

Lactic Acid Bacteria from Vagina

Subjects: **Microbiology**

Contributor: Auttawit Sirichoat , Ana Belén Flórez , Lucía Vázquez , Pranom Buppasiri , Marutpong Panya , Viraphong Lulitanond , Baltasar Mayo

During the selection process of probiotics for vaginal applications, twenty-five lactic acid bacteria (LAB) isolates from human vagina belonging to six different species were tested for antimicrobial resistance by a microdilution method. Gene-specific PCR amplifications proved the strains carry no acquired antibiotic resistance genes, except for a *tet(W)* gene present in two tetracycline-susceptible *Bifidobacterium bifidum* strains. Genome analysis of a selected set of strains showed no other acquired resistance determinants. The *tet(W)* of *B. bifidum* was inactive by the insertion of two guanine residues in the middle of the gene. Surprisingly, the inactive gene became active and functional very easily, providing resistance to tetracycline and remaining stable afterward. LAB intended to be used in health applications must be free of acquired antimicrobial resistance genes; these could be spread and transferred to human pathogens.

lactic acid bacteria

antibiotic resistance

vaginal microbiota

genome analysis

tet(W)

1. Isolation, Identification and Typing of Vaginal LAB

Twenty-five vaginal LAB isolates with clear acidification halos on MRS agar supplemented with 0.5% CaCO₃ were recovered. All were Gram-positive rods, catalase negative, and γ -hemolytic. They were subsequently identified at the species level by 16S rRNA gene sequencing and sequence comparison. Seven isolates were identified as belonging to *Lactobacillus crispatus*, six to *Lactobacillus salivarius*, four to *Lactobacillus jensenii*, four to *Lactobacillus paracasei*, two to *Lactobacillus reuteri*, and two to *Bifidobacterium bifidum* (Table 1). A fingerprinting analysis by combining the results of RAPD-PCR and rep-PCR techniques detected 21 different strains among the 25 isolates, distributed as follows: *L. crispatus* (six strains), *L. salivarius* (five), *L. jensenii* (three), *L. paracasei* (three), *L. reuteri* (two), and *B. bifidum* (two strains) (Supplementary Figure S1).

Table 1. Minimum inhibitory concentration (MIC) values of 16 antibiotics to the vaginal LAB species and strains of this study.

Species	Strain	Antibiotic (MIC as $\mu\text{g mL}^{-1}$)															
		GEN	KAN	STR	NEO	TET	ERY	CLI	CHL	AMP	PEN	VAN	QDA	LIN	TMP	CIP	RIF
<i>L. crispatus</i>	VA20-32AN	2	16	2	16	2	0.06	0.25	4	1	0.5	0.5	1	4	>64	16	1

