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Safety Assessment of Commercial *Enterococcus* Probiotics in Korea

Lee, Kieun, Minyoung Lee, and Yeonhee Lee*

Culture Collection of Antimicrobial Resistant Microbes, Department of Biology, Seoul Women's University, Seoul 139-774, Korea

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There have been concerns about possible pathogenicity and antimicrobial resistance in *Enterococcus*, which constitute more than 50% of probiotics in the worldwide market. In this study, *Enterococcus* in sixteen products manufactured by ten different companies was tested for the presence of six virulence genes and two vancomycin resistance genes. Results in this study showed the safety of *Enterococcus* on the Korean market and the importance of screening *vanA*, *vanB*, *agg*, *cylA*, *esp*, and *gelE*. Pulse-field gel electrophoresis showed that the sixteen isolates tested in this study are originated from three strains.

Keywords: Probiotic, *Enterococcus*, antimicrobial resistance, safety, virulence factor

Recently, many investigators have speculated that commensal bacteria including lactic-acid-producing bacteria (LAB) may act as reservoirs of antimicrobial resistance genes for human pathogens [8]. Since most LAB are intrinsically resistant to many antimicrobial agents, no particular safety concern is associated with intrinsic type of resistance. However, plasmid-associated antimicrobial resistance, especially in *Enterococcus*, is suspected to have a possibility of the resistance spreading to other more harmful species and genera in the gastrointestinal tract [4]. In addition to antimicrobial resistance, virulence factors such as hemolysin, cytolysin production, and the capacity for adhesion in *Enterococcus* are known to be transmissible by highly efficient gene transfer mechanisms [2]. From these reasons, new *Enterococcus* strains have been prohibited to be on the market [3]. In Korea, *Enterococcus* constitutes more than 50% of the total probiotic market and is easily found in *kimchi* [7, 10]. However, most of them are labeled without the strain number, causing big concerns about their safety as well as violation of the intellectual property. This study was conducted to assess the safety of *Enterococcus* on the market in Korea and the similarity among these.

Sixteen products containing *Enterococcus*, manufactured by ten different companies, were purchased from local supermarkets and total sixteen *Enterococcus* isolates were obtained using Enterococcosel medium (Difco, Detroit, MI, U.S.A.). DNA was isolated from each isolate with cetyltrimethyl ammonium bromide (CTAB) and genes for 16S rRNA were amplified with PCR using a primer set (27F, 5'-AGAGTTTGATCCTGGCTCAG-3'; 1088R, 5'-GCTCGTTGCGGGACTTAACC-3') designed by Suzuki and Giovannoni [15] with 30 cycles of denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec, and polymerization at 72°C for 45 sec. Vancomycin resistance genes (*vanA* and *vanB*) and virulence genes (*agg*, *cylA*, *efaAfs*, *efaAfm*, *esp*, *gelE*) were amplified as shown in Table 1. Quinolone resistance determining regions (QRDRs) in *gyrA* were amplified as described in a previous paper [12]. After PCR products were confirmed on 1% agarose gel, DNA fragments were extracted with the gel extraction kit (Qiaquick; Qiagen, Valencia, CA, U.S.A.) and sequenced with Sanger's method in an API Prism 310 Genetic Analyzer (PE Applied BioSystems, Foster City, GA, U.S.A.). DNA sequences were compared with data in GenBank (http://www.ncbi.nlm.nih.gov/). Type strain *E. faecium* ATCC 19434 and *E. faecalis* ATCC 29212 were included as control strains. Four clinical isolates of *E. facium* and four clinical isolates of *E. faecalis* were provided by the Culture Collection of Antimicrobial Resistant Microbes (CCARM) in Korea. Minimal inhibitory concentrations (MICs) were determined by the agar dilution method following the Clinical and Laboratory Standards Institute's (CLSI) guidelines for veterinary microorganisms [1]. Pulsed-field gel electrophoresis (PFGE) was performed following the method of others [9] with a small modification using 20 U SmaI (MBI Fermentas, Hanover, MD, U.S.A.) to digest DNA in the CHEF-DR III system (Bio-Rad, Richmond, CA, U.S.A.). Digested DNA fragments were separated with 1 pulse with an initial time for 1 sec and a final time for 20 sec for 20 h. After electrophoresis, DNA fragments were stained with

*Corresponding author
Phone: 82-2-970-5664; Fax: 82-2-970-5901;
E-mail: yhlee@swu.ac.kr

Table 1. PCR conditions for amplifying antimicrobial resistance and virulence genes in *Enterococcus* isolates.

