* generally carried by pathogenic *E. faecium* strains (Fisher and Phillips, 2009; Lee et al., 2019). In our previous study, we used a multiplex PCR assay to report the absence of these genes in the probiotic

strains (Amachawadi et al., 2018). Although 22 probiotic strains carried *bopD* gene, none carried the *fsrABC* operon, which controls the expression of the *bopD* gene (Bourgogne et al., 2006). The non-functional *bopD* gene due to lack of the *fsrABC* operon has been previously reported in human probiotic strains of *E. faecium* (Natarajan and Parani, 2015; Ghattargi et al., 2018). Similarly, the *acm* gene, which encodes for an adhesin that binds to the host collagen in seven of the 15 *acm*-positive strains, is likely to be non-functional due to the presence of non-sense mutation. Probiotic strains of *E. faecium* have been reported to carry non-functional *acm* genes (Natarajan and Parani, 2015; Ghattargi et al., 2018; Urshev and Yungareva, 2021). All 22 strains carried *upps* (encodes for undecaprenyl pyrophosphate synthase) and *cdsA* (encodes for phosphatidate cytidylyltransferase) genes, which play a role in the evasion of immune system. A number of strains carried several genes (*acm, efaA, ebpA, ebpB, ebpC, srtC*, and *bopD*) involved in adhesion to host proteins and other biotic and abiotic surfaces and in biofilm formation (Franz et al., 2011). Although adhesion is a critical process in an infectious process, adherence is also one of the main criteria for the selection of potential probiotics. Adherence to the gut epithelial cells and subsequent colonization extend the persistence of probiotic strains in the intestinal tract (Ouwehand et al., 1999; Ferreira et al., 2011). Investigation on the presence of virulence genes and their contributions to virulence in enterococci from several sources has shown that the occurrence of individual virulence factors is strain specific (Abriouel et al., 2008; Franz et al., 2011). More often, the strains become virulent by acquiring specific virulence-associated genes via mobile genetic elements (Leavis et al., 2007; van Schaik et al., 2010), possibly increasing their fitness to adapt to the animal or human host. For example, a *E. faecium* genetic lineage that developed and spread globally is hospital-adapted (CC 17) because of acquisition of a large pathogenicity island of > 60 kbp, *esp* gene, vancomycin-resistance gene, and *acm* gene (van Schaik et al., 2010). Studies have shown separate clustering of and distinct genomic differences between clinical and nonclinical *E. faecium* strains (Kim and Marco, 2014; Beukers et al., 2017). Genomes of clinical strains are significantly larger than non-clinical strains because of acquisition of mobile genetic elements, virulence, and AMR genes (Kim and Marco, 2014).

Because *E. faecium* is Gram-positive, it is intrinsically resistant to low levels of aminoglycoside (Moellering and Weinberg, 1971). The presence of *aac(6′)-Ii,* which encodes for 6′-*N*-aminoglycoside acetyltransferase that cleaves the 6′-amino group of aminoglycoside antibiotics, in all 22 strains of *E. faecium* suggests moderate level of aminoglycoside resistance (Chow, 2000). Additionally, two cattle strains (P and Q) possessed *aph(3′)-III* and *ant(6)-Ia* genes that encode for aminoglycoside modifying enzymes, 3′-aminoglycoside *O*-phosphotransferase type IIIa and aminoglycoside nucleotidyltransferases, respectively, which allow the organisms to resistant to high concentrations of aminoglycosides (Costa et al., 1993). However, both strains were phenotypically susceptible to kanamycin (MIC < 1.024 µg/mL), suggesting that the genes were likely non-functional. The comparison of the phenotypic susceptbility and resistance to genotypic data showed presence of both related and unrelated genes in the 22 probiotic strains. The strains that were phenotypically resistant to kanamycin and lincomycin carried AMR genes related to the antibiotic class. However, the seven strains pan-susceptbile to NARMS Gram positive panel contained two AMR genes, *aac(6′)-Ii* and *msrC,* which likely means that they were nonfunctional. The strains that were phenotypically resistant to chloramphenicol, ciprofloxacin, daptomycin, and penicillin did not have related AMR genes. It is possible that the organisms had nonspecific efflux pumps. Tyson et al. (2018) have compared phenotypic resistance with genotypic data, derived from WGS of 100 *E. faecium* strains from animal and food sources, and have reported high degree of concordance for the 11 antibiotics tested (Tyson et al., 2018).

