Antimicrobial resistance of *Enterococcus faecium* strains isolated from commercial probiotic products used in cattle and swine[1,2]

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ABSTRACT: Probiotics, an antibiotic alternative, are widely used as feed additives for performance benefits in cattle and swine production systems. Among bacterial species contained in probiotics, *Enterococcus faecium* is common. Antimicrobial resistance (**AMR**), particularly multidrug resistance, is a common trait among enterococci because of their propensity to acquire resistance and horizontally transfer AMR genes. Also, *E. faecium* is an opportunistic pathogen, and in the United States, it is the second most common nosocomial pathogen. There has been no published study on AMR and virulence potential in *E. faecium* contained in probiotic products used in cattle and swine in the United States. Therefore, our objectives were to determine phenotypic susceptibilities or resistance to antimicrobials, virulence genes (*asa1*, *gelE*, *cylA*, *esp*, and *hyl*) and assess genetic diversity of *E. faecium* isolated from commercial products. Twenty-two commercially available *E. faecium*-based probiotic products used in cattle (n = 13) and swine (n = 9) were procured and *E. faecium* was isolated and species confirmed. Antimicrobial susceptibility testing to determine minimum inhibitory concentrations was done by micro-broth dilution method using National Antimicrobial Resistance Monitoring Systems Gram-positive Sensititre panel plate (CMV3AGPF), and categorization of strains as susceptible or resistant was as per Clinical Laboratory and Standards Institute's guidelines. *E. faecium* strains from 7 products (3 for swine and 4 for cattle) were pan-susceptible to the 16 antimicrobials tested. Strains from 15 products (6 for swine and 9 for cattle) exhibited resistance to at least one antimicrobial and a high proportion of strains was resistant to lincomycin (10/22), followed by tetracycline (4/22), daptomycin (4/22), ciprofloxacin (4/22), kanamycin (3/22), and penicillin (2/22). Four strains were multidrug resistant, with resistant phenotypes ranging from 3 to 6 antimicrobials or class. None of the *E. faecium* strains were positive for any of the virulence genes tested. The clonal relationships among the 22 *E. faecium* strains were determined by pulsed-field gel electrophoresis (**PFGE**) typing. A total of 10 PFGE patterns were observed with 22 strains and a few of the strains from different probiotic products had identical (100% Dice similarity) PFGE patterns. In conclusion, the *E. faecium* strains in a few commercial probiotics exhibited AMR to medically-important antimicrobials, but none contained virulence genes.

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

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INTRODUCTION

Probiotics or direct-fed microbials are widely used as feed additives in cattle and swine production systems (Krehbiel *et al.*, 2003; Cho *et al.*, 2011). The use of probiotics is a sustainable alternative to antibiotics because performance benefits are attained or claimed with a concurrent reduction in the overall exposure of gut flora to antimicrobials. Most probiotic products include one or more species of the following bacterial genera: *Lactobacillus, Enterococcus, Bifidobacterium, Pediococcus, Bacillus, Leuconostoc,* and *Propionibacterium,* and many also include species of fungi, *Saccharomyces* and *Aspergillus*. Although probiotics are generally recognized as safe, there are two possible concerns with the safety of bacterial species: 1) they may carry virulence genes and produce virulence factors that could cause infections (Senok *et al.*, 2005; Boyle *et al.*, 2006), and 2) they may carry antimicrobial resistance (**AMR**) genes; therefore, they may be a source of AMR and could potentially transfer AMR genes to other bacteria, including pathogenic bacteria (Ashraf and Shah, 2011).

Among probiotic bacteria used in cattle and swine products, *Enterococcus faecium* is a common species in many commercial products. Enterococci, particularly *E. faecium,* are opportunistic pathogens, and in the United States, it is the second most prevalent nosocomial pathogen (Arias and Murray, 2012). In Europe, according to the Qualified Presumption of Safety (**QPS**) approach (similar to "Generally Recognized as Safe" status in the United States), established by the European Food Safety Authority (EFSA, 2012), the nature of any antibiotic resistant determinant contained in a microorganism should be determined prior to approval of the product. There has been no published study on the AMR of *E. faecium* contained in probiotic products used in cattle and swine. Therefore, our objectives were to determine phenotypic susceptibility or resistance to antimicrobials, virulence genes, and genetic diversity of *E. faecium* isolated from commercial probiotic products.

