Journal of Food Protection, Vol. 63, No. 10, 2000, Pages 1369–1376

Effect of Conjugated Bile Salts on Antibiotic Susceptibility of Bile Salt–Tolerant *Lactobacillus* and *Bifidobacterium* Isolates

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MS 99-65: Received 17 March 1999/Accepted 17 July 1999

ABSTRACT

Virtually every antibiotic may cause in vivo alterations in the number, level, and composition of the indigenous microbiotae. The degree to which the microbiotae are disturbed depends on many factors. Although bile may augment antibiotic activity, studies on the effect of bile on the antibiotic susceptibility of indigenous and exogenous probiotic microorganisms are lacking. It was against this background that the antibiotic susceptibility of 37 bile salt–tolerant *Lactobacillus* and 11 *Bifidobacterium* isolates from human and other sources was determined in the presence of 0.5% wt/wt oxgall (conjugated bile salts). Oxgall did not affect the intrinsic resistance of lactobacilli to metronidazole (5 μg), vancomycin (30 μg), and co-trimoxazole (25 μg), whereas it resulted in a complete loss of resistance to polymyxin B (300 μg) and the aminoglycosides gentamicin (10 μg), kanamycin (30 μg), and streptomycin (10 μg) for most strains studied ($P < 0.001$). Oxgall did not affect the intrinsic resistance of bifidobacteria to metronidazole and vancomycin, whereas polymyxin B and co-trimoxazole resistance was diminished ($P < 0.05$) and aminoglycoside resistance was lost ($P < 0.001$). Seven lactobacilli, but no bifidobacteria strain, showed unaltered intrinsic antibiotic resistance profiles in the presence of oxgall. Oxgall affected the extrinsic susceptibility of lactobacilli and bifidobacteria to penicillin G (10 μg), ampicillin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), and rifampicin (5 μg) in a source- and strain-dependent manner. Human strain–drug combinations of lactobacilli ($P < 0.05$) and bifidobacteria ($P < 0.01$) were more likely to show no change or decreased susceptibility compared with other strain-drug combinations. The antimicrobial activity spectra of polymyxin B and the aminoglycosides should not be considered limited to gram-negative bacteria but extended to include gram-positive genera of the indigenous and transiting microbiotae in the presence of conjugated bile salts. Those lactobacilli (7 of 37) that show unaltered intrinsic and diminished extrinsic antibiotic susceptibility in the presence of oxgall may possess greater upper gastrointestinal tract transit tolerance in the presence of antibiotics.

Lactobacillus and *Bifidobacterium* species constitute a significant proportion of the indigenous (colonization resistance) and exogenous (transiting) microbiotae of the healthy human gastrointestinal *(11)* and female genitourinary tracts *(12).* This has prompted their use as probiotic nutritional and bacteriotherapeutic modalities in the management (prophylaxis and therapy) of infection and antibiotic-associated diarrhea *(12, 40).* Virtually every antibiotic, depending on the intraluminal drug concentration and antimicrobial spectrum, may cause in vivo alterations in the number, level, and composition of these species *(14, 15).* In addition, in vivo antibiotic exposure may predispose toward acquisition of resistance determinants *(14)* and to the development of a residuum of persistently resistant strains that have the potential to rebound and predominate rapidly on resumption of antibiotic use *(47).* Thus, the performance of antimicrobial susceptibility testing may be regarded as both a necessary selection criterion for probiotic cultures *(18)* and an effective guide for specific antimicrobial therapy *(44).* Its use ensures the absence of undesirable, such as enterococcal vancomycin resistance, and atypical antibiotic resistance among microbial adjuncts and bacteriotherapeutic agents *(1, 45),* while enabling the design of antibiotic regimens that affect to a lesser extent the indigenous and exogenous microbiotae *(52).*

