Antibiotic Susceptibility of *Lactobacillus* and *Bifidobacterium* Species from the Human Gastrointestinal Tract

Susana Delgado, Ana Belén Flórez, Baltasar Mayo

Instituto de Productos Lácteos de Asturias (CSIC), Carretera de Infiesto s/n, 33300 Villaviciosa, Asturias, Spain

Abstract. One hundred and twenty-two strains of *Bifidobacterium* and *Lactobacillus* species have been tested against 12 antibiotics and two antibiotic mixtures by a commercial system (Sensititre Anaero3; Treck Diagnostic Systems). The upper limits of some minimum inhibitory concentrations (MICs) were completed on MRS agar plates by the NCCLS procedure. All strains were sensitive to chloramphenicol and imipenem and most of the strains were resistant to metronidazole. Bifidobacteria isolates were susceptible to cefoxitin, whereas about half of the lactobacilli were resistant. Approximately 30% of the *Bifidobacterium* isolates were resistant to tetracycline, as well as five *Lactobacillus* strains belonging to four different species. None of the tested *Bifidobacterium* isolates was resistant to vancomycin, whereas a species-dependent resistance was found among the lactobacilli. Single strains of *Bifidobacterium longum*, *Bifidobacterium pseudocatenulatum*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, and *Lactobacillus brevis* were resistant to erythromycin and/or clindamycin. Most of the observed resistances seemed to be intrinsic, but some others could be compatible with transmissible determinants.

Lactic acid bacteria (LAB) species, including lactobacilli and bifidobacteria, are indigenous members of the gastrointestinal microbiota of humans and enjoy a time-honored reputation as health promoters. In fact, these bacteria have a "generally regarded as safe" (GRAS) status and are frequently uses as probiotics [12]. Bacterial strains intended to be used as probiotics in food systems have to be systematically examined for antibiotic susceptibility in order to avoid the spread of antibiotic-resistant determinants by the food chain [19]. The presence of several resistance genes in many LAB strains from the human gastrointestinal tract (GIT) has been firmly stated [2, 14, 18]. Some determinants have been found to be transferred even into the GIT of mammals [17]. Furthermore, it should also be considered that, although rarely, LAB species, including probiotic strains, have been reported to cause infections in humans [9], thus antibiotic resistance hampering treatment of the diseases.

In recent years several studies concerning antibiotic susceptibility of intestinal LAB species from humans have been undertaken [3, 4, 13, 15, 16, 20]. However, due to the multiplicity of methods used and the unrelatedness of the strains, there is still a lack of agreement in the resistance-susceptibility breakpoints for most antibiotics in LAB [4, 7].

In this paper we report on the level of susceptibility to several antimicrobial agents of some *Lactobacillus* and *Bifidobacterium* species isolated from the human GIT of healthy individuals, which could eventually be used as probiotics. Antibiotic resistance was used as a negative criterion in the selection process of the strains. At the same time, the survey was regarded as an overview of the level of antibiotic resistance in LAB from the human GIT.

Materials and Methods

Bacterial strains, media, and culture conditions. One hundred and twenty-two strains of *Bifidobacterium* and *Lactobacillus* species, isolated from the feces of eight individuals, were screened for antibiotic susceptibility in this study. They have been isolated as part