Over the years, the public health implication of vancomycin-resistant *E. faecium* in human clinical strains is well recognized (Treitman et al., 2005; Deshpande et al., 2007). None of the probiotic strains in our study carried genes for vancomycin resistance. However, they carried genes encoding resistance for other major clinically important antibiotics (aminoglycosides, tetracyclines, macrolides, lincosamide, streptogramin B, and phenicol). The WGS analysis of a probiotic strain *E. faecium* LBB.E81, another human probiotic strain, revealed the presence of *aac(6′)-Ii* and *msrC* (Urshev and Yungareva, 2021).

Swine and cattle probiotic *E. faecium* strains belonged to diverse sequence types. Some of the STs have been previously reported in *E. faecium* strains from various sources (ST696 from human clinical case and waste water, ST94 from human clinical case and waste water, ST296 from waste water, ST178 from waste water, and ST160 in National collection of type culture; Gouliouris et al., 2018). Phylogenetic analysis revealed close clustering of *E. faecium* strains belonging to the same source with a few exceptions. Some of the 22 probiotic strains clustered closely with cattle and human fecal strains, which suggest movement of *E. faecium* strains across different species and the ability of the strains to adapt to various ecological niches. Swine and cattle probiotic strains identified as *E. lactis* clustered separately along with two human fecal (CP025685) and CP040878) and one cattle fecal (MJDY01) strains. The two human and one cattle fecal strains are now listed as *E. lactis* in the updated NCBI database.

In conclusion, 14 of the 22 probiotic strains were identified as *E. lactis,* not as *E. faecium*. None of the strains investigated carried any of the major virulence genes characteristic of the clinical *E. faecium* strains, suggesting that these probiotic strains are unlikely to initiate opportunistic infection. However, a number of strains carried several genes involved in adhesion to host proteins and other biotic and abiotic surfaces, which may be a beneficial feature for probiotic strains. Close clustering of the probiotic strains with other human and cattle fecal strains, and the presence of AMR genes suggest the potential of these strains to survive in different ecological niche and transfer AMR genes to medically important antimicrobials to other gut bacteria. The study also illustrates the utility of WGS in assessing the safety of probiotic strains.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

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Conflict of Interest Statement

None of the authors has any conflict of interest with the publication of the study.

Literature Cited

Abriouel, H., N. B. Omar, A. C. Molinos, R. L. López, M. J. Grande, P. Martínez-Viedma, E. Ortega, M. M. Cañamero, and A. Galvez. 2008. Comparative analysis of genetic diversity and incidence of virulence factors and antibiotic resistance among enterococcal populations from raw fruit and vegetable foods, water and soil, and clinical samples. *Int. J. Food Microbiol*. 123:38–49. doi:10.1016/j.ijfoodmicro.2007.11.067.

Amachawadi, R. G., F. Giok, X. Shi, J. Soto, S. K. Narayanan, M. D. Tokach, M. D. Apley, and T. G. Nagaraja. 2018. Antimicrobial resistance of *Enterococcus faecium* strains isolated from commercial probiotic products used in cattle and swine. *J. Anim. Sci*. 96:912–920. doi:10.1093/jas/sky056.

Arias, C. A., and B. E. Murray. 2012. The rise of the *Enterococcus*: beyond vancomycin resistance. *Nat. Rev. Microbiol*. 10:266–278. doi:10.1038/nrmicro2761.

Bankevich, A., S. Nurk, D. Antipov, A. A. Gurevich, M. Dvorkin, A. S. Kulikov, V. M. Lesin, S. I. Nikolenko, S. Pham, A. D. Prjibelski, et al. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol*. 19:455–477. doi:10.1089/cmb.2012.0021.