Species	Strain	Antibiotic (MIC as $\mu\text{g mL}^{-1}$)															
		GEN	KAN	STR	NEO	TET	ERY	CLI	CHL	AMP	PEN	VAN	QDA	LIN	TMP	CIP	RIF
	VA27-7	4	32	64	8	1	1	2	8	2	2	1	1	4	32	64	4
	VA27-9	1	16	2	2	2	0.03	0.5	4	2	0.5	0.5	1	4	64	32	2
	VA28-12	1	16	2	2	2	0.06	0.5	4	2	0.5	0.5	2	4	64	32	2
	VA32-17	2	64	2	8	4	0.03	0.5	2	1	1	0.5	1	2	>64	64	8
	VA32-17AN	4	128	32	4	2	0.25	0.5	4	1	0.5	1	1	2	16	32	4
	VA50-4AN	≤ 0.5	32	1	2	4	0.12	0.12	4	4	1	0.5	1	4	>64	32	4
<i>L. jensenii</i>	VA04-1AN	≤ 0.5	4	2	1	0.25	≤ 0.016	0.12	4	0.25	0.12	1	0.5	1	>64	8	0.25
	VA04-2AN	≤ 0.5	4	4	1	0.5	0.03	0.12	2	0.5	1	1	0.5	2	>64	8	0.25
	VA15-2AN	≤ 0.5	≤ 2	1	≤ 0.5	1	≤ 0.016	≤ 0.03	2	0.06	0.06	0.5	0.5	0.5	>64	8	0.25
	VA16-11	≤ 0.5	8	1	2	4	0.06	0.25	4	0.06	≤ 0.03	2	0.5	2	>64	16	0.5
Breakpoint ($\mu\text{g mL}^{-1}$)		16	16	16	-	4	1	4	4	2	-	2	-	-	-	-	-
<i>L. salivarius</i>	VA09-4	8	64	16	4	2	0.25	0.25	2	1	0.25	128	0.25	0.5	≤ 0.12	1	2
	VA16-20	≤ 0.5	4	2	0.5	1	0.06	0.06	2	0.5	0.12	>128	0.5	0.5	0.25	0.5	0.5
	VA37-13	≤ 0.5	4	≤ 0.5	≤ 0.5	0.5	0.06	0.06	2	0.25	0.12	>128	0.5	0.5	0.25	≤ 0.25	0.5
	VA40-10	128	>1024	>256	256	2	1	1	4	1	0.25	>128	1	1	1	4	0.5
	VA40-12AN	4	128	32	4	2	0.25	0.25	4	0.5	0.25	>128	1	0.5	0.25	1	1
	VA40-	4	128	32	4	2	0.25	0.5	4	0.5	0.25	>128	1	0.5	≤ 0.12	1	1

Species	Strain	Antibiotic (MIC as $\mu\text{g mL}^{-1}$)															
		GEN	KAN	STR	NEO	TET	ERY	CLI	CHL	AMP	PEN	VAN	QDA	LIN	TMP	CIP	RIF
	14AN																
	Breakpoint ($\mu\text{g mL}^{-1}$)	16	64	64	-	8	1	4	4	4	-	n.r.	-	-	-	-	-
<i>L. paracasei</i>	VA02-1AN	≤ 0.5	16	8	1	2	0.12	0.06	8	1	0.25	>128	1	4	0.5	4	0.5
	VA24-4	1	16	8	4	4	0.12	0.06	4	0.5	0.25	>128	1	2	0.25	4	0.5
	VA26-3	≤ 0.5	16	8	2	2	0.12	0.06	4	1	0.25	>128	1	2	1	2	0.5
	VA27-8	1	32	16	8	2	0.06	0.06	8	0.5	0.25	>128	1	4	0.25	4	0.5
	Breakpoint ($\mu\text{g mL}^{-1}$)	32	64	64	-	4	1	4	4	4	-	n.r.	-	-	-	-1	-
<i>L. reuteri</i>	VA15-3	≤ 0.5	⁻¹ 4	2	≤ 0.5	8	0.12	≤ 0.03	⁻¹ 4	1	2	>128	1	2	>64	32	0.25
	VA24-5	≤ 0.5	16	4	≤ 0.5	16	0.06	≤ 0.031	4	2	8	>128	0.5	4	>64	32	0.25
	Breakpoint ($\mu\text{g mL}^{-1}$)	8	64	64	-	32	1	⁻¹ 4	4	2	-	n.r.	-	-	-	-	-
<i>B. bifidum</i>	VA07-1AN	8	64	>256	16	1	≤ 0.016	0.06	1	≤ 0.03	≤ 0.03	0.5	0.5	0.5	16	8	2
	VA07-2AN	32	64	>256	32	1	≤ 0.016	≤ 0.03	1	≤ 0.03	≤ 0.03	1	0.5	0.5	16	8	1
	Breakpoint ($\mu\text{g mL}^{-1}$)	64	-	128	-	8	1	1	4	2	-	2	-	-	-	-	-

