Antibiotic resistance characteristics of potential probiotic *Lactobacillus* strains

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Abstract. Antibiotic-resistant bacteria are increasingly common and threaten human health. Recently, antibiotic resistance in food associated bacteria become an emerging threat to this state. These bacteria may act as reservoirs of antibiotic resistance genes and transfer to commensal or pathogenic bacteria in the human intestines. Therefore, either probiotics or starter cultures have to be evaluated for antibiotic interaction. In this study, the antibiotic susceptibility of seven *Lactobacillus* strains was determined. The MIC values revealed that all strains were resistant to tetracycline, ciprofloxacin, vancomycin and aminoglycoside antibiotics group (gentamicin, neomycin, kanamycin and streptomycin). However, the results from PCR analysis showed that *par*C gene for ciprofloxacin resistance was only present in *Lb. fermentum* SMC2; *van*X gene, responsible for the vancomycin resistance, was found in two strains *Lb. plantarum* AS34 and TJ26. Most strains showed susceptibilityto at least one type of protein synthesis inhibit antibiotics: chloramphenicol, erythromycin and clindamycin. Four strains *Lb. brevis* NCTH24, *Lb. casei* PK2, *Lb. fermentum* SBV2, and *Lb. plantarum* NCDC3 did not carry any antibiotic resistance genes which indicates these antibiotic resistances are intrinsic and nontransmissible.

Keywords: Antibiotic resistance genes, *Lactobacillus*, probiotics.

Classification numbers: 1.2.1, 1.3.2, 1.4.8.

1. INTRODUCTION

Antibiotic resistance (AR) has been developing at an alarming rate and has become a global public health concern. In recent years, studies on the dissemination of antibiotic resistances have focused mainly on clinically relevant bacterial species. More recently, it was speculated that food bacteria may carry antibiotic resistance genes which might be transferred to other bacteria when they enter the intestines. According to the European Food Safety Authority (EFSA) guidelines, bacteria intended for human consumption do not carrying any transmissible antibiotic resistance genes [1].

Lactobacillus are lactic acid bacteria of high biotechnological and natural significance. They are often used in foods fermentation and preservation. In addition, some lactobacilli exist naturally in human intestine and play important roles in maintaining the host colonic health,

intestinal microecological balance and strengthening the immune function. Many *Lactobacillus* species possessing intrinsically antibiotics resistance, may benefit patients whose normal intestinal microbiota have become unbalanced or greatly reduced in numbers due to antimicrobial agent's treatment. However, potentially transmissible plasmid and transposon-located antibiotic resistance genes have been also reported in certain *Lactobacillus* strains. The *tet*S gene in the probiotic strain *Lb. plantarum* CCUG 43738 was found to be located on a 14 kbp plasmid [2]. The *erm*(B) in *Lb. johnsonii* G41, which seems to have become inserted into the chromosome from a plasmid-encoded *erm(*B) locus of *Enterococcus faecalis* [3]. Moreover, chloramphenicol-resistance *cat* gene has been found in *L. plantarum* [4]. Different erythromycin resistance genes (*erm*B, *erm*A, *erm*C, *erm*T) and at least 11 tetracycline resistance genes (*tet*W, *tet*M, *tet*S, t*et*O, *tet*Q, *tet*36, *tet*Z, *tet*O/W/32/O/W/O, *tet*W/O, *tet*K, and *tet*L) have been detected to date in lactobacilli [5, 6]. Aminoglycoside resistance genes, such as *aac*(6)-*aph*(2), *ant*(6), and *aph*(3)-IIIa [7], and β-lactam resistance genes (*blaZ*) were found much less frequently in lactobacilli [8]. This study aimed to investigate the antibiotic resistance of seven *Lactobacillus* strains having potential application as probiotics, to common 14 antibiotics.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

*Lactobacillu*s strains including: *Lb. plantarum*AS34 (isolated from breast milk)*, Lb. plantarum* NCDC3, *Lb. plantarum* TJ26 and *Lb. brevis* NCTH24 (isolated from fermented pork meat), *Lb. fermentum* SBV2 and *Lb. fermentum* SMC2 (isolated from fermented milk), *Lb. casei* PK2 (isolated from feces of breast-fed infant) were obtained from FIRI's culture collection. All strains were appreciated for probiotic characteristics such as tolerance to low pH and bile salt resistance, persistence in the gastrointestinal tract, adhesion to simulated intestinal mucus and growth inhibition of enteric pathogens. All strains were preserved 40 % glycerol stocks at - 80 ^{o}C and activated by growing in de Man, Rogosa Sharpe (MRS) broth at 37 °C for 24 h.