Belloso Daza, M. V., C. Cortimiglia, D. Bassi, and P. S. Cocconcelli. 2021. Genome-based studies indicate that the *Enterococcus faecium* Clade B strains belong to *Enterococcus lactis* species and lack of the hospital infection associated markers. *Int. J. Syst. Evol. Microbiol*. 71(8):004948. doi: 10.1099/ijsem.0.004948

Beukers, A. G., R. Zaheer, N. Goji, K. K. Amoako, A. V. Chaves, M. P. Ward, and T. A. McAllister. 2017. Comparative genomics of *Enterococcus* spp. isolated from bovine feces. *BMC Microbiol*. 17:52. doi:10.1186/s12866-017-0962-1.

Bourgogne, A., S. G. Hilsenbeck, G. M. Dunny, and B. E. Murray. 2006. Comparison of OG1RF and an isogenic fsrB deletion mutant by transcriptional analysis: the Fsr system of *Enterococcus faecalis* is more than the activator of gelatinase and serine protease. *J. Bacteriol*. 188:2875–2884. doi:10.1128/JB.188.8.2875-2884.2006.

Chen, C. C., M. S. Kong, M. W. Lai, H. C. Chao, K. W. Chang, S. Y. Chen, Y. C. Huang, C. H. Chiu, W. C. Li, P. Y. Lin, et al. 2010. Probiotics have clinical, microbiologic, and immunologic efficacy in acute infectious diarrhea. *Pediatr. Infect. Dis. J*. 29:135–138. doi:10.1097/inf.0b013e3181b530bf.

Chen, L., J. Yang, J. Yu, Z. Yao, L. Sun, Y. Shen, and Q. Jin. 2005. VFDB: a reference database for bacterial virulence factors. *Nucleic Acids Res*. 33(Database issue):D325–D328. doi:10.1093/nar/gki008.

Chow, J. W. 2000. Aminoglycoside resistance in enterococci. *Clin. Infect. Dis*. 31:586–589. doi:10.1086/313949.

Costa, Y., M. Galimand, R. Leclercq, J. Duval, and P. Courvalin. 1993. Characterization of the chromosomal aac(6')-Ii gene specific for *Enterococcus faecium*. *Antimicrob. Agents Chemother*. 37:1896–1903. doi:10.1128/AAC.37.9.1896.

Deshpande, L. M., T. R. Fritsche, G. J. Moet, D. J. Biedenbach, and R. N. Jones. 2007. Antimicrobial resistance and molecular epidemiology of vancomycin-resistant enterococci from North America and Europe: a report from the SENTRY antimicrobial surveillance program. *Diagn. Microbiol. Infect. Dis*. 58:163–170. doi:10.1016/j.diagmicrobio.2006.12.022.

EFSA. 2012. Scientific Opinion on Lactiferm® (*Enterococcus faecium*) as a feed additive for weaned piglets and calves. *EFSA J*. 10(2574). doi:10.2903/j.efsa.2012.2574.

FDA. 2017. *The National Antimicrobial Resistance Monitoring System: NARMS Integrated Report, 2015*, Laurel, MD.

Feedstuffs.Com. 2021. Direct-fed microbial, enzymes and forage additive compendium. https://www.feedstuffs.com/story-direct-fed-microbial-enzyme-forage-additive-compendium-46-125834

Feil, E. J., B. C. Li, D. M. Aanensen, W. P. Hanage, and B. G. Spratt. 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J. Bacteriol*. 186:1518–1530. doi:10.1128/JB.186.5.1518-1530.2004.

Ferreira, C. L., L. Grześkowiak, M. C. Collado, and S. Salminen. 2011. In vitro evaluation of *Lactobacillus gasseri* strains of infant origin on adhesion and aggregation of specific pathogens. *J. Food Prot*. 74:1482–1487. doi:10.4315/0362-028X.JFP-11-074.