Correspondence to: B. Mayo; *email:* baltasar.mayo@ipla.csic.es

of the dominant LAB populations on MRS agar (VWR International, Darmstadt, Germany) containing 0.25% cyseine (Sigma Chemical, St. Louis, MO). The strains were grouped by their carbohydrate fermentation profiles using a commercial kit (PhenePlate system, PhP, Stockholm, Sweden) and classified by amplification, sequencing, and comparison of a stretch of their 16S rDNA gene, using two universal primers based on prokaryotic conserved regions of the 16S rRNA gene, as previously described [21]. Around 10% of the isolates proved to be replicates by RAPD (data not shown). The strains were assigned to the following species: 47 *Bifidobacterium longum*, 16 *Bifidobacterium bifidum*, 11 *Bifidobacterium pseudocatenulatum*, 2 *Bifidobacterium catenulatum*, 20 *Lactobacillus gasseri*, 8 *Lactobacillus delbrueckii*, 7 *Lactobacillus casei/Lactobacillus paracasei*, 5 *Lactobacillus rhamnosus*, 2 *Lactobacillus acidophilus*, and single strains of *Lactobacillus plantarum*, *Lactobacillus parabuchneri*, *Lactobacillus brevis*, and *Lactobacillus vaginalis*. Incubations were performed at 37°C in an anaerobic chamber (Mac500, Down Whitley Scientific, West Yorkshire, UK) containing an anoxic atmosphere (10% H_2, 10% CO_2, 80% N_2).

Determination of the Minimum Inhibitory Concentration (MIC). MICs to 12 antibiotics and two antibiotic mixtures were tested with the Sensititre Anaero3 kit (Trek Diagnostic Systems, East Grinstead, UK) following the recommendations of the supplier. In short, colonies from solid media were used to make a 0.5 McFarland suspension in Brucella standard broth, and 100 µL of this suspension was transferred to the same medium supplemented with haemin and vitamin K1, given an approximate concentration of 1×10^6 CFU/mL. One hundred microliters of this suspension was inoculated into each well of the Sensititre Anaero3 plate. Because this system does not have an adequate range of concentrations for some antibiotics, the upper limits of the MICs were determined by the standardized NCCLS agar dilution technique in MRS-cystein agar (VWR International) plates [1], although a higher inoculum was needed to obtain enough growth. The antibiotics analyzed include inhibitors of the cell wall synthesis (β-lactams: penicillin G, amoxicillin, amoxicillin plus clavulanic acid, piperacillin, piperacillin plus tazobactam and imipenem; cephalosporins: cefoxitin; and glycopetides: vancomycin), protein synthesis (chloramphenicol, clindamycin, erythromycin, and tetracycline), and nucleic acid synthesis (metronidazole and moxifloxacin).

Results and Discussion

The distribution of MICs to the several antibiotics inhibiting cell wall synthesis (β-lactams: penicillins, cephalosporins, and carbapenems; and the glycopeptide vancomycin) are summarized in Table 1. All tested strains were susceptible to the lower concentration of piperacillin and piperacillin plus tazobactam (all MICs ≤ 16 µg/mL). MICs to all other antibiotics showed variability. MICs to the amoxicillin–clavulanic acid mixture were always lower and followed the same pattern as those for amoxicillin alone. Thus, for reasons of clarity, only MICs to the latter were included in Table 1. For the same reason, MICs to imipinem were not summarized in the table as all were lower than 0.25 µg/ mL. The real MIC to piperacillin was further evaluated in a set of 10 different bifidobacteria and lactobacilli isolated by the E-test technique (AB Biodisk, Solna,

Sweden) and found to vary between 0.032 and 0.50 µg/ mL (data not shown).

All bifidobacteria and lactobacilli strains assayed were susceptible to penicillin and amoxicillin (MICs lower than 4 µg/mL), except for those of *L. plantarum* and *L. brevis* strains, and in clinical terms none of the strains should be considered resistant (clinical resistant breakpoint by the NCCLS ≥ 4 µg/mL). Cefoxitin was the only β-lactam for which a high number of resistant strains were found. All strains of *L. casei/L. paracasei* (7), *L. rhamnosus* (5), *L. acidophilus* (2), *L. plantarum* (1), *L. brevis* (1), *L. vaginalis* (1), and a few *L. gasseri* strains (4 out of 20). The resistance of the lactobacilli to this antibiotic has been repeatedly reported [3, 7, 15]. On the contrary, only two *B. longum* isolates showed MICs of 32 and 64 g/mL, respectively. This result differs from those of Charteris et al. [4], who found most of the bifidobacteria isolates resistant by an overlay disc diffusion test. Cell wall impermeability seems to be the principal mechanism of resistance to inhibitors of the cell wall synthesis (penicillins and cephalosporins), as anaerobic microorganisms lacks cytochrome-mediated electron transport [6]. But the cooperation of nonspecific mechanisms (multidrug transporters, general stress-induced response, mutation on penicillin binding proteins) may also account for differences between strains.