2.2. Methods

2.2.1. Determination of the minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined using the microdilution method described by ISO10932/IDF223 [9]. The assay was repeated 3 times.

Based on the EFSA guidelines (2018), a strain was recorded to be resistant to an antibiotic, if its MIC value was higher than the reference cut–off value. If its MIC value was equal or lower than the reference cut–off value, the strain was considered susceptible [1]. According to the European Commission (EUC), a strain with a MIC value equal to the reference breakpoint was classified as resistant [10].

2.2.2. DNA extraction and detection of antibiotic resistance genes

Genomic DNA from *Lactobacillus* strains was extracted and purified using a G – Spin^{TM} Kit (Korea). Antibiotic resistance genes were detected by polymerase chain reactions (PCR) with gene specific primers (Table 2) [11]. The PCR mixture consisted of 2 µL of bacterial DNA, 1 µL of each primer (IDT- USA), 12.5 µL of 2X PCR master mix (i-Taq TM – iNtRON Biotechnology, Korea) and nuclease-free water up to a total volume of 25 µL. The PCR

condition and cycles are listed in Table 2. The amplicons were analyzed on 1 % agarose gel to confirm the DNA fragment size.

Table 1. Microbiological breakpoints (µg/mL) for *Lactobacillus* species.

Antibiotic	*Lb. fermentum*	*Lb. plantarum*	*Lb. casei*	*Lb. brevis*
Erythromycin	1[a]	1[a]	1[a]	4[b]
Chloramphenicol	4[a]	8[a]	4[a]	16[b]
Trimethoprim	32[b]	32[b]	32[b]	32[b]
Rifampicin	32[b]	32[b]	32[b]	32[b]
Tetracycline	8[a]	32[a]	4[a]	16[b]
Teicoplanin	n.r[a]	n.r[a]	n.r[a]	n.r[a]
Clindamycin	4[a]	4[a]	4[a]	4[a]
Ciprofloxacin	4[b]	4[b]	4[b]	4[b]
Gentamicin	16[a]	16[a]	32[a]	1[b]
Neomycin	32[b]	32[b]	32[b]	32[b]
Ampicillin	2[a]	2[a]	4[a]	2[b]
Kanamycin	64[a]	64[a]	64[a]	32[b]
Vancomycin	4[b]	4[b]	4[b]	4[b]
Streptomycin	64[a]	16[b]	64[a]	16[b]

[a]: EFSA, 2018; [b]: EUC, 2005; n.r: not required

Table 2. Gene specific primers and conditions for polymerase chain reaction detection.

Antibiotic	AR gene	Primer (5'→3')	Annealing temperature (°C)	Cycles	Amplicon size (bp)
Erythromycin	*erm*(B)	F:GAAAAGRTACTCAACCAAATA R:AGTAACGGTACTTAAATTGTTT AC	52	35	642
	erm(B)-I	F: CATTTAACGACGAAACTGGC R: GGAACATCTGTGGTATGGCG	55	30	405
	erm(C)	F: TCAAAACATAATATAGATAAA R:GCTAATATTGTTTAAATCGTCA AT	50	35	642
Tetracycline	*tet*(M)	F: GTTAAATAGTGTTCTTGGAG R: CTAAGATATGGCTCTAACAA	55	30	576
	tet(K)	F: TTAGGTGAAGGGGTTAGGTGC R: GCAAACTCATTCCAGAAGCA	55	30	697
	tet(W)	F: GAGAGCCTGCTATATGCCAGC R: GGGCGTATCCACAATGTTAAC	64	30	168
Streptomycin	*ant*(6)	F: ACTGGCTTAATCAATTTGGG R: GCCTTTCCGCCACCTCACCG	58	30	597
	*aad*A	F: ATCCTTCGGCGCGATTTTG R: GCAGCGCAATGACATTCTTG	56	35	282
	*aad*E	F: ATGGAATTATTCCCACCTGA R: TCAAAACCCCTATTAAAGCC	5	35	565
Gentamicin	*aph*(6')-*aph*(2'')	F: CCAAGAGCAATAAGGGCATA R: CACTATCATAACCACTACCG	60	30	220