MATERIALS AND METHODS

Probiotic Products

Twenty-two (n = 22) commercially available *E. faecium*-containing probiotic products used in swine (n = 9) and cattle (n = 13) in the United States were purchased. All products were stored as per manufacturer's guidelines. In order to maintain confidentiality of the commercial products, a letter code (A to V) was assigned to each product.

Isolation and Identification of E. faecium

Approximately, 1 g of the probiotic product was suspended in 9 mL of phosphate-buffered saline and vortexed to obtain a uniform suspension. Fifty microliters of the probiotic suspension were spread-plated onto M-*Enterococcus* agar and incubated at 42°C for 24 h. Putative colonies (pin-point red, pink, or metallic red) were selected from the plate and streaked onto blood agar plates (Remel, Lenexa, KS) and incubated overnight at 37°C for 24 h. The presumptive isolates that were Gram positive cocci were tested for esculin hydrolysis for genus confirmation. The test was done by inoculating a single colony into 100 µL of Enterococcosel broth in a 96-well microtiter plate (Becton and Dickson, Franklin Lakes, NJ) and incubating at 37°C for 4 h. Species confirmation of the esculin-positive isolates was carried out using a polymerase chain reaction (PCR) procedure designed to identify *E. faecium* and three other species (*faecalis, gallinarum,* and *cassiliflavus*) of enterococci (Jackson *et al.*, 2004). The DNA was isolated by suspending a single colony in nuclease-free water with 5% Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA) and boiled for 10 min. An ATCC strain of *E. faecium* (ATCC 19434; American Type Culture Collection, Manassas, VA) was used as a positive control in the assay. The primers (forward and reverse) were supplied by Invitrogen Life Technologies (Carlsbad, CA). The isolated strains were also subjected to matrix-assisted laser desorption/ionization time-of-flight (**MALDI-TOF**) mass spectrometry (Bruker Daltonics Inc., Billerica, MA) for species confirmation (Clark *et al.*, 2013).

Antimicrobial Susceptibility Determinations

Isolates confirmed as *E. faecium* were subjected to antimicrobial susceptibility testing to determine minimum inhibitory concentrations (**MIC**) of antimicrobials by the micro-broth dilution method according to the Clinical Laboratory and Standards Institute (CLSI, 2013). Bacterial inocula concentrations were adjusted to 0.5 McFarland turbidity standards by mixing individual bacterial colonies with demineralized water (Trek Diagnostic Systems, Cleveland, OH). A 50-µL aliquot of the bacterial inoculum was added to Mueller-Hinton broth (11 mL) (Trek Diagnostics Systems, Cleveland,

OH) and vortexed. Then, 100 µL of the Mueller-Hinton broth containing bacterial inoculum were dispensed into Gram-positive NARMS panel plates (CMV3AGPF, Trek Diagnostics Systems, Cleveland, OH) with the aid of the Sensititre automated inoculation delivery system (Trek Diagnostics Systems). Plates were incubated for 18 h at 37°C. The breakpoints were recorded as resistant, intermediate, or sensitive based on the CLSI guidelines (CLSI, 2013). *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 strains served as reference quality control strains.

Detection of Virulence Genes

Isolates confirmed as *E. faecium* were tested by PCR for the virulence genes, *asa1* (aggregation substance), *gelE* (gelatinase), *cylA* (cytolysin), *esp* (enterococcal surface protein), and *hyl* (hyaluronidase), by a multiplex PCR assay. The DNA template was prepared as before and *E. faecalis* MMH594 (positive for the 5 virulence genes; Amachawadi *et al.*, 2015) was used as a positive control. Master mixes, primers and running conditions for the multiplex PCR were as described by Vankerckhoven *et al.* (2004).