Fisher, K., and C. Phillips. 2009. The ecology, epidemiology and virulence of *Enterococcus*. *Microbiology (Reading)*. 155(Pt 6):1749–1757. doi:10.1099/mic.0.026385-0.

Food and Agricultural Organization of the United Nations/World Health Organization-FAO/WHO. 2002. *Guidelines for the Evaluation of Probiotics in Food, Report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food*, London, Ontario, Canada.

Franz, C., M. Huch, H. Abriouel, and A. Gálvez. 2011. Enterococci as probiotics and their implications in food safety. *Int. J. Food Microbiol*. 151:125–140. doi: 10.1016/j.ijfoodmicro.2011.08.014.

Ghattargi, V. C., M. A. Gaikwad, B. S. Meti, Y. S. Nimonkar, K. Dixit, O. Prakash, Y. S. Shouche, S. P. Pawar, and D. P. Dhotre. 2018. Comparative genome analysis reveals key genetic factors associated with probiotic property in *Enterococcus faecium* strains. *BMC Genomics* 19:652. doi:10.1186/s12864-018-5043-9.

Ghorbani, G. R., D. P. Morgavi, K. A. Beauchemin, and J. A. Leedle. 2002. Effects of bacterial direct-fed microbials on ruminal fermentation, blood variables, and the microbial populations of feedlot cattle. *J. Anim. Sci*. 80:1977–1985. doi:10.2527/2002.8071977x.

Gouliouris, T., K. E. Raven, C. Ludden, B. Blane, J. Corander, C. S. Horner, J. Hernandez-Garcia, P. Wood, N. F. Hadjirin, M. Radakovic, et al. 2018. Genomic surveillance of *Enterococcus faecium* reveals limited sharing of strains and resistance genes between livestock and humans in the United Kingdom. *mBio*. 9(6):e01780-18. doi: 10.1128/mBio.01780-18.

Hanchi, H., W. Mottawea, K. Sebei, and R. Hammami. 2018. The genus *Enterococcus*: between probiotic potential and safety concerns—an update. *Front. Microbiol*. 9:1791. doi: 10.3389/fmicb.2018.01791

Hoge, C. W., J. Adams, B. Buchanan, and S. D. Sears. 1991. *Enterococcal bacteremia*: to treat or not to treat, a reappraisal. *Rev. Infect. Dis*. 13:600–605. doi:10.1093/clinids/13.4.600.

Ibal, J. C., H. Q. Pham, C. E. Park, and J. H. Shin. 2019. Information about variations in multiple copies of bacterial 16S rRNA genes may aid in species identification. *PLoS One* 14:e0212090. doi:10.1371/journal.pone.0212090.

Jaureguy, F., L. Landraud, V. Passet, L. Diancourt, E. Frapy, G. Guigon, E. Carbonnelle, O. Lortholary, O. Clermont, E. Denamur, et al. 2008. Phylogenetic and genomic diversity of human bacteremic *Escherichia coli* strains. *BMC Genomics* 9:560. doi:10.1186/1471-2164-9-560.

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

Kim, E. B., and M. L. Marco. 2014. Nonclinical and clinical *Enterococcus faecium* strains, but not *Enterococcus faecalis* strains, have distinct structural and functional genomic features. *Appl. Environ. Microbiol*. 80:154–165. doi:10.1128/AEM.03108-13.

Larsen, M. V., S. Cosentino, O. Lukjancenko, D. Saputra, S. Rasmussen, H. Hasman, T. Sicheritz-Pontén, F. M. Aarestrup, D. W. Ussery, and O. Lund. 2014. Benchmarking of methods for genomic taxonomy. *J. Clin. Microbiol.* 52:1529–1539. doi:10.1128/JCM.02981-13.

Leavis, H. L., R. J. Willems, W. J. van Wamel, F. H. Schuren, M. P. Caspers, and M. J. Bonten. 2007. Insertion sequence-driven diversification creates a globally dispersed emerging multiresistant subspecies of *E. faecium*. *PLoS Pathog.* 3:e7. doi:10.1371/journal.ppat.0030007.