Antibiotic	AR gene	Primer (5'→3')	Annealing temperature (°C)	Cycles	Amplicon size (bp)
	aac(6')Ie-aph(2'')Ia	F: CAGAGGCTTGGGAAGATGAAG R: CCTCGTGTAATTCATGTTCTGGC	58	30	348
Kanamycin Neomycin	*aph*(3')-III	F: GCCGATGTGGATTGCGAAAA R: GCTTGATCCCCAGTAAGTCA	60	30	292
Kanamycin	*ant*(2'')-I	F: GGGGGCGTCATGGAGGAGTT R: TATCGCGACCTGAAAGCGGC	67	35	329
Chloramphenicol	*cat*A	F: GGATATGAAATTTATCCTC R: CAATCATCTACCCTATGAAT	50	30	486
	cat	F: TTAGGTTATTGGGATAAGTTA R: GCATGRTAACCATCACAWAC	48	30	300
Neomycin	*aph*(3'')-I	F: AACGTCTTGCTCGAGGCCGCG R: GGCAAGATCCTGGTATCGGTCT CGG	68	35	670
Ampicillin	*bla*Z	F: ACTTCAACACCTGCTGCTTTC R: TAGGTTCAGATTGGCCCTTAG	58	35	240
	*mec*A	F: GGGATCATAGCGTCATTATTC R: AGTTCTGCAGTACCGGATTTGC	58	35	1429
	bla	F: CATARTTCCGATAATASMGCC R: CGTSTTTAACTAAGTATSGY	51	35	297
Ciprofloxacin	*gyr*A	F: GAYTATGCWATGTCAGTTATTGT R: GGAATRTTRGAYGTCATACCA AC	45	35	451
	*par*C	F: TATTCYAAATAYATCATTCARGA R: GCYTCNGTATAACGCATMGCCG	50	35	286
Clindamycin	*lnu*(A)	F:GCTGGCTGGGGGGTAGATGTAT TAACTGG R:GCTTCTTTTGAAATACATGGTATTT TTCGATC	55	25	323
	lnu(B)	F: CCTACCTATTGTTTGTGGAA R:ATAACGTTACTCTCCTATTTC	54	30	925
Vancomycin	*van*E	F: TGTGGTATCGGAGCTGCAG R: GTCGATTCTCGCTAATCC	52	30	513
	*van*X	F: TCGCGGTAGTCCCACCATTCGTT R: AAATCATCGTTGACCTGCGTTAT	55	30	454
Trimethoprim	*dfr*A	F: CTTTTCTACGCACTAAATGTAAG R: CATTATCAATAATTGTCGCTCAC	50	30	474
	*drf*D	F:GGAAGGGCTTTACCTGACAGGA AG R: CGACATAAGGCAAGAACATAAC ATA	50	30	175
Rifampicin	*rpo*B	F: TAACCGTGGTGCTTGGCTDGAA TWYGAAAC R:ATCAAACCAATGTTAGGNCCT TCWGGDGTTTC	59	30	1100

3. RESULTS AND DISCUSSION

3.1. Antibiotic susceptibility testing of *Lactobacillus* strains

The MIC values of 14 antibiotics for 7 *Lactobacillus* strains were shown in Table 3. The data compared to the breakpoints in the EFSA and EUC guidelines, showed that all tested strains

are resistant to aminoglycoside antibiotics: gentamicin, neomycin, kanamycin and streptomycin – except strain *Lb. casei* PK2; tetracycline; ciprofloxacin and vancomycin. In the study of Zhou *et al*. [12], all probiotic *Lactobacillus* strains were resistant to the aminoglycoside antibiotics. However, Huiling Guo *et al*. [11] have reported that 22 strains of *Lb. plantarum* and *Lb. casei* were sensitive to gentamicin, neomycin, streptomycin, tetracycline and resistant to vancomycin. According to Hikmate Abriouel *et al*. [13], most *Lactobacillus* species were intrinsically resistant to aminoglycosides (neomycin, kanamycin, streptomycin and gentamicin), glycopeptides (vancomycin and teicoplanin) and ciprofloxacin.