All tested bifidobacteria were very susceptible to vancomycin, as were all isolates of *L. gassseri*, *L. delbrueckii*, and *L. acidophilus*. However, resistance at a high level was found in strains of *L. casei/L. paracasei*, *L. rhamnosus*, *L. plantarum*, *L. parabuchneri*, *L. brevis*, and *L. vaginalis*, which showed MICs ≥ 256 µg/mL. The level of resistance found in these species is in accordance with previous observations [7, 10]. Intrinsic resistance in lactobacilli to this glycopeptide is attributed to the synthesis of modified cell wall peptidoglycan precursors that terminate in lactate [11]. This type of resistance does not seem to pose a problem since it is different from the inducible, transferable mechanism observed in other bacteria.

Table 2 summarizes the distribution of MICs to several antibiotics inhibiting the synthesis of proteins. None of the assayed strains studied showed resistance to chloramphenicol (most MICs of ≤ 4 µg/mL). Although most of the isolates of both bifidobacteria and lactobacilli had a low MIC to tetracycline, several resistant strains appeared. The 15 isolates that were only examined for a concentration of 16 µg/mL (Table 2), were further confirmed as highly resistant by an E-test assay (data not shown). The two *L. acidophilus* isolates and the single *L. plantarum* one had the highest MICs to this antibiotic: over 256 µg/mL. Intermediate levels of resistance were encountered in 11 *Bifidobacterium*

Table 1. Distribution of MICs to several antibiotics inhibiting cell wall synthesis (β-lactams: penicillins and cephalosporins and the glycopeptide vancomycin) in *Bifidobacterium* and *Lactobacillus* species of the human gastrointestinal tract

Number of isolates for which the MIC (µg/mL) was as follows:

Penicillin G

Antibiotic	Species	Number of strains	≤ 0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256
Penicillin G	*B. longum*	47	6	21	17	2		1							
	B. bifidum	16	14	2											
	B. pseudocatenulatum	11	6	1	3	1									
	B. catenulatum	2	2												
	L. gasseri	20	13	7											
	L. delbrueckii	8	8												
	L. casei/L. paracasei	7		4	1	1		1							
	L. rhamnosus	5		1	2	2									
	L. acidophilus	2			1	1									
	L. plantarum	1							1						
	L. parabuchneri	1			1										
	L. brevis	1								1					
	L. vaginalis	1			1										

Amoxicillin

Antibiotic	Species	Number of strains	≤ 0.25	0.5	1	2	4	8	16	32	64
Amoxicillin	*B. longum*	47	35	10	1	1					
	B. bifidum	16	16								
	B. pseudocatenulatum	11	7	4							
	B. catenulatum	2	2								
	L. gasseri	20	9	11							
	L. delbrueckii	8	8								
	L. casei/L. paracasei	7		5	2						
	L. rhamnosus	5		3	2						
	L. acidophilus	2		2							
	L. plantarum	1		1							
	L. parabuchneri	1		1							
	L. brevis	1	1								
	L. vaginalis	1			1						

Cefoxitin

Antibiotic	Species	Number of strains	≤ 0.5	1	2	4	8	16	32	≥ 64
Cefoxitin	*B. longum*	47	2	17	9	9	7	1	1	1
	B. bifidum	16	10	6						
	B. pseudocatenulatum	11	2	1	2	3	3			
	B. catenulatum	2	1			1				
	L. gasseri	20	1	1		1	7	7	1	3
	L. delbrueckii	8		2		2	1	3		
	L. casei/L. paracasei	7								7
	L. rhamnosus	5								5
	L. acidophilus	2								2
	L. plantarum	1							1	
	L. parabuchneri	1			1					
	L. brevis	1								1
	L. vaginalis	1								1