Whole bile tolerance constitutes another important selection criterion for probiotic adjuncts and bacteriotherapeutic agents *(13).* Bile is a digestive secretion essential for lipid metabolism, which enters the descending portion of the duodenum from the liver and gall bladder. It is primarily composed of conjugated, insoluble, amphiphilic, steroidal bile acids and is inhibitory to many gastrointestinal bacteria, the magnitude of which is determined primarily by the bile salts' concentration and ionic strength *(32).* Bile may also contain antibiotics undergoing excretion and/or enterohepatic circulation, such as tetracycline, erythromycin, rifampicin, ampicillin, and metronidazole *(27, 42, 53)*, or come into contact with antibiotics administered orally. Lactobacilli and bifidobacteria strains have been shown to exhibit strain variation in bile salt tolerance *(7, 19, 30)* and antibiotic susceptibility *(3, 4, 8, 9, 14, 15, 24, 31, 33–35, 37, 39, 41, 51).* However, their tolerance to combinations of bile and antibiotic has not been determined. In this study, we examined the effect of the presence of 0.5% wt/wt oxgall (conjugated bile salts) on the antibiotic susceptibility of

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TABLE 1. *Origin of lactobacilli and bifidobacteria in this study*

Species	Strain	Origin	Source
L. acidophilus	1748	Human pharynx	Aarla[a]
	Ki	Human fecal isolate	NIZO[b]
	VVM2	Human fecal isolate	NIZO
	243	Traditional Greek cheese	ACA-DC[c]
	245	Traditional Greek cheese	ACA-DC
L. crispatus	33820T	Human fecal isolate	CUP[d]
L. gasseri	221	Feces of human breast–fed infant	CUP
L. johnsonii	332	Nonhuman isolate	ATCC[e]
L. casei	3026	Feces of human breast–fed infant	CUP
	121	Feces of human breast–fed infant	CUP
	F17	Human gastrointestinal isolate	NIZO
	F19	Human gastrointestinal isolate	NIZO
	F38	Human gastrointestinal isolate	NIZO
	334T	Emmenthal cheese	CUP
	212.1	Brine of Feta cheese	CUP
	212.2	Brine of Feta cheese	CUP
	212.3	Brine of Feta cheese	CUP
	212.4	Brine of Feta cheese	CUP
	206	Nonhuman isolate	NCFB[f]
L. rhamnosus	GG	Adult human fecal isolate	Aarla
	86	Dairy product isolate	NCFB
	207	Dairy product isolate	NCFB
	243T	Dairy product isolate	NCFB
	244	Dairy product isolate	NCFB
	252	Dairy product isolate	NCFB
	330	Dairy product isolate	NCFB
	1051	Dairy product isolate	NCFB
	1657	Dairy product isolate	NCFB
	1856	Dairy product isolate	NCFB
	1858	Dairy product isolate	NCFB
	2773	Dairy product isolate	NCFB
L. fermentum	KLD	Human gastrointestinal isolate	Aarla
	53608	Porcine isolate	Aarla
L. reuteri	20016T	Adult human fecal isolate	CUP
L. delbrueckii ssp. *bulgaricus*	233	Greek yogurt	ACA-DC
	235	Greek yogurt	ACA-DC
	2317	Greek yogurt	ACA-DC
B. bifidum	11863	Human isolate	NIZO
	35914	Human isolate	NIZO
	Bo	Human infant feces	NIZO
	2715	Human infant feces	NCFB
	1453	Human infant feces	NCFB
B. breve	15698	Human infant feces	ATCC
	15701	Human infant feces	NIZO
B. infantis	27920	Human infant feces	ATCC
	15702	Human infant feces	NIZO
	25962	Human infant feces	ATCC
B. animalis	25527T	Rat feces	ATCC

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lactobacilli and bifidobacteria of known bile salt tolerance. It is anticipated that the study will provide a better understanding of the tolerance of these microorganisms to antibiotic exposure in vivo.

MATERIALS AND METHODS

Strains. The source and origin of strains are given in Table 1. Stock cultures of lactobacilli and bifidobacteria were maintained

TABLE 2. *Antimicrobial agents and associated interpretative zone diameters for disc diffusion antibiotic susceptibility testing*