Gene	Responsible for	Primer sequence	PCR condition			Size (bp)	Ref.
			Denaturation	Annealing	Extension		
vanA	Vancomycin resistance	5'-GCTATTCAGCTGTACTC-3' 5'-CAGCGGCCATCATACGG-3'	95°C	52°C	72°C	783	[12]
vanB	Vancomycin resistance	5'-CATCGCCGTCCCCGAATTTCAAA-3' 5'-GATGCGGAAGATACCGTGGCT-3'	95°C	52°C	72°C	297	[12]
esp	Evading immunogenicity	5'-TTGCTAATGCTAGTCCACGACC-3' 5'-GCGTCAACACTTGCATTGCCGAA-3'	95°C	63°C	72°C	933	[11]
gelE	Hydrolysis of gelatin, collagen, hemoglobin	5'-ACCCCGTATCATTGGTTT-3' 5'-ACGCATTGCTTTTCCATC-3'	95°C	52°C	72°C	419	[14]
agg	Cell adhesion	5'-AAGAAAAAGAAGTAGACCAAC-3' 5'-AAACGGCAAGACAAGTAAATA-3'	94°C	52°C	72°C	1,553	[5]
efaAfs	Antigen of bacterial endocarditis	5'-GACAGACCCTCACGAATA-3' 5'-AGTTCATCATGCTGTAGTA-3'	94°C	52°C	72°C	705	[13]
efaAfm	Antigen of bacteria endocarditis	5'-AACAGATCCGCATGAATA-3' 5'-CATTTCATCATCTGATAGTA-3'	94°C	52°C	72°C	735	[13]
cylA	Activating cytolysin	5'-TGGATGATAGTGATAGGAAGT-3' 5'-TCTACAGTAAATCTTTCGTCA-3'	94°C	57°C	72°C	517	[6]

10 μg/ml ethidium bromide and analyzed with Gel Compar (Applied Maths, Kortrijk, Belgium). Similarity of more than 85% was considered to be the same group.

Among sixteen *Enterococcus* food isolates, four were labeled as *E. facium*, eight were as *Streptococcus facium*, and six were as *Strep. faecalis*, and none was labeled with a strain number. All of these were identified as *E. faecium*. Food isolates were susceptible to six antimicrobial agents but not to erythromycin and norfloxacin (Table 2). Ten isolates among sixteen food isolates were intermediately resistant to quinolone (MIC=8 μg/ml) and all of the sixteen isolates were intermediately resistant to erythromycin (MIC= 4 μg/ml), whereas type strain *E. faecium* ATCC 19434 was intermediately resistant to quinolones. Since quinolone intermediately resistant food isolates had the same DNA sequence in QRDR of the type strain, they were determined to have no quinolone resistance mutation. Compared with this result, clinical isolates were resistant to quinolone (MIC=64 μg/ml) and erythromycin (MIC>128 μg/ml). Genes for *vanA*, *vanB*, *esp*, *gelE*, *agg*, and *cylA* were not detected in food isolates but were detected in clinical isolates. As expected, *efaAfs* were detected in all *E. faecalis*, whereas *efaAfm* were detected in all *E. faecium*.

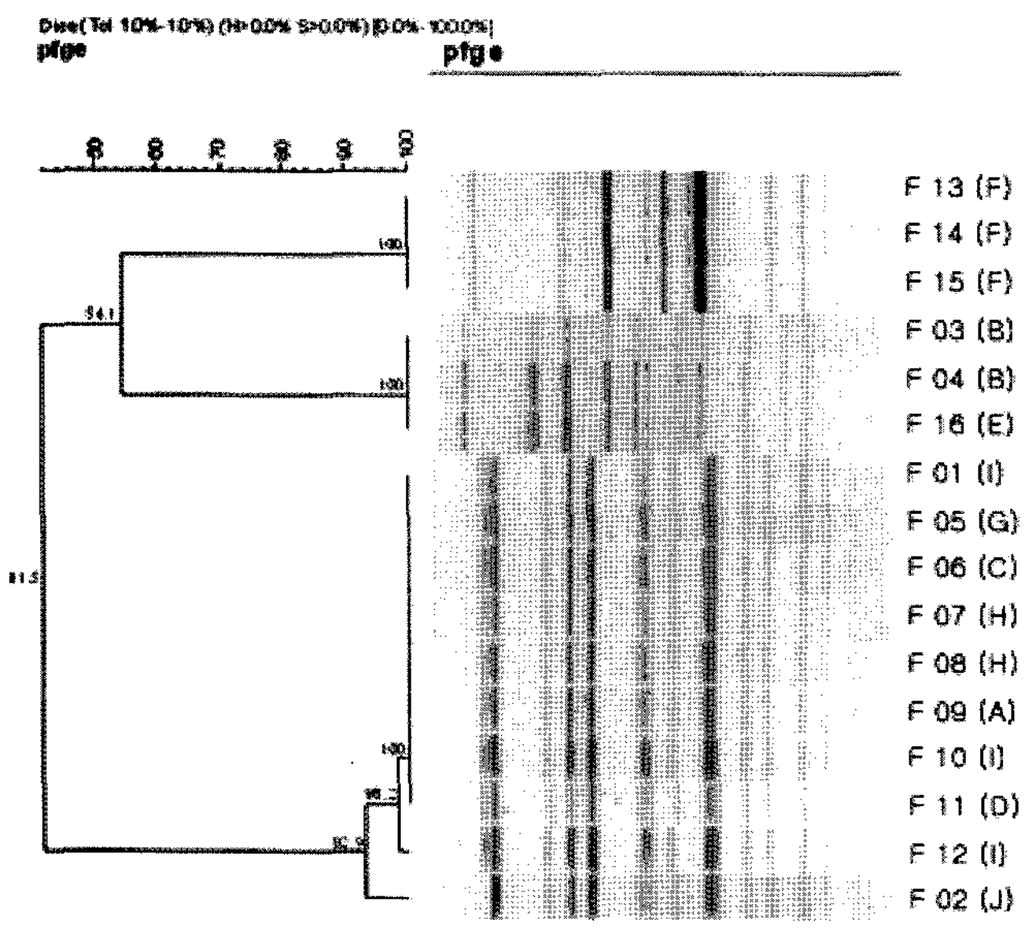

Fig. 1. Pulsed-field gel electrophoresis of sixteen food isolates of *E. faecium* manufactured by ten different companies.
A–J, companies.