The presence of genes coding for the commonest AR genes spread among LAB was investigated by PCR. No genes involved in resistance to chloramphenicol (*cat*), β -lactams (*bla*), aminoglycosides [*aac(6')*-*aph(2")* and *aad(E)*], macrolides [*erm(A)*, *erm(B)*, *erm(C)*, *erm(F)*, *mef(A)*], tetracycline [*tet(M)*, *tet(O)*, *tet(S)*, *tet(K)*, *tet(L)*], clindamycin (*isaA*) or vancomycin (*vanA*) were ever detected in any of the isolates (data not shown). In contrast, PCR analyses for genes encoding ribosomal protection proteins (RPP) causing tetracycline resistance using the degenerate primer pairs DI-DII and Tet1-Tet2 (Supplementary Table S1) produced a positive amplification when DNA from two tetracycline-susceptible *B. bifidum* isolates was used as a template (Figure 1A). Amplification with gene-specific primers gave a positive result only for the *tet(W)* gene (Figure 1B). Amplicon sequencing and sequence comparison further proved the presence in these isolates of a *tet(W)* gene highly homologous to those present in many Gram-positive and Gram-negative bacteria.

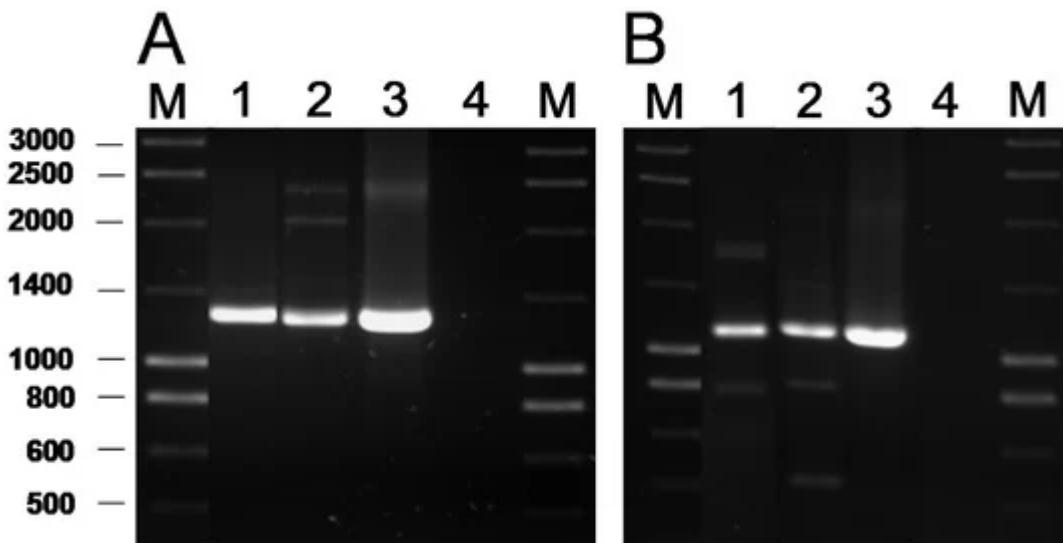


Figure 1. PCR amplification of tetracycline resistance genes using the universal primers Tet1 and Tet2 targeting a segment of 1,300 bp of the genes encoding RPP (A) and 1,200 bp of the *tet(W)* gene with the specific primer pair tetWF-Tet2 (B). Key of samples: Lane 1, DNA from *B. bifidum* VA07-1AN; lane 2, *B. bifidum* VA07-2AN; lane 3, *Leuconostoc mesenteroides* subsp. *mesenteroides* LbE16 (positive control) [1]; line 4, blank (no template DNA). M, molecular weight marker.