Antimicrobial group	Antimicrobial		Interpretative zone diameter (mm)[a]		
	Name	Disc conc. (μg)	R	MS	S
Group 1—inhibitors of cell wall synthesis					
Penicillins	Penicillin G	10	≤19	20–27	≥28
	Ampicillin	10	≤12	13–15	≥16
Glycopeptides	Vancomycin	30	≤14	15–16	≥17
Group 2—inhibitors of cytoplasmic membrane function					
Polymyxins	Polymyxin B	300	≤8	9–11	≥12
Group 3—inhibitors of nucleic acid synthesis					
Co-trimoxazole	Co-trimoxazole	25	≤10	11–15	≥16
Rifampicins	Rifampicin	5	≤14	15–17	≥18
Nitroimidazoles	Metronidazole	5	≤14	15–17	≥18
Group 4—inhibitors of protein synthesis					
Aminoglycosides	Gentamicin	10	≤12	—	≥13
	Kanamycin	30	≤13	14–17	≥18
	Streptomycin	10	≤11	12–14	≥15
Tetracyclines	Tetracycline	30	≤14	15–18	≥19
Single antibiotics	Chloramphenicol	30	≤13	14–17	≥18
Macrolides	Erythromycin	15	≤13	14–17	≥18

[a] Susceptibility expressed as R, resistant; MS, moderately susceptible; or S, susceptible *(15).*

TABLE 3. *Effect of 0.5% w/w oxgall on intrinsic antibiotic resistance of bile salts–tolerant lactobacilli and bifidobacteria*

Antibiotic	Number of resistant strains			
	Lactobacillus[a]		*Bifidobacterium*[b]	
	Without oxgall	With oxgall[c]	Without oxgall	With oxgall
Metronidazole	37	37	11	11
Vancomycin	34	34	11	11
Co-trimoxazole	32	29	3	0*
Polymyxin B[d]	34	10***	11	8*
Gentamicin	30	7***	10	0***
Kanamycin[e]	37	15***	11	1***
Streptomycin[f]	30	9***	9	0***

[a] Thirty-seven strains tested, paired-sample comparison using two-tailed Student *t* test with *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

[b] Eleven strains tested, paired-sample comparison using two-tailed Student *t* test with *, $P < 0.05$; **, $P < 0.01$, and ***, $P < 0.001$.

[c] Seven lactobacilli were intrinsically resistant to all antibiotics in the presence of oxgall, including Ki, VVM2, 33820T, 121, 334T, 212.2, and 212.4.

[d] Three additional lactobacilli were resistant to polymyxin B in the presence of oxgall, including F19, 244, and 1051; resistance was shared by all bifidobacteria except Bo, 2715, and 25962.

[e] Eight additional lactobacilli were kanamycin resistant in the presence of oxgall, including F19, F38, 221, 212.3, 244, 1051, 1858, and 233, *Bifidobacterium bifidum* strain Bo was kanamycin resistant in the presence of oxgall; *Bifidobacterium bifidum* strain 35914 was kanamycin resistant in the presence of oxgall.

[f] Two additional lactobacilli were streptomycin resistant in the presence of oxgall, including 1748 and 233.

at −20°C on glass beads in MRS medium *(25)* and TPY broth *(48)* that contained glycerol (40% vol/vol), respectively. MRS medium was obtained from Oxoid (Unipath Ltd., Basingstoke, UK), and TPY broth was prepared as described by Scardovi *(48).* Strains were anaerobically transferred (BBL GasPak System, Beckton Dickinson & Co., Paramus, NJ) at 37°C for 48 h three times before assay. They have previously been characterized in terms of gastric transit tolerance *(16),* bile salt tolerance *(7, 20),* and Caco-2 cell adhesion capacity *(21, 22, 46).*

Antibiotics and susceptibility testing. Antibiotic susceptibility discs were obtained from Oxoid (Unipath) and stored in sealed containers with a desiccant at 4°C. Antibiotic susceptibility was determined semiquantitatively by soft agar overlay disc diffusion, without prediffusion or preincubation, on MRS or TPY agar as previously described *(10, 14, 17)* in the presence and absence of 0.5% wt/wt oxgall (Sigma Chemical Co., St. Louis, Mo.). Briefly, Petri plates (9 cm), containing 15 ml of agar, were overlaid with 4 ml of soft agar seeded with 200 μl of an active culture at 45°C. This provided a moderately heavy inoculum of about $10^6 - 10^7$ viable cells per ml of agar overlay. Petri plates were allowed to stand at room temperature for 15 min before dispensing antibiotic-containing discs with the Oxoid MKII Disc Dispensing System (Unipath). Inhibition zone diameters were measured after anaerobic incubation at 37°C for 24 h using sliding callipers. The results (mean of three determinations) are expressed in terms of resistance, moderate susceptibility, or susceptibility, according to interpretative standards described previously *(14)* (Table 2). The precision and accuracy of the antimicrobial susceptibility test procedure were monitored using *Escherichia coli* ATCC 25922, which was of known antibiotic susceptibility and capable of growth on MRS, TSB (soybean casein digest), and WCA (Wilkens Chalgren Anaerobe) agars.