Lebreton, F., W. van Schaik, A. M. McGuire, P. Godfrey, A. Griggs, V. Mazumdar, J. Corander, L. Cheng, S. Saif, S. Young, et al. 2013. Emergence of epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains. *mBio* 4(4):e00534-13. doi: 10.1128/mBio.00534-13

Lee, T., S. Pang, S. Abraham, and G. W. Coombs. 2019. Antimicrobial-resistant CC17 *Enterococcus faecium*: the past, the present and the future. *J. Glob. Antimicrob. Resist.* 16:36–47. doi:10.1016/j.jgar.2018.08.016.

Lodemann, U., K. Hübener, N. Jansen, and H. Martens. 2006. Effects of *Enterococcus faecium* NCIMB 10415 as probiotic supplement on intestinal transport and barrier function of piglets. *Arch. Anim. Nutr.* 60:35–48. doi:10.1080/17450390500468099.

Mamuad, L. L., S. H. Kim, A. A. Biswas, Z. Yu, K. K. Cho, S. B. Kim, K. Lee, and S. S. Lee. 2019. Rumen fermentation and microbial community composition influenced by live *Enterococcus faecium* supplementation. *AMB Express* 9:123. doi:10.1186/s13568-019-0848-8.

Masucci, F., G. De Rosa, F. Grasso, F. Napolitano, G. Esposito, and A. Di Francia. 2011. Performance and immune response of buffalo calves supplemented with probiotic. *Livest. Sci.* 137(*1*):24–30. doi:10.1016/j.livsci.2010.09.019.

Moellering, R. C., Jr., and A. N. Weinberg. 1971. Studies on antibiotic syngerism against *Enterococci*. II. Effect of various antibiotics on the uptake of 14 C-labeled streptomycin by *Enterococci*. *J. Clin. Invest.* 50(*12*):2580–2584. doi: 10.1172/JCI106758.

Morandi, S., P. Cremonesi, M. Povolo, and M. Brasca. 2012. *Enterococcus lactis* sp. nov., from Italian raw milk cheeses. *Int. J. Syst. Evol. Microbiol.* 62(Pt 8):1992–1996. doi:10.1099/ijs.0.030825-0.

Murray, B. E. 1990. The life and times of the *Enterococcus*. *Clin. Microbiol. Rev.* 3:46–65. doi:10.1128/CMR.3.1.46.

Nascimento, M., A. Sousa, M. Ramirez, A. P. Francisco, J. A. Carriço, and C. Vaz. 2017. PHYLOViZ 2.0: providing scalable data integration and visualization for multiple phylogenetic inference methods. *Bioinformatics* 33:128–129. doi:10.1093/bioinformatics/btw582.

Natarajan, P., and M. Parani. 2015. First complete genome sequence of a probiotic *Enterococcus faecium* strain T-110 and its comparative genome analysis with pathogenic and non-pathogenic *Enterococcus faecium* genomes. *J. Genet. Genomics* 42:43–46. doi:10.1016/j.jgg.2014.07.002.

Nocek, J. E., W. P. Kautz, J. A. Leedle, and J. G. Allman. 2002. Ruminal supplementation of direct-fed microbials on diurnal pH variation and in situ digestion in dairy cattle. *J. Dairy Sci.* 85:429–433. doi:10.3168/jds.S0022-0302(02)74091-5.

Nocek, J. E., W. P. Kautz, J. A. Leedle, and E. Block. 2003. Direct-fed microbial supplementation on the performance of dairy cattle during the transition period. *J. Dairy Sci.* 86:331–335. doi:10.3168/jds.S0022-0302(03)73610-8.

Noguchi, N., H. Nakaminami, K. Nakase, and M. Sasatsu. 2011. Characterization of enterococcus strains contained in probiotic products. *Biol. Pharm. Bull.* 34:1469–1473. doi:10.1248/bpb.34.1469.