4. Genome Analysis for AR Genes

Based on the phenotype and genotype results (Table 1; Supplementary Figure S1), six strains were subjected to genome sequencing: *L. crispatus* VA50-4AN (resistant to kanamycin, ampicillin, and trimethoprim), *L. jensenii* VA04-2AN (resistant to trimethoprim), *L. salivarius* VA40-10 (resistant to gentamicin, kanamycin, streptomycin, neomycin, and vancomycin), *L. paracasei* VA02-1AN (resistant to chloramphenicol and vancomycin), *L. reuteri* VA24-5 (resistant to vancomycin and trimethoprim), and *B. bifidum* VA07-1AN (resistant to streptomycin). Supplementary Table S2 shows the general features of their genomes. Their size was, in all cases, around 2.2 Mbp but the number of contigs obtained after assembly ranged from 17 to 300. Supplementary Table S3 summarizes some of the key genetic features of the genomes of the sequenced strains. Genes coding for penicillin binding proteins (PBP) were found in all the genomes, although with different numbers and types for the distinct species. Mutations in PBPs encoding-genes known to confer AR were not identified. One gene coding for a D-alanine-D-alanine ligase (Ddl) was detected in each of the strains. In several LAB species, the presence of phenylalanine at the enzyme active site in Ddl is correlated with intrinsic resistance to vancomycin [2]. In addition, in each of the strains, 9-32 genes were classified by the RAST server as belonging to the category “Virulence, Disease, and Defence”, subcategory “Resistance to Antibiotic and Toxic Compounds”. The majority of these genes encoded components dedicated to homeostasis or resistance to heavy metals, such as copper, mercury, and the cobalt-zinc-cadmium triad. Genes encoding elongation factors, efflux pumps, DNA gyrases, and topoisomerases were also included by RAST in this subcategory.

By comparing the genome sequences against the databases CARD, ResFinder, and ARG-ANNOT, no genes known to be involved in AR in *L. jensenii* VA04-2AN (resistant to trimethoprim), *L. paracasei* VA02-1AN (resistant to chloramphenicol and vancomycin), and *L. reuteri* VA24-5 (resistant to trimethoprim and vancomycin) were detected. The only positive correlation between phenotype and genotype was the presence of a conserved phenylalanine (F) residue in the active site of the Ddl ligase, corresponding to amino acid 261 of the *Leuconostoc mesenteroides* enzyme [2], in the deduced sequence of all vancomycin-resistant (Vm^r) strains, while the susceptible (Vm^s) strains were characterized by the presence of a tyrosine (Y) residue at this position (Figure 2).

<i>L. crispatus</i> VA50-4AN Vm ^s	...GVGQITNAKGSFYTYENKYDDNSTSKLQIPADLPQE...
<i>L. jensenii</i> VA04-2AN Vm ^r	...GVGQIINAKGSFYSYKNKYDDSSTTLQIPADLPAE...
<i>L. paracasei</i> VA02-1AN Vm ^r	...YSAIEIKVRSGWYDFKHKFQAGYTDIFITPPKLDDED...
<i>L. reuteri</i> VA24-5 Vm ^r	...LGAVRVPEDDLFYDYENKFDVASGVVFELPVKLPQY...
<i>L. salivarius</i> VA40-10 Vm ^r	...LGAIIRIPESDDFYDYNNKFDVASGVVFEMPIKLPEK...

Figure 2. Alignment of amino acid sequences around the active site of D-Ala-D-Ala ligases of the five *Lactobacillus* spp. strains sequenced. Strains with phenylalanine (F) at the enzyme active site (green) show a vancomycin-resistant phenotype, while those having a tyrosine (Y) (pale blue) display a vancomycin-susceptible phenotype.

Genome analysis of *L. crispatus* VA50-4AN, *L. salivarius* VA40-10, and *B. bifidum* VA07-1AN identified no genes known to be involved in aminoglycoside resistance. Therefore, mutations in key genes, such as those coding for the ribosomal S12 protein and others acting on the 16S rRNA molecule, were therefore sought by comparing the DNA and deduced protein sequences from our strains with those in databases. No amino acid differences were observed in the sequences of the ribosomal protein S12 for *L. crispatus* VA50-4AN and *L. salivarius* VA40-10 from those belonging to susceptible strains of the same species. Further, alignment of the deduced amino acid sequences for the 16S rRNA guanine(527)-N(7)-methyltransferase (RsmG) proteins of the sequenced strains, showed heterogeneity at several positions between themselves and with respect to sequences in databases. In particular, the RsmG sequence of *L. crispatus* VA50-4AN showed one amino acid change at position 38 (N → H), while that of *B. bifidum* VA07-1AN showed three amino acid changes at positions 105 (E → A), 150 (G → D), and 206 (R → G), and that of *L. salivarius* VA40-10 showed six exclusive amino acid changes at positions 12 (G → E), 67 (D → N), 186 (N → D), 199 (I → V), 208 (Q → K), and 209 (V → I). However, by comparing RsmG sequences from resistant and susceptible strains, none of the changes considered could be associated with streptomycin resistance.