Statistical methods. Results are expressed as the mean and standard deviation of three determinations. Statistical analysis comprised significance testing of the difference between means

TABLE 4. *Effect of 0.5% w/w oxgall on susceptibility of intrinsically and nonintrinsically resistant lactobacilli and bifidobacteria to penicillin G and ampicillin*

Genus	Antibiotic susceptibility[a]	No. strains	Zone diameter (mm)[b] Penicillin G Without oxgall	Penicillin G With oxgall	Ampicillin Without oxgall	Ampicillin With oxgall
Lactobacillus	Intrinsically resistant	7[c]	33.90 (4.83) BC	23.90 (5.00) ABC	33.43 (5.18) AB	26.36 (3.12) BC
	Nonintrinsically resistant	30	33.40 (5.07)	35.74 (6.17) C	30.91 (3.83) AB	33.04 (5.27) B
Bifidobacterium	Nonintrinsically resistant	11	27.25 (2.55) C	27.71 (1.60) A	31.75 (3.24) C	35.70 (1.00) C

[a] Metronidazole, vancomycin, co-trimoxazole, polymyxin B, gentamicin, kanamycin, and streptomycin.
[b] Data are presented as the mean (standard deviation), $n = 3$.
[c] Ki, VVM2, 33820T, 121, 334T, 212.2, and 212.4
[d] Paired-sample comparison within and between rows that share the same letter are significantly different with A, $P < 0.05$, B, $P < 0.01$, and C, $P < 0.001$, using the two-tailed Student t test.

using a two-tailed Student's t test at the following levels: $P < 0.05$, $P < 0.01$, and $P < 0.001$.

RESULTS

Effect of oxgall on intrinsic antibiotic susceptibility. Few *Lactobacillus* and *Bifidobacterium* strains failed to retain intrinsic resistance to the aminoglycosides in the presence of oxgall ($P < 0.001$) (Table 3). Oxgall also changed intrinsic resistance of a few lactobacilli (and some bifidobacteria) to polymyxin B. These results show that the antimicrobial spectra of aminoglycosides and polymyxin B include gram-positive genera of the indigenous microbiota in the presence of physiological levels of conjugated bile salts.

Bile salt tolerance and resistance. Using oxgall-mediated changes in intrinsic aminoglycoside susceptibility as a measure of bile tolerance, only a few strains are considered to be intrinsically resistant to oxgall. Four human (Ki, VVM2, 33820T, and 121) and three dairy (334, 212.2, and 212.4) lactobacilli showed unaltered intrinsic antibiotic susceptibility profiles in the presence of oxgall, and when assayed for susceptibility to penicillin and ampicillin (inhibitors of cell wall synthesis), they did not show increased susceptibility in the presence of oxgall (Table 4).

Effect of oxgall on extrinsic antibiotic susceptibility. Oxgall significantly affected so-called extrinsic susceptibility in a source- and strain-dependent manner, with human lactobacilli- ($P < 0.05$) and bifidobacteria-drug combinations ($P < 0.01$) being more likely to show no change or decreased antibiotic susceptibility in the presence of oxgall compared with other strain-drug combinations (Table 5). That lactobacilli showed altered extrinsic antibiotic susceptibility in the presence of oxgall enabled a categorization of strains on this basis as shown in Table 6. Among those strains used commercially, *Lactobacillus fermentum* KLD and *Lactobacillus rhamnosus* GG showed no change in extrinsic antibiotic susceptibility, whereas *Lactobacillus acidophilus* 1748 was markedly less susceptible in the presence of oxgall ($P < 0.05$). In addition, the commercially promising *Lactobacillus casei* 212.3 *(6)* is less susceptible in the presence of oxgall ($P < 0.05$).