Ogier, J. C., and P. Serror. 2008. Safety assessment of dairy microorganisms: the *Enterococcus* genus. *Int. J. Food Microbiol.* 126:291–301. doi:10.1016/j.ijfoodmicro.2007.08.017.

Ouwehand, A. C., P. V. Kirjavainen, C. Shortt, and S. Salminen. 1999. Probiotics: mechanisms and established effects. *Int. Dairy J.* 9(1):43–52. doi: 10.1016/S0958-6946(99)00043-6

Overbeek, R., R. Olson, G. D. Pusch, G. J. Olsen, J. J. Davis, T. Disz, R. A. Edwards, S. Gerdes, B. Parrello, M. Shukla, et al. 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res.* 42(Database issue):D206–D214. doi:10.1093/nar/gkt1226.

Pang, D. G., H. J. Yang, B. B. Cao, T. T. Wu, and J. Q. Wang. 2014. The beneficial effect of *Enterococcus faecium* on the in vitro ruminal fermentation rate and extent of three typical total mixed rations in northern China. *Livestock Sci.* 167:154–160. doi: 10.1016/j.livsci.2014.06.008.

Pollmann, M., M. Nordhoff, A. Pospischil, K. Tedin, and L. H. Wieler. 2005. Effects of a probiotic strain of *Enterococcus faecium* on the rate of natural chlamydia infection in swine. *Infect. Immun.* 73:4346–4353. doi:10.1128/IAI.73.7.4346-4353.2005.

Rambaut, A. 2012. FigTree v1. 4.

Rice, L. B. 2008. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J. Infect. Dis.* 197:1079–1081. doi:10.1086/533452.

Scharek, L., J. Guth, K. Reiter, K. D. Weyrauch, D. Taras, P. Schwerk, P. Schierack, M. F. Schmidt, L. H. Wieler, and K. Tedin. 2005. Influence of a probiotic *Enterococcus faecium* strain on development of the immune system of sows and piglets. *Vet. Immunol. Immunopathol.* 105:151–161. doi:10.1016/j.vetimm.2004.12.022.

Taras, D., W. Vahjen, M. Macha, and O. Simon. 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.* 84:608–617. doi:10.2527/2006.843608x.

Treitman, A. N., P. R. Yarnold, J. Warren, and G. A. Noskin. 2005. Emerging incidence of *Enterococcus faecium* among hospital isolates (1993 to 2002). *J. Clin. Microbiol.* 43:462–463. doi:10.1128/JCM.43.1.462-463.2005.

Tyson, G. H., J. L. Sabo, C. Rice-Trujillo, J. Hernandez, and P. F. McDermott. 2018. Whole-genome sequencing based characterization of antimicrobial resistance in *Enterococcus*. *Pathog. Dis.* 76(2):fty018. doi: 10.1093/femspd/fty018.

Urshev, Z., and T. Yungareva. 2021. Initial safety evaluation of *Enterococcus faecium* LBB.E81. *Biotechnol. Biotechnol Equip.* 35(1):11–17. doi: 10.1080/13102818.2020.1840438.

van Schaik, W., J. Top, D. R. Riley, J. Boekhorst, J. E. Vrijenhoek, C. M. Schapendonk, A. P. Hendrickx, I. J. Nijman, M. J. Bonten, H. Tettelin, et al. 2010. Pyrosequencing-based comparative genome analysis of the nosocomial pathogen *Enterococcus faecium* and identification of a large transferable pathogenicity island. *BMC Genomics* 11:239. doi:10.1186/1471-2164-11-239.

Wirth, T., D. Falush, R. Lan, F. Colles, P. Mensa, L. H. Wieler, H. Karch, P. R. Reeves, M. C. Maiden, H. Ochman, et al. 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Mol. Microbiol.* 60:1136–1151. doi:10.1111/j.1365-2958.2006.05172.x.

Zankari, E., H. Hasman, S. Cosentino, M. Vestergaard, S. Rasmussen, O. Lund, F. M. Aarestrup, and M. V. Larsen. 2012. Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67:2640–2644. doi:10.1093/jac/dks261.