As expected, the genome analysis confirmed the presence of *tet(W)* in *B. bifidum* VA07-1AN; this gene was also unequivocally identified by searches in the three AR databases used. The *tet(W)* gene in *B. bifidum* VA07-1AN was located in a contig of 76,748 bp. Figure 3 shows the genetic organization of the 40-kbp left extreme of the contig that included the *tet(W)* gene. The *tet(W)* sequence of VA07-1AN (1922 bp) was almost identical to that described for *Bifidobacterium longum* LTBL16 (CP034089.1). Similar *tet(W)* sequences have also been found in the chromosome of strains belonging to other species such as *B. bifidum* L22 (NG_048301.1), *Lachnospiraceae*

bacterium KGMB03038 (CP041667.1), and *Ruminococcus* sp. JE7A12 (CP039381.1); and in plasmids, such as pTZC1 from *Cutibacterium acnes* TP-CU389 (LC473083.1). Compared to *tet(W)* in *B. longum* LTBL16, the *tet(W)* in *B. bifidum* VA07-1AN contained an insertion of two extra guanine residues (GG) after nucleotide 731 in the ORF resulting in a frameshift, which produced only a short peptide—289 amino acids long compared to 639 residues for the functional Tet(W). This likely explains the susceptibility of VA07-1AN to tetracycline. The *tet(W)* gene was flanked by ORFs coding for proteins showing the greatest homology to others from *B. longum* in the upstream region, and proteins typical of *B. bifidum* in the downstream region (Supplementary Table S4).

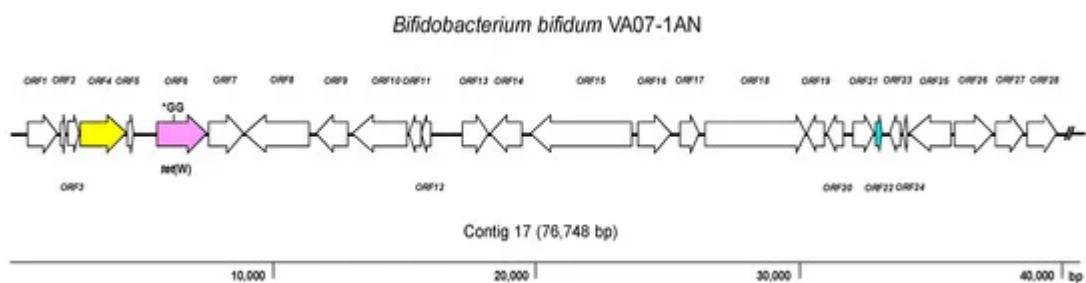


Figure 3. Diagram showing the genetic organization of ORFs in the contig harboring the *tet(W)* gene of *Bifidobacterium bifidum* VA07-1AN. Color key: purple, *tet(W)* gene (the position of the GG insertion disrupting the ORF is indicated); yellow, conjugation-associated gene; pale blue, gene encoding a transcription regulator; white, genes involved in other processes. The broken line symbol indicates the contig extends beyond this point.

The CARD database further identified in the genome of *B. bifidum* VA07-1AN a single nucleotide polymorphism (SNP) point mutation in the *rpsL* gene (encoding the ribosomal S12 protein), a variation causing an amino acid substitution (K → R) at position 43 of the protein (Figure 4). This amino acid change has been associated with streptomycin resistance in many species [3].