Among three *Lb. plantarum* strains, only strain NCDC3 exhibited the phenotype resistance to three antibiotics: erythromycin, chloramphenicol and trimethoprim. Regard to *Lb. fermentum* species, strain SBV2 was sensitive to clindamycin, ampicillin and resistant to erythromycin, chloramphenicol, trimethoprim whereas strain SMC2 was resistant to clindamycin, ampicillin and sensitive to the latter.

Table 3. The MIC values (µg/ml) of antibiotics for *Lactobacillus* strains.

Antibiotic	*Lb. fermentum*		*Lb.plantarum*			*Lb. casei*	*Lb. brevis*
	SBV2	SMC2	NCDC3	AS34	TJ26	PK2	NCTH24
Erythromycin	**4***	0.125	**16**	0.125	0.125	0,5	1
Chloramphenicol	**16**	0.5	**16**	0.5	0.5	4	**32**
Trimethoprim	**> 128**	1	**> 128**	1	1	**32**	**> 128**
Rifampicin	> 16	0.125	> 16	0.125	0.125	0.25	> 16
Tetracycline	**> 64**	**> 64**	**> 64**	**> 64**	**> 64**	**8**	**> 64**
Teicoplanin	> 32	> 32	> 32	> 32	> 32	2	> 32
Clindamycin	0.25	**> 16**	**> 16**	1	**> 16**	1	**> 16**
Ciprofloxacin	**> 64**	**> 64**	**> 64**	**> 64**	**> 64**	**> 64**	**> 64**
Gentamycin	**> 128**	**> 128**	**> 128**	**> 128**	**> 128**	128	**> 128**
Neomycin	**> 64**	**> 64**	**> 64**	**> 64**	**> 64**	**> 64**	**> 64**
Ampicillin	2	**> 16**	**8**	**> 16**	**> 16**	0.5	**8**
Kanamycin	**> 128**	**> 128**	**> 128**	**> 128**	**> 128**	**> 128**	**> 128**
Vancomycin	**> 32**	**> 32**	**> 32**	**> 32**	**> 32**	**> 32**	**> 32**
Streptomycin	**> 128**	**> 128**	**> 128**	**> 128**	**> 128**	16	**> 128**

* The bold data represent that the MIC values is higher than microbiological breakpoints

Among the tested *Lactobacillus* strains, *Lb. casei PK2* was sensitive to many antibiotics: erythromycin, chloramphenicol, clindamycin, ampicillin and streptomycin. In contrast, *Lb. brevis* NCTH24 was resistant to most antibiotics and only sensitive to erythromycin.

3.2. Detection for antibiotic resistance genes in *Lactobacillus* strains

The antibiotic resistance phenotype can be encoded by gene. Moreover, according to Anisimova *et al*. [14], some strains possessing antibiotic – sensitive phenotype, may still carry

non – expressed antibiotic resistance genes. Therefore, in this study, 28 genes associated with resistance to 14 antibiotics were tested in all *Lactobacillus* strains.

Table 4. Detection of antibiotic resistance genes in *Lactobacillus* strains by PCR.