Vancomycin

Antibiotic	Species	Number of strains	≤ 2	4	8	16	32	64	128	≥ 256
Vancomycin	*B. longum*	47	46	1						
	B. bifidum	16	4	12						
	B. pseudocatenulatum	11	10	1						
	B. catenulatum	2	2							
	L. gasseri	20	10	4	6					
	L. delbrueckii	8	6	2						
	L. casei/L. paracasei	7								7
	L. rhamnosus	5								5
	L. acidophilus	2	2							
	L. plantarum	1								1
	L. parabuchneri	1								1
	L. brevis	1								1
	L. vaginalis	1								1

Table 2. Distribution of MICs to several antimicrobials inhibiting protein synthesis for Bifidobacterium and Lactobacillus species of the human gastrointestinal tract

Chloramphenicol

Antibiotic	Species	Number of strains	≤ 2	4	8	16	32	64
Chloramphenicol	B. longum	47	45	2				
	B. bifidum	16	16					
	B. pseudocatenulatum	11	9	2				
	B. catenulatum	2	2					
	L. gasseri	20	1	13	3	3		
	L. delbrueckii	8	4	2	2			
	L. casei/L. paracasei	7	5	1	1			
	L. rhamnosus	5	3	1	1			
	L. acidophilus	2	2					
	L. plantarum	1	1					
	L. parabuchneri	1		1				
	L. brevis	1				1		
	L. vaginalis	1				1		

Tetracycline

Antibiotic	Species	Number of strains	≤ 2	4	8	16	32	64	128	256	≥ 256
Tetracycline	B. longum	47	28	2	2	8*		1	3	3	
	B. bifidum	16	8		1	3*		4			
	B. pseudocatenulatum	11	9			2*					
	B. catenulatum	2	1			1					
	L. gasseri	20	5	12	3						
	L. delbrueckii	8	7		1						
	L. casei/L. paracasei	7	7								
	L. rhamnosus	5	5								
	L. acidophilus	2								2	
	L. plantarum	1								1	
	L. parabuchneri	1		1							
	L. brevis	1				1*					
	L. vaginalis	1				1*					

Erythromycin

Antibiotic	Species	Number of strains	≤ 1	2	4	8	16	32	64	128	256	512	≥ 1024
Erythromycin	B. longum	47	31	2	1	4	4	4	1				
	B. bifidum	16	16										
	B. pseudocatenulatum	11	8			2				1			
	B. catenulatum	2	1			1							
	L. gasseri	20	19	1									
	L. delbrueckii	8	8										
	L. casei/L. paracasei	7	5		2								
	L. rhamnosus	5	3										2
	L. acidophilus	2											2
	L. plantarum	1	1										
	L. parabuchneri	1	1										
	L. brevis	1					1						
	L. vaginalis	1					1						

Clindamycin

Antibiotic	Species	Number of strains	≤ 0.5	1	2	4	8	16	32	64	128	≥ 256
Clindamycin	B. longum	47	34				3	2	2		3	3
	B. bifidum	16	16									
	B. pseudocatenulatum	11	10					1				
	B. catenulatum	2	2									
	L. gasseri	20	1			4	9	6				
	L. delbrueckii	8	8									
	L. casei/L. paracasei	7	6	1								
	L. rhamnosus	5	3									2
	L. acidophilus	2										2
	L. plantarum	1	1									
	L. parabuchneri	1	1									
	L. brevis	1								1		
	L. vaginalis	1	1									

*These strains were found to be highly resistant (MICs between 128 and ≥ 256 μ g/ml^{-1}) by an E-test assay (not shown).