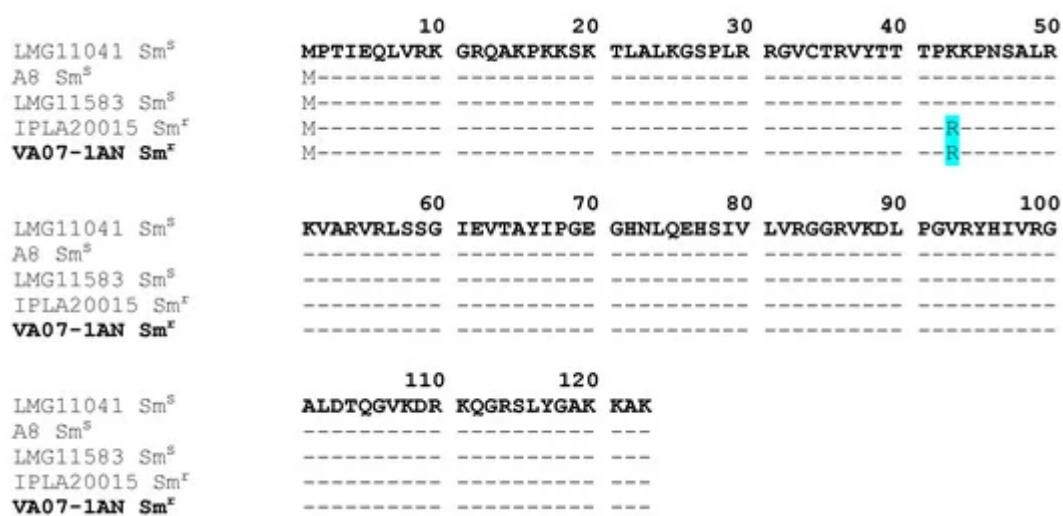


Figure 4. Alignment of the deduced amino acid sequence of S12 ribosomal proteins encoded by the *rpsL* gene from streptomycin-resistant (Sm^r) and -susceptible (Sm^s) *Bifidobacterium bifidum* strains. The amino acid

replacement K→R at position 43 in the resistant strains is highlighted in pale blue. In bold, the strain of this study (VA07-1AN).

5. Restoration of the Tetracycline Resistance Phenotype in *B. Bifidum* VA07-1AN

When the susceptibility of *B. bifidum* VA07-1AN to tetracycline was assayed using the MICE test, colonies growing within the inhibition halo were noted. Identification and typing showed them to be tetracycline-resistant variants of VA07-1AN. After plating on antibiotic-containing and antibiotic-free plates, about 0.6% of the colonies from an overnight culture were found to be tetracycline-resistant. Amplification and sequencing of *tet(W)* genes from 13 tetracycline-resistant variants showed the addition of one guanine nucleotide in most revertants to the guanine stretch where the two Gs disrupting the ORF had been inserted (as in R-1; Figure 5). Other mutations consisting of both nucleotide insertions and deletions in the vicinity of the stretch of Gs were occasionally seen (as in R-11; Figure 5). In either case, there was a net gain of one nucleotide, which, together with the two Gs that disrupted the *tet(W)* ORF, created a new codon that opened the reading frame of Tet(W) producing a functional protein that provided tetracycline resistance. The MIC of tetracycline in the tetracycline-resistant variants ranged from 48 to 96 μ g mL⁻¹. In contrast, growing the antibiotic-resistant variants in the absence of tetracycline for about 80-100 generations showed no tetracycline-susceptible revertants, demonstrating high stability of the mutations that restored the resistant phenotype.