*Pulsed-Field Gel Electrophoresis (*PFGE*)*

The PFGE analyses of *E. faecium* strains were performed as per Murray *et al.* (1990) with minor modifications. A single colony of the strain was inoculated into 5 mL of brain heart infusion broth and incubated overnight at 37°C. One milliliter of the overnight culture (absorbance of 1 to 1.2 at 600 nm) was transferred into 1.5 mL Eppendorf tube and bacterial cells were pelleted by centrifugation at 9,300 ×*g* for 1 min, followed by suspension of the pellet in 200 µL of 0.85% NaCl. The plugs were prepared by mixing 200 µL of the cell suspension with 200 µl of 1.6% SeaKem gold agarose. The plugs were lysed by transferring them into a 10-mL lysis solution [6 mM Tris-HCl, pH 7.4, 100 mM EDTA, 1 M NaCl, 0.5% sodium lauroyl sarcosine, 0.5% Brij, 0.2% deoxycholate, lysozyme (500 µg/mL), and RNaseA (20 µg/mL)] for 4 h at 37°C with gentle shaking. Then, the plugs were transferred to ESP buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% SDS, and 50 µg/mL proteinase K) and incubated overnight at 50°C with gentle shaking. The plugs were washed with TE dilute (10 mM Tris-HCl, pH-7.4, 0.1 mM EDTA) for three times at room temperature for 10 min each and stored at 4°C until used.

Restriction digestion of the plugs was carried out with 2 µL of *Sma*I in 100 µL of 10× buffer by placing a small slice of agarose plug (1 mm wide slices) in a 1.5-mL centrifuge tube and incubated at 25°C for 4 h. The digested plugs were transferred onto an agarose gel (1% SeaKem Gold Agarose prepared in 0.5× Tris-borate-EDTA buffer) in the wells and the remaining spaces in the wells were then filled with the agarose gel. Then the gel was placed in an electric field device CHEF-DR II (Bio-Rad, Richmond, CA) at 200 V. The gel running conditions and pulse times were as follows: for block 1, an initial time of 3.5 s and a final time of 25 s for 12 h; and for block 2, an initial time of 1 s and a final time of 5 s for 8 h. After the last pulse time, the gel was stained with 0.0001% (or 1 µg/mL) ethidium bromide for 30 min followed by destaining 3 times for 20 min each in distilled water. Gel images were captured using a GEL DOC 2000 system (Bio-Rad).

The banding patterns were analyzed and compared by using BioNumerics version 3.0 (Applied Maths, Austin, TX). Isolates were grouped based on identical banding patterns (100% Dice similarity). We used the band-based Dice similarity coefficient (clustering with 1.5% for optimization and 1.5% for band comparison) and the unweighted-pair group mathematical average algorithm method [unweighted-pair group method using average linkages (UPGMA)]. A strain of *Salmonella* Braenderup H9812 was used as the standard.

Statistical Analysis

Data were analyzed using STATA SE version 12.1 (Stata Corp., College Station, TX). The Sensititre data for 16 antimicrobials were considered resistant or susceptible to *E. faecium* based on their breakpoints, and outcomes were categorized as binary for further analyses and to obtain multidrug resistant profiles (resistant to ≥ 3 antimicrobial classes). Bivariate descriptive statistics on binary outcomes were expressed as proportions with exact 95% confidence intervals. The 95% confidence intervals for the MIC50 and MIC90 were estimated for frequency distributions of MIC for each antimicrobial.

RESULTS

Twenty-two (n = 22) *E. faecium* isolates were obtained from swine (n = 9) and cattle (n = 13) probiotic products. All 22 isolates were confirmed as *E. faecium* by PCR, and MALDI-TOF mass

spectrometry correctly identified all 22 strains as *E. faecium*. None of the 22 strains contained any of the 5 virulence genes (*asa1*, *gelE*, *cylA*, *esp*, and *hyl*) tested.