Table 3. Distribution of MICs to the DNA synthesis inhibitor metronidazole and the fluoroquinolone moxifloxacin for *Bifidobacterium* and *Lactobacillus* species of the human gastrointestinal tract

Antibiotic	Species	Number of strains	Number of isolates for which the MIC ($\mu g/ml^{-1}$) was as follows:							
			≤ 0.50	1	2	4	8	16	32	≥ 64
Metronidadozole	*B. longum*	47	4	19	6	2	3	1		12
	B. bifidum	16		6	7	3				1
	B. pseudocatenulatum	11		2	4	3	1	1		
	B. catenulatum	2						1		1
	L. gasseri	20								20
	L. delbrueckii	8								8
	L. casei/L. paracasei	7								7
	L. rhamnosus	5								5
	L. acidophilus	2								2
	L. plantarum	1								1
	L. parabuchneri	1								1
	L. brevis	1								1
	L. vaginalis	1								1

Antibiotic	Species	Number of strains	≤ 0.12	0.25	0.50	1	2	4	8	≥ 16
Moxifloxacin	*B. longum*	47	1		8	20	5	8	2	3
	B. bifidum	16				7	1		4	4
	B. pseudocatenulatum	11	2		1	1	2		2	3
	B. catenulatum	2							1	1
	L. gasseri	20				1	5	8		6
	L. delbrueckii	8		1	1				4	2
	L. casei/L. paracasei	7		1	4		2			
	L. rhamnosus	5		3	2					
	L. acidophilus	2					2			
	L. plantarum	1								1
	L. parabuchneri	1				1				
	L. brevis	1								1
	L. vaginalis	1								1

isolates (MICs between 64 and 256 μg/mL). Our results confirm the findings of Matteuzzi et al. [16], who showed that tetracycline has a very variable effect against bacteria of this genus, while all were susceptible to chloramphenicol. The number of tetracycline-resistant bacteria in the GIT has been correlated with the use of this antibiotic [18], suggesting that the increasing antibiotic pressure posed by the wide use of antibiotics in veterinary and human medicine is certainly contributing to the dissemination of resistances into intestinal bacteria.

The findings for tetracycline should apply also to erythromycin and clindamycin, for which most MICs were very low but a few high resistant strains were found. Among the 46 strains of lactobacilli analyzed, two of five *L. rhamnosus* isolates and the two *L. acidophilus* strains, which were resistant to tetracycline, showed high resistance to both erythromycin and clindamycin (MIC ≥ 1024 and ≥ 256 μg/mL, respectively). High resistance to erythromycin and/or clindamycin was also found in several bifidobacterial isolates, one of which displayed resistance to tetracycline (MIC ≥16 μg/mL). These results differs from those published elsewhere [4, 13, 16], in which bifidobacteria of human and commercial origin were shown to be sensitive to these two broad spectrum antibiotics.

Metronidazole is an antibiotic frequently used in cases of anaerobic infections of the digestive tract, being the agent of choice for *Clostridium difficile*-induced pseudomembranous colitis [8]. In accordance with other surveys [3, 7], all lactobacilli strains were found to be resistant to metronidazole (MIC ≥ 32 μg/mL) (Table 3), indicating that this antibiotics is not active in this genus. Resistance of lactobacilli to metronidazole might be, as in other LAB species, because of the absence of hydrogenase activity [5]. Susceptibility to metronidazole was variable in bifidobacterial strains. Some of them were very sensitive whereas others had intermediate to high resistance, a result which has been reported before [13, 20]. The MICs to the fluoroquinolone moxifloxacin

were randomly distributed from less than 0.12 to ≥16 μg/mL, and no clear pattern could be seen (Table 3).

Multiple resistances are not common in intestinal lactobacilli and bifidobacteria and were only observed in a couple of *L. acidophilus* strains (resistant to tetracycline, erythromycin, and clindamycin). Only a minority of intestinal lactobacilli and bifidobacterial species showed antibiotic resistances (against tetracycline, erythromycin, and/or clindamycin). However, for the results to be confident more strains should be analyzed for several species. Some of the observed resistances (cefoxitin, vancomycin, metronidazole) seemed to be intrinsic and the level is genus- or species-dependent. This small antibiotic-resistant fraction justifies an antibiotic-susceptibility assay to avoid the inclusion of resistant strains in the formulation of probiotics. A phenotypic test can not confirm the presence or absence of transferable resistance genes, but some of the observed levels are compatible with common transmissible determinants.

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