DISCUSSION

Bile plays a central role in specific *(38)* and nonspecific *(36)* defense mechanisms of the gut and in doing so has the potential to augment the toxicity of an antibiotic administered orally or undergoing excretion or enterohepatic circulation. We have previously shown that lactobacilli *(14)* and bifidobacteria *(15)* intended for use as probiotics may be considered intrinsically resistant to metronidazole, vancomycin, co-trimoxazole (lactobacilli only), polymyxin B, and the aminoglycosides gentamicin, kanamycin, and streptomycin. In this study, most strains from both genera failed to retain intrinsic resistance to the aminoglycosides in the presence of oxgall ($P < 0.001$) (Table 3). It is thought that oxgall-enhanced cell envelope permeability circumvented the lack of cytochrome-driven antibiotic uptake *(2),* thereby changing aminoglycoside susceptibility. The bacterial uptake of aminoglycosides has previously been shown to be facilitated in the presence of inhibitors of cell wall synthesis, such as β-lactams and vancomycin, and is considered to form the basis of antibacterial synergism between aminoglycosides and β-lactam antibiotics *(23, 29).* Bactericidal synergy between penicillin or ampicillin and aminoglycosides constitutes the basis for the choice of these antibiotics in the treatment of serious infections involving antibiotic-tolerant lactobacilli *(5).* Oxgall also changed intrinsic resistance of most lactobacilli (and some bifidobacteria) to polymyxin B, a cationic peptide-like antibiotic known to display membrane-disruptive properties *(49, 50),* by a process also thought to involve cell membrane disruption. These results are considered clinically significant, since the antimicrobial spectra of aminoglycosides and polymyxin B are considered to be limited to gram-negative genera *(53).*

The recognition that aminoglycoside inhibition of protein synthesis was enhanced by oxgall prompted a distinction to be made between *Lactobacillus* strains as conjugated bile salts ''tolerant'' or truly ''resistant'' (i.e., total tolerance). Using oxgall-mediated changes in intrinsic aminoglycoside susceptibility as a measure of bile tolerance or resistance, only a few strains are considered intrinsically resistant to oxgall. The group comprises four human (Ki, VVM2, 33820T, and 121) and three dairy (334, 212.2, and

TABLE 5. *Effect of 0.5% w/w oxgall on extrinsic antibiotic susceptibility of lactobacilli and bifidobacteria*

		No. isolates showing altered susceptibility with added oxgall[a]							
		Human[b]				Other[b]			
Antibiotic	Genus	No change	Decreased	Increased	Total	No change	Decreased	Increased	Total
Penicillin	*Lactobacillus*	3	6	4	13	9	7	8	24
	Bifidobacterium	5	—	3	8[c]	0	0	1	1
Ampicillin	*Lactobacillus*	4	6	3	13	11	6	7	24
	Bifidobacterium	3	—	6	9[c]	0	0	1	1
Tetracycline	*Lactobacillus*	3	8	2	13	6	10	8	24
	Bifidobacterium	8	1	1	10	1	0	0	1
Chloramphenicol	*Lactobacillus*	3	9	1	13	9	6	9	24
	Bifidobacterium	5	0	5	10	0	0	1	1
Erythromycin	*Lactobacillus*	5	7	1	13	8	5	11	24
	Bifidobacterium	4	0	6	10	0	0	1	1
Rifampicin	*Lactobacillus*	0	12	1	13	7	11	6	24
	Bifidobacterium	9	1	0	10	0	1	0	1
Overall	*Lactobacillus*	18***	48	12**	78***	50***	45	49**	144***
	Bifidobacterium	34*	2*	21*	58***	1*	1*	4*	6***

[a] Results are expressed as the number of isolates that showed "no change" (<3.0 mm difference), "decreased" or "increased" (>3.0 mm difference) zone diameter in the presence of 0.5% w/w oxgall.
[b] Paired comparisons between human and "other" (dairy and other) sources of strains were made using two-tailed Student *t* test with *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$.
[c] Eight of ten and nine of ten drug-strain combinations were determined, respectively.