Phenotypic Susceptibilities to Antimicrobials

Minimum inhibitory concentrations for each swine (n = 9) and cattle (n = 13) *E. faecium* strain are presented in Tables 1 and 2, respectively. Strains from 7 products (3 swine and 4 cattle) were pan susceptible to the 16 antimicrobials included in the panel. All 22 strains were susceptible, based on CLSI breakpoints, to gentamicin, linezolid, nitrofurantoin, quinupristin/dalfopristin, streptomycin, tigecycline, tylosin, and vancomycin. *E. faecium* from 15 products (6 swine and 9 cattle) exhibited resistance to at least one antimicrobial. A high number of strains was resistant to lincomycin (11/22), followed by tetracycline (4/22), daptomycin (4/22), ciprofloxacin (4/22), kanamycin (3/22), and penicillin (2/22). The number of strains resistant and MIC50 and MIC90 for all strains are presented in Table 3. Four strains (1 swine and 3 cattle strains) were multidrug resistant (**MDR**), with resistant phenotypes ranging from 3 to 6 antimicrobials (Table 4). More often, the MDR strains were resistant to chloramphenicol, erythromycin, kanamycin, lincomycin, penicillin, and tetracycline.

PFGE Typing

The clonal relationships of the 22 *E. faecium* strains determined by PFGE typing are shown in Fig. 1. A total of 10 PFGE patterns were observed with 22 strains. Nine swine *E. faecium* strains shared 6 PFGE types and 13 cattle strains shared 7 PFGE types. Several strains from cattle or swine probiotic products had identical banding patterns (100% Dice similarity). Among cattle probiotic products, strains from L and N, O and T, P, Q, R, and S, and J and K were of the same PFGE types. Similarly, among swine probiotic products, strains from B, G and I, and strains from D and F were of the same PFGE types. Three swine strains (B, G, and I) had identical PFGE patterns as two cattle strains (O and T). The swine strain, E, shared same PFGE pattern with two cattle strains, L and N. Another swine strain (H) had identical banding pattern of a cattle strain (U).

DISCUSSION

Probiotics have been a popular feed additive in food animal production systems because performance benefits are obtained or claimed without the concern of generating AMR in gut bacteria associated with the use of antimicrobials in feeds (Thacker, 2013). Because probiotics contain live

Table 1. Antimicrobial susceptibilities of *Enterococcus faecium* strains isolated from commercially available swine probiotic products

Antimicrobials	Concentration range (μg/mL)	Breakpoints (μg/mL)[1]	Swine probiotic products								
			A	B	C	D	E	F	G	H	I
Chloramphenicol	2–32	≥32	2	4	4	8	4	4	4	4	4
Ciprofloxacin	0.12–4	≥4	4	1	0.5	1	1	1	1	0.5	1
Daptomycin	0.25–16	N/A[2]	16	8	2	4	4	2	4	2	8
Erythromycin	0.25–8	≥8	1	2	4	2	2	2	2	4	2
Gentamicin	128–1,024	>500	128	128	128	128	128	128	128	128	128
Kanamycin	128–1,024	≥1,024	256	512	128	128	128	256	512	256	512
Lincomycin	1–8	≥8	1	8	4	8	4	8	8	4	8
Linezolid	0.5–8	≥8	1	2	2	2	2	2	2	2	2
Nitrofurantoin	2–64	≥128	64	64	64	64	64	64	64	64	64
Penicillin	0.25–16	≥16	0.25	4	4	2	4	2	4	4	4
Quinupristin/Dalfopristin	0.5–32	≥4	0.5	1	2	2	1	2	1	2	1
Streptomycin	512–2,048	>1,000	512	512	512	512	512	512	512	512	512
Tetracycline	1–32	≥16	32	1	1	1	1	1	1	1	1
Tigecycline	0.015–0.5	N/A[3]	0.06	0.06	0.12	0.06	0.06	0.06	0.06	0.06	0.06
Tylosin	0.25–32	≥32	1	4	2	4	2	4	4	4	4
Vancomycin	0.25–32	≥32	2	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

[1]Breakpoints established by Clinical and Laboratory Standards Institute (2013).