Strain	Antibiotic	Antibiotic resistance gene
Lb. fermentum SBV2		-
*Lb. fermentum*SMC2	Ciprofloxacin	*par*C
Lb. plantarum NCDC3		-
Lb.plantarum AS34	Vancomycin	*van*X
Lb. plantarum TJ26	Vancomycin	*van*X
Lb. casei PK2		-
Lb. brevis NCTH24		-

The results showed that all phenotypically antibiotic–sensitive strains did not contain silent antibiotic resistance genes. Huiling Guo *et al*. [11], has reported that eleven of the thirty – three *Lactobacillus* strains were sensitive to one of the antibiotics: erythromycin, ciprofloxacin and rifampicin but they carried antibiotic resistance genes: *erm*B, *gry*A and *rop*B, respectively.
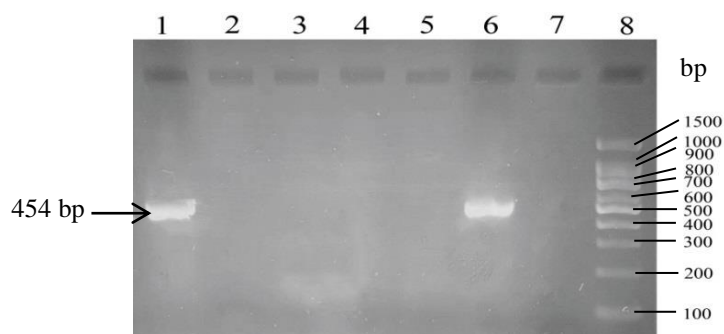

Figure 1. The results of PCR amplification of *van*X (454 bp) gene in 1 % agarose gel.

Lane: 1 -Lb. plantarum AS34, 2 - Lb. fermentum SBV2, 3 - Lb. casei PK2, 4 - Lb. fermentumSMC2, - Lb. brevis NCTH24, - Lb. plantarum TJ26, - Lb. plantarum NCDC3, 8- Marker.

Seven *Lactobacillus* strains were phenotypically resistant to a variety of antibiotics, however only two antibiotic resistance genes were detected in them. Particularly, the *par*C gene, conferring resistance to ciprofloxacin, was found only in *Lb. fermentum* SMC2 and the *van*X gene was identified in two strains *Lb. plantarum* AS34 and TJ26. In the study of Anisimova *et al*. [14], nineteen of twenty were resistant to ciprofloxacin but four strains carried *par*C gene.

The vancomycin resistance has been common in *Lactobacillus, Pediococci* and *Leuconostoc* species. It has been reported to be intrinsic, chromosomally encoded and not inducible or transferable in lactobacilli [15, 16]. The *van*X gene was found in more than 90 % *Lb. plantarum* [12] and 75 % of *Lactobacillus* strains [14]. Among the genes of the vancomycin resistance cluster, only *van*A gene is considered transferable via conjugation within the plasmid

DNA or the conjugative transposon [17, 18]. In our work, no PCR products were amplified with primer of *van*A gene.

Four strains including *Lb. casei* PK2, *Lb. fermentum* SBV2, *Lb. plantarum* NCDC3 and *Lb. brevis* NCTH24 did not carry antibiotic resistance genes, show that these antibiotic resistance characteristics are intrinsic and nontransferable. Antibiotic resistance may be naturally occurring involves the absence of the target or the presence of low-affinity targets, low cell permeability, antibiotic inactivation of the antibiotics and the presence of efflux mechanisms [19]. According to the European Food Safety Authority, the intrinsic antibiotic resistance bacteria are considered a safety to use as feed additive or as production organisms [20].

4. CONCLUSIONS

Among 14 antibiotics, seven *Lactobacillus* strains were phenotypically resistant to many antibiotics: Gentamicin, neomycin, kanamycin, streptomycin, tetracycline, ciprofloxacin, vancomycin and sensitive to at least one type of protein synthesis inhibit antibiotics: chloramphenicol, erythromycin and clindamycin. The antibiotic resistance genes were detected in the *Lb. fermentum* SMC2 (*par*C gene) and two strains *Lb. plantarum* AS34 and TJ26 (*van*X gene). Excepting the resistance to ciprofloxacin and vancomycin in these strains, the other antibiotic resistance characteristics are not encoded by genes and not transmitted to other bacteria.

CRediT authorship contribution statement. Dinh Huy Son: Formal analysis, Writing the manuscript. Dang Thu Huong: Methodology, Investigation, Editing. Loc Tu Anh: Formal analysis. Nguyen La Anh: Methodology, Supervision

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