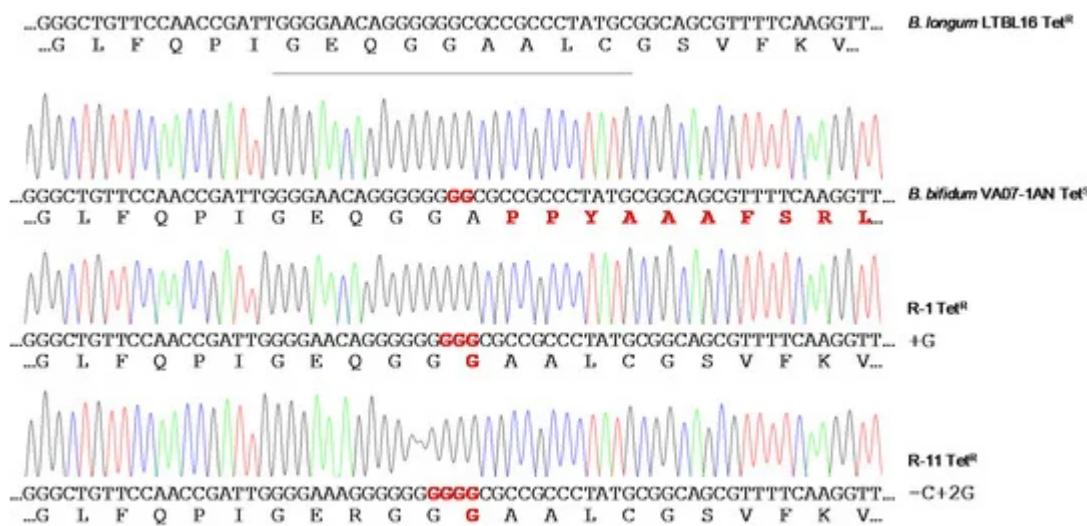


Figure 5. Chromatograms of amplicons of the *tet(W)* gene from the original tetracycline-susceptible strain *B. bifidum* VA07-1AN and two representative tetracycline-resistant revertants (R-1 and R-11). Nucleotide sequences and the corresponding deduced amino acid sequences are displayed below each of the chromatograms. DNA and protein differences with canonical sequences of the *tet(W)* gene from the tetracycline-resistant *Bifidobacterium longum* LTBL16 strain (on top of the figure) are highlighted in red.

6. Discussion

LAB contribute to the maintenance of vaginal health via the production of substances (mainly organic acids) that acidify the environment and inhibit the development of pathogens [4]. However, there is an increasing concern that LAB may act as reservoirs of AR determinants, from which they could ultimately be transferred to pathogens [5][6]. Indeed, the existence of lactobacilli and bifidobacteria strains resistant to several antibiotics, by either acquiring mutations or exogenous genes, has been repeatedly reported [7][8][9][10][11]. Therefore, during the selection of probiotics, the susceptibility of lactobacilli and bifidobacteria to antibiotics has to be assessed and the absence in the selected strains of transferable AR genes should be assured [12].

Studies reporting lactobacilli and bifidobacteria to be generally susceptible to tetracycline, erythromycin, chloramphenicol, penicillin, ampicillin, clindamycin, quinupristin-dalfopristin, linezolid, and rifampicin have been published over the last 15 years [10][13][14]. In agreement, the phenotypic analysis of the present isolates showed them to be susceptible to these antibiotics, with the exception of ampicillin and chloramphenicol—to which one and three isolates, respectively, were associated with MIC values higher than EFSA's cut-offs [15]. In contrast, nine isolates showed resistance to one or more aminoglycosides (gentamicin, kanamycin, streptomycin, and neomycin). Resistance to aminoglycosides may occur based on several mechanisms, which include (i) enzymatic modification and inactivation of the antibiotics mediated by aminoglycoside acetyltransferases, nucleotidyltransferases, or phosphotransferases, (ii) increased efflux, (iii) decreased permeability, and (iv) modifications of the 30S ribosomal subunit interfering with the binding of this class of antibiotics [16]. However, most of the MIC values recorded in this study were just one dilution higher than the corresponding cut-off. These small MIC differences might be explained by the normal variation associated with the microdilution assay [17]. Accordingly, none of the aminoglycoside resistance genes searched for by PCR, including the widespread *aac(6')-aph(2")* and *aad(E)* genes [18], were found in any of the isolates. The genome analysis further discarded the presence of acquired resistances in the sequenced strains, comprising genes and well-characterized mutations involved in aminoglycoside resistance. Given the lack of cytochrome-mediated drug transport, aminoglycoside resistance has been claimed as an intrinsic feature of LAB and other anaerobic bacteria [19]. However, large differences in the MIC values for aminoglycosides even in strains from the same species have been reported in the literature [10][13][14]. The cooperation of other non-specific mechanisms, such as increased membrane impermeability, enhanced activity of unspecific efflux pumps and multi-drug transporters, or the presence of defective cell wall autolytic systems, may further account for differences in MICs between different species and strains [20].