[2]N/A = not applicable. A susceptibility breakpoint of ≤ 4 μg/mL for daptomycin exists but no resistant breakpoint has been established. In this study, isolates with a minimal inhibitory concentration ≥ 8 μg/mL were categorized as resistant.

[3]A susceptibility breakpoint of ≤0.25 μg/mL for tigecycline exists but no resistant breakpoint has been established. In this study, isolates with a minimal inhibitory concentration ≥ 0.5 μg/mL were categorized as resistant.

Table 2. Antimicrobial susceptibilities of *Enterococcus faecium* strains isolated from commercially available cattle probiotic products

Antimicrobials	Concentration range (µg/mL)	Breakpoints (µg/mL)[1]	Cattle probiotic products												
			J	K	L	M	N	O	P	Q	R	S	T	U	V
Chloramphenicol	2–32	≥32	32	32	8	4	4	4	8	4	8	8	4	4	32
Ciprofloxacin	0.12–4	≥4	0.5	0.5	1	0.5	1	1	4	4	4	4	1	0.5	2
Daptomycin	0.25–16	N/A[2]	2	4	4	4	2	8	4	4	4	4	4	4	4
Erythromycin	0.25–8	≥8	8	8	4	2	2	2	2	2	2	4	4	4	4
Gentamicin	128–1,024	>500	128	128	128	128	128	128	128	128	128	128	128	128	128
Kanamycin	128–1,024	≥1,024	1024	1024	256	128	128	512	256	128	128	256	512	128	1024
Lincomycin	1–8	≥8	8	8	4	8	4	8	1	1	1	1		4	8
Linezolid	0.5–8	≥8	2	2	2	2	2	2	2	2	2	2	2	2	2
Nitrofurantoin	2–64	≥128	64	64	64	64	64	64	64	32	64	64	64	32	64
Penicillin	0.25–16	≥16	16	16	4	1	4	4	1	1	1	2	4	4	8
Quinupristin/dalfopristin	0.5–32	≥4	2	2	1	2	1	1	0.5	0.5	0.5	0.5	2	2	1
Streptomycin	512–2,048	>1,000	512	512	512	512	512	512	512	512	512	512	512	512	512
Tetracycline	1–32	≥16	32	32	1	1	1	1	1	1	1	1	1	1	32
Tigecycline	0.015–0.5	N/A[3]	0.12	0.12	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.12	0.12	0.06	0.06
Tylosin	0.25–32	≥32	8	8	2	4	2	4	1	1	1	1	4	2	4
Vancomycin	0.25–32	≥32	0.5	0.5	0.5	2	0.5	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5

[1]Breakpoints established by Clinical and Laboratory Standards Institute (2013).

[2]N/A = not applicable. A susceptibility breakpoint of ≤4 µg/mL for daptomycin exists but no resistant breakpoint has been established. In this study, isolates with a minimal inhibitory concentration ≥ 8 µg/mL were categorized as resistant.

[3]A susceptibility breakpoint of ≤0.25 µg/mL for tigecycline exists but no resistant breakpoint has been established. In this study, isolates with a minimal inhibitory concentration ≥ 0.5 µg/mL were categorized as resistant.

bacteria and almost all bacterial species are intrinsically resistant or become resistant by mutation or acquisition by gene transfer to one or more antimicrobials, it is of interest to determine whether bacterial species in commercial products are resistant to antimicrobials, particularly to medically-important antimicrobials (WHO, 2012; CDC, 2013). Among probiotic bacterial species, we chose to focus on *E. faecium* because a number of commercial products contain the species, but more importantly,

Table 3. Antimicrobial susceptibilities and minimum inhibitory concentrations of *Enterococcus faecium* strains isolated from commercially available cattle and swine probiotic products