Sixteen isolates could be grouped into three with PFGE with similarity higher than 90%: group I, ten isolates from six companies; group II, three isolates from the same company; group III, three isolates from two companies (Fig. 1). The antibiograms (Table 2) coincided with the PFGE results: MICs of isolates in Group A, less than 0.25 μg/ml for tetracycline and 1 μg/ml for vancomycin; MICs of isolates in Group B, less than 0.25 μg/ml for tetracycline and 0.5 μg/ml for vancomycin; MICs of isolates in Group C, 0.5 μg/ml for tetracycline and 1 μg/ml for vancomycin.

Based on the results in this study, we concluded that *Enterococcus* probiotics on the market in Korea at present are safe to be used. However, careful monitoring of resistance and pathogenicity as well as labeling probiotics with a strain number needs to be implemented as soon as possible for safety.

Table 2. MICs of *Enterococcus* isolates and the presence of virulence and resistance genes detected with PCR.

Source	Isolate No.	AMP	CHL	ERY	LEV	NOR	TEI	TET	VAN	*vanA*	*vanB*	*agg*	*cylA*	*efaAfs*	*efaAfm*	*esp*	*gelE*	PFGE group
Product	F01	2	8	4	2	8	1	0.5	1	–	–	–	–	–	+	–	–	C
	F02	2	8	4	4	8	1	0.5	1	–	–	–	–	–	+	–	–	C
	F03	1	4	4	1	4	0.5	<0.25	0.5	–	–	–	–	–	+	–	–	B
	F04	1	4	4	1	4	0.5	<0.25	0.5	–	–	–	–	–	+	–	–	B
	F05	2	4	4	2	8	1	0.5	1	–	–	–	–	–	+	–	–	C
	F06	2	4	4	2	8	1	0.5	1	–	–	–	–	–	+	–	–	C
	F07	2	4	4	2	8	2	0.5	1	–	–	–	–	–	+	–	–	C
	F08	2	4	4	2	8	2	0.5	1	–	–	–	–	–	+	–	–	C
	F09	2	4	4	2	8	2	0.5	1	–	–	–	–	–	+	–	–	C
	F10	2	4	4	2	8	2	0.5	1	–	–	–	–	–	+	–	–	C
	F11	2	4	4	2	8	2	0.5	1	–	–	–	–	–	+	–	–	C
	F12	2	4	4	2	8	2	0.5	1	–	–	–	–	–	+	–	–	C
	F13	2	4	4	1	2	1	<0.25	1	–	–	–	–	–	+	–	–	A
	F14	2	4	4	1	2	1	<0.25	1	–	–	–	–	–	+	–	–	A
	F15	2	4	4	2	2	1	<0.25	1	–	–	–	–	–	+	–	–	A
	F16	1	4	4	2	2	1	<0.25	0.5	–	–	–	–	–	+	–	–	B
Human	P01	>128	16	>128	8	8	64	<0.25	>128	+	–	–	–	–	+	+	–	
	P02	32	8	>128	8	64	<0.25	<0.25	>128	–	+	–	–	–	+	+	–	
	P03	32	8	>128	8	64	<0.25	<0.25	>128	–	+	–	–	–	+	+	–	
	P04	64	8	>128	8	64	0.5	<0.25	>128	–	+	–	–	–	+	+	–	
	P05	1	8	>128	8	4	>128	64	>128	+	–	+	–	+	–	–	+	
	P06	1	8	>128	8	2	<0.25	0.5	32	–	+	+	–	+	–	–	+	
	P07	2	8	1	8	2	<0.25	64	4	–	–	–	–	+	–	+	+	
	P08	1	8	1	8	2	<0.25	8	2	–	–	–	+	+	–	–	+	

AMP, Ampicillin; CHP, Chloroamphenicol; ERY, Erythromycin; LEV, Levofloxacin; NOR, Norfloxacin; TEI, Teicoplanin; TET, Tetracycline; VAN, Vancomycin; *vanA*, a gene for vancomycin resistance; *vanB*, a gene for vancomycin resistance; *agg*, aggregation protein involved in adherence to eukaryotic cells; *cyl*, a gene for activation of cytolysin; *efaAfs*, a gene for cell adhesion in *En. faecalis*; *efaAfm*, a gene for cell adhesion in *En. faecium*; *esp*, a gene for enterococcal surface protein; *gelE*, a gene for gelatinase, extracellular metalloendopeptidase; -, not detected; +, detected.

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