Resistance to the aminoglycoside streptomycin has largely been associated with mutations in chromosomal genes, for example, in *rpsL* that codes for the ribosomal protein S12 [21], or in *rsmG* that codes for the 16S rRNA guanine(527)-N(7)-methyltransferase (RsmG) [22]. Comparison of the deduced proteins from streptomycin-susceptible and -resistant strains of the different lactobacilli species analyzed revealed random differences between the RsmG sequences. However, none of them could be consistently associated with streptomycin resistance. In contrast, a mutation in *rpsL* causing an amino acid change at position 43 (K → R) was observed in *B. bifidum* VA07-1AN. The same amino acid replacement has been reported in other streptomycin-resistant strains of bifidobacteria [23] and many other species [3], suggesting this to be the most likely explanation for the high resistance to streptomycin shown by VA07-1AN.

Strong resistance to vancomycin is an intrinsic feature in certain *Lactobacillus* phylogroups and other LAB species such as *Leuconostoc* spp. [24] caused by an amino acid replacement in the active site of the DdIa ligase (F261Y), as it has been experimentally demonstrated for *Leuconostoc mesenteroides* [2] and *L. reuteri* [25].

Although cut-offs for trimethoprim and ciprofloxacin in LAB and bifidobacteria have yet to be defined, strains of most of the present species were associated with quite high MICs. In fact, the resistance of most *Lactobacillus* species to these antibiotics has been repeatedly reported [10][14]. Folate auxotrophy in lactobacilli is generally accepted as the intrinsic cause of resistance to trimethoprim [26]. Similarly, the reduced affinity of DNA gyrase (GyrA) and topoisomerase IV (ParC) variants for ciprofloxacin and other fluoroquinolones saw in some LAB species has been determined responsible for their insensitivity to this class of antibiotics [27]. Further, the presence of active multidrug efflux systems could contribute to an increase in the MIC for ciprofloxacin in some strains [7]. Since no genes coding for β -lactamases has ever been detected in LAB, non-specific mechanisms, as already discussed for the aminoglycosides, might contribute to the increased MIC of ampicillin in *L. crispatus* VA50-4AN, as has been reported for *L. reuteri* [28].

The vaginal lactobacilli in the present study were very susceptible to tetracycline—even though many LAB strains are resistant to it [9][18]. Unexpectedly, PCR analysis detected the presence of *tet(W)* in the two tetracycline-susceptible *B. bifidum* strains. This gene has been found to be disseminated among gut-dwelling bacteria of different species from humans and animals [29]. The genome analysis of *B. bifidum* VA07-1AN showed the gene to contain an insertion of two Gs bases at its center, shifting the ORF and rendering a shorter non-functional peptide. The presence of silent tetracycline resistance genes in bifidobacteria has been reported elsewhere [30][31]. The reactivation of a silent tetracycline resistance phenotype has also been reported for *Bifidobacterium animalis* subsp. *lactis* Bb12 [31]. Silent AR genes could, therefore, represent a hazard, even more so when they can be easily reactivated and the restored gene remains stable afterward. Therefore, the use of strains harboring such genes in food and feed systems should be avoided.

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