Antimicrobials	Swine strains (n = 9)			Cattle strains (n = 13)		
	Number of strains resistant	MIC50* (µg/mL)	MIC90** (µg/mL)	Number of strains resistant	MIC50 (µg/mL)	MIC90 (µg/mL)
Chloramphenicol	0	4	8	3	8	32
Ciprofloxacin	1	1	4	4	1	4
Daptomycin	3	4	16	1	4	4
Erythromycin	0	2	4	2	4	8
Gentamicin	0	128	128	0	128	128
Kanamycin	0	256	512	3	256	1,024
Lincomycin	5	6	8	6	4	8
Linezolid	0	2	2	0	2	2
Nitrofurantoin	0	64	64	0	64	64
Penicillin	0	4	4	2	4	16
Quinupristin/dalfopristin	0	1.5	2	0	1	2
Streptomycin	0	512	512	0	512	512
Tetracycline	1	1	32	3	1	32
Tigecycline	0	0.06	0.12	0	0.06	0.12
Tylosin	0	4	4	0	2	8
Vancomycin	0	0.5	2	0	0.5	1.7

*MIC50 = MIC values at which 50% of the isolates are inhibited.

**MIC90 = MIC values at which 90% of the isolates are inhibited.

Table 4. Phenotypic resistance patterns of *Enterococcus faecium* strains isolated from commercially available cattle and swine probiotics

Animal species	Product code	Phenotypic resistance pattern	Total number of antimicrobials
Swine	A	Ciprofloxacin, Daptomycin, Tetracycline	3
	B	Daptomycin, Lincomycin	2
	C	None	0
	D	Lincomycin	1
	E	None	0
	F	Lincomycin	1
	G	Lincomycin	1
	H	None	0
	I	Daptomycin, Lincomycin	2
Cattle	J	Chloramphenicol, Erythromycin, Kanamycin, Lincomycin, Penicillin, Tetracycline	6
	K	Chloramphenicol, Erythromycin, Kanamycin, Lincomycin, Penicillin, Tetracycline	6
	L	None	0
	M	Lincomycin	1
	N	None	0
	O	Daptomycin, Lincomycin	2
	P	Ciprofloxacin	1
	Q	Ciprofloxacin	1
	R	Ciprofloxacin	1
	S	None	0
	T	Lincomycin	1
	U	None	0
	V	Chloramphenicol, Kanamycin, Lincomycin, Tetracycline	4

this species has the propensity to become resistant to antimicrobials, including multidrug resistance, because of genetic mutation or acquisition of resistance via mobile genetic elements, (Miller *et al.*, 2014). Additionally, *E. faecium* is a major nosocomial pathogen and is often associated with multiple antibiotic resistant infections of blood stream, surgical wounds, and urinary tract (Palmer *et al.*, 2010). None of the 22 strains of *E. faecium* examined possessed any of the virulence genes characteristic of virulent enterococci (Murray, 1990). Previous studies with *E. faecium* strains from human probiotics have also been shown to contain no virulence genes (Eaton and Gasson, 2001; Semedo *et al.*, 2003; Vankerckhoven *et al.*, 2008).

All 22 *E. faecium* strains in this study were susceptible to many of the clinically-relevant antibiotics such as vancomycin, gentamicin, tigecycline, linezolid, nitrofurantoin, quinupristin/dalfopristin, streptomycin, and tylosin. However, *E. faecium* from a few probiotic products ($n = 15$) were resistant to tetracycline, daptomycin, ciprofloxacin, kanamycin, and penicillin. A high number of strains (11/22) were resistant to lincomycin, an antibiotic that is widely used in swine but not used in cattle or in humans (Burch, 2013). Of the 9 swine probiotic *E. faecium* strains, five were resistant to lincomycin and 6 of 13 cattle strains were also resistant (≥8 µg/mL). Four *E. faecium* strains (one swine and three cattle strains) were categorized as MDR because