TABLE 6. *Categorization of lactobacilli according to the magnitude of change in extrinsic antibiotic susceptibility in the presence of 0.5% w/w oxgall*

Oxgall-mediated zone diameter change[a]	*P* value	Origin of lactobacilli		
		Human isolates	Dairy isolates	Other
Decrease (>3 mm)[b]	$P < 0.001$	VVM2, 121, F17	245, 334T	206
	$P < 0.01$	F38, 33820T	212.2	
	$P < 0.05$	F19, 1748 Ki	1051, 212.3	
No change (<3 mm)		20016T, KLD, GG	243, 332, 212.1	53608
		221, 3026	212.4, 207, 244	
			233, 235, 2317	
Increase (>3 mm)[b]	$P < 0.001$		252, 1858	
	$P < 0.01$		330, 1856, 2773	
	$P < 0.05$		86, 243T, 1657	

[a] Extrinsic susceptibility to penicillin, ampicillin, tetracycline, chloramphenicol, erythromycin, and rifampicin.
[b] Paired-sample comparison between means with and without oxgall using two-tailed Student *t* test. Note: Only *Bifidobacterium bifidum* strain Bo showed altered (increase, $P < 0.05$) extrinsic antibiotic susceptibility in the presence of oxgall.

212.4) strains that show unaltered intrinsic and extrinsic (penicillin and ampicillin) antibiotic susceptibility in the presence of oxgall (Table 4). These strains are considered likely to possess greater upper gastrointestinal tract transit tolerance during antibiotic treatment and may constitute a potentially important group for application in prophylaxis and therapy of antibiotic-associated diarrhea.

Oxgall significantly affected so-called extrinsic antibiotic susceptibility in a source- and strain-dependent manner, with human lactobacilli ($P < 0.05$) and bifidobacteria ($P < 0.01$) being more likely to show no change or decreased antibiotic susceptibility in the presence of oxgall than isolates from other sources (Table 5). The recognition that lactobacilli showed altered variation in extrinsic antibiotic susceptibility in the presence of oxgall prompted a categorization of strains on this basis as shown in Table 6. Among those strains used commercially, *L. fermentum* KLD and *L. rhamnosus* GG show no change in extrinsic antibiotic susceptibility, whereas *L. acidophilus* 1748 is markedly less susceptible in the presence of oxgall ($P < 0.05$). In addition, the commercially promising *L. casei* 212.3 *(6)* is less susceptible in the presence of oxgall ($P < 0.05$). It is envisaged that strains 1748 and 212.3 may persist for longer in vivo than strains KLD and GG during antibiotic exposure.

Although, the mechanism of bile salt resistance among lactobacilli and bifidobacteria is not fully understood, it is thought that the cell wall acts as a barrier against bile salts. The magnitude of this barrier effect has previously been shown to be incomplete in *Lactobacillus reuteri (26), L. acidophilus (43),* and *E. coli (28).* In this regard, oxgall addition (0.15 or 0.3%) to lactose-supplemented (2%) MRS or peptonized milk nutrient broth significantly increased β-galactosidase activity of whole cells of *L. reuteri (26)* and *L. acidophilus (43)* without affecting specific growth rate or causing leakage of enzyme. In addition, de Valdez et al. *(26)* observed decreased viability (1 to 2 log cycles) of *L. reuteri* to freeze-drying after growth in the presence of oxgall and a further strain-dependent decrease in viability when oxgall was also present in the freezing medium. Partial detachment of the cell membrane from the cell wall and the expulsion of mesosomelike elements into the space between the cell wall and concentrated cytoplasm were associated with diminished viability during freeze-drying. Sodium glycocholate and sodium taurocholate have also been shown to increase the β-glucuronidase activity of *E. coli* and *Clostridium perfringens,* suggesting increased permeability of cells in the presence of bile salts *(28).*

ACKNOWLEDGMENTS

The authors acknowledge funding under the EU FLAIR Programme (Project no. CT91–0053), the support of Drs. Hoestra and Cornelese on behalf of the European Commission, and their colleagues for contributing strains to the project culture collection. This paper is dedicated to our late colleague Dr. Anton Weerkamp (NIZO, the Netherlands): *Prima quae vitam dedit hora, carpit.*

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