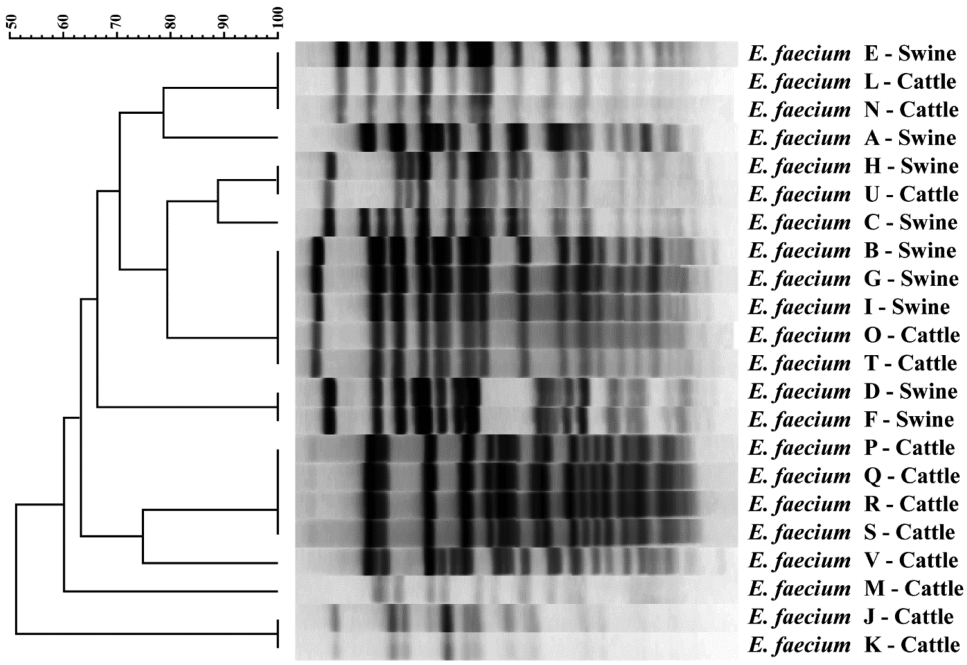

Figure 1. Pulsed-field gel electrophoresis patterns of genomic DNA of *Enterococcus faecium* strains isolated from commercially available swine ($n = 9$) and cattle ($n = 13$) probiotics.

of resistance to ≥ 3 classes (phenicols, macrolides, aminoglycosides, lincosamides, beta-lactams, and tetracyclines) of antimicrobials (chloramphenicol, erythromycin, kanamycin, lincomycin, penicillin, and tetracycline). The MDR trait is more often associated with the promiscuity of acquiring genetic elements through horizontal gene transfer and share some adaptive genetic traits, such as AMR determinants and mobile genetic elements (Mikalsen *et al.*, 2015). In enterococci, horizontal gene transfer facilitates the adaptation by enabling acquisition of AMR and virulence determinants thereby provide selective advantages and promote colonization in the gut (Manson *et al.*, 2010; Palmer *et al.*, 2010; Mikalsen *et al.*, 2015). Susceptibilities of 128 strains of *E. faecium* isolated from human clinical cases and commercial probiotic products were tested for 16 antimicrobials and two isolates were phenotypically resistant to erythromycin and one of them was positive for *erm*(B) gene (Vankerckhoven *et al.*, 2008). Blandino *et al.* (2008) have reported that *E. faecium* isolated from human probiotic products in Italy were susceptible to all the tested antibiotics, which included vancomycin, ampicillin, cefaclor, cefotaxime, erythromycin, ciprofloxacin, and gentamicin. A strain of *E. faecium* isolated from a European probiotic product was resistant to vancomycin using disc diffusion method, but later shown to be vancomycin susceptible by broth dilution and PCR (Temmerman *et al.*, 2003). *Enterococcus* strains used in probiotics in Japan have shown resistance to tetracyclines and beta-lactams (Yamaguchi *et al.*, 2013). Generally, the prevalence of AMR genes in enterococcal strains from clinical samples is greater than food or environmental isolates (Abriouel *et al.*, 2008).

A few studies have examined the transferability of AMR determinants from *E. faecium* to other organisms (Mater *et al.*, 2008; Rizotti et al., 2009; Amachawadi *et al.* 2011). Transfer of *vanA,* which encodes for vancomycin resistance, from enterococci to a probiotic strain of *Lactobacillus acidophilus* has been shown to occur in vitro and in orally-inoculated mice (Mater *et al.*, 2008). Rizotti et al. (2009) have reported on the transferability of *tet*(M) gene from multiple species of *Enterococcus,* including *E. faecium,* to other species within the genus (to *faecalis*) or to *Listeria innocua,* which raises the possibility of spread of tetracycline resistance to potentially pathogenic bacteria. Because enterococci are often found in the same habitat as *Listeria* species, dissemination of AMR by horizontal spread of resistance is possible on farms or food processing facilities (Ashraf and Shah, 2011). Previous studies have focused on the role of commensal lactic acid bacteria as reservoir of AMR genes and their propensity for horizontal gene transfer (Eaton and Gasson, 2001; Toomey *et al.*, 2010, Vignaroli *et al.*, 2011; Jahan *et al.*, 2015). The presence of the transferable resistance and(or) virulence determinants in strains of enterococci indeed fuels the debate about their use in probiotics (Hummel *et al.*, 2007). A study on pyrosequencing-based comparative genome analysis of *E. faecium* strains revealed that the genes involved in environmental persistence, colonization, and virulence can be easily acquired by *E. faecium* (van Schaik *et al.*, 2010).

Pulsed-field gel electrophoresis, a commonly used method for genotyping of enterococci, was used to determine the diversity among probiotic *E. faecium* strains (Kuhn *et al.*, 1995; Tomayko and Murray, 1995). Interestingly, *E. faecium* strains from several cattle and swine probiotic products had identical banding patterns (100% Dice similarity). A few strains from both cattle and swine probiotic products from different manufacturers shared 100% Dice similarity, indicating that they belonged to the same PFGE type. Similarly, in a PFGE analysis of human probiotic *E. faecium* strains, a number of strains had identical banding patterns indicating they belonged to the same PFGE type and a couple of probiotic strains clustered with clinical strains of *E. faecium*, indicating that the clinical strains may be reisolations of probiotic strains (Vankerckhoven *et al.*, 2008). The PFGE typing, although commonly used to track strains in disease outbreaks, is not very discriminatory. It is possible that use of more than one restriction enzyme in the generation of DNA fragments may distinguish the strains that were shown to be identical among the cattle or swine probiotic strains (Murray *et al.*, 1990; Davis *et al.*, 2003). Other methods for assessing genetic diversity, such as multilocus sequence typing, single nucleotide polymorphism analysis, or whole genome sequencing may be more discriminatory than PFGE.

In the United States, probiotic products and or bacterial strains contained in the probiotics are not regulated and not subject to the FDA oversight. However, in the European Union, the use of probiotics is strictly regulated and the word "probiotic" is not used unless a product receives approval for a health claim (https://www.statnews.com/2016/01/21/probiotics-shaky-science/). Although our culture-based isolation, identification, and screening procedures have identified the presence of antibiotic resistance determinants, further studies are needed to apply culture independent methods for genotypic

analysis, such as DNA array or whole genome sequencing, for comprehensive identification of AMR genes in the probiotic strains.

SUMMARY AND CONCLUSIONS

Although *E. faecium* strains (n = 22) isolated from commercial swine and cattle probiotic products were susceptible to many of the antimicrobials, some (n = 15) were resistant to a few medically-important antimicrobials, such as ciprofloxacin, daptomycin, kanamycin, penicillin, and tetracycline. One swine and three cattle strains were multidrug resistant because of resistance to ≥ 3 classes of antimicrobials and resistance phenotypes ranged from 3 to 6 antimicrobials, including erythromycin, kanamycin, penicillin, and tetracycline. None of the *E. faecium* strains contained virulence genes. In conclusion, *E. faecium* strains from a few probiotic products that exhibited phenotypic resistance to medically-important antimicrobials have the potential to be a source of AMR in the gut. The lack of virulence genes in any of the *E. faecium* strains indicates that the probiotic strains are not likely to exhibit virulence and cause infection in